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# The effect of metformin on vascular function and AMP-activated protein kinase activation in Type 2 diabetes

A thesis submitted to

**The Faculty of Medicine**

For the degree of

DOCTOR OF MEDICINE

By

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## ***Abstract***

### ***Introduction***

Obesity, metabolic syndrome and Type 2 diabetes are clinical states associated with insulin resistance and an increased risk of vascular disease. The prevalence of Type 2 diabetes is reaching epidemic proportions and there is a growing need for new therapeutic targets. There is a significant body of research that demonstrates an association between obesity, insulin resistance, endothelial dysfunction and accelerated atherosclerosis, but the molecular mechanisms underlying this link are incompletely understood. Biguanides and thiazolidinediones are widely used in the treatment of Type 2 diabetes to improve glycaemia but the precise site, mode and extent of their actions remain uncertain. For example, in the United Kingdom Prospective Diabetes Study (UKPDS), metformin treatment reduced the incidence of myocardial infarction by 39% in comparison with conventional treatment which is more than would have been predicted by the difference in achieved HbA1c (0.6%). In addition, when the metformin group was compared with a group treated with sulphonylurea or insulin, to control for glycaemia, there was still a significant reduction in the incidence of stroke and any other diabetes-related end-point. The enzyme AMPK is a novel therapeutic target in Type 2 diabetes and the development of specific tissue specific AMPK activators is an attractive prospect for the future. We therefore investigated the role of AMPK in the action of biguanides and thiazolidinediones. Accordingly, the aims of this project were to first determine if metformin improves vascular endothelial function and large artery stiffness in patients with Type 2 diabetes. In parallel, the thesis examines if metformin exerts its beneficial effects in patients with Type 2 diabetes in association with altered AMPK activity in human adipose. Finally, the thesis determines if insulin sensitizers such as metformin and the thiazolidinediones, acting directly on vascular endothelial cells, increase NO production by increasing AMPK activity thus

accounting for beneficial effects on endothelial function and, in metformin's case, cardiovascular outcome.

### ***Methods***

Twenty men with Type 2 diabetes were randomised to metformin (500 milligrams three times daily for ten weeks) and gliclazide (80 milligrams twice daily for ten weeks) in a double-blinded, glycaemia controlled, cross-over design. There was a six week run in and washout period. At the end of each ten week phase of therapy routine and non routine blood sampling (including plasma ADMA), PWV and an adipose biopsy were performed to determine resistance artery function and adipose AMPK activity. The effects of metformin, its closely related analogue phenformin and rosiglitazone were characterised in cultured human aortic endothelial cells (HAECs). AMPK activity was assessed using a peptide kinase assay and the quantification of phosphorylation of the AMPK substrate, acetyl-CoA carboxylase (ACC) in HAECs incubated in rosiglitazone, phenformin or metformin. Nitric oxide (NO) release was evaluated with a Sievers NO meter.

### ***Results***

The mean age and BMI of subjects in the clinical study was 56.5 years and 31kg/m<sup>2</sup>. Mean HbA1c after metformin therapy was 8.3% compared with 7.8% following gliclazide therapy. There was no significant difference between resistance artery function and PWV. ADMA was marginally lower following metformin therapy ( $p=0.019$ ). AMPK activity was 2 fold higher following metformin therapy when compared with gliclazide therapy ( $p=0.002$ ). Stimulation of human aortic endothelial cells with phenformin and rosiglitazone resulted in the time- and dose-dependent stimulation of AMP-activated protein kinase activity and NO production with concomitant phosphorylation of endothelial NO synthase at Ser1177. Infection of endothelial cells with a virus encoding a dominant negative AMPK mutant attenuated both phenformin and rosiglitazone-stimulated

Ser1177 phosphorylation and NO production. Furthermore, the stimulation of AMPK and NO synthesis by rosiglitazone was unaffected by the peroxisome proliferator-activated-receptor-inhibitor, GW9662. Incubation of HAECS with metformin, however, had no effect on AMPK activity or NO synthesis.

### ***Conclusion***

Taken together, the data in this thesis provides further insight into the molecular mechanisms underlying the interactions between insulin sensitizing drugs, the enzyme AMPK and both the human vascular endothelium and adipose. Further work leading to an increased understanding of the molecular mechanisms by which anti-diabetic drugs activate AMPK in both the vascular endothelium and adipose tissue may identify novel therapeutic targets in patients with Type 2 diabetes.

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***Declaration***

I declare that the work presented in this thesis has been carried out by myself, unless otherwise stated. It is entirely of my own composition and has not, in whole, or in part been submitted for any other degree. The work described in this thesis was carried out under the supervision of Professor John Connell, Dr Ian Salt and Dr Steve Cleland in the Division of Cardiovascular and Medical Sciences, Glasgow.

**James Graham Boyle**

**August 2009**

### ***Definitions***

ACC	Acetyl-coA carboxylase
ACH	Acetylcholine
ADMA	Asymmetrical dimethylarginine
AICAR	5-aminoimidazole -4-carboxamide-1-β -D-ribofuranoside
AMPK	AMP-activated protein kinase
BAEC	Bovine aortic endothelial cell
BH <sub>4</sub>	Tetrahydrobiopterin
BMI	Body mass index
CaM	Calmodulin
CAMKK	Calcium/CaM-dependent protein kinase kinase
cGMP	Cyclic guanosine monophosphate
CPT1	Carnitine palmitoyltransferase 1
eNOS	Endothelial nitric oxide synthase
EDHF	Endothelium derived hyperpolarising factor
ET-1	Endothelin-1
FAD	Flavin adenine dinucleotide
FFA	Free fatty acids
FMN	Flavin mononucleotide
G6Pase	Glucose-6-phosphatase
GSK3	Glycogen-synthase kinase 3
HAEC	Human aortic endothelial cell
HDL	High density lipoprotein
HOMA	Homeostatic Model Assessment
HUVEC	Human umbilical vein endothelial cells

IL-6	Interleukin 6
iNOS	Inducible nitric oxide synthase
ICAM-1	Intercellular adhesion molecule-1
IR	Insulin receptor
IRS	Insulin receptor substrate
KO	Knock out
LDL	Low density lipoprotein
L-NAME	NG-Nitro-L-Arginine Methyl Ester
MAPK	Mitogen activated protein kinase
NADPH	Nicotinamide adenine dinucleotide phosphate
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
PDK-1	Phosphoinositide -dependent Kinase 1
PEPCK	Phosphoenolpyruvate carboxykinase
PGI2	Prostacyclin
PI3K	Phosphoinositol 3-kinase
PIP3	Phosphatidylinositol(3,4,5)trisphosphate
PKB	Protein kinase B
PKC	Protein kinase C
PWV	Pulse wave velocity
ROS	Reactive oxygen species
SDMA	Symmetrical dimethylarginine
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus
TNF- $\alpha$	Tumour necrosis factor-alpha

VCAM-1	Vascular cell adhesion molecule-1
VEGF	Vascular endothelial growth factor
VSMC	Vascular smooth muscle cell
WBC	White blood cells

## **Publications**

### **Full**

**Boyle JG**, Logan PJ, Ewart MA, Reihill JA, Ritchie SA, Connell JMC, Cleland SJ, Salt IP. Rosiglitazone stimulates nitric oxide synthesis in human aortic endothelial cells via AMP-activated protein kinase and LKB1. (2008) *Journal of Biological Chemistry* 283(17):11210-7

### **Abstract**

**Boyle JG**, Salt IP, McConnachie A, Jones GC, Small M, Connell JMC, Sattar N, Cleland SJ (2008). Effects of metformin versus gliclazide on plasma asymmetric dimethylarginine (ADMA) levels of subjects with Type 2 diabetes: a randomised, double-blind, glycaemia controlled crossover study. *Diabetologia* 51 (Suppl.)

**Boyle JG**, Salt IP, Jones GC, Small M, Sattar N, Connell JMC, Cleland SJ (2008). Metformin increases AMP-activated protein kinase activity in human adipose tissue of subjects with Type 2 diabetes. *Diabetes UK Type 2 Diabetes Research Award*. *Diabetic Medicine* 26 (Suppl.).

Salt IP, **Boyle JG**, Ewart MA, Reihill JA, Connell JMC, Cleland SJ (2007) Rosiglitazone stimulates nitric oxide production in cultured human aortic endothelial cells via AMP-activated protein kinase. *Diabetes UK Basic Science Poster Award Shortlist*. *Diabetic Medicine*, 24 (Suppl.), 122-129

**Boyle JG, Salt IP, Cleland SJ, Connell JMC** (2006) The acute stimulation of nitric oxide synthesis by rosiglitazone in human aortic endothelial cells is independent of the PPAR gamma receptor but is dependent on the fuel sensing enzyme AMPK. *Endocrine Abstracts* (Vol. 11).

**Boyle JG, Cleland SJ, Salt IP, Connell JMC** (2005) Rosiglitazone and phenformin, but not metformin activates AMP-activated protein kinase in human aortic endothelial cells. *Endocrine Abstracts* (Vol. 9)

*Presentations to learned societies containing work undertaken for this thesis*

**Oral communications**

The role of the AMP-activated protein kinase in the management of Type 2 diabetes.

*Caledonian Endocrine Society Prize Lecture*

**Boyle JG**

Caledonian Endocrine Society Meeting

Peebles, December 2008

Effects of metformin versus gliclazide on plasma asymmetric dimethylarginine (ADMA) levels of subjects with Type 2 diabetes: a randomised, double-blind, glycaemia controlled crossover study.

**Boyle JG, Salt IP, McConnachie A, Jones GC, Small M, Connell JMC, Sattar N, Cleland SJ.**

44<sup>th</sup> EASD Annual Meeting

Rome, September 2008

Metformin increases AMP-activated protein kinase activity in human adipose tissue of subjects with Type 2 diabetes.

*Recipient of the Diabetes UK Type 2 Diabetes Research Award*

**Boyle JG, Salt IP, Jones GC, Small M, Sattar N, Connell JMC, Cleland SJ**

Diabetes UK Annual Professional Conference, March 2008

Rosiglitazone and phenformin, but not metformin activates AMP-activated protein kinase in human aortic endothelial cells.

**Boyle JG**, Cleland SJ, Salt IP, Connell JMC

RCPSG Research Symposium, November 2005

The role of insulin sensitizers and AMP-activated protein kinase in the regulation of endothelial function

**Boyle JG**, Salt IP, Connell JMC, Cleland SJ

32<sup>nd</sup> Meeting of the British Hypertension and Vascular Research Group,

Gleneagles, January 2005

***Poster communications***

Rosiglitazone stimulates nitric oxide production in cultured human aortic endothelial cells via AMP-activated protein kinase

IP Salt, **JG Boyle**, MA Ewart, JA Reihill, JMC Connell, SJ Cleland

*Diabetes UK Basic Science Poster Award Shortlist*

Diabetes UK Annual Professional Conference

Glasgow, March 2007

Rosiglitazone stimulates AMP-activated protein kinase mediated nitric oxide synthesis in cultured human aortic endothelial cells

**JG Boyle**, IP Salt, SJ Cleland, JMC Connell

42<sup>nd</sup> EASD Annual Meeting

Copenhagen, September 2006

The acute stimulation of nitric oxide synthesis by rosiglitazone in human aortic endothelial cells is independent of the PPAR gamma receptor but is dependent on the fuel sensing enzyme AMPK

**JG Boyle, IP Salt, SJ Cleland, JMC Connell**

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Rosiglitazone and phenformin, but not metformin activates AMP-activated protein kinase in human aortic endothelial cells.

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Phenformin activates AMP-activated protein kinase and stimulates nitric oxide in human aortic endothelial cells

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*Chapter 1 Introduction*

## ***1.1 Diabetes Mellitus, Insulin Resistance and the Metabolic Syndrome***

### ***1.1.1 Diabetes Mellitus***

The key clinical features of diabetes mellitus were recognised as long ago as around 1500BC when Hindu scholars first described an illness featuring the familiar combination of polyuria, polydipsia, body wasting and the less familiar description of urine sweet enough to attract flies and ants (Howlett and Bailey, 2007). Today, diabetes mellitus is a chronic disease that we can treat but not cure. There are an estimated 2.2 million patients with diabetes in the United Kingdom (UK) with a further 500,000 of the UK population undiagnosed (Information Centre Quality Outcomes Framework Data 2005/06). Diabetes Mellitus is estimated to cost the NHS £5.2 billion pounds per year. This represented a staggering 9.2% of the annual budget in 1997 (Currie *et al* 1997). Type 2 Diabetes (T2DM), the most prevalent form of the disease, today affects some 250 million people worldwide with more than 380 million predicted by the year 2025.

The major cause of morbidity and mortality in T2DM is cardiovascular disease (Zimmet and Alberti 1997, Laakso 1999). The proportion of cardiovascular disease attributable to T2DM has increased over the last 50 years (Fox *et al* 2007). In addition to cardiovascular disease; cerebrovascular, peripheral vascular and renovascular disease all contribute significantly to both vascular morbidity and mortality in diabetes mellitus (DeFronzo *et al* 2004). The cornerstone of all forms of diabetes mellitus is abnormal insulin physiology. In Type 1 diabetes this constitutes insulin deficiency. In T2DM this constitutes resistance to insulin's metabolic actions.

### ***1.1.2 Insulin Resistance***

Insulin resistance can be defined generically as reduced biological action of insulin at a certain concentration. The gold standard for investigating and quantifying insulin resistance is the hyperinsulinaemic euglycaemic clamp, so called because it measures the amount of glucose

necessary to compensate for an increased insulin level without causing hypoglycaemia (DeFronzo *et al* 1979). Given the complicated nature of the "clamp" technique (and the potential dangers of hypoglycaemia in some patients), alternatives have been sought to simplify the measurement of insulin resistance. One example is the Homeostatic Model Assessment (HOMA) which employs fasting insulin and glucose levels to calculate insulin resistance (Wallace *et al* 2004).

Sensitivity to insulin-mediated glucose disposal varies more than six-fold in the general population (Yeni-Komshian *et al* 2000). When patients with insulin resistance cannot maintain the degree of hyperinsulinaemia needed to overcome the insulin resistance, T2DM develops (Warram *et al* 1990, Lilloja *et al* 1993). However, the vast majority of patients with insulin resistance continue to secrete the large amount of insulin required to overcome this defect in insulin action, thereby maintaining normal or near-normal glucose tolerance.

The mechanisms responsible for insulin resistance are poorly understood. Several factors appear to contribute to a patient's metabolic susceptibility to insulin resistance. Age, physical activity, drugs, as well as ethnicity, racial status and family propensity are all important. At a more mechanistic level metabolic susceptibility to insulin resistance has been related to the maldistribution of adipose tissue such as visceral adiposity (Lewis *et al* 2002). In fact, the mechanisms by which patients develop visceral adiposity have recently been the subject of intensive research. Visceral adipose tissue is very metabolically active and recent discoveries have shown that visceral adiposity is associated with abnormalities in the release of a variety of factors from adipose tissue including the adipocytokines and free fatty acids (FFA) (Hotamisligil *et al* 1993, Considine *et al* 1996, Boden *et al* 1997, Arita *et al* 1999). It has now been established that there is a close and dynamic relationship between insulin resistance and visceral adiposity and that insulin sensitivity is modulated by adiposity (Campbell *et al* 1993, Banerji *et al* 1995, Ludvik *et al* 1995, Abate *et al* 1996). This has

led to significant interest in the adipocytokines and FFA, which are proposed to play a pivotal role in the regulation of insulin signalling and action.

### ***1.1.2.1 Tumour necrosis factor – alpha***

Tumour necrosis factor – alpha (TNF- $\alpha$ ) is expressed as a 26kDa cell surface protein and while it is produced in the most part by macrophages, it is also produced in adipose tissue (Kern *et al* 1995, Wajant *et al* 2003). TNF- $\alpha$  expression is increased in the adipose tissue of obese animals and humans (Hotamisligil *et al* 1993, Hotamisligil *et al* 1995). Moreover, TNF- $\alpha$  expression is positively correlated to BMI, percentage body fat, and hyperinsulinaemia (Kern *et al* 1995). It has also been shown than weight loss decreases TNF- $\alpha$  levels (Kern *et al* 1995). Infusion of anti-TNF- $\alpha$  antibodies has been demonstrated to improve insulin sensitivity in animals, but is less effective in patients with obesity, impaired glucose tolerance or T2DM (Ofei *et al* 1996, Paquot *et al* 2000). Moreover, the use of recombinant soluble TNF- $\alpha$  receptor improves insulin sensitivity in insulin resistant animals (Hotamisligil *et al* 1993). Models using knock-out of the genes encoding TNF- $\alpha$  protein or its receptor in mice show improved insulin sensitivity (Uysal *et al* 1997).

### ***1.1.2.2 Leptin***

Leptin is a 16kDa protein secreted by adipocytes in proportion to the total amount of adipocytes present (Considine *et al* 1996). Leptin is proposed to act as a signal that informs the hypothalamus of the body's fat reserve. In patients without visceral adiposity or insulin resistance it is proposed that as food is eaten, absorbed and stored, serum leptin concentrations increase, cross the blood-brain barrier and promote satiety (Mizuno *et al* 1998, Schwartz *et al* 1996, Schwartz *et al* 1998). Children with frameshift mutations in the leptin (ob) gene are leptin deficient and display hyperphagia, obesity and hyperinsulinaemia (Farooqi *et al* 2002).

### **1.1.2.3 Interleukin-6**

Interleukin-6 (IL-6) is a 26 kDa protein produced by adipose tissue among many other tissues (Mohamed-Ali *et al* 1997, Podor *et al* 1989). Adipose tissue has been postulated to contribute a significant proportion of the total circulating IL-6 (Mohammed-Ali *et al* 1997, Yudkin *et al* 1999). Elevated IL-6 concentrations are associated with insulin resistance and obesity (Yudkin *et al* 1999). The addition of IL-6 to cell lines and mice has been shown to inhibit insulin action in skeletal muscle, hepatic and adipose tissue (Senn *et al* 2002, Rotter *et al* 2003, Kim *et al* 2004, Nieto-Vasquez *et al* 2008). IL-6 pre treatment inhibited the insulin stimulated signalling cascade in both hepatic and adipose tissue with a reduction in insulin stimulated glycogen formation and glucose uptake demonstrated (Senn *et al* 2002, Rotter *et al* 2003). Furthermore, the addition of IL-6 to mice attenuated both insulin related suppression of hepatic gluconeogenesis and insulin stimulated glucose uptake in skeletal muscle (Kim *et al* 2004, Nieto-Vasquez *et al* 2008).

### **1.1.2.4 Adiponectin**

Adiponectin is a protein secreted exclusively and abundantly by adipose tissue (Scherer *et al* 1995), with plasma levels negatively correlated with insulin resistance, obesity and cardiovascular disease (Arita *et al* 1999, Weyer *et al* 2001, Hotta *et al* 2000). This is in contrast to many of the other major adipocytokines such as TNF- $\alpha$ , leptin and IL-6, whose levels have been shown to increase with increasing adiposity. Adiponectin exists in 3 or more different higher order complexes: high molecular weight form (HMW, 12 to 36mer), low molecular weight form (hexamer) and trimeric form. The ratio of HMW to the other forms of adiponectin serves as an independent predicting factor for metabolic disorders (Fisher *et al* 2005, Wang *et al* 2007). Adiponectin levels are reduced in patients with insulin resistance (Yudkin *et al* 1999, Weyer *et al* 2001). Interestingly, studies of the Pima Indian population have shown that in normal healthy individuals low levels of adiponectin predict the likelihood of developing T2D later in their life (Lindsay *et al* 2002).

### **1.1.2.5 Free Fatty Acids**

Visceral adiposity is associated with elevated FFA due to increased activity of hormone-sensitive lipase. Accumulation of intracellular lipid in the cell is proposed to contribute to insulin resistance at a cellular level and increases in FFA concentration are frequently found in patients with T2DM due to increased release from adipose tissue and reduced skeletal muscle uptake (Kelley *et al* 1994, Boden *et al* 1997, Dresner *et al* 1999). Free fatty acids stimulate reactive oxygen species (ROS) production and inhibit the insulin-stimulated signalling cascade (Muniyappa *et al* 2007, Chinen *et al* 2007). Consequently, visceral adiposity and its associated effect on FFA and the adipocytokines may be important in the pathogenesis of insulin resistance (Lebovitz *et al* 2005, Miles *et al* 2005).

### **1.1.3 The Metabolic Syndrome**

More recently a ‘pre-diabetes’ clinical state termed the metabolic syndrome has been described which combines increased adiposity, hypertension, insulin resistance and dyslipidaemia (Alberti *et al* 2005) and is associated with increased cardiovascular risk (Hu *et al* 2004). There have been numerous definitions of the metabolic syndrome which has led to confusion and subsequent criticism. In both Adult Treatment Panel III (Grundy *et al* 2005) and the International Diabetes Federation (IDF), (Alberti *et al* 2005) formulations of the risk of the metabolic syndrome include the following surrogates:

- Serum triglyceride >1.7mmol/l
- High density lipoprotein (HDL) <1.0mmol/l (men), <1.3mmol/l (women)
- Blood pressure (BP) greater than or equal to 130/85 mmHg OR on medication
- Fasting serum glucose greater than or equal to 5.6mmol/l
- Increased waist circumference (population and sex dependent thresholds)

Each criteria requires at least 3 surrogates for the diagnosis of metabolic syndrome with the IDF combining lipids as one surrogate criteria and requiring waist circumstance as one of the 3 surrogates.

It is often considered uncertain whether the presence of the metabolic syndrome adds to cardiovascular disease prediction beyond the contribution of more than the sum of its individual risk factor parts. In a prospective study involving patients with and without diabetes who were followed up for an average of 11 years, the risk of incident coronary heart disease associated with the syndrome was no greater than that explained by its components (McNeil *et al* 2005). Moreover, in the secondary analysis of the West of Scotland Coronary Prevention Study (WOSCOPS) study (Sattar *et al* 2003) demonstrated that the metabolic syndrome was not a significant predictor of coronary heart disease when corrected for its component factors. This evidence suggests that the metabolic syndrome is essentially just the equivalent of the sum of its component parts and brings into question its value to the clinician in the day to day practice of a diabetes clinic.

## **1.2 Insulin**

### **1.2.1 Insulin: Structure and function**

Insulin consists of 2 linked polypeptide chains secreted by the  $\beta$  cells of the pancreas in response to glucose and amino acids (Sanger 2001). Insulin is the most potent anabolic hormone known, promoting the synthesis and storage of carbohydrates, lipids and proteins and inhibiting their degradation and release back into the circulation. The major sites of action are skeletal muscle, hepatocytes and adipocytes (Borge *et al* 2002). It is now also recognised that insulin receptors are present in many other tissues including the vascular endothelium (Steinberg *et al* 2002).

### **1.2.2 Insulin receptor tyrosine kinase**

The insulin receptor is a tetrameric membrane glycoprotein comprised of two  $\alpha$  and two  $\beta$  subunits that belongs to the large class of tyrosine kinase receptors (Saltiel *et al* 2002). The insulin receptor (IR) is expressed in most tissues of the body (including muscle, liver, adipose and the endothelial cell surface). Predictably, the IR has a high affinity for insulin and is necessary for insulin action (Zeng *et al* 1996, Kido *et al* 2001). When insulin binds to its membrane receptor this results in the stimulation of an intra-cellular signalling cascade in insulin sensitive tissues. To be more precise, insulin binds to the extracellular  $\alpha$ -subunit inducing a conformational change resulting in receptor autophosphorylation (Hubbard *et al* 1994, Hubbard *et al* 1997). This in turn enables interaction with and subsequent phosphorylation of the insulin receptor substrate (IRS) family of proteins (White *et al* 1985, White *et al* 1985, White *et al* 1998).

### **1.2.3 Insulin receptor substrate proteins**

The process of insulin signalling is incompletely understood. It is currently the subject of intensive research effort and a large number of proteins have been implicated in the process (Saltiel *et al* 2002). The IRS group of proteins have been the focus of a significant body of research recently due

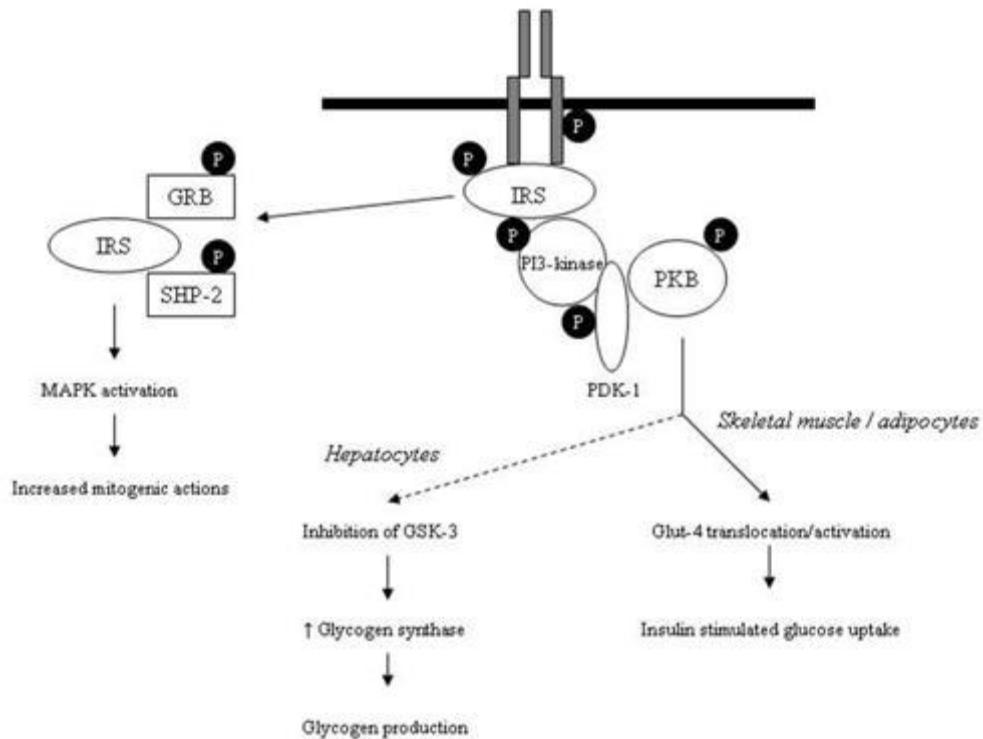
to their key role in insulin signalling. Of the 4 members identified (IRS-1, IRS-2, IRS-3, IRS-4), IRS-1 and IRS-2 have been subject to the most research. While all 4 members are present in rats, only IRS-1, 2, and 4 are present in humans (White 2002). The exact role of each member is yet to be determined.

IRS-1 is thought to be important in mediating the metabolic and mitogenic effects of insulin in peripheral tissues such as skeletal muscle and adipose tissue as well as being key in insulin action in the endothelium (Abe *et al* 1998). IRS-1 is not thought to have a significant role in insulin action in the liver (Yamauchi *et al* 1996). Mice lacking IRS-1 are insulin resistant but not overtly diabetic (Tamemoto *et al* 1994, Yamauchi *et al* 1996). Novel IRS-1 phosphorylation sites have been identified that regulate the action of insulin, and have the potential to be modified in pathological states and facilitate altered signalling (Zhang *et al* 2008). Key phosphorylation sites identified on IRS-1 include the tyrosine residues 612 and 632 required for IRS-1 / phosphatidylinositol 3-kinase (PI3-K) interaction (Montagnani *et al* 2002). IRS-Ser312 (akin to residue Ser307 in mice) is proposed to inhibit the downstream insulin signalling cascade (Danielsson *et al* 2005). Moreover, Ser636 and Ser318 appear to act as a stimulator of insulin action and a negative feedback regulator respectively (Weigert *et al* 2005, Luo *et al* 2007). Furthermore, IRS-1 gene polymorphisms are associated with the development of T2DM in humans (Sesti *et al* 2001).

IRS-2 is thought to have an important role in the mediation of insulin action in the liver and adipose tissue (Withers *et al* 1998, Withers *et al* 1999). IRS-2 is thought to mediate pancreatic beta cell function and therefore insulin secretion (Diabetes *et al* 2000). IRS-2 should, therefore, be important in the pathogenesis in both Type 1 diabetes (T1DM) and T2DM as defective insulin secretion is common to both conditions in time. Indeed, mice lacking the IRS-2 protein exhibit diabetes (Withers *et al* 1998).

#### **1.2.4 Insulin signalling cascade in skeletal muscle, hepatocytes and adipocytes**

Insulin binding to its receptor results in receptor autophosphorylation on tyrosine residues and subsequent phosphorylation of IRSs. This allows association of IRS-1 with the regulatory subunit of PI3K. PI3K consists of an 85kDa regulatory subunit and an 110kDa catalytic subunit that interacts with IRS-1 upon activation by the IR (Montagnani et al 2002) via an SH2 domain. Activation of catalytic subunit of PI3K results in the production of phosphatidylinositol(3,4,5)trisphosphate (PIP<sub>3</sub>) and activation of the downstream kinase phosphoinositide - dependent kinase-1 (PDK1), (Bevan 2001). Protein kinase B (PKB) or Akt (v-akt murine thymoma viral oncogene homologue), a serine/threonine kinase is then activated in response to phosphorylation by PDK1 at site threonine 308 (DeFronzo et al 2004). Mammalian TOR (mTOR) is the target for the immunosuppressant drug rapamycin and acts as an intracellular nutrient sensor that controls protein synthesis and growth (Brown et al 1994). Indeed, PKB is also phosphorylated at site serine 473 by mTOR and its associated protein, rictor (which form the complex mTORc2), (Sarbassov et al 2005). PKB also regulates the activity of mTOR with PKB activating mTORc1 (complex of mTOR and its associated protein raptor), (Vander Harr et al 2007). PKB also phosphorylates and inactivates nutrient sensing protein, tuberous sclerosis complex 2 (TSC2) (Potter et al 2002). Indeed, TSC2 has been shown to inhibit cell growth and size and antagonise insulin signalling (Ito and Rubin 1999, Gao and Pan 2001, Potter et al 2001, Tapon et al 2001). In skeletal muscle and adipose tissue activation of PKB results in translocation of GLUT-4 to the plasma membrane thereby allowing the uptake of glucose in the cell (Kohn et al 1996, Sano et al 2003), (figure 1.1). In hepatic tissue, PKB-mediated phosphorylation inactivates glycogen synthase kinase 3 (GSK-3) resulting in activation of glycogen synthase and therefore glycogen synthesis (Sutherland et al 1993, Cross et al, 1995, Van Weeren et al 1998), (figure 1.1). In addition, the inhibition of GSK3 by PKB is a key step in insulin induction of glycogen synthesis in muscle (McManus et al 2005). Moreover, GSK-3 is also important for the regulation of hepatic gene transcription by insulin. For example, GSK-3 regulates



**Figure 1.1 Insulin signalling in skeletal muscle, adipocytes and hepatocytes.** Insulin binding to its receptor results in receptor autophosphorylation on tyrosine residues and subsequent phosphorylation of IRSs. This allows association of IRS-1 with the regulatory subunit of PI3K. Activation of catalytic subunit of PI3K results in the production of PIP<sub>3</sub> and activation of the downstream kinase PDK1. PKB or Akt is then activated in response to phosphorylation by PDK1. In skeletal muscle and adipose tissue activation of PKB results in translocation of GLUT-4 to the plasma membrane thereby allowing the uptake of glucose in the cell. In hepatic tissue, PKB-mediated phosphorylation inactivates GSK-3 resulting in activation of glycogen synthase and therefore glycogen synthesis. GRB2 and SHP2 adaptor proteins interact with IRS molecules and activate mitogen activated protein kinases (MAPK) and stimulate mitogenic responses in the form of gene transcription.

the transcription of PEPCK and G6Pase genes, two rate controlling steps in gluconeogenesis (Lochhead *et al* 2001). Thus, the IRS/PI3K/PDK1/PKB pathway is very important to insulin's ability to regulate metabolism and promote growth.

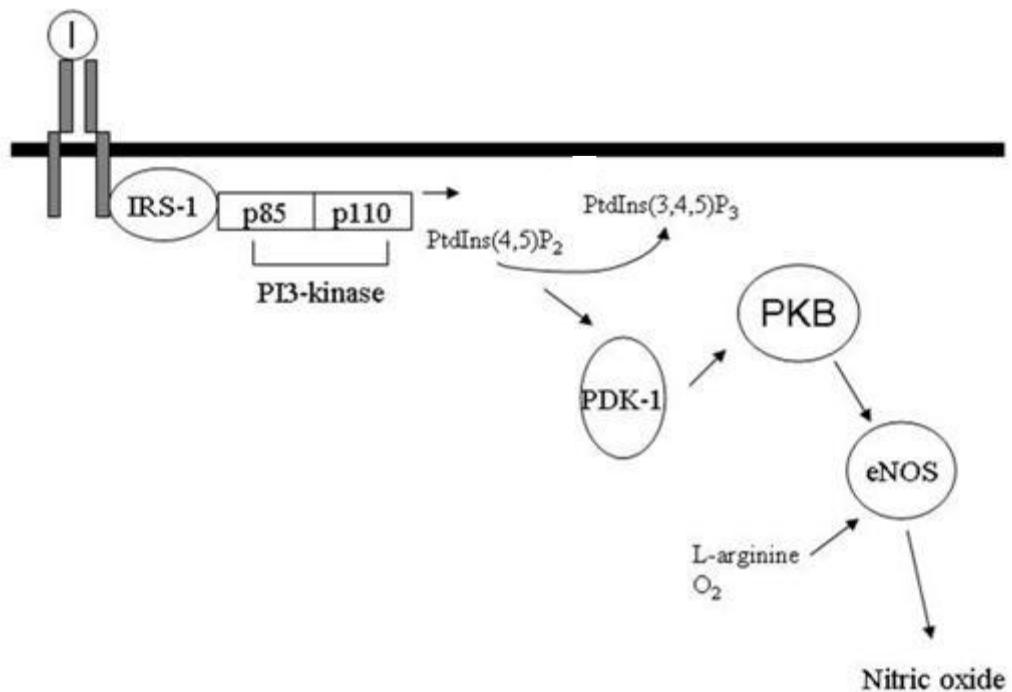
While it is recognised that the IRS group of proteins mediate a significant proportion of the insulin stimulated signalling events through PI3K it is also important to note that insulin also has the ability to regulate a number of adaptor proteins (GRB2 and SHP2). These adaptor proteins interact with IRS molecules and activate mitogen activated protein kinases (MAPK) and stimulate mitogenic responses in the form of gene transcription. (Bevan 2001, Coggins *et al* 2001, DeFronzo *et al* 2004).

Insulin resistant states therefore lead to less insulin stimulated glucose uptake as well as less inhibition of hepatic gluconeogenesis (Yki-Jarvinen 1995). This combination results in hyperglycaemia and the development of impaired glucose tolerance and ultimately the diagnosis of T2DM.

### **1.2.5 Insulin signalling cascade in the vascular endothelium**

Insulin receptors have been demonstrated to be expressed at the endothelial cell surface (40,000 IR per cell) and insulin stimulates an intracellular signalling cascade in the human endothelium with resultant nitric oxide production (NO) (Zeng and Quon 1996).

As with the other insulin-sensitive tissues, the IRS proteins are key components of the insulin signalling cascade in the vascular endothelium (Figure 1.2). Indeed, overexpression of IRS-1 in endothelial cells causes a 3-fold increase in NO production in response to a maximally stimulating dose of insulin compared to wild type controls not infected with IRS-1 (Montagnani *et al* 2002)



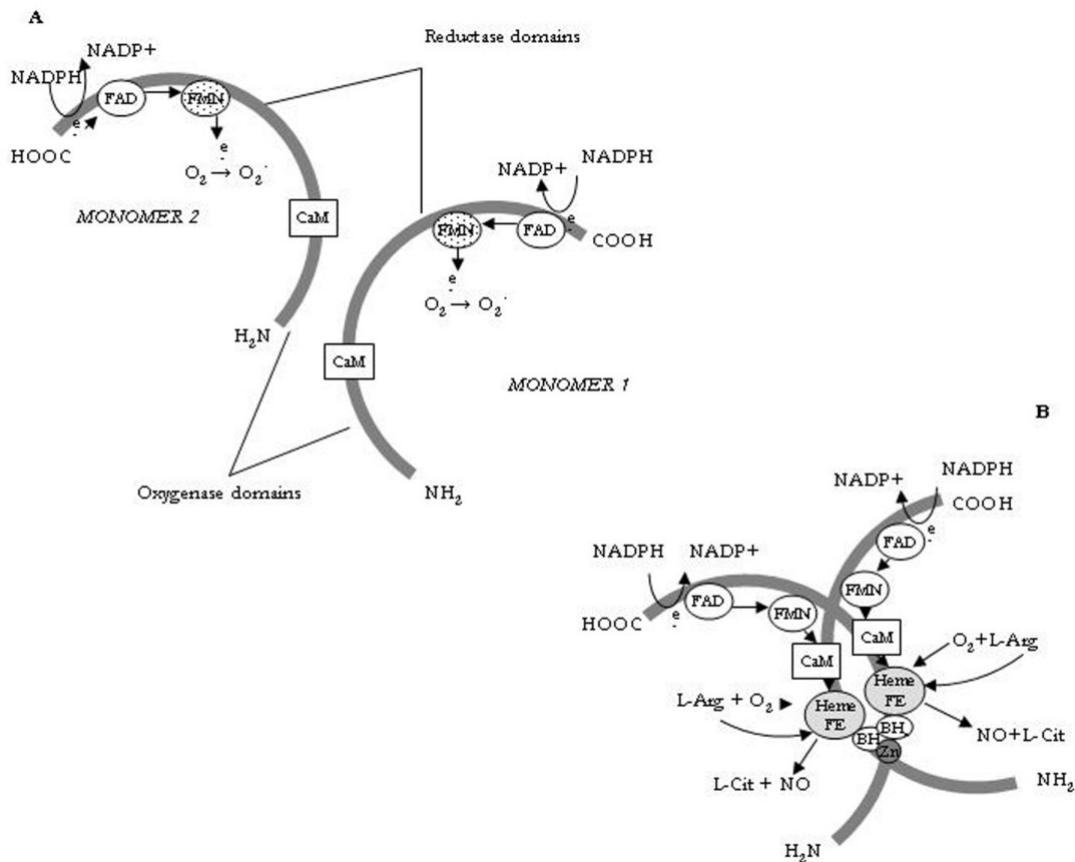
**Figure 1.2 Schematic diagram of insulin signalling in the endothelium**

Insulin (I) binds to its receptor and stimulates an intracellular signalling cascade involving IRS-1, PI3K, PIP<sub>3</sub> production and PDK-1 activation. PKB is activated leading to increased eNOS activity.

while over-expression of a mutant IRS-1 inhibited insulin-stimulated NO production and PI3K recruitment to IRS-1 (Montagnani *et al* 2002). Furthermore, PI3K, PDK-1 and PKB are also components of the insulin signalling cascade in the vascular endothelium. The key role of PI3K in insulin stimulated NO production in endothelial cells is demonstrated in studies using the PI3K inhibitor wortmannin (Zeng and Quon 1996, Hartell *et al* 2005). PDK-1 activity is also required for insulin stimulated NO production in bovine aortic endothelial cells (BAEC) (Montagnani *et al* 2002). PKB directly phosphorylates endothelial NO synthase (eNOS) at residue serine 1177 and increases enzyme activity in endothelial cells (Dimmeler *et al* 1999). Insulin stimulated eNOS activation is independent of calcium concentration but requires PKB mediated eNOS Ser1177 phosphorylation (Montagnani *et al* 2001). The regulation of eNOS is complex, as eNOS exists as a monomer but following the binding of essential cofactors including tetrahydrobiopterin (BH<sub>4</sub>), calmodulin (CaM), flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) an active enzyme dimer is formed resulting in NO and L-citrulline synthesis from L-arginine, O<sub>2</sub> and nicotinamide adenine dinucleotide phosphate (NADPH), (reviewed in Forstermann and Munzel 2006, figure 1.3).

The importance of eNOS to insulin action has been reinforced in knockout studies involving mice. For example, eNOS deficient mice are insulin resistant, hypertensive and have defects in fatty acid metabolism and dyslipidaemia. They also have impaired insulin stimulated glucose uptake (Duplain *et al* 2001, Nisoli *et al* 2005, Le Gouill *et al* 2007).

The adipocytokines are thought to be important in the pathogenesis of T2DM and it has been suggested that they play a role in the insulin signalling cascade in the vascular endothelium. Adiponectin has also been demonstrated to increase nitric oxide production (Hattori *et al* 2003) by



**Figure 1.3 Endothelial Nitric Oxide Synthase activation**

**Panel A:** Endothelial nitric oxide synthase is synthesised as a monomer. Each monomer contains both a reductase and an oxidase domain. Monomers bind calmodulin and are able to transfer electrons from NADPH through FAD and FMN and have a limited capacity to synthesise  $O_2^-$ . Monomers are unable to bind BH<sub>4</sub> or L-arginine and therefore unable to produce NO. The binding of haem allows eNOS dimerisation and the formation of a functional enzyme capable of producing NO. This also allows the interaction of the reductase and oxidase domains allowing transfer of electrons from the flavins to the haem of the opposite monomer. This dimer (in the absence / relative deficiency of BH<sub>4</sub> and L-arginine) can produce  $O_2^-$  more effectively.

**Panel B:** When sufficient BH<sub>4</sub> and L-arginine are available, NO and L-citrulline are produced. (Adapted from Forstermann and Munzel 2006).

a pathway involving PI3K and PKB activation by AMPK (Ouchi *et al* 2004, Chen *et al* 2003). Furthermore, when TNF- $\alpha$  is added to endothelial cells there is inhibition of insulin-stimulated NO production with impairment of the insulin stimulated signalling cascade (Kim *et al* 2001). Finally, the exposure of endothelial cells to IL-6 results in inhibition of insulin-stimulated NO production with impairment of the insulin stimulated signalling cascade (Andreozzi *et al* 2007).

### **1.3 AMP activated protein kinase**

#### **1.3.1 Structure of AMPK**

AMP activated protein kinase (AMPK) is an enzyme ubiquitously expressed in mammalian tissues, consisting of 3 subunits ( $\alpha$ - [catalytic],  $\beta$ - [regulatory],  $\gamma$ -[regulatory]) with 2-3 genes encoding each subunit (Towler and Hardie 2007). The  $\alpha$  subunits contain a protein kinase catalytic domain at the N-terminus and a C-terminal regulatory domain that is involved in binding to the  $\beta$  and  $\gamma$  subunits (Hardie 2003). All three subunits are encoded by multiple genes ( $\alpha 1$ ,  $\alpha 2$ ,  $\beta 1$ ,  $\beta 2$ ,  $\gamma 1$ ,  $\gamma 2$ ,  $\gamma 3$ ) yielding 12 heterotrimeric combinations, with splice variants adding to the diversity (Hardie 2004). All possible combinations of the heterotrimer appear to be expressed in mammals. The potential for a number of different heterotrimeric combinations can lead to tissue-specific enzyme diversity (Towler and Hardie 2007). It is still unclear what cellular consequences the composition of AMPK isoforms may have on the function of each AMPK complex in relation to their particular localisation and specific downstream targets. For example a recent study investigated the isoform composition of AMPK complexes in human skeletal muscle and found that only 3 of the 12 theoretically possible AMPK complexes were present ( $\alpha 2\beta 2\gamma 1$ ,  $\alpha 2\beta 2\gamma 3$  and  $\alpha 1\beta 2\gamma 1$ ), (Birk *et al* 2006, Treebak *et al* 2007).

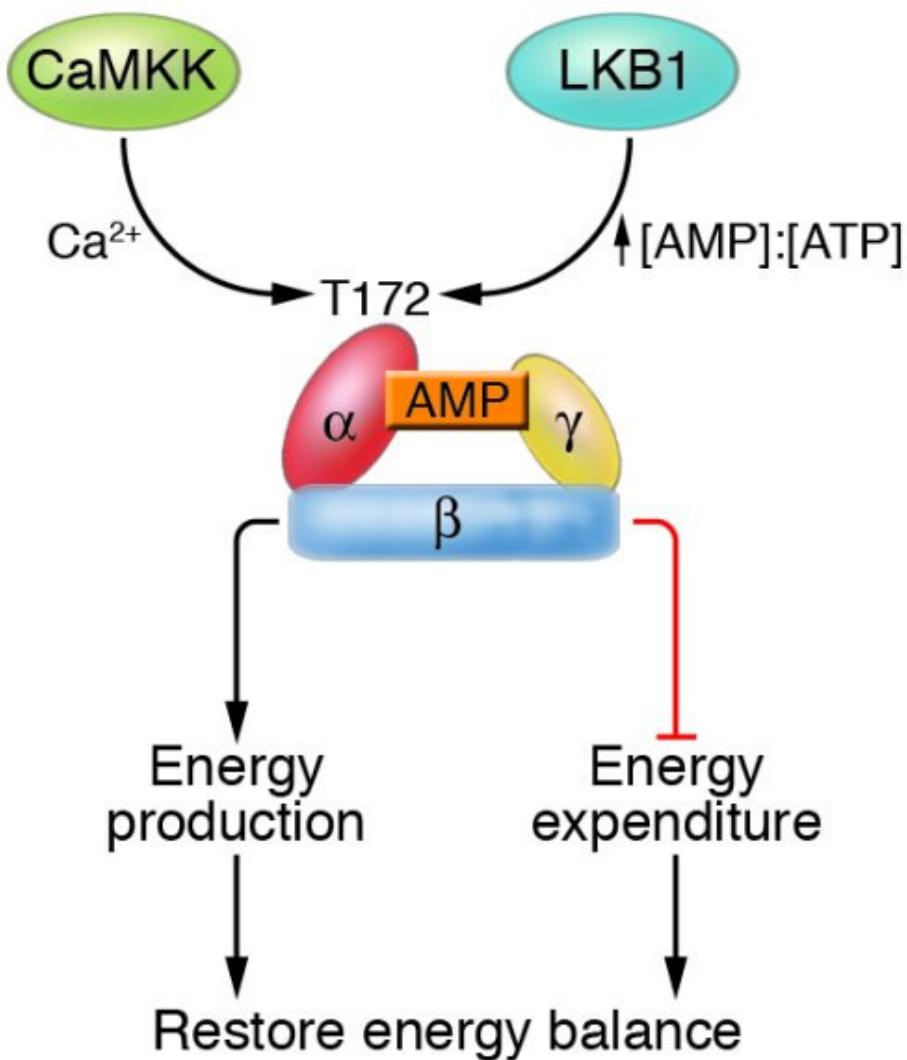
#### **1.3.2 Regulation of AMPK**

To sustain metabolism, intracellular ATP levels must be maintained within a narrow range. This is achieved by the regulation of cellular catabolic and anabolic pathways. This may be co-ordinated by AMPK which acts as a sensor of cellular energy status, a fuel gauge or metabolic master switch (Winder *et al* 1999, Hardie DG 2003). AMPK can be allosterically activated by AMP (Munday *et al* 1988). Aminoimidazole carboxamide ribonucleotide (AICAR) is an adenosine analogue which activates AMPK through direct binding, followed by allosteric modification. It is initially taken up by adenosine transporters and subsequently phosphorylated by adenylate kinase to produce the

AMP analogue ZMP within the cell. ZMP mimics AMP in AMPK signalling but may induce some actions that are independent of AMPK (Merrill *et al* 1997). Furthermore, AMPK is activated in response to environmental or nutritional stress factors, which deplete intracellular ATP levels including heat shock, hypoxia, ischaemia, glucose deprivation or prolonged exercise (Hardie 2004, Musi *et al* 2001). This results in an increase in the AMP:ATP ratio which inhibits dephosphorylation of AMPK and therefore potentiates AMPK mediated phosphorylation by an upstream kinase LKB-1 (a tumour suppressor gene whose germline mutations in humans are the cause of Peutz-Jeghers syndrome) which is considered to be constitutively active (Hardie 2004), (Figure 1.4). Under conditions in which cellular energy demands are increased or when fuel availability is decreased, intracellular ATP is reduced and AMP levels rise (Hardie 2004). In cells, adenylate kinase maintains the reaction



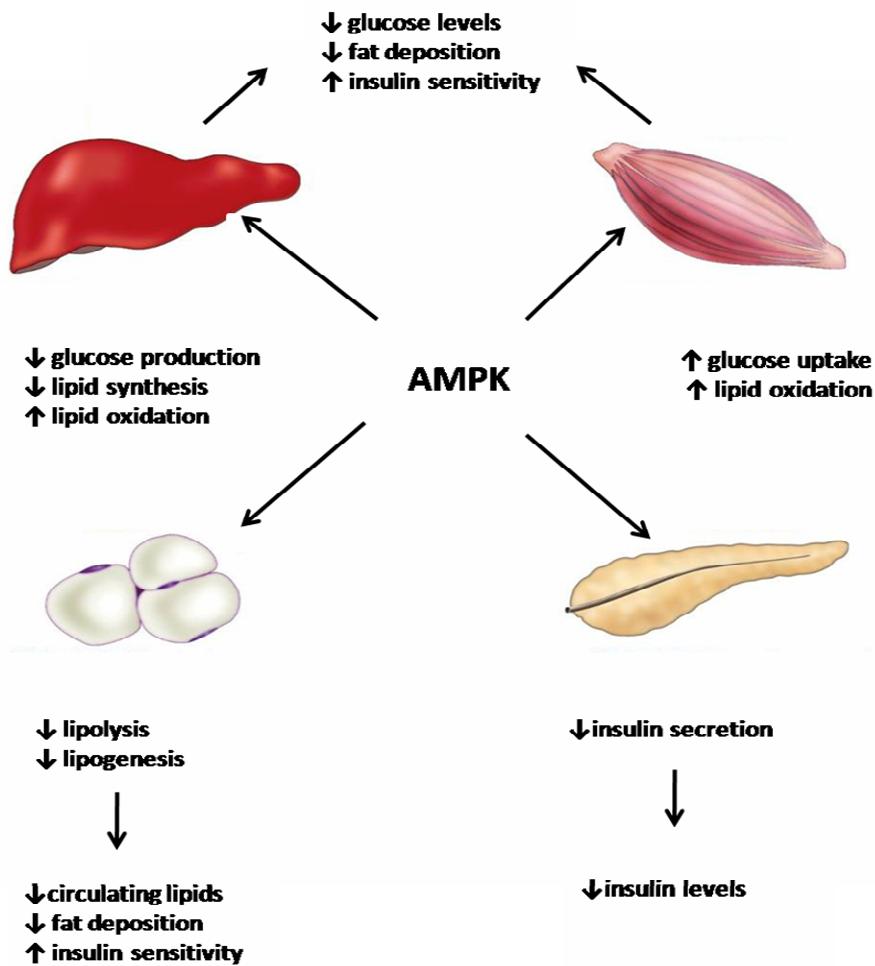
close to equilibrium such that the AMP:ATP ratio is proportional to the square of the ADP:ATP ratio and is therefore considered to be a more sensitive indicator of compromised energy status than the ADP:ATP ratio. In addition to increased AMP, AMPK is activated by increased intracellular calcium concentration, mediated by calcium/CaM-dependent protein kinase kinase (CaMKK), which phosphorylates and activates AMPK at Thr-172, the same site phosphorylated by LKB1 (Towler and Hardie 2007, Long and Zierath 2006). Furthermore, binding of AMP inhibits the dephosphorylation of Thr-172 by protein phosphatases and high concentrations of ATP inhibit the activation of AMPK by antagonising binding of AMP (Sanders *et al* 2007, Riek *et al* 2008).



**Figure 1.4 Structure and regulation of AMPK.** AMPK is a heterotrimeric complex consisting of an  $\alpha$ ,  $\beta$ , and  $\gamma$  subunits and is activated by the upstream kinases CaMKK and LKB1 via phosphorylation of Thr172. Activation of AMPK by CaMKK is dependent on intracellular calcium whereas LKB1 activity is constitutive. Generally, AMPK restores energy balance by activating processes that produce energy (e.g., lipid oxidation and glucose uptake) while inhibiting those that consume energy (for example fatty acid, cholesterol and protein synthesis) (Long and Zierath 2006).

### **1.3.3 Role of AMPK in Energy Homeostasis**

Activation of AMPK triggers catabolic pathways that produce energy, while turning off anabolic pathways that consume energy thereby maintaining cellular energy stores. For example, AMPK inhibits lipid and triglyceride synthesis in both liver and adipose tissue and stimulates skeletal muscle glucose uptake, glycolysis and fatty acid oxidation (Towler and Hardie 2007), (Figure 1.5). Stimulation of fatty acid oxidation occurs when AMPK directly phosphorylates and inactivates the downstream target, acetyl-coA carboxylase (ACC). ACC synthesizes malonyl-CoA which inhibits the rate-controlling step of fatty oxidation involving the transfer of fatty acids into the mitochondria by the enzyme carnitine palmitoyltransferase 1 (CPT1) (Trumble *et al* 1995, Ruderman *et al* 1999). During exercise, activated AMPK inhibits ACC to reduce malonyl-CoA concentration, thereby driving the entry of fatty acids into the mitochondria for  $\beta$ -oxidation to restore energy balance (Winder *et al* 1996). The ability of AMPK to induce lipid oxidation and thus the amount of fat deposited in skeletal muscle and liver is considered an important feature of AMPK activation. It suggests that AMPK and insulin signalling pathways may oppose each other and that AMPK activation is insulin sensitising. Therefore it may be that AMPK plays an important role in controlling the whole body energy homoeostasis including the regulation of plasma glucose levels and body weight. The control of AMPK activity provides an attractive target for therapeutic intervention in metabolic disorders such as T2DM and obesity. Indeed, a number of physiological and pharmacological factors that are beneficial in these disorders have been shown to act, at least in part, through the activation of AMPK. The importance of AMPK in regulating whole body energy metabolism is also emphasized by studies on AMPK  $\alpha 2$  knockout (KO) mice. Deletion of  $\alpha 2$  results in mild insulin resistance and an insulin-secretory defect (Viollet *et al* 2003, Jorgensen *et al* 2004). Glucose intolerance in the  $\alpha 2$  KO mice is not due to a defect in skeletal-muscle glucose uptake, but probably results from both impaired insulin action and inhibition of insulin secretion (Viollet *et al* 2003). The molecular basis for this defect is not understood. In a recent study, AMPK



**Figure 1.5 Role of AMPK in the regulation of whole-body glucose homeostasis.** Stimulation of AMPK activates ATP-generating processes, while inactivating ATP-consuming processes. In skeletal muscle, acute activation of AMPK increases glucose uptake and lipid oxidation. Activation of AMPK inhibits glucose and lipid synthesis in the liver but increases lipid oxidation. Lipolysis and lipogenesis in adipose tissue are also reduced by AMPK activation. Together, activation of AMPK in skeletal muscle, liver, and adipose tissue results in decreased circulating glucose, reduced plasma lipid, and ectopic fat accumulation, as well as enhanced insulin sensitivity and therefore makes AMPK an attractive target for the treatment of T2DM. Activation of pancreatic AMPK is associated with decreased insulin secretion, likely a protective measure to prevent hypoglycaemia although this needs to be considered in pharmaceutical targeting of AMPK for the treatment of T2DM (Adapted from Long and Zierath 2006).

$\alpha 2$  knockout mice fed with a high-fat diet have been shown to exhibit increased body weight coupled with an increase in fat when compared with wild-type animals (Villena *et al* 2004). At present, the mechanism underlying this response is not known.

### ***1.3.4 Role of AMPK in the regulation of peripheral tissues***

#### ***1.3.4.1 Skeletal muscle***

A large proportion of insulin stimulated glucose disposal occurs in skeletal muscle and insulin resistance at this site is thought to contribute to the pathogenesis of T2DM (Petersen *et al* 2007). Insulin increases glucose uptake in muscle by stimulating the translocation of glucose transporter GLUT4 from intracellular vesicles to the cell surface. It has been shown that muscular AMPK activation stimulates muscle glucose uptake either by exercise or by AICAR. Exercise stimulates glucose uptake independently of the traditional insulin pathway but the molecular mechanisms remain incompletely understood (Ploug *et al* 1984). Electrical contraction of skeletal muscle increases glucose uptake and increase AMPK activity (Hutber *et al* 1997). Furthermore, activation of AMPK with AICAR increases glucose uptake in the skeletal muscle of rodents (Merill *et al* 1997). Interestingly, AICAR-mediated glucose uptake is not attenuated by inhibition of the insulin-dependent PI3K pathway with wortmannin and, like exercise, appears to utilise separate pathways from insulin stimulated glucose uptake in skeletal muscle (Merill *et al* 1997). AICAR can effectively increase glucose transport and GLUT4 translocation in skeletal muscle in patients with T2DM (Koistinen *et al* 2003). Thus, an AMPK-dependant increase in glucose transport is observed in skeletal muscle of both rodents and humans. Moreover, in AMPK  $\alpha 2$  knockout mice the glucose lowering effects of AICAR are abolished (Mu *et al* 2001). There is, however, conflicting evidence as to whether AMPK is required for exercise induced glucose uptake in skeletal muscle (Jorgensen *et al* 2004, Sakamoto *et al* 2005). Activation of AMPK with metformin is associated with increased glucose uptake in isolated rat skeletal muscle (Zhou *et al* 2001). Significantly, metformin increases

AMPK activity, ACC phosphorylation and glucose disposal in isolated skeletal muscle from patients with T2DM (Musi *et al* 2002). Therefore, the beneficial metabolic effects of metformin in T2DM patients may also be partly mediated by the activation of AMPK in skeletal muscle.

### **1.3.4.2 Heart**

In myocardial ischaemia and left ventricular dysfunction an increase in the AMP/ATP ratio results in an increase in AMPK activity (Russell *et al* 2004). In rat cardiac myocytes AMPK increases FFA uptake and increased translocation of GLUT4 (leading to increased glucose uptake) in a manner that is independent of the PI3K pathway. (Russell *et al* 1999, Luiken *et al* 2003). Indeed, in isolated hearts of AMPK knockout mice glucose uptake following ischaemia was reduced when compared to wild type mice (Xing *et al* 2003). Intriguingly, it has been recently demonstrated that an increase in AMPK activity by acute metformin therapy results in decreased myocardial injury in both diabetic and non-diabetic mice after ischaemia and reperfusion (Calvert *et al* 2008). Moreover, chronic metformin therapy in mice improves left ventricular function and survival after ischaemia and reperfusion (Gundewar *et al* 2009). This evidence suggests that AMPK may play a role in cardiac ischaemia and therapeutic targeting of AMPK in patients with angina, acute coronary syndrome or myocardial infarction may be of value. The important role of AMPK in the heart is further reinforced by the finding of a novel mutation (Arg531Gly) in the AMPK  $\gamma 2$  subunit (*PRKAG2* gene) to be responsible for Wolff–Parkinson–White Syndrome which is associated with ventricular pre-excitation and the early onset of atrial fibrillation and conduction disease (Gollob *et al* 2001). Indeed, transgenic mice with a cardiac specific expression of this mutation were found to have cardiac hypertrophy, impaired contractile function, electrical conduction abnormalities and marked glycogen accumulation (Davies *et al* 2006).

### **1.3.4.3 Liver**

AMPK suppresses both liver gluconeogenesis and lipogenesis. AMPK also increases fatty acid oxidation and in turn reduces hepatic lipid deposition (Carling 2004, Khan *et al* 2005). Activating AMPK in the liver is therefore an attractive target for those with T2DM as it could improve glycaemia, body weight and the lipid profile. Euglycaemia can be maintained if hepatic gluconeogenesis matches insulin mediated glucose uptake in peripheral tissue and when insulin resistance creates an imbalance, T2DM can ensue (DeFronzo *et al* 1989, Basu *et al* 2000). Hepatic gluconeogenesis is regulated in part by the enzymes phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase) (Saltiel *et al* 2001). The role of AMPK in inhibiting hepatic gluconeogenesis is thought to be related to its ability to mimic insulin and downregulate the genes encoding both PEPCK and G6Pase (Lochhead *et al* 2000). As previously stated, GSK-3 is important for the regulation of hepatic gene transcription by insulin and regulates the transcription of PEPCK and G6Pase genes, two rate controlling steps in gluconeogenesis (Lochhead *et al* 2001). Indeed, AMPK activation increases GSK-3 phosphorylation and reduces PEPCK gene expression in the liver of rodents (Horike *et al* 2008). Furthermore, liver AMPK  $\alpha 2$  or LKB1 knockout mice exhibit hyperglycaemia perhaps due to the increased activity of PEPCK and G6Pase (Shaw *et al* 2005, Andreeli *et al* 2006). In rat hepatocytes, activation of AMPK by AICAR or metformin increases fatty acid oxidation via inhibition of ACC (Zhou *et al* 2001). Moreover, when AMPK is activated by metformin in cultured human hepatocytes intracellular lipid content is reduced. This effect is abrogated after infection of hepatocytes with adenoviruses expressing a dominant negative mutant AMPK (Zang *et al* 2004).

### **1.3.4.4 Adipose Tissue**

Visceral adiposity is associated with elevated FFA due to increased activity of hormone-sensitive lipase which is the rate limiting step of lipolysis (Meijssen *et al* 2001). Accumulation of

intracellular lipid is proposed to contribute to insulin resistance at a cellular level. Insulin is thought to control the release of FFA from adipose tissue by inhibition of hormone-sensitive lipase (Meijssen *et al* 2001). FFA are also reported to stimulate reactant oxygen species (ROS) production which in turn inhibits insulin signalling (Muniyappa *et al* 2007, Chinen *et al* 2007). Consequently, visceral adiposity and its associated effect on FFA may be important in the pathogenesis of T2DM by causing insulin resistance in skeletal muscle and liver in addition to inhibiting insulin secretion. (Bayes *et al* 2004, Lebovitz *et al* 2005, Miles *et al* 2005). AMPK is thought have important role in adipose tissue by inhibiting lipolysis and lipogenesis. Indeed, AMPK has been implicated in the regulation of lipolysis through direct phosphorylation of hormone-sensitive lipase (Garton *et al* 1989). Lipolysis and lipogenesis are inhibited in rat adipocytes when using the AMPK activator AICAR, (Sullivan *et al* 1994). It is also suggested that when the regulation of lipid metabolism by AMPK is absent, then weight gain and insulin resistance prevail. Indeed when AMPK  $\alpha 2$  knockout mice were fed with a high-fat diet they were shown to exhibit increased body weight, increased adiposity and a degree of insulin resistance when compared with wild-type mice (Viollet *et al* 2003, Jorgensen *et al* 2004, Villena *et al* 2004). It has also been recently shown that glucocorticoids inhibit AMPK activity in adipose tissue of insulin resistant patients with Cushing's syndrome (Kola *et al* 2008). Moreover, metformin has been found to prevent the glucocorticoid effects on *ex vivo* cultured human adipocytes (Christ-Crain *et al* 2008). The effect of insulin sensitizers such as metformin or the thiazolidinediones on AMPK activity in human adipose tissue has not yet been investigated. Further work is required to examine whether metformin exerts its beneficial metabolic effects in patients with T2DM in part by increasing AMPK activity in human adipose.

#### **1.3.4.5 Pancreas**

Evidence suggests that AMPK has a role in the release of insulin perhaps as a sensor of cellular energy store. High glucose concentrations stimulate insulin release and it has been proposed that an

increase in glucose increases in the cellular ATP/ADP ratio. This in turn activates ATP-sensitive K<sup>+</sup> channels, causing depolarization of the plasma membrane, opening of voltage gated calcium channels, an influx of calcium ions and subsequent exocytosis of secretory granules which is a critical step in the secretion of insulin (Rahan *et al* 1990). An increase in glucose levels represses AMPK activity in β cell lines (Salt *et al* 1998, Leclerc *et al* 2004), whereas when AMPK is activated by AICAR, insulin release in response to high glucose is attenuated (Zhang *et al* 1995). Furthermore, overexpression of a constitutively active form of AMPK results in a reduction in insulin release from β cell lines while overexpression of a dominant negative form of AMPK leads to an increase in insulin release (Da Silva Xavier *et al* 2003). Moreover, when metformin is added to β cell lines, AMPK is activated and insulin secretion in response to glucose is attenuated (Leclerc *et al* 2004). The activation of pancreatic AMPK is therefore associated with decreased insulin secretion which could have a negative effect on glycaemic control in patients with T2DM. This effect may, however, be a protective effect during periods of hypoglycaemia, where AMPK is activated and insulin secretion falls to maintain euglycaemia. Nevertheless, the exact role of AMPK in insulin release remains incompletely understood and further work is required before AMPK can be targeted by drug therapy.

#### **1.3.4.6 Hypothalamus**

The hypothalamus plays a key role in the regulation of energy balance. It does so by using AMPK as a fuel gauge that can respond to both hormones, anorexia or an excess of food. (Schwartz *et al* 2000, Andersson *et al* 2004). Injection of leptin into rodents inhibits AMPK activity in the hypothalamus whereas administration of ghrelin stimulates AMPK activity (Andersson *et al* 2004). Moreover, fasting stimulates AMPK activity whereas hyperglycaemia and refeeding inhibits AMPK activity in the hypothalamus (Minokowski *et al* 2004). Therefore, it appears that activating AMPK can stimulate food intake through its action in the hypothalamus. While this effect probably

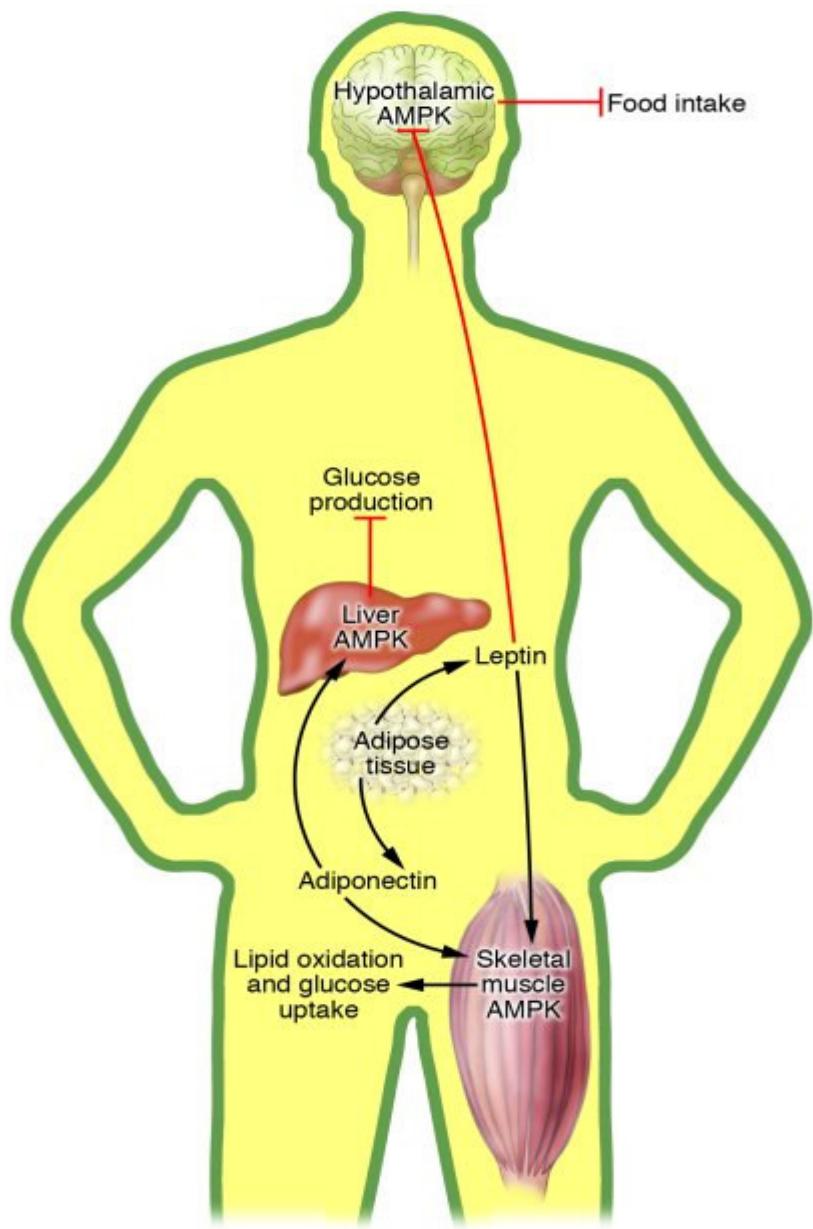
represents a physiological response in glucose homeostasis, the prospect of patients gaining weight through the pharmacological targeting of AMPK for the treatment of T2DM is unattractive and along with AMPK's documented role in the pancreas, presents a significant challenge to the pharmaceutical industry.

#### **1.3.4.7 Vascular Endothelium**

AMPK has an important role in the release of NO from the vascular endothelium. AICAR stimulates eNOS Ser1177 phosphorylation and NO production in human aortic endothelial cells (HAECS) (Morrow *et al* 2003). Indeed, AMPK activation has been demonstrated to stimulate muscle glucose uptake in a NO-dependent manner and NO production has been proposed to be largely responsible for insulin-independent glucose uptake during exercise (Chen *et al* 2000, Fryer *et al* 2000, Hawley *et al* 2002). AMPK also has a role in angiogenesis. Vascular endothelial growth factor (VEGF) is a key regulator of angiogenesis and stimulates differentiation, survival, migration, proliferation and vascular permeability (Ferrara *et al* 2003). VEGF rapidly stimulates endothelial NO synthesis and does so in an AMPK dependent manner (Reihill *et al* 2007). The ability of insulin sensitizers, such as metformin and the thiazolidinediones, to act directly on vascular endothelium via AMPK is discussed in sections **1.6** and **1.7** respectively.

#### **1.3.4.8 Interaction with the adipocytokines**

The adipocytokines such as leptin and adiponectin are thought to be important in the pathogenesis of T2DM and it has been suggested that AMPK plays a role in mediating their metabolic and vascular effects.



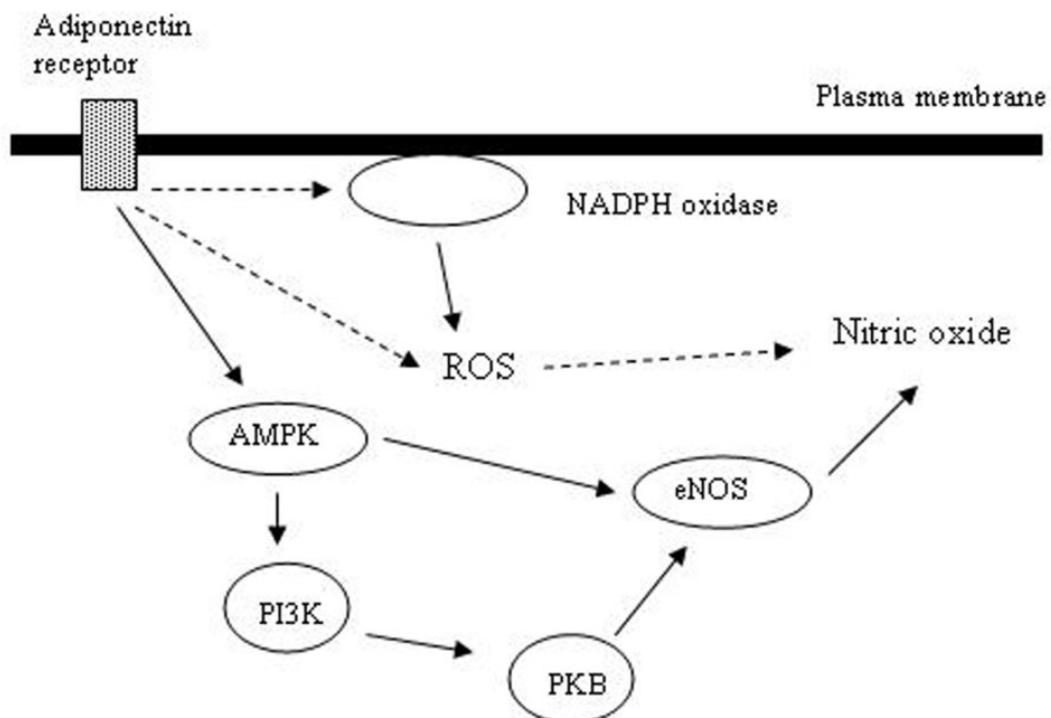
**Figure 1.6 Integration of intertissue signalling by AMPK.** Leptin reduces food intake through inhibition of hypothalamic AMPK. Leptin increases skeletal muscle fatty acid oxidation via direct activation of AMPK and the hypothalamus–sympathetic nervous system axis. The involvement of hypothalamic AMPK in leptin-induced skeletal muscle lipid oxidation and glucose uptake is unknown. Adiponectin is secreted by adipose tissue and increases skeletal muscle lipid oxidation and glucose uptake, as well as suppressing liver glucose production by activating AMPK. (Adapted from Long and Zierath 2006)

#### **1.3.4.8.1 Leptin**

The adipocytokine leptin diminishes adiposity by reducing food intake (Halaas *et al* 1995) and improves insulin sensitivity (Khan *et al* 2000). Leptin induces fatty acid oxidation and glucose uptake in peripheral tissues, two metabolic responses that are also triggered by AMPK activation (Muoio *et al* 1996, Haque *et al* 1999). AMPK may therefore be a candidate in the mediation of leptin responses with skeletal muscle, liver and the hypothalamus being cited as peripheral targets (Minokoshi *et al* 2002), (Figure 1.6). AMPK is also thought to regulate food intake via the hypothalamus. Indeed, in contrast to the effect on peripheral tissues, leptin inhibits AMPK activity in the hypothalamus. This is thought to be important for the hormone's ability to reduce food intake and promote satiety (Minokoshi *et al* 2004).

#### **1.3.4.8.2 Adiponectin**

Plasma levels of adiponectin negatively correlate to insulin resistance, obesity and cardiovascular disease. The effects of the adiponectin are partly accounted for by adiponectin induced AMPK activation which results in stimulation skeletal muscle fatty acid oxidation and glucose uptake (Tomas *et al* 2002, Yamauchi *et al* 2002). Furthermore, activation of AMPK by adiponectin suppresses hepatic gluconeogenesis. (Yamauchi *et al* 2002). Adiponectin has also been demonstrated to increase nitric oxide production (Hattori *et al* 2003), by a pathway proposed to involve the direct phosphorylation of eNOS by AMP-activated protein kinase (AMPK), and also by a parallel pathway involving PI3K and PKB activation by AMPK (Chen *et al* 2003, Tan *et al* 2004, Ouchi *et al* 2004, Chen *et al* 2005), (figure 1.7). Furthermore, adiponectin has been shown to reduce infarct size and improve left ventricular function and coronary blood flow following ischaemia and reperfusion of isolated rat hearts by a mechanism involving AMPK and NO release (Gonon *et al* 2008). Moreover, AMPK may be necessary for adiponectin to exert its cardio protective effect as ischaemia and reperfusion caused a larger myocardial infarct and enhanced



**Figure 1.7 Potential signalling mechanisms by which adiponectin exerts its effects in human endothelial cells.** Adiponectin stimulates NO production via a signalling cascade initiated by binding to either isoform of its receptor. AMPK is activated (with a parallel signalling pathway utilising PKB activated also) with resultant eNOS activation (Adapted from Goldstein and Scala 2004).

myocardial apoptosis in adiponectin knockout mice when compared with wild type mice (Shibata *et al* 2005). Adiponectin knockout mice have been shown to have progressive cardiac remodelling in a pressure-overloaded condition due to reduced AMPK signalling and increased insulin resistance when compared with wild type mice (Liao *et al* 2005). Therefore, AMPK appears to be important in mediating both the metabolic and the vascular effects of adiponectin but as yet the exact signalling mechanisms have not been clarified.

## **1.4 Vascular function and endothelium**

### **1.4.1 Background**

Atherosclerosis is the process of disruption of normal vascular homeostasis leading to occlusive vascular disease. It is one of the most common diseases of the modern age, and is increasing in incidence and prevalence. This places a great burden on the health care resources of the world (Bonow *et al* 2002). Atherosclerosis predominantly manifests as ischaemic heart disease, cerebrovascular disease and peripheral vascular disease. Endothelial dysfunction is proposed to be the triggering event of this process (Hansson 2005, Ross 1999). The vascular endothelium is an important mediator of the tone of smooth muscle surrounding the artery and therefore affects local blood flow as well as blood pressure (Behrendt *et al* 2002). Many factors associated with endothelial dysfunction have been identified which predispose to the development of cardiovascular disease (Grundy *et al* 1999). These include diabetes, hypertension, family history, hypercholesterolemia and cigarette smoking.

### **1.4.2 Vascular anatomy**

#### **1.4.2.1. The vascular tree**

The arterial vascular tree is composed of a series of bifurcating vessels, which progressively reduce in diameter with each generation. All arteries have three layers, termed the tunica intima, the tunica media and tunica adventitia. These three layers are clearly defined in the large arteries, and become progressively less distinct as the size of artery decreases. The largest arteries are termed elastic arteries, and include the aorta and its major branches - the innominate, subclavian, and the proximal common carotid and pulmonary arteries. These vessels are characterised by a tunica media (muscular layer) rich in elastic tissue (Mulvany *et al* 1990). This elastic layer provides resilience, absorbing energy during systole and releasing this energy during diastole, aiding forward propulsion of blood through the circulation (O'Rourke *et al* 2002). Loss of elasticity is pathological

and a natural part of ageing. As vessel size decreases, the quantity of elastic tissue within the *tunica media* decreases. At this point, vessels are termed muscular, or medium-sized, arteries. The adventitia of these vessels contains many more nerve-endings than in elastic arteries, reflecting the increased importance of autonomic control of these vessels. Small arteries retain the three layers of the arterial wall, becoming progressively less distinct to the level of the capillaries, which are characterised by a single cell wall (Ross 1999).

#### **1.4.2.2 The resistance artery**

Resistance to flow (impedance) is related to vessel size (McDonald *et al* 1959). Pressure measurements made along the vascular tree have shown that the majority of the pre-capillary drop in blood pressure occurs in vessels 100 - 500 micrometers ( $\mu\text{m}$ ) in size (Bohlen 1986, Mulvany 1990). Thus, this calibre of vessel accounts for the majority of peripheral resistance within the vasculature, and consequently are termed resistance arteries (Schiffrin 1992, Davis *et al* 1986). The peak of resistance within the vasculature coincides with a fall in blood pressure. The *tunica media* of the resistance artery consists of circumferentially arranged smooth muscle cells and connective tissue, with the *intima* consisting of longitudinally arranged endothelial cells. The *adventitia* consists of collagen, sympathetic nerves and fibroblasts (Mulvany 2002). Although physically discrete, the vascular endothelium (the *intima*) and the vascular smooth muscle layer (the *media*) are functionally coupled, as detailed in later sections of this thesis. A great deal of scientific effort has been directed towards a better understanding of the means by which endothelial cells influence the activity of the smooth muscle cells of the *tunica media*.

### **1.4.3. Vascular physiology and the pulse wave**

#### **1.4.3.1. Autoregulation**

The vasculature is constrained by tight haemodynamic limitations. The majority of organs rely on mean arterial pressure to maintain perfusion. Excursions of pressure above such a pressure leads to vessel rupture and potentially life-threatening haemorrhage. Conversely, a drop in blood pressure may lead to a reduction in perfusion of vital organs. Blood pressure must therefore be kept relatively stable within tolerable levels. Maintenance of pressure homeostasis is achieved by a variety of mechanisms. The larger arteries which are elastic and muscular are unable to respond acutely to changes of pressure. Regulation is therefore predominantly achieved by changes in cardiac output and systemic resistance. The latter is achieved by a change of the physical properties of resistance arteries (ie. resistance artery calibre) within various peripheral vascular beds (Nichols *et al* 2001). This implies a change in the tone of the smooth muscle of the resistance artery *tunica media* and implies the involvement of the resistance artery endothelium. Endothelial cells of resistance arteries are therefore of critical importance in the maintenance of normal blood pressure, and endothelial dysfunction has been implicated as a primary mediator of the development of vascular disease.

#### **1.4.3.2. The pulse wave**

The assessment, by palpation, of the arterial pulse wave is one of the most fundamental examinations performed in clinical practice. In recent years there has been a resurgent appreciation of the information that can be obtained from the measurement and analysis of the pulse pressure wave. The basic properties of the radial and carotid pulse waves were described in the late 19th Century (Mahomed 1872). The relationship between the physical properties of arterial walls and the pulse wave had been identified by the early 1920s (Bramwell *et al* 1922).

#### **1.4.3.3. Pulse wave morphology**

The central arterial pulse wave is composed two major components; a forward travelling wave generated by left ventricular systolic contraction and a reflected wave, arriving from the periphery. The reflected wave adds to the amplitude of the forward travelling wave by the principle of superimposition. The properties of the forward travelling wave are dependent upon the physical and mechanical properties of the central large arteries. The properties of the reflected wave are dependent on physical and mechanical properties of the whole arterial tree, the transmission velocity of the incident wave, and the distance to the major reflecting site(s) (Nichols *et al* 2002). Within the vascular tree, the majority of reflections occur at bifurcations, although the tapering observed in larger arteries is also a source of reflections.

An increase in the stiffness of arterial walls causes an increase in the transmission velocity of both the incident pressure wave and the reflected wave. As the overall pulse wave morphology is dependent on the superimposition of the incident and reflected pressure waves, an early return of the reflected component leads to an increase in the amplitude of the pulse pressure.

#### **1.4.3.4. Definition and measurement of arterial stiffness**

‘Arterial stiffness’ is a general term that requires definition. There are several measurable physical parameters of the arterial wall that may contribute to the degree of stiffness of the wall including pressure, diameter and height of the vessel wall (O’Rourke 1995). In the general literature, arterial stiffness is used to describe the overall rigidity of the arterial walls (MacKenzie *et al* 2002). Several established techniques are used to measure arterial stiffness. Pulse Wave Velocity is used to measure arterial stiffness in this thesis and detailed discussion of this technique can be found in

#### **2.2.2.3.4**

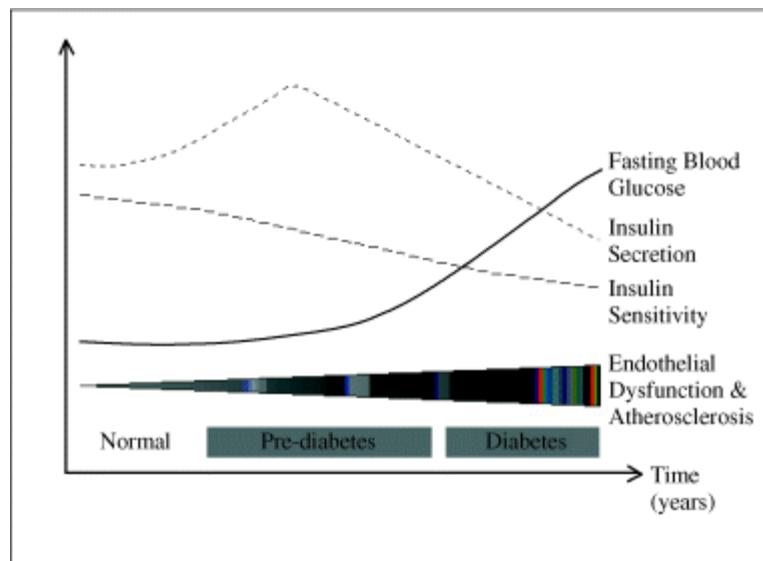
#### **1.4.4. The vascular endothelium**

##### **1.4.4.1 Introduction**

All blood vessels are lined with a monolayer of cells collectively termed the vascular endothelium. For many years this layer of cells was thought to be relatively inert. It is now recognised, however, that these cells are metabolically active with important paracrine, endocrine and autocrine functions, indispensable for the maintenance of vascular homeostasis (Vallance *et al* 2001, Bonetti *et al* 2003). Abnormal endothelial cell function is a key step in the development of vascular disease (Stenhouwer *et al* 1997). The relevant normal physiology of the endothelium of the resistance artery will be outlined, and the mechanisms and implications of endothelial dysfunction considered.

##### **1.4.4.2 Insulin resistance, diabetes mellitus, endothelial dysfunction and atherosclerosis.**

The vascular endothelium is a highly active organ that secretes a number of vasoconstrictor substances (endothelin-1) and vasodilator substances (nitric oxide and prostacyclin). The monolayer of endothelial cells that line the luminal surface of the vasculature play a central role in vascular homeostasis and their dysfunction is thought to play a pivotal role in the development of atherosclerosis (Wheatcroft *et al* 2003). Endothelial dysfunction is found in people with insulin resistance, obesity and T2DM but the molecular mechanisms that underlie this association are poorly characterised (Cleland *et al* 2000). A key player in vascular homeostasis is NO. NO is produced in endothelial cells from L-arginine which is catalysed by the enzyme eNOS (Muniyappa *et al* 2007). NO regulates vascular tone, inhibits cell adhesion, platelet aggregation and smooth muscle proliferation, giving an overall anti-thrombogenic effect (Wheatcroft *et al* 2003). Obesity and metabolic syndrome are associated with increased cardiovascular risk compared with healthy controls which may be present years before hyperglycaemia is clinically apparent (figure 1.8). Endothelial dysfunction is the initiating step leading to atherosclerosis. Endothelial injury or dysfunction (e.g. a reduction in bio-available NO leads to an inflammatory response at which time



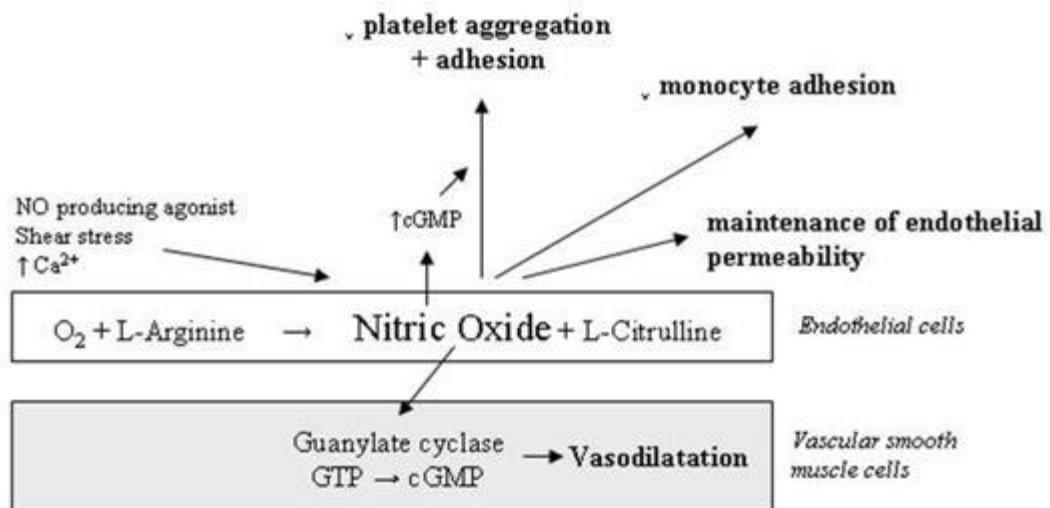
**Figure 1.8 Metabolic changes in the evolution of Type 2 diabetes.**

A schematic diagram representing the metabolic changes seen during the evolution of T2DM. The vasculature may be exposed to an increasingly atherogenic environment for many years before diabetes becomes clinically apparent (adapted from Wheatcroft *et al* 2003).

there is a loss of balance and the production of vasoconstrictor substances predominates (Hattori *et al* 2004). After injury, the expression of cell adhesion molecules (E-selectin, P-selectin, L-selectin, intercellular adhesion molecule-1 [ICAM-1] and vascular cell adhesion molecule-1 [VCAM-1] on the surface of the endothelium increases (Ross 1999). White blood cells bind to the cell adhesion molecules and the endothelium become pro-coagulant with increased cell permeability (Ross 1999). White blood cells invade the arterial media and then intima where they differentiate into macrophages and promote arterial remodelling. The macrophages subsequently take up lipids and become foam cells (Ross 1999). Ultimately the foam cells degrade to become fatty streaks and then the lipid core of an atherosclerotic plaque resulting in reduced luminal diameter (Boisvert *et al* 1998). The lipid core is covered by a fibrous cap which can rupture into the lumen causing transient or permanent occlusive thrombosis formation of the vessel which may result in acute coronary syndrome, transient ischaemic attacks, myocardial infarction and cerebrovascular accidents (Ross 1999). Macrovascular complications occur in T2DM and give rise to coronary artery, cerebrovascular and peripheral vascular disease (DeFronzo *et al* 2004).

#### **1.4.4.3 Nitric oxide**

Initial experiments suggesting the importance of the endothelium as a regulator of vascular tone resulted in the identification of the endothelium-derived relaxing factor (EDRF) as NO. This work to identify NO was subsequently recognised with the Nobel Prize for Physiology or Medicine 1998 (Raju 2000). NO is synthesised within endothelial cells during conversion of the amino acid L-arginine to L-citrulline by eNOS (Vallance 2001). The endothelium controls vascular tone and plays a key role in maintaining vascular homeostasis. This process is mediated by release of NO, prostacyclin, and endothelium derived hyperpolarizing factors. Nitric oxide has an overall anti-atherogenic effect on the vasculature, regulating blood pressure, inhibiting leukocyte activation, vascular smooth muscle proliferation, and platelet aggregation (Figure 1.9) (Moncada *et al* 1993,



**Figure 1.9** Endothelium derived nitric oxide synthesis and action (Adapted from Moncada and Higgs 1993).

Shimokawa *et al* 1995). NO is a potent vasodilator and there is clear evidence that basal NO dependent vasodilatation occurs in humans (Moncada *et al* 1993).

NO is generated from L-arginine by a family of NOS (Harrison 1997) Three different isoforms of NOS occur in humans. Endothelial (eNOS, NOS III) and neuronal (nNOS, NOS I) isoforms are constitutively expressed and synthesise small amounts of nitric oxide (NO) under basal conditions as well as following stimulation by various stimuli e.g. insulin, shear stress, acetylcholine (Ach) or a rise in intracellular calcium concentration (Michel and Feron 1997). nNOS and iNOS are soluble whereas eNOS is membrane bound (Liu *et al* 1995).

NO production by eNOS requires numerous cofactors including FMN, FAD, BH<sub>4</sub> and CaM (Moncada 1993). The half-life of NO is short (less than 4 seconds). It is rapidly metabolised to nitrite and then to nitrate before being excreted in the urine (Moncada 1993). NO passes from the endothelial cell to the vascular smooth muscle cell by diffusion. Once in the smooth muscle cell, it activates guanylate cyclase leading to an increase in intracellular guanosine-3,5-monophosphate (cGMP) concentrations, leading to a reduction in smooth muscle tone and vasodilation (Vallance *et al* 2001). The importance of NO as a mediator of vascular tone was initially demonstrated in experiments in both rabbit aorta *ex vivo* and the human forearm *in vivo*, in which application or infusion of the eNOS inhibitor, L-NMMA, was shown to produce vasoconstriction (Vallance *et al* 1989). Abnormalities of endothelium-mediated vascular function have since been reported in a large number of studies in various cardiovascular disease states, including heart failure (Maguire *et al* 1998), hyperlipidaemia (Chowienczyk *et al* 1992), diabetes (Schofield *et al* 2000) and hypertension (Taddei *et al* 2002). An impairment of endothelium-derived NO-mediated reduction in smooth muscle tone in states of cardiovascular disease and endothelial dysfunction may be

caused by either decreased formation or accelerated breakdown of NO. Each of these will be addressed in turn.

#### ***1.4.4.3.1 Decreased formation of NO***

eNOS activity within the endothelial cell may also be modulated by circulating factors including insulin. Insulin has important physiological effects on the endothelium, increasing NO availability, stimulating vasodilatation and is proposed to act in an anti-atherogenic manner (Muniyappa *et al* 2007). Insulin-stimulated endothelial dependent vasodilatation is impaired in insulin resistance (Cleland *et al* 2000, Wheatcroft *et al* 2003). Cultured human umbilical vein endothelial cells (HUVECs) demonstrate acutely increased activity of eNOS and consequent NO production in response to insulin incubation (Zeng *et al* 1996, Yki-Jarvinen *et al* 1998). A fall in the circulating concentration of insulin, or a decreased sensitivity of intracellular signalling mechanisms could lead to a reduction in eNOS activity and therefore in NO production. eNOS may be inhibited by endogenous products of arginine metabolism with some similar properties to L-NMMA, particularly asymmetric dimethylarginine (ADMA) (Vallance *et al* 2001). ADMA, a guanidino-substituted analogue of L-arginine, is a potent endogenous competitive inhibitor of eNOS. ADMA is produced by methylation of L-arginine which is catalysed by protein arginine methyltransferases (Achan *et al* 2003). Symmetric dimethylarginine is also produced by methylation of L-arginine but does not exert effects on the production of NO by NOS. The predominant mode of elimination of ADMA occurs via enzymatic degradation by dimethyl arginine dimethyl amino hydrolase (DDAH) to citrulline and dimethylamine (Achan *et al* 2003). Increased levels of ADMA reduce NO formation and are associated with endothelial dysfunction. Plasma levels of ADMA have been found to be increased in hypercholesterolaemia (Boger *et al* 1998), hypertriglyceridaemia (Lundman *et al* 2001), hyperhomocyst(e)inemia (Stuhlinger *et al* 2003), obesity (McLaughlin *et al* 2006), Type 1 diabetes mellitus (Altinova *et al* 2007), Type 2 diabetes mellitus (Abbasi *et al* 2001),

gestational diabetes (Mittermayer *et al* 2002) and, most recently, polycystic ovary syndrome (Heutling *et al* 2008). There is a close relationship between concentrations of ADMA and insulin resistance (Stuhlinger *et al* 2002). Indeed, in several prospective studies, ADMA has been noted to be an independent predictor of cardiovascular events (Valkonen *et al* 2001, Mittlemayer *et al* 2006, Krzyzanowska *et al* 2007). These substances may act as endogenous competitive inhibitors of eNOS and provide a credible mechanism by which NO bioavailability may be impaired in states of cardiovascular disease.

#### **1.4.4.3.2 Accelerated breakdown of NO**

Accelerated degradation of NO by reactive oxygen species is probably the major mechanism impairing NO bioavailability in states of cardiovascular disease (Harrison 1997, Behrendt *et al* 2002). The most important mediators of increased oxidative stress are increased generation of superoxide anion, and oxidised LDL cholesterol which react with endothelial NO before it reaches the vascular smooth muscle cell and therefore reduce total NO-mediated vasodilation (Kojda *et al* 1999).

#### **1.4.4.4 Prostacyclin**

Prostacyclin (PGI2) is the major metabolite of arachidonic acid produced by cyclooxygenase (COX-1) in the endothelium. PG12 acts as a vasodilator. Clinical and experimental models of diabetes are associated with decreased secretion of PGI2 (Feletou and Vanhoutte 2006, Muniyappa *et al* 2007)

#### **1.4.4.5 Endothelium derived hyperpolarising factor**

NO has been shown to be an important endothelium-dependent mediator of vascular tone in relatively large arteries and larger arterioles. There are, however, certain arteries in which

endothelium-mediated vasodilation is predominately affected by endothelium-dependent hyperpolarisation of vascular smooth muscle cells which persists in the presence of inhibitors of eNOS and prostacyclin (Feletou *et al* 1988, Garland *et al* 1992). This suggested that a separate mechanism is partially responsible for endothelium dependent vasodilation of these arteries. The exact identity of the chemical (or other) mediator(s) involved in this mechanism is unclear at present. This non-prostanoid, non-NO dependent endothelium-dependent hyperpolarisation characterised by the EDHF pathway does not appear to represent any one substance, and it is therefore likely that there are several endothelium-derived factors responsible (Feletou and Vanhoutte 2006).

The relative importance of the EDHF mediated mechanisms to NO mediated mechanisms alters with vessel size, and appears to increase as vessel size decreases (Shimokawa *et al* 1996). At the level of the aorta, reduced NO bioavailability is proposed to be the main marker for endothelial dysfunction. In resistance arteries, NO, prostacyclins and EDHFs are thought to be involved in mediating endothelial function (Kang *et al* 2007).

#### ***1.4.4.6 Measurement of endothelial function***

Several established techniques are used to measure endothelial function. Wire myography is used to measure endothelial function in this thesis and detailed discussion of this technique can be found in 2.2.2.4.1

## **1.5 Metformin**

### **1.5.1 Galegine (*isoamylene guanidine*)**

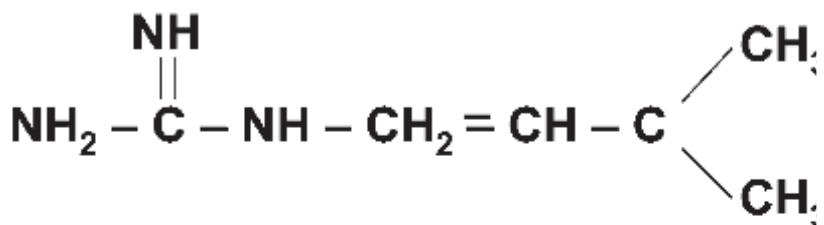
*Galega officinalis* has been used since medieval times for medicinal purposes by French monks to control what was described as “intense urination” (Witters 2001). The therapeutic properties of *Galega Officinalis* for the management of patients with diabetes was ultimately developed in the early 1900’s with the knowledge that the plant was a source of guanidine which induced glucose lowering biological effects. Guanidine proved to be too toxic and the less toxic derivative galegine or isoamylene guanidine was identified (Figure 1.10). Guanidine was subsequently shown to reduce blood glucose levels in both animals and humans (Bailey *et al* 2004). The clinical application of Galegine was limited, however, due to its short duration of action, differing efficacy and safety concerns (Bailey *et al* 2004).

### **1.5.2 The discovery of metformin**

Following a hiatus in research into diabetes after the discovery of insulin, work by the French clinician Sterne and colleagues lead to the discovery of dimethylbiguanide (metformin), (Sterne 1964). Sterne also made some important discoveries in metformin’s mechanism of action. He demonstrated that the presence of the pancreas and diabetes (Sterne 1964) was required for metformin to lower blood glucose.

At a similar time other investigators in the United States were working on the more potent biguanide phenformin (phenylethylbiguanide) (Unger *et al* 1960). (Figure 1.11). A typically effective dose of phenformin would be around 100 mg rather than 2000 mg with metformin. Reports of phenformin associated lactic acidosis (Walker *et al* 1959) and evidence of increased cardiovascular mortality in those with T2DM with phenformin (Goldner *et al* 1971) resulted in

## GaleGINE



## Metformin

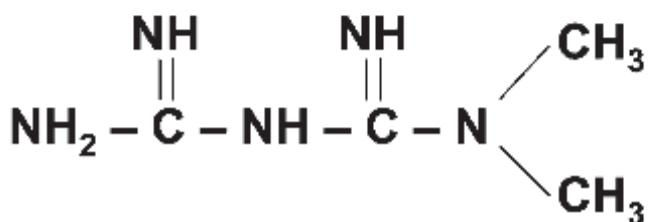
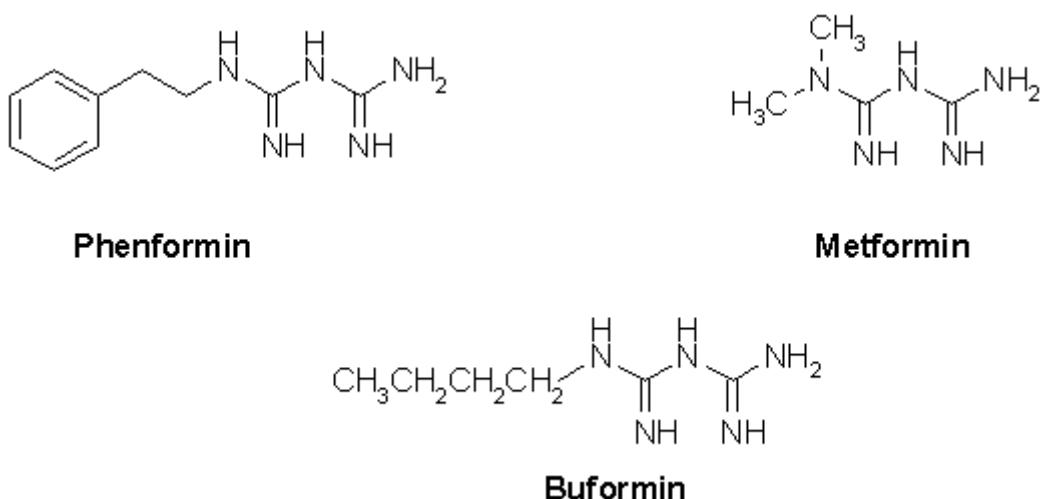


Figure 1.10 Structure of galeGINE ( $\beta$ -methyl-2-butenylguanine/isoamylene guanidine) and metformin (dimethylbiguanide)



**Figure 1.11 Chemical structure of biguanides.** Buformin (butylbiguanide) is less potent than phenformin but there are case reports of associated lactic acidosis (Krishnamurthy *et al* 2004) and this drug is not used in the United Kingdom.

phenformin's withdrawal from clinical use from most countries in the 1970's. This proved to be a major challenge to the introduction of metformin and it was not introduced into clinical practice in the United States until 1995, almost 40 years after it was first introduced as a treatment of T2DM in Europe in 1957. This coincided with evidence that the risk of lactic acidosis associated with metformin is considerably lower than other biguanides and is probably no higher than other anti-diabetic drugs when prescribed correctly (Misbin 2004). The spectre of increased cardiovascular mortality associated with biguanides in 1971 was finally dispelled when the results of the UK Prospective Diabetes Study (UKPDS) were published in 1998.

### ***1.5.3 Structure and action of metformin***

As outlined above, three biguanides have been used widely in patients with T2DM: metformin, phenformin and buformin; but only metformin remains part of today's worldwide pharmacopoeia. While there are close similarities between these drugs the unique properties of metformin explain its longer lasting appeal. Both metformin and phenformin are guanidine derivatives with one mono-substitution as already shown in Figure 1.10. As shown phenformin has a phenyl ethyl ring substituted at the terminal amino group rather than metformin's two methyl groups. Metformin is of smaller molecular weight, more chemically stable, freely soluble in water and does not undergo substantial metabolism *in vivo* (Karttunen *et al* 1983).

Metformin acts by countering insulin resistance, which is thought to occur principally in liver and muscle (Hundal *et al* 2000). Metformin acts mainly to reduce hepatic gluconeogenesis and also to increase peripheral insulin sensitivity in peripheral insulin sensitive tissues such as muscle and adipose. Finally, metformin has protective effects on the cardiovascular system which cannot be fully explained by its blood glucose lowering properties (UKPDS 1998). There is evidence to suggest that metformin's beneficial effects are attributable to disruption of respiratory chain

oxidation in mitochondria (El Mir *et al* 2000, Owen 2000), enhanced insulin tyrosine kinase receptor activity (Dominguez *et al* 1996), enhanced GLUT4 translocation to the membrane (Matthaei *et al* 1993, Hundal *et al* 1992) and finally the focus of this thesis, activation of AMPK (Zhou *et al* 2001, Musi *et al* 2002).

### **1.5.4 Efficacy**

As detailed above metformin has been used in the treatment of patients with T2DM since 1957 in Europe and 1995 in the United States. It is now the most commonly prescribed oral hypoglycaemic worldwide. This section outlines the therapeutic profile of metformin and further details effects on blood glucose, weight, lipid profile and the focus of this thesis, the cardiovascular system.

#### **1.5.4.1 Glucose lowering**

The UKPDS study showed that the addition of metformin to diet resulted in a dramatic improvement in glycaemic control over a few years compared with patients on diet alone (UKPDS 1998). Evidence also suggests that the hypoglycaemic properties of metformin are dose dependent with the largest study of 451 patients concluding that 2000 mg of metformin was the optimal daily dose with a mean change in HbA<sub>1C</sub> from baseline -0.7% after 14 weeks (Garber *et al* 1997). In Europe the maximum recommended dose is 3000 mg and in the UKPDS study a median daily dosage of 2550 mg was used (UKPDS 1998).

Before considering how metformin reduces blood glucose in patients with T2DM it is important to consider how glucose is regulated in normal physiology. Following a meal, insulin concentrations rise and stop glucose production in the liver and lipolysis in the adipocytes. Glucose uptake peripherally in insulin sensitive tissue such as skeletal muscle and adipocytes increases as the elevated insulin concentrations improve GLUT4 translocation to their plasma membranes. In T2DM

this mechanism of regulating blood glucose is disrupted years before diagnosis (DeFronzo *et al* 1992). The liver, skeletal muscle and adipocytes become less responsive to the insulin which results in a compensatory hyperinsulinaemia. This can control blood glucose levels for a period until the pancreas can no longer secrete enough insulin to maintain normal blood glucose levels and the patient develops T2DM (DeFronzo *et al* 1992).

The principal way in which metformin exerts its glucose lowering effects is by reversing insulin resistance and not, like the sulphonylurea class of drugs, by increasing insulin secretion. This occurs by reducing hepatic glucose production (Johnson *et al* 1993, Tikkainen *et al* 2004), improving peripheral insulin action (Johnson *et al* 1993), increasing intestinal glucose uptake (Bailey *et al* 1994, Mithieux *et al* 2006) and reducing lipolysis in adipocytes (Perriello *et al* 1994, Abbasi *et al* 1998, Tikkainen *et al* 2004). Exactly how metformin improves insulin action, however, is not completely understood after more than 50 years of clinical use.

#### **1.5.4.2 Weight**

The vast majority of patients with T2DM are overweight or obese (Daousi *et al* 2006). There is considerable variability between studies on the effect of metformin on weight loss but reductions in body weight are often observed. Two meta-analyses of controlled studies, however, concluded that there was no difference in metformin, placebo or diet on body weight or BMI in patients with T2DM (Johansen 1999, Saenz *et al* 2005). This conclusion is reinforced by the UKPDS study, the longest randomised evaluation of metformin effects on weight in overweight patients, where after 10 years, weight was not significantly different between metformin and placebo (UKPDS 1998). There was, however, a significant difference in weight in a recent meta-analysis (Saenz *et al* 2005) comparing metformin with sulphonylurea and in the UKPDS study (UKPDS 1998).

#### **1.5.4.3 Lipid profile**

Metformin is associated with modest improvements in the lipid profile. This is usually reflected in reductions in total cholesterol, LDL – cholesterol and mainly triglycerides. HDL – cholesterol is generally unaffected by metformin (Wulffele *et al* 2004). While the beneficial effects of metformin on the lipid profile would tend to reduce cardiovascular risk when the reductions are entered into the UKPDS risk engine, the overall benefits to 10 year risk of macrovascular end-points are modest.

#### **1.5.4.4 Blood pressure**

In some animal models, metformin appears to have a vascular action. In spontaneously hypertensive rats, it has been reported to reduce blood pressure (BP), possibly via modulation of adrenergic tone (Peuler *et al* 1999). In man, blood pressure responses to intravenous infusions of norepinephrine and angiotensin II are blunted in the presence of metformin (McAuley *et al* 1997). Reductions in blood pressure have been observed in patients treated with metformin (Chan *et al* 1993) but the overwhelming body of evidence including a meta-analysis suggest that metformin does not alter BP in man (Saenz *et al* 2005).

#### **1.5.4.5 Macrovascular Benefits**

T2DM confers a 2 to 4 fold greater risk of heart disease and stroke when compared with the general population. Moreover, life expectancy in patients with T2DM is thought to fall by anything from 5 to 10 years. The United Kingdom Prospective Diabetes Study (UKPDS) study, initiated in 1977 and reported in 1998, was the first randomised trial to demonstrate improved outcome with metformin treatment. Randomisation to metformin was associated with dramatic relative risk reductions in diabetes-related death (-42%), myocardial infarction (-39%), stroke (-42%) any diabetes related end-point (-32%) and all cause mortality (-36%) when compared with diet. This is in contrast to the relative risk reductions with sulphonylurea or insulin in diabetes-related death (-20%), myocardial

infarction (-21%), stroke (+14%) any diabetes related end-point (-7%) and all cause mortality (-8%) when compared with diet. The benefits observed and could not be explained by differences in glycaemia. Mean HbA<sub>1c</sub> after follow up in the diet group and metformin group were 8% and 7.4% respectively and there was no clear difference in the glucose lowering effect between metformin, sulphonylurea and insulin. Given that the glucose lowering effects on metformin, sulphonylurea and insulin were similar, it has been proposed that metformin must confer additional vascular benefits beyond those of glycaemia alone. The reason why metformin has beneficial cardiovascular effects in overweight diabetic patients in UKPDS remains unanswered.

#### ***1.5.4.6 Endothelial function***

Despite 50 years of research the exact mechanisms of action of metformin remain poorly understood and have yet to be fully elucidated. It is likely that no single cellular effect can explain metformin's cardioprotective effect. As outlined above it is unlikely that changes in the usual risk factors of hyperglycaemia, dyslipidaemia, obesity and hypertension with metformin are enough to account for its cardiovascular benefits and therefore other mechanisms must therefore be involved.

There is evidence that metformin improves endothelial function. In a placebo controlled study in 44 patients with T2DM metformin improved endothelial-dependent vasodilation as assessed by forearm plethysmography during an infusion of acetylcholine (Mather *et al* 2001). This is completed by evidence from a randomised placebo controlled cross-over study in patients with insulin T2DM in which there were significant reductions in the circulating cell adhesion molecules VCAM-1 and E-selectin, reflecting suppression of endothelial activation unexplained by changes in glucose (De Jager *et al* 2005). These results have also been reproduced in patients with impaired glucose tolerance (Caballero *et al* 2004), metabolic syndrome (Vitale *et al* 2005), polycystic ovary syndrome (Diamanti-Kandarakis *et al* 2005, Orio *et al* 2005), cardiac syndrome X (Jadhav *et al*

2006) as well as the first degree relatives of patients with T2DM (De Aguiar *et al* 2006). There is also, however, conflicting evidence demonstrating no effect on endothelial function with metformin (Natali *et al* 2004).

#### **1.5.4.7 AMPK**

Recent clinical studies have shown that the effects of metformin may go beyond improving HbA1c and may include reductions in cardiovascular end points in T2DM. Perhaps the most intriguing development in elucidating metformin's cellular mechanism of action in recent years is the proposed involvement of AMPK. The metabolic and cardio-protective effects of metformin may be attributable to the activation of AMPK and its downstream pathways.

The precise mechanisms of how metformin activates AMPK are still poorly understood. Metformin has not been shown to bind directly to AMPK or regulate phosphorylation and dephosphorylation in cell-free assays (Hawley *et al* 2002). One hypothesis is that it activates AMPK by weakly inhibiting complex I of the respiratory chain resulting in an increase in the AMP/ATP ratio (El-Mir *et al* 2000, Owen *et al* 2000, Hawley *et al* 2002). The increase in the AMP/ATP ratio inhibits dephosphorylation of AMPK thereby potentiating the phosphorylation of AMPK by LKB1, whose activity is thought to be constitutive (Hawley *et al* 2003). There is also contrasting evidence suggesting that AMPK can be activated by metformin without changes in the AMP/ATP ratio (Hawley *et al* 2002). Interestingly, it has been shown that LKB1 is necessary for metformin to activate AMPK in the liver and in turn decrease blood glucose in obese rodents (Shaw *et al* 2005).

Metformin is not metabolised but is transported by at least two organic cation transporters. Organic cation transporter-1 (OCT-1) is mainly expressed in the cell membrane of hepatocytes and is thought to play an important role in the uptake of metformin from intestinal cells (Wang *et al* 2002,

Dressler *et al* 2002, Kirmura *et al* 2005). Organic cation transporter-2 (OCT-2) is mainly expressed in the cell membrane of the distal renal tubules and is thought to play an important role in the renal tubular excretion of metformin. In contrast, phenformin enters cell membranes by passive diffusion without the need for active transport. In mouse hepatocytes, deletion of OCT-1 resulted in attenuation of the effects of metformin on AMPK phosphorylation and gluconeogenesis (Shu *et al* 2007). It should be noted that there was significant metformin uptake and AMPK activation despite deletion of OCT-1 suggesting that other mechanisms such as passive diffusion or other as yet, undiscovered transporters may contribute to metformin's action in liver or indeed in other tissues. It is important to note that in OCT-1 deficient mice the glucose lowering effects of metformin were abolished suggesting that OCT-1 in the liver plays an important role in mediating metformin's ability to reduce hepatic glucose production (Shu *et al* 2007). Moreover, it has been recently observed that OCT-1 mediates lactic acidosis, the major adverse effect of metformin (Wang *et al* 2003). When mice were given metformin, the blood lactate concentration significantly increased in wild type mice compared with OCT-1 knockout mice. As previously stated, metformin is a weak inhibitor of complex I of the respiratory chain (Hawley *et al* 2002) and this mechanism of action is likely to explain the life-threatening side effect of lactic acidosis that led to the withdrawal of the sister drug, phenformin, from human use. Recently, genetic polymorphisms of OCT-1 have been found to be associated with variations in metformin's action. Several nonsynonymous and deletion variants have been identified in the human OCT-1 gene (encoded by SLC22A1). It is noteworthy that 2 variants that reduce transport of metformin, 420del and R61C, are common polymorphisms of OCT-1 with allele frequencies of 19% and 7.2% respectively in those of European descent (Shu *et al* 2003). In addition the effects of metformin on glucose tolerance tests were significantly lower in 12 healthy volunteers carrying reduced function polymorphisms of OCT-1 (Shu *et al* 2007). Identification of polymorphisms in genes encoding cation transporter proteins may, therefore explain differences in T2DM patients' tolerance and response to metformin therapy (Jonker *et al*

2004). Interestingly, in a recently published retrospective study, the 420del and R61C variants failed to have any effect on metformin ability to lower HbA1c in a large cohort of patients with T2DM (Zhou *et al* 2009). Large scale prospective studies, however, are necessary to confirm the clinical relevance of personalised metformin therapy based on OCT-1 gene polymorphisms. Nevertheless, the prospect of the pharmacological tissue specific targeting of influx transporters and AMPK in the management of T2DM is an attractive one (Shu *et al* 2007).

Metformin increases skeletal muscle glucose disposal and decreases hepatic glucose production. Metformin has been shown to activate AMPK in myocytes and skeletal muscle cells (Zhou *et al* 2001, Yang *et al* 2006). Therapeutic doses of metformin have been shown to increase AMPK activity with an associated decrease in ACC activity in serial muscle biopsies in patients with T2DM (Musi *et al* 2002). Levels of metformin in the skeletal muscle of OCT-1 knockout mice and wild type mice are no significantly different, suggesting that the uptake of metformin in skeletal muscle may involve other transporters (Wang *et al* 2003, Shu *et al* 2007). Metformin has been reported to activate AMPK in hepatocytes (Zhou *et al* 2001) and 3T3-LI adipocytes (Huypens *et al* 2005), yet an effect of metformin on AMPK in human liver or adipose tissue has not been investigated. Indeed, as is the case with skeletal muscle, the transporter(s) involved in the uptake of metformin in adipose tissue remains unknown.

It has recently been reported that metformin stimulates NO synthesis in an AMPK-dependent manner in BAECs (Zou *et al* 2004). This is in contrast to data that suggests that the incubation of BAECs with phenformin activates AMPK without altering Ser1177 phosphorylation (Mount *et al* 2005). Further work has demonstrated that metformin acts to phosphorylate AMPK and ACC in HUVECs (Hattori *et al* 2006). However in HAECs, metformin failed to show any effect on AMPK activity within 2 hours (Morrow *et al* 2003). The reasons for these conflicting data remain elusive

but may be explained by species- and tissue-specific factors. Moreover, the neutral results with metformin may reflect the reduced lipophilicity compared with phenformin, such that phenformin rapidly accumulates in cells and subsequently has a more rapid effect on metabolism compared with metformin. Therefore, a longer incubation period with a higher concentration of metformin may be required to stimulate AMPK and NO synthesis in HAECS. Furthermore, the neutral results with metformin may also be explained by the possibility of cells either carrying reduced function polymorphisms of OCT-1 or not expressing OCT-1 at all.

Exactly how metformin improves insulin action, lowers body weight, improves the lipid profile, improves endothelial function and reduces the incidence of myocardial infarction remains incompletely understood. Further work is required to examine if biguanides mediate these effects by stimulating AMPK activity in human adipose tissue and vascular endothelial cells.

#### **1.5.4.8 ADMA**

Metformin has been reported to decrease the plasma ADMA concentrations in poorly controlled subjects with Type 2 diabetes in an open labelled, uncontrolled study without a placebo arm; the fall in ADMA concentration was associated with improved fasting plasma glucose, although the study failed to demonstrate a relationship between the decrease in ADMA concentrations and improvement in glycaemic control (Asagami *et al* 2002). Importantly, no assessment of vascular function was made in this study. Furthermore, in a recent study, a subgroup of women with PCOS who received metformin demonstrated reductions in ADMA concentration independent of a reduction in BMI and fasting blood glucose (Heutling *et al* 2008). There was a marginal improvement in carotid intima media thickness in favour of metformin therapy. However, once again, this was also an uncontrolled study involving a small subset of patients without the benefit of a placebo arm and it is clear that more data are required to clarify whether metformin mediated

reduction in ADMA concentration is genuine and, if so, whether this change can lead to beneficial effects on vascular function in overweight subjects with Type 2 diabetes. Interestingly, patients with T2DM randomised to the second generation sulphonylurea glibenclamide were found to have no change in ADMA concentration after six months of therapy (Kelly *et al* 2007). Further work is required to determine if metformin therapy improves vascular endothelial function in association with lower circulating concentrations of ADMA.

## 1.6 Sulphonylureas

The sulphonylureas are derived from sulphonic acid and urea and were introduced to the pharmacopeia in 1950s with gliclazide being the most popular in the United Kingdom at present (Gerich *et al* 1989). All sulphonylureas contain a central *S*-phenylsulfonylurea structure with p-substitution on the phenyl ring and various groups terminating the urea N' end group. The sulphonylureas are effective pancreatic insulin secretagogues that can rapidly improve glycaemic control (Haupt *et al* 1971). They control blood glucose levels by directly stimulating insulin secretion in the pancreatic beta cells (Gorus *et al* 1988). Sulphonylureas bind to a receptor associated with the ATP-sensitive potassium channel ( $K_{ATP}$ ; a 140 kDa membrane protein) on the plasma membrane which results in closure of the channel (Sturgess *et al* 1985, Schmid-Antomarchi *et al* 1987). This in turn depolarizes the membrane and triggers the opening of voltage-sensitive calcium channels, leading to the rapid influx of calcium. Increased intracellular calcium causes an alteration in the cytoskeleton, and stimulates translocation of insulin-containing secretory granules to the plasma membrane and the exocytotic release of insulin (Hu *et al* 2000). Differences in the binding, retention in islets and drug half lives result in different patterns of insulin release (Hu *et al* 2000).

Gliclazide for example affects first and second phase of insulin secretion and therefore has the advantage of affecting fasting blood glucose and post prandial blood glucose levels (Korytkowski *et al* 2002). In general, experience suggests the sulphonylureas lower blood glucose by around 3mmol/l corresponding to a drop in HbA<sub>1C</sub> of around 1.5 to 2% (Inzucchi *et al* 1998). Sulphonylureas are predictably associated with weight gain. In UKPDS, there was an increase in body weight of 2 to 3kg which was maximal within 1 year of the study and maintained for the entire ten year follow up. In terms of blood pressure and plasma lipid profile, sulphonylureas are generally neutral (Inzucchi *et al* 1998). Improved glycaemic control reduces the progression of

microvascular complications but the effect of sulphonylureas on macrovascular complications is less clear. In the UKPDS study, where sulphonylureas or insulin were compared with conventional therapy in patients with T2DM (with median results of HbA<sub>1C</sub> of 7% and 7.9% respectively), there was a significant 25% reduction in all microvascular end-points as well as a significant 12% reduction in any diabetes-related endpoint. The effect of sulphonylureas on macrovascular disease is less certain. In UKPDS, there was no difference in all cause mortality or diabetes-related death. It is important to note that there was a reduction in myocardial infarction and cardiovascular mortality that failed to reach statistical significance ( $p = 0.052$ ). Overall there was no significant difference between those treated with insulin or sulphonylureas and it was concluded that lowering blood glucose alone is insufficient to reduce macrovascular events in this high risk group. This is in contrast to the reduction in myocardial infarction and cardiovascular mortality in obese patients with T2DM randomised to metformin which could not be fully explained by a reduction in glucotoxicity. Sulphonylureas are not thought to activate AMPK, indeed the third generation sulphonylurea, glimepiride had no effect on AMPK activity when incubated with cultured adipocytes and muscle cells (Inukai *et al* 2005). Thus, the sulphonylureas are an appropriate control in studies examining metformin's ability to stimulate AMPK in patients with T2DM.

### **1.7 Thiazolidinediones**

The peroxisome proliferator activated receptor- $\gamma$  (PPAR- $\gamma$ ) agonists such as the thiazolidinediones (TZDs), rosiglitazone and pioglitazone, are an important class of compounds used in the management of T2DM. TZDs are thought to work by the binding to and activating of the PPAR- $\gamma$  receptor (Lehmann *et al* 1995). The PPAR- $\gamma$  receptor is a nuclear receptor that is thought to have a regulatory role in the differentiation of cells and is present in many tissues including adipose, liver, muscle and the vascular endothelium. Indeed, TZDs main action is to promote differentiation of pre-adipocytes to adipocytes and redistribute lipids. The ability of TZDs to lower blood glucose is well established and they have similar efficacy to other oral hypoglycaemic drugs. They lower glucose by lowering insulin resistance in muscle and adipose, increasing insulin related glucose disposal and reducing hepatic glucose production (Semple *et al* 2006). While TZDs are effective in reducing blood glucose levels they also cause weight gain and peripheral oedema (Nissen *et al* 2007).

The ability of TZDs to reduce cardiovascular events in patients with diabetes has attracted much interest. A number of studies have demonstrated that TZDs have the capacity to improve endothelial function in non-diabetic subjects (Wang *et al* 2004, Hetzel *et al* 2005) and subjects with T2DM (Satoh *et al* 2003, Natali *et al* 2004, Pitrosh *et al* 2004). The beneficial effects of TZDs have been reported to be independent of glycaemia, inferring a direct effect on vascular tissues (Satoh *et al* 2003, Pitrosh *et al* 2004). The endothelial effects of TZDs may be relatively rapid; indeed rosiglitazone has been reported to improve endothelial function within 24 h in healthy human subjects (Hetzel *et al* 2005).

The mechanism of action by which TZDs improve vascular endothelial function remains uncertain. Recent studies have suggested that TZDs also directly improve NO bioavailability in endothelial

cells (Calnek *et al* 2003, Cho *et al* 2004, Polikandritois *et al* 2005, Kim *et al* 2006). Previous studies have demonstrated that incubation of HUVECs with rosiglitazone for 48h stimulated NO synthesis and phosphorylation of eNOS at Ser 1177 (Polikandritois *et al* 2005). Similarly, troglitazone has been reported to stimulate NO synthesis within 3h and eNOS phosphorylation at the equivalent site (Ser 1179) in BAEC's (Cho *et al* 2004). In HUVECs, rosiglitazone-stimulated NO synthesis and eNOS Ser 1177 phosphorylation were reported to be completely inhibited in the presence of the PPAR- $\gamma$  inhibitor, GW9662 (Polikandritois *et al* 2005). In BAECs, however, troglitazone-stimulated NO synthesis and eNOS Ser 1179 phosphorylation was only partially inhibited in the presence of the PPAR- $\gamma$  inhibitor, BADGE (Cho *et al* 2004). Several protein kinases have been demonstrated to phosphorylate eNOS at Ser 1177 in endothelial cells, including protein kinase B (PKB) and AMPK (Fulton *et al* 1999, Dimmeler *et al* 1999; Chen *et al* 1999, Morrow *et al* 2003), but the protein kinase and signalling mechanism responsible for phosphorylation of eNOS in response to TZDs is as yet undetermined. TZDs have been demonstrated to activate AMPK in muscle, liver and adipose (Fryer *et al* 2002, Saha *et al* 2004, Konrad *et al* 2005; Lebrasseur *et al* 2006). Indeed, rosiglitazone associated AMPK activation has been shown to be via an AMP/ATP dependent pathway in muscle (Hawley *et al* 2002). TZDs can also activate AMPK by stimulating the release and expression of circulating adiponectin from adipose tissue or indirectly by increasing the cellular AMP/ATP ratio by a similar mechanism to metformin (Yamauchi *et al* 2002, Tomas *et al* 2002, Brunmair *et al* 2004). Further work is required to examine if TZDs mediate their beneficial effects by stimulating AMPK in vascular endothelial cells.

Despite the TZD related improvements in vascular biology and endothelial function described above, it is important to note that metformin remains the only oral hypoglycaemic drug to demonstrate good evidence for cardiovascular protection in a randomised trial. Since UKPDS reported in 1998, the thiazolidinediones had been proposed to have beneficial cardiovascular

effects. This was assessed by randomisation to pioglitazone to high risk patients with T2DM in the Prospective Pioglitazone Clinical Trial in Macrovascular Events (PROactive) study (Dormandy *et al* 2005). The PROactive study was a prospective randomised controlled trial in 5238 patients with T2DM who have evidence of cardiovascular disease. Patients were randomised to 45mg of pioglitazone versus placebo and continued to take other glucose lowering drugs with an absolute reduction in HbA1c of 0.5% in the pioglitazone group versus placebo. However, pioglitazone was not associated with cardiovascular benefit as defined by the primary composite cardiovascular end-point of all-cause mortality, non-fatal myocardial infarction, stroke, acute coronary syndrome, amputation or revascularisation (Dormandy *et al* 2005). Indeed, there was only a modest 10% reduction that failed to reach statistical significance ( $p=0.92$ ). It is worth noting that the “main” secondary end-point of all cause mortality and non-fatal myocardial infarction was reduced by 16% and did reach statistical significance ( $p=0.027$ ).

There has been concern that rosiglitazone may be associated with additional cardiovascular risk in patients with Type 2 diabetes mellitus. This was related to a recent meta-analysis suggested an increase in adverse cardiovascular outcomes in patients randomised to rosiglitazone compared to any other treatment (Nissen *et al* 2007). The meta-analysis included 42 randomised trials (duration of 24 weeks or more) and found that randomisation to metformin was associated with a significant ( $p=0.03$ ) increase in the risk of myocardial infarction of 43%, while a 64% increase in cardiovascular death just failed to reach statistical significance ( $p=0.06$ ). The meta-analysis was limited, however, by a small event rate and the inclusion of a number of small trials. Following publication of this meta-analysis, the European Medicines Evaluation Agency (EMEA) for Medicinal Products for Human Use adopted a scientific opinion in January 2008 recommending the inclusion of a new warning stating that the use of rosiglitazone in patients with ischaemic heart disease and/or peripheral arterial disease is not recommended. In the United States, the Federal Drug

Administration (FDA) also issues a “black box” warning stating that the drug could increase the risk of heart attacks.

Until very recently, no adequately powered randomised cardiovascular morbidity and mortality trial evaluating rosiglitazone has been published. The largest randomised examining the effects of rosiglitazone on cardiovascular morbidity and mortality has now been published (Home *et al* 2009). Rosiglitazone Evaluated for Cardiac Outcomes and Regulation of Glycaemia in Diabetes (RECORD). The results of the trial were largely consistent with those of a non pre-specified interim analysis published in 2007 (Home *et al* 2007). In the trial's 4447 patients with type 2 diabetes, who were randomized to receive either rosiglitazone on top of either metformin or sulphonylurea (n=2220) or a metformin and sulphonylurea combination (n=2227), the rate of cardiovascular hospitalisation or death from cardiovascular causes was 14.5% in both groups over a mean follow-up of 5.5 years. The rosiglitazone versus active control hazard ratio (HR) for that primary end point met RECORD's prospectively stated criteria for non-inferiority. There were no significant differences between the groups in risk of the individual end points of cardiovascular death, myocardial infarction, or stroke, although the HR for myocardial infarction was elevated by a non significant 14% in the rosiglitazone group.

As previously documented in the PROactive study, there was increase in the incidence of heart failure with a HR of 2.10 (1.35–3.27, p=0.001). This data were supported in a recent meta-analysis that reported that TZDs increased the risk of developing heart failure (relative risk, 1.72, P=0.002) (Lago *et al* 2007). It was, however, acknowledged that the RECORD study had limited statistical power for individual components of the primary end point because it was not designed to address these questions. In addition, the risk of any bone fracture was increased with rosiglitazone by 57% (p<0.0001) overall and by 82% in women and 23% in men. The authors stated the rosiglitazone is

not recommended for patients with a history of heart failure or with previous problems that might have led to myocardial dysfunction and recommended that rosiglitazone should be used with caution in women at high risk of fractures. They also acknowledged that although the evidence was insufficient to rule out a small increased risk of myocardial infarction caused by rosiglitazone when compared with other glucose-lowering agents, rosiglitazone does not increase overall cardiovascular morbidity or mortality. It is clear that the molecular mechanisms by which rosiglitazone and other thiazolidinediones have their effects on the cardiovascular system are complex and it remains to be seen if either the EMEA or the FDA review their position on the cardiovascular safety of rosiglitazone following the publication of the RECORD data.

### ***1.8 Objectives***

Biguanides and thiazolidinediones are widely used in the treatment of Type 2 diabetes but the site and precise mode of their action remain uncertain. The prevalence of Type 2 diabetes is reaching epidemic proportions and there is a growing need for new therapeutic targets. AMPK is a novel therapeutic target and the development of specific tissue specific AMPK activators is an attractive prospect for the future. The aims of this thesis are:

- i. To determine if metformin improves vascular endothelial function in association with lower circulating concentrations of ADMA.
- ii. To determine if metformin exerts beneficial metabolic effects in patients with T2DM in association with altered AMPK activity in human adipose.
- iii. To determine if insulin sensitizers such as metformin and the thiazolidinediones, acting directly on vascular endothelial cells, increase NO production by increasing AMPK activity thus accounting for beneficial effects on endothelial function and, in metformin's, case cardiovascular outcome.

*Chapter 2 Materials and Methods*

## ***2.1 Materials***

All reagents used were of the highest quality available and were obtained from the following suppliers:

### ***2.1.1 General Reagents***

#### **Acros Organics, Loughborough, Leicestershire, UK**

Glacial acetic acid (nitrogen flushed)

Sodium iodide (nitrogen flushed)

Sodium nitrite

#### **Amersham Biosciences, Little Chalfont, Buckinghamshire, UK**

Amersham ECL Western Blotting detection kit

Protein G Sepharose

#### **Axxora UK Ltd, Nottingham, UK**

GW9662

#### **BDH Laboratory Supplies, Poole, Dorset, UK**

Calcium chloride

Coomassie Brilliant Blue G -250

Disodium hydrogen phosphate

Magnesium sulphate

Orthophosphoric acid

Potassium chloride

Sodium dihydrogen orthophosphate

Sodium dodecyl sulphate (SDS)

Sodium pyrophosphate

**Calbiochem, Beeston, Nottingham, UK**

A23187 (from *Streptomyces chartreusensis*)

**Fisher Scientific, Loughborough, Leicestershire, UK**

Dimethylsulphoxide (DMSO)

Ethanol

D-Glucose

Glycine

4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES)

Methanol

Potassium dihydrogen phosphate

Tris (hydroxymethyl) -aminothane (Tris base)

**GlaxoSmithkline Stevenage, UK**

Rosiglitazone was a generous gift from Dr Phil Amberry

**Melford Laboratories Ltd, Chelsworth Ipswich, UK**

Dithiothreitol (DTT)

**National Diagnostics, Hull, UK**

Ecoscint<sup>TM</sup> A scintillation solution

**Riedel de Häen, Seelze, German**

Ammonium persulphate

Ethylenediamine tetraacetic acid (EDTA)

Glycerol

Sodium hydrogen carbonate

**Schleicher & Schuell, Brunswick Way, London, UK.**

Protran Nitrocellulose membrane

Whatman 3MM Filter paper

Whatman P-81 phosphocellulose paper

**Severn Biotech Ltd., Kidderminster, Worcestershire, UK**

30% (w/v) Acrylamide (Acrylamide to Bis-Acrylamide ratio 19:1)

**Sigma-Aldrich Chemical Company Ltd., Poole, Dorset, UK**

Adenosine 5'-triphosphate (ATP)

L-arginine

Benzamidine

Bovine serum albumin (BSA)

Brij-35

Bromophenol blue

Caesium chloride

Ethylene glycol-bis (a -aminoethylether)-N,N,N',N'-tetra acetic acid (EGTA)

D-Mannitol

NG-Nitro-L-Arginine Methyl Ester (L-NAME)

N,N,N',N'-tetramethylethylenediamine (TEMED)

Oxypurinol

Phenylmethylsulfonyl fluoride (PMSF)

Sodium chloride

Sodium fluoride

Sodium orthovanadate

1:1 tri-n-octylamine

Triton X-100

Trypsin inhibitor, from soyabean (SBTI)

Tween 20

Wortmannin

Metformin

Phenformin

### ***2.1.2 Antibodies***

The following antibodies were used in this study, with the conditions used for immunoblotting included.

#### **Cell Signalling Technology, Hitchin, Hertfordshire, UK.**

**Anti-phospho-AMPK $\alpha$  (Thr172),** catalogue number 2531. A rabbit polyclonal antibody raised against a synthetic phospho-peptide (KLH-coupled) corresponding to residues surrounding Thr172 of human AMPK- $\alpha$ . The antibody detects levels of both human  $\alpha$ -1 and  $\alpha$ -2 isoforms of the catalytic subunit (diluted 1: 1000 in 5% w/v BSA, incubated at 4°C overnight).

**Anti-phospho-Acetyl-CoA carboxylase (Ser 80)**, catalogue number 3661. A rabbit polyclonal antibody raised against a synthetic phospho-peptide (KLH-coupled) corresponding to residues surrounding Ser80 of human Acetyl CoA carboxylase (diluted 1: 500 in 5% w/v milk powder solution, incubated at 4°C overnight).

**Anti-phospho-eNOS (Ser1177)**, catalogue number 9571. A rabbit polyclonal antibody raised against a synthetic phospho-peptide (KLH-coupled) corresponding to residues surrounding Ser1177 of human eNOS, (diluted 1: 1000 in 5% w/v BSA, incubated at 4°C overnight).

**Sigma-Aldrich Chemical Company Ltd., Poole, Dorset, UK**

**Anti-Endothelial Nitric Oxide Synthase**, catalogue number N2643. A rabbit antibody raised against a synthetic peptide (KLH-coupled) corresponding to amino acids 596-609 of the bovine eNOS sequence. The immunogenic sequence is identical to the human eNOS sequence, (diluted 1: 5000 in 1% w/v BSA, incubated at 4°C overnight).

**Upstate Cell Signaling Solutions, Lake Placid, New York.**

**Anti-PKB $\alpha$** , catalogue number 07-416. A rabbit polyclonal antibody raised against a 15 residue synthetic peptide (C -RPHFPQFSYSASGTA) corresponding to the C-terminal residues (466-480) of rat PKB $\alpha$  (this sequence is strongly conserved in humans). The antibody has species cross-reactivity with human PKB $\alpha$ , (diluted 1: 1000 in 5% w/v BSA, incubated at 4°C overnight).

**Anti-phospho-eNOS (Thr495)**, catalogue number 07-384. A rabbit antibody raised against a synthetic phosphopeptide (KLH-coupled) corresponding to amino acids 489-501

**Sheep subunit isoform specific anti-AMPK $\alpha$ 1 and anti-AMPK $\alpha$ 2 antibodies** were a generous gift from Prof Grahame Hardie, University of Dundee, and have been described previously (Woods *et al.*, 1996), (diluted 1: 1000 in 5% w/v milk powder solution, incubated at 4°C overnight)

### **2.1.3 Radioactive materials**

Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, UK

[ $\gamma$ -<sup>32</sup>P] adenosine 5'-triphosphate

### **2.1.4 Peptides**

SAMS peptide (HMRSAMSGLHLVKRR) was purchased from Pepceuticals Ltd, Nottingham, UK.

### **2.1.5 Adenoviruses**

Control and dominant negative AMPK adenovirus (Ad. $\alpha$ 1-DN) were a generous gift from Dr Fabienne Foufelle, Paris, France and have been described previously (Morrow *et al.*, 2003).

### **2.1.6 Cell Culture Plastics**

Corning, NY, USA

Costar® 25ml, 10ml, 5ml, 1ml sterile pipettes

75cm<sup>2</sup> tissue culture flasks

150cm<sup>2</sup> tissue culture flasks

Falcon, Becton Dickinson Labware, Franklin Lakes, NJ, USA

6-well tissue culture plates

12-well tissue culture plates

100mm tissue culture plates

### **2.1.7 General Buffers**

**ECL reagent protocol:** **Solution a:** 0.1M Tris-HCl (pH 8.5), 2.5mM Luminol, 80nM Coumaric acid. **Solution b:** 0.1M Tris-HCl (pH 8.5), 0.06% (v/v) hydrogen peroxide.

**Electrode buffer:** 25mM Tris, 192mM glycine, 0.1% (w/v) SDS

**HEPES-Brij buffer:** 50mM HEPES-NaOH, pH 7.4, 1mM DTT, 0.02% (v/v) Brij 35

**IP buffer:** 50mM Tris -HCl, pH 7.4 at 4°C, 150 mM NaCl, 1% (v/v) Triton X-100, 1% (v/v) glycerol, 50mM NaF, 5mM Na<sub>4</sub>P<sub>2</sub>O<sub>7</sub>, 1mM Na<sub>3</sub>VO<sub>4</sub>, 1mM EDTA, 1mM EGTA, 1mM DTT, Complete protease inhibitor cocktail.

**KRH buffer:** 119mM NaCl, 20mM Hepes-NaOH pH 7.4, 5mM NaHCO<sub>3</sub>, 4.8mM KCl, 2.5mM CaCl<sub>2</sub>, 1.2mM MgSO<sub>4</sub>, 5mM Glucose, 1.2mM, NaH<sub>2</sub>PO<sub>4</sub>, 0.1mM arginine

**Wire Myography buffer (KPSS):** 118.4mM KCl, 1.2mM MgSO<sub>4</sub>, 24.9mM NaHCO<sub>3</sub>, 2.5mM CaCl<sub>2</sub>, 11.1mM glucose, 0.023mM EDTA pH 7.4.

**Lysis buffer:** 50mM Tris -HCl pH7.4 at 4°C, 50mM NaF, 1% (v/v) Triton X-100, 250mM mannitol, 1mM Na<sub>4</sub>P<sub>2</sub>O<sub>7</sub>, 1mM Na<sub>3</sub>VO<sub>4</sub>, 1mM EDTA, 1mM EGTA, 1mM DTT, Complete protease inhibitor cocktail.

**Biopsy collection buffer (PSS):** 118.4mM NaCl, 4.7mM KCl, 1.2mM MgSO<sub>4</sub>, 24.9mM NaHCO<sub>3</sub>, 2.5mM CaCl<sub>2</sub>, 11.1mM glucose, 0.023mM EDTA pH 7.4.

**TBS:** 20mM Tris-HCl pH 7.4, 137 mM NaCl

**TBST:** 20mM Tris-HCl pH 7.4, 137 mM NaCl, 0.1% (v/v) Tween-20

**Transfer Buffer:** 25mM Tris, 192mM glycine, 20% (v/v) methanol

**4 x sample buffer:** 200mM Tris-HCl (pH 6.8), 400mM DTT, 8% (w/v) SDS, 0.4% (w/v) bromophenol blue, 40% (v/v) glycerol

## **2.2 Methods**

### ***2.2.1 Identification and recruitment of subjects***

Twenty men with Type 2 diabetes (duration > 6 months) were recruited from Diabetes Clinics of the North Glasgow University National Health Service Trusts. North Glasgow University Hospitals National Health Service Trust ethics committee approved the study. After full explanation of experimental procedures aided by a subject information sheet, written informed consent was obtained. Each subject was issued with details of the study as well as the investigators' contact telephone numbers. The subject's General Practitioner was informed of their involvement in the study. The inclusion and exclusion criteria for the study are outlined below:

#### ***Inclusion criteria:***

BMI range 27-40 kg/m<sup>2</sup>

HbA1<sub>c</sub> > 7%, <11% (DCCT) at screening

Previously treated with diet alone or oral monotherapy (i.e. metformin or sulphonylurea). Subjects on monotherapy had discontinued medication during the six-week run-in period

#### ***Exclusion criteria:***

Subjects on warfarin treatment

Subjects treated with insulin currently or in the previous 12 months

Previous intolerance of metformin or sulphonylurea

Presence of contra-indication to metformin therapy for example renal disease or congestive cardiac failure

## **2.2.2 Study protocol**

### **2.2.2.1 Study design**

The study had a randomised, double blind, glycaemia-controlled crossover design (Figure 2.1). Patients on monotherapy discontinued medication during a six-week run-in period. After this period, patients were randomised to receive metformin (500mg three times daily) or gliclazide (80mg twice daily with a lunchtime placebo capsule to ensure blinding) for ten weeks, aiming for a similar reduction in HbA1c. Each drug had a two week dose titration as follows:

**Week 1:** Gliclazide 80mg once daily with breakfast, metformin 500mg once daily with breakfast.

**Week 2:** Gliclazide 80mg once daily with breakfast + dummy capsule at lunch, metformin 500mg twice daily with breakfast and lunch.

**Week 3 – 10:** Gliclazide 80mg twice daily at breakfast and evening meal + dummy capsule at lunch, metformin 500mg three times daily with breakfast, lunch and evening meal.

Subjects were asked to inform the investigators of any medication started or discontinued during the study period. No specific advice on lifestyle was given at the time of randomisation.

### **Study randomisation**

Randomisation and tablet supply was co-ordinated by the hospital pharmacy. Metformin and gliclazide capsules of identical appearance were manufactured by the Western Infirmary pharmacy, Glasgow. A computerised randomisation list was made. Randomisation codes were put into sealed envelopes and stored by the pharmacist of the Western Infirmary, Glasgow. Medication bottles

were numbered, and allocation was done in sequence. Unblinding was performed at the end of the study period.

### ***Subject visits***

The study required subjects to attend the Clinical Investigation and Research Unit (CIRU), Division of Cardiovascular Sciences, Western Infirmary, University of Glasgow on a total of nine occasions:

***Week 0 - Screening visit***

***Week 1 - Start of phase 1***

***Week 5 - Interim visit***

***Week 10 - End of phase 1 (with biopsy)***

***Week 12 - Stitch removal***

***Week 16 - Start of phase 2***

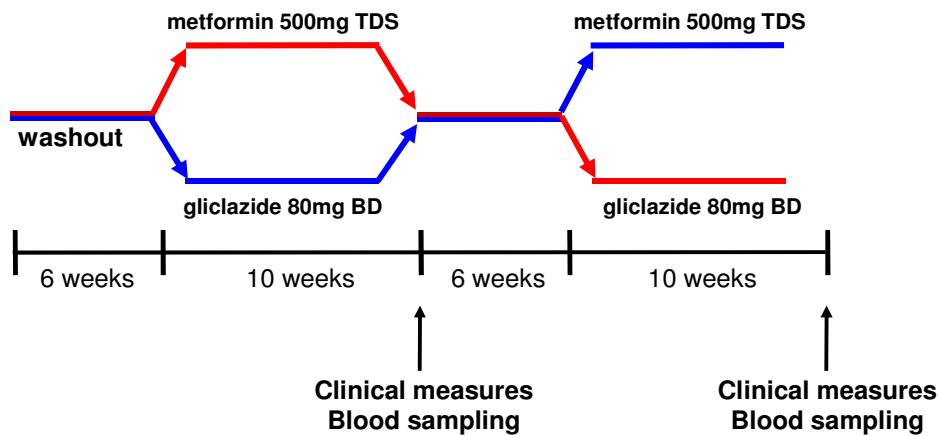
***Week 21 - Interim visit***

***Week 26 - End of phase 2 (with biopsy)***

***Week 28 - Stitch removal***

Patients were contacted by telephone at two weeks and attended the CIRU for a brief assessment at five weeks during each phase to check on any side effects and to assess glycaemic control. Any patient with significant osmotic symptoms or a fasting blood glucose of >15mM would have been withdrawn from the study. Patients were then required to attend the CIRU at 0830hrs at the end of the ten week study phase having fasted from midnight (and having abstained from alcohol, caffeine and moderate/heavy exercise in the preceding 72 hours) for clinical measures, adipose biopsy and blood sampling for biochemical analysis. Taxis were available to transfer volunteers to and from

the CIRU. Snacks were provided at the CIRU when the study protocol was completed. Following a six-week washout phase, the groups were crossed over (Figure 2.1).



**Figure 2.1: Crossover design**

### **2.2.2.2 Clinical and morphometric measurements**

#### **2.2.2.2.1 Body mass index**

Body weight was measured using analogue scales (Seca, Germany) to within 1kg in light clothing without shoes. The weighing scales were calibrated regularly. Height was measured barefoot using a stadiometer to within 0.5 cm. BMI was calculated as follows: weight (kg)/height(m)<sup>2</sup>.

#### **2.2.2.2.2 Blood pressure**

Blood pressure recordings were taken by a semiautomatic sphygmomanometer (Dinamap Critikon, Johnson and Johnson Professional Products Ltd, Bracknell, UK) at screening and the end of each

ten week phase. Readings were taken supine after thirty minutes rest. The average of 3 measurements was calculated.

### ***2.2.2.3 Clinical procedures***

#### ***2.2.2.3.1 Buttock biopsy***

Subjects were invited to attend the CIRU at 0830, having fasted from midnight. With the subject lying prone, one buttock was exposed and the area sterilised with 10% (v/v) iodine. A strict aseptic technique was employed. 1% lignocaine hydrochloride was then instilled subcutaneously to cover an area of 6cm x 4cm. Once the area was anaesthetised, an elliptical incision was made in the buttock and a 3cm x 0.75 cm segment of skin and a diamond shaped segment of adipose tissue was removed. A 2g portion of adipose tissue was cut from the margin of the specimen, snap frozen in liquid nitrogen and stored at -70°C. Four skin stitches were then inserted to close the wound. The volunteer was then given a light meal and asked to refrain from exertion for five days while keeping the wound clean. Ten days later the stitches were removed.

#### ***2.2.2.3.2 Analysis of routine blood samples***

Fasting venous blood samples for urea, creatinine, electrolytes, total cholesterol, triglycerides, HDL-cholesterol, LDL-cholesterol, glucose, liver function tests and HbA1C were collected between 0830hrs and 0930hrs from the antecubital fossa and placed immediately into the appropriate containers. Patients were in a recombinant position for at least ten minutes prior to the procedure. The samples were then analysed as routine samples in the Biochemistry Department, Gartnavel General Hospital, Glasgow.

### **2.2.2.3.3 Analysis of non-routine blood samples**

Venous blood was collected between 0830hrs and 0930hrs into EDTA-containing tubes, centrifuged (500 x g, 4°C) and plasma removed into microcentrifuge tubes and stored at -70°C until analysis. Total adiponectin was determined using ELISA (R&D Systems, Abingdon, UK). Plasma concentrations of ADMA and related arginine metabolites were measured using an optimized and fully validated HPLC method, based on that previously described (Teerlink 2005), with certain modifications as recently described (Blackwell *et al* 2007). All assays were performed at the Biochemistry department at Glasgow Royal Infirmary under the direction of Professor Naveed Sattar.

### **2.2.2.3.4 Pulse wave velocity (PWV)**

A relatively simple and direct measure of arterial stiffness, PWV is determined by measurement of the time taken for the pulse wave to traverse the distance between two fixed measuring points. The PWV is therefore derived as:

distance [m] / time [s], in m/s.

There are a number of potential confounding factors to be considered when measuring the PWV. The measurement required is of the velocity of the forward wave, and reflected waves may interfere with the accuracy of the calculation. Measurements must therefore be taken from the foot of the waveform (the upstroke of the pressure wave), which is generated entirely by the forward wave. Another potential problem is the accuracy of measurement of the distance between measuring points. Out of necessity, the measurement (using standard techniques) is an approximation of the actual distance, as the actual length of the artery cannot be measured. This particular problem is addressed with new techniques for the measurement of PWV, using magnetic resonance imaging

(MRI) (Mohiaddin *et al* 1993 and MacGowan *et al* 2002). Such techniques may become less constrained by time, expense and portability in the future and therefore more widely useable. A simple and well-established method of measurement of the PWV is to use a semi-automated device for analysis of the pressure waveforms at the two measurement sites (Asmar *et al* 1995). The upstroke of the wave is identified at each of these sites, and the time between the two upstroke points is measured. Once the distance between measuring points has been identified and entered, the velocity of the pulse wave is calculable by the software.

An advantage of this method of PWV measurement over some of the available derived techniques (in which different pulse waves are measured at the same relative point in the cardiac cycle using ECG gating of data acquisition) is that the pressure waves measured at each point stem from the same ventricular systolic contraction. This eliminates potential errors arising from beat-to-beat variability of cardiac output and consequent variability of the pulse wave morphology. This directly observed method of PWV measurement has been used to demonstrate that an increase in PWV is observed in subjects with hypertension (Blacher *et al* 1991, Asmar *et al* 2001), polycystic ovary syndrome (Kelly *et al* 2002), diabetes (Lehmann *et al* 1992), atherosclerosis (Wada *et al* 1994), end-stage renal failure (London *et al* 2000) the elderly (Meaume *et al* 2001) and even acutely after smoking a single cigarette (Mahmud *et al* 2003).

In this thesis pulse wave velocity was assessed by using SphygmoCor which uses the principle of applanation tonometry to record the pulse waveform at radial and carotid sites. This technique is non-invasive and extremely well tolerated. Total duration of the PWV protocol is around 20-30 minutes and it was performed after each ten week phase of treatment in a temperature controlled room at 23°C. A minimum of three readings were taken on each study day. After allowing the volunteer to rest prone for 15 minutes, a highly-sensitive TY-306 pressure transducer (Fukuda Co,

Tokyo, Japan) was applied lightly to the radial artery, enough to flatten the arterial wall but not to occlude blood flow. A stable pressure flow waveform was sampled for 10 seconds. Brachial artery blood pressure was measured concurrently by a semi-automated oscillometric device. An aortic waveform was then generated using validated transfer functions. By combining measurement of waveforms from carotid and femoral sites in conjunction with ECG input and a measurement of the distance between the two sites, pulse wave velocity can also be calculated. PWV was calculated by an automated device from the measurement of the pulse transit time and the distance between the two recordings sites at the carotid and femoral arteries [PWV = distance(m)/transit time(s)]

#### ***2.2.2.4 Laboratory procedures***

##### ***2.2.2.4.1 Wire myography***

###### ***2.2.2.4.1.1 Introduction***

A technique for the investigation of the properties of small arteries with diameters down to approximately 100 µm was first suggested in 1972 by Bevan and Osher (Bevan and Osher 1972). This technique was developed by Mulvany and Halpern, with the method for investigation of a single vessel of diameter 100 – 1000 µm being published in 1977 (Mulvany and Halpern 1977). Over subsequent years this technique has been developed to allow investigation of initially two, and then four vessels simultaneously. This technique allows small vessels to be mounted as cylinder or ring preparations, using a relativelyatraumatic technique to preserve vascular endothelial functional integrity. The technique allows measurement of isometric responses (Mulvany 1999, Angus 2000) and is termed Mulvany-Halpern (wire) myography.

#### ***2.2.2.4.1.2 Preparation of arteries***

A single investigator, Angela Spiers, was blinded to treatment allocation, and undertook all myography studies. After the gluteal biopsy was dissected it was placed immediately in biopsy collection buffer (PSS) prewarmed to 37°C and transported to the laboratory. In the myography laboratory, resistance arteries were dissected from the biopsy. Where possible, four segments of artery, (200-400 µm diameter and 2mm in length) were mounted as ring preparations on two 40 µm stainless steel wires in a four-channel small vessel myograph. (Danish MyoTechnology, Aarhus, Denmark). One wire was attached to an isometric force transducer and the other to a movable micrometer. The vessels were then bathed twice in a physiological salt solution, KPSS (PSS solution with KCl substituted for NaCl on an equimolar basis). The temperature was maintained at 37 °C, and pH maintained at 7.4, with a gas mixture of 5% (v/v) CO<sub>2</sub> and 95%(v/v) O<sub>2</sub> being bubbled throughout the experiment.

#### ***2.2.2.4.1.3 Normalization of vessels***

Following a rest period of 30 minutes, each artery was stretched at one minute intervals to determine the resting tension-internal circumference (L) relationship. The La Place equation P=T/r (P is the effective pressure, T is the wall tension and r is the internal radius), was used to determine L<sub>100</sub>. This is the calculated internal diameter of the vessel would have *in vivo* when relaxed and subjected to a transmural pressure of 100mmHg (13.3Kpa). To achieve optimal contraction, each vessel was then set to the normalised internal diameter L<sub>1</sub> = 0.9L<sub>100</sub>.

#### ***2.2.2.4.1.4 Wire myography protocol***

Small vessel myography is now an established technique used in the measurement of small vessel contractility and relaxation, particularly in response to vasoactive agents. The measurement of insulin-mediated vasorelaxation by assessing its effect on NE-induced vasoconstriction has been

described previously by our own and other groups. Following the normalisation procedure described above, the vessels were maintained in physiological salt solution (PSS) at 37°C for a further 60 minutes. The vessels were then exposed twice to KPSS (PSS solution with KCL substituted for NaCl on an equimolar basis). Vessels were subsequently incubated in PSS for 30 min (washout) before a cumulative concentration response curve (CRC) to norepinephrine (NE) from  $10^{-9}$  M to  $3 \times 10^{-5}$  M was obtained. After a further 30 min washout, a plateau contraction was obtained with  $10^{-5}$  μM NE before a CRC to acetylcholine (ACh) from  $10^{-9}$  M to  $3 \times 10^{-5}$  M was performed. In this manner, a quantitative assessment of endothelium-dependent vasodilation can be obtained. Studies in our laboratory have previously shown that insulin causes relaxation of resistance arteries, and that this is attenuated in vessels from insulin resistant subjects (Kelly *et al* 2002). Accordingly, CRC to NE was repeated following incubation with insulin (1nM, 1h). Finally vessels were pre-incubated with insulin as before with the addition of 1mM L-NMMA to all vessels. A final concentration curve to NE was then obtained.

#### **2.2.2.4.1.5 Wire myography analyses**

In this thesis, one parameter was used to compare myography data between phases. Responses are expressed as a vessel contraction response, the change in active effective pressure (kPa), calculated as change in isometric tension from resting divided by the normalised internal radius, pD<sub>2</sub> (which is the negative logEC<sub>50</sub> of the concentration of agonist (norepinephrine) required to produce 50% of the maximal contractile response for 1uM norepinephrine).

#### **2.2.2.4.2 Cell culture**

##### **2.2.4.2.1 Preparation of HAEC media**

HAECs were cultured in large vessel endothelial cell growth medium supplemented with amphotericin B, gentamicin and an endothelial cell supplement (containing foetal bovine serum, heparin, human epidermal growth factor, human basic fibroblast growth factor, and hydrocortisone), supplied by TCS Cellworks (Boltolph Claydon, Buckinghamshire, UK). Media was prepared by mixing the components together and equilibrating at 37°C, 95% air and 5% CO<sub>2</sub> in a humidified incubator for a minimum of 40 minutes. The final serum concentration of the media was 2% (v/v) and the glucose concentration was 5mM.

##### **2.2.4.2.2 Growth of HAECs**

Cryopreserved HAECs (approximately 500,000 cells per vial) from either TCS Cellworks or Promocell (Heidelberg, Germany) were rapidly thawed at 37°C and seeded into 10 x 25cm<sup>2</sup> flasks containing 4ml/flask of prepared medium. The media was changed after 24 hours, and every 48 hours thereafter. The cells were maintained at 37°C, 95% air and 5% CO<sub>2</sub> in a humidified incubator and passaged at 70% confluence.

##### **2.2.4.2.3 Passage of HAECs**

Media was discarded and replaced with 4ml/flask 0.05% (w/v) Trypsin-EDTA (InvitrogenTM). Flasks were returned to the humidified incubator for 1 minute, and subsequently lightly tapped to aid cell detachment. Complete medium (4ml) was added to the flasks and the resultant suspension was transferred to 50 ml centrifuge tubes. The cell suspension was centrifuged for 5min at 157g and the supernatant discarded. The cells were resuspended in medium and distributed equally between the desired number of plates or flasks. On average the cells from one confluent 75cm<sup>2</sup>

flask were seeded into 5 x 10cm dishes. The medium was replaced 24 hours after trypsinisation, and every 48 hours thereafter. HAEC were utilised for experiments between passages 3 and 6. In no instances were cells used after passage 6.

#### ***2.2.4.2.4 Quiescence of HAECs***

Cells were quiesced for 3-6 hours prior to experiments. Medium containing no endothelial supplement, gentamicin or amphotericin B was used during this time.

#### ***2.2.4.2.5 Measurement of nitric oxide production in HAECs***

##### ***2.2.4.2.5.1 Detection of nitric oxide***

NO production was measured in cell culture media as follows. The detection of NO using the Sievers 280A Nitric Oxide Analyser is based on the reaction of NO with dissolved oxygen to form nitrite ( $\text{NO}_2^-$ ) and nitrate ( $\text{NO}_3^-$ ) in liquid, with nitrite being the predominant product in cell culture systems. The Sievers 280A analyser determines the amount of NO produced from the amount of nitrite present in the liquid sample injected into the purge vessel. Under the reducing conditions in the purge vessel (see Figure 2)  $\text{NO}_2^-$  is reduced to form NO. The reducing agent was prepared by dissolving 60mg of sodium iodide in 2ml of deionised water. This was added to 4ml of nitrogen flushed acetic acid in the purge vessel and nitrogen gas was bubbled through to purge the NO present from solution.



***Figure 2.2 Nitrite is reduced to form Nitric oxide***

The chemical reaction between sodium iodide and acetic acid results in reducing conditions that cause the reduction of nitrite ( $\text{NO}_2^-$ ) to NO in the liquid sample injected into the meter's purge vessel.

A photomultiplier measures any chemiluminescence that has resulted from the reaction of ozone with the NO produced in the purge vessel and produces a signal. This signal is converted to an electrical potential and displayed in mV by the NO analyser. The electrical potential displayed is related to the amount of nitrite present in the original liquid sample injected into the purge vessel. On each day of sample analysis a standard curve was prepared. A standard solution of 100mM  $\text{NaNO}_2$  stock was prepared using deionised water and serial dilutions were made to give 10nM, 100nM, 1 $\mu\text{M}$  and 10 $\mu\text{M}$  standards. A calibration curve was generated from the amount of NO produced from the reduction of nitrite present in the standard solutions. HAEC culture medium of unknown nitrite concentration was analysed against this standard curve by computer analysis and the amount of NO present determined (in nM).

#### ***2.2.4.2.5.2 Preparation of adenoviruses and infection of human endothelial cells***

Control and Dominant negative AMPK adenovirus (Ad. $\alpha$ 1-DN) were a generous gift from Dr Fabienne Foufelle, Paris, France and were propagated and purified as described previously (Morrow *et al* 2003). Human endothelial cells were infected with 10 pfu/cell adenovirus in complete medium and the cells cultured for 48 h prior to experimentation.

#### ***2.2.4.2.5.3 Sample preparation for NO analysis***

HAECs cultured in 12-well plates were quiesced (section 2.2.4.2.4), the media subsequently removed and cells incubated in 0.5 ml/well KRH buffer in a 37°C water bath for 1h. The medium was then removed and replaced with fresh KRH buffer (0.5 ml/well) in the presence of various

concentrations of test substances (metformin, phenformin and rosiglitazone). An 80µL sample was taken to ascertain a basal level of NO in the cell media. After incubation for various durations with metformin, phenformin or rosiglitazone, 80µL samples of media were removed and the amount of NO determined. The appropriate control experiments were performed in the presence of the eNOS inhibitor, N(G)-nitro-L-arginine methyl ester (L-NAME, 0.1 mM for 1 hour), calcium ionophore (A23187, 3 µM for 15 minutes) and insulin (1µM for 15 minutes). The dose of L-NAME used in all experiments is similar to the physiological plasma concentration of L-arginine (Goumas *et al* 2001). The dose and duration of A23187 and insulin stimulation was based on previous investigations in our laboratory (Morrow *et al* 2003). Data is presented as L-NAME-sensitive NO synthesis as a percentage difference in NO between the basal and stimulated HAEC media.

#### **2.2.4.2.6 General methods**

##### **2.2.4.2.6.1 HAEC lysate preparation**

HAECs cultured in 100mm dishes were quiesced for 3 - 4 hours at 37°C, 95% (v/v) air, 5% (v/v) CO<sub>2</sub>, in serum free medium. The medium was replaced with 5ml KRH buffer and cells placed in a 37°C incubator for 1hour. The medium was replaced with 5 ml of fresh KRH buffer containing metformin, phenformin or rosiglitazone and incubated for various durations at 37°C. The medium was replaced with 0.5 ml of lysis buffer (see section 2.1.7). The cell extract was then scraped off and transferred to a microcentrifuge tube. Extracts were vortex-mixed and centrifuged (17530g, 4°C, 3 minutes). The supernatants were then stored at -20°C prior to use.

##### **2.2.4.2.6.2 Adipose tissue lysate preparation**

From the gluteal adipose sample approximately 2g was snap frozen in liquid nitrogen and stored in -80°C. When all samples had been collected the samples were thawed for batch analysis. Adipose

lysates were prepared as follows. 0.5ml of lysis buffer (section 2.1.7.) was added to each microcentrifuge tube and then the tissue was homogenised by 20 passes with a Dounce homogeniser. Homogenates were then centrifuged at 805g at 4°C for 5 minutes. The resulting infranatant was aspirated and stored at -80° prior to use. The resultant supernatant and pellet was discarded.

#### ***2.2.4.2.6.3 Gel electrophoresis***

Endothelial cell lysates were resolved on sodium dodecyl sulphate (SDS)/polyacrylamide gel electrophoresis (SDS-PAGE), carried out using the Bio-Rad mini-protean II system. The resolving gel consisted of approximately 10% acrylamide (percentage can vary depending on the molecular mass of the protein under study), 375mM Tris - HCl (pH 8.8), 0.1% (w/v) SDS, polymerised with 0.1% (w/v) ammonium persulphate and 0.05% (v/v) TEMED. The stacking gel consisted of 5% acrylamide, 125mM Tris - HCl (pH 6.8), 0.1% (w/v) SDS, polymerised with 0.1% (w/v) ammonium persulphate and 0.05% (v/v) TEMED. Lysate samples were mixed with 4 x sample buffer, and heated at 95°C for 5 minutes. Samples were loaded into the wells in the stacking gel. Broad range pre-stained molecular weight markers (15µl, New England Biolabs) were added to a minimum of one well. Gels were immersed in electrode buffer and proteins were resolved using a potential difference of 80V through the gel. Once the protein samples had passed through the stacking gel a constant voltage of 100V - 150V was applied (generally 120V).

#### ***2.2.4.2.6.4 Immunoblotting***

After gel electrophoresis, proteins were transferred to nitrocellulose using the Bio-Rad trans-blot apparatus. Nitrocellulose and Whatmann 3MM filter paper was cut to size using scissors and a ruler and then equilibrated in transfer buffer. The gel was laid on the nitrocellulose and sandwiched

between 2 sheets of filter paper and 2 sponges. This was placed in a transfer cassette and transfer of proteins was carried out at 400mA for 2 hours or 40mA overnight in a transfer tank.

#### ***2.2.4.2.6.5 Immunodetection of proteins***

Nitrocellulose membrane was incubated with TBST containing 5% (w/v) non-fat milk powder, for 1 hour at room temperature to block non-specific binding sites. The membrane was washed 3 times over 15 minutes with TBST before the primary antibody was added. The concentration of primary antibody was determined by the manufacturer's data sheet and was diluted to the required concentration in TBST containing either milk powder or BSA. The nitrocellulose was incubated with shaking with the antibody as determined by manufacturer data sheet but typically this was at 4°C overnight. After the incubation with primary antibody was completed, the nitrocellulose was washed 3 times with TBST and the nitrocellulose incubated with secondary antibody for at least 1 hour. The secondary antibody used were species specific and HRP-linked raised to the (tail) Fc-region of the primary antibody IgG molecule, and were used at a 1:2000 dilution in TBST containing 5% (w/v) non-fat milk powder. After incubation with the secondary antibody was completed, the nitrocellulose was again washed 3 times with TBST and twice with TBS. Immunlabelled proteins were visualised using the ECL (enhanced chemico-luminescence, section 2.1.7) system. Solution 1 (2ml) was added to solution 2 (2ml), mixed, and incubated with the nitrocellulose for 1 minute. The nitrocellulose was exposed to film in an X-ray cassette and developed using a Kodak X-Omat S film processor. The films were then underwent densitometric analysis using NIH image software (Scion Image).

#### ***2.2.4.2.6.6 Immunoprecipitation of AMPK from lysates***

AMPK was immunoprecipitated from endothelial cell and adipose tissue lysates using isoform-specific anti-AMPK antibodies. AMPK $\alpha$ 1 and  $\alpha$ 2 subunit) antibodies (2 $\mu$ g / lysate) was pre-bound to 10 $\mu$ l per sample of 25% (w/v) protein G Sepharose in IP buffer (section 2.1.7) by incubation for 1 h at 4 °C on a roller mixer. The mixture was then centrifuged (17530g 1min; 4 °C) and the bead were washed with 5 x 1 ml of IP buffer prior to the addition of the lysate (100 $\mu$ g). Beads and lysate were incubated together for 2 hours at 4°C. Beads were again washed 2 times in IP buffer and 2 times with high salt IP buffer (containing 1M NaCl ) then a final wash with HEPES-Brij assay buffer. All washes were at 4°C. The final pellet was then resuspended in HEPES-Brij assay buffer to be assayed for AMPK activity.

#### ***2.2.4.2.6.7 Preparation of Bradford Reagent and protein assays***

Protein concentration of lysates was determined by the method of Bradford (Bradford 1976). Bradford reagent was prepared (5% [v/v] ethanol, 5.5% [v/v] phosphoric acid, 40mg/l Coomassie G), filtered and stored protected from light. To determine the protein concentration in lysate sample a 5 $\mu$ l aliquot of each sample was added to 95 $\mu$ l of sterile water and 1ml of a protein assay reagent. After mixing and equilibration, the absorbance was read in a spectrophotometer at a wavelength of 595nm measured against a reference sample containing 1ml protein assay reagent and 100 $\mu$ l dH<sub>2</sub>O. A standard curve of 2-6 $\mu$ g/ml bovine serum albumin (BSA) was prepared and the protein concentration of lysates determined by comparing values to the standard curve. This was adapted from the method of Bradford (Bradford 1976).

#### ***2.2.4.2.6.8 AMPK assays***

Cell lysates were prepared as described in section 2.2.4.2.6.1 and 2.2.4.2.6.2. AMPK activity was determined as follows. AMPK immunoprecipitations were carried out using 100 $\mu$ g of protein

lysate, 2 $\mu$ g of anti  $\alpha$ 1 AMPK antibody, and protein G sepharose beads (see section 2.2.4.2.6.6). Reaction mixtures (20 $\mu$ l) containing 5 $\mu$ l HEPES-Brij assay buffer, 5 $\mu$ l 1mM AMP in HEPES-Brij buffer, 5 $\mu$ l of 1mM SAMS peptide in HEPES-Brij buffer and 5  $\mu$ l of the immunoprecipitated lysate (pellet) resuspended in HEPES-Brij assay buffer were prepared on ice. The reaction initiated by the addition of 5 $\mu$ l of [ $\gamma$ - $^{32}$ P] ATP solution (1mM [ $\gamma$ - $^{32}$ P] ATP with a specific activity in the range 250,000 to 500,000 cpm/pmol, 25mM MgCl<sub>2</sub>). Assays were performed in triplicate including blank reactions prepared by substituting HEPES-Brij buffer for substrate peptide. After incubation at 30°C for 10min, 15 $\mu$ l of the reaction mixture was removed and spotted onto 1cm<sup>2</sup> squares of Whatmann P-81 phosphocellulose paper, and the paper dropped into 1% (v/v) phosphoric acid. The P-81 paper squares were washed twice with phosphoric acid and then rinsed with water and dried. The incorporation of  $^{32}$ P into the substrate peptide was determined by immersion in 5 ml toluene-based scintillation fluid (Ecoscint™) and liquid scintillation counting. Units of AMPK activity were expressed as nmol of [ $\gamma$ - $^{32}$ P] incorporated into substrate peptide per min at 30 °C. In the case of adipose tissue AMPK assays experiments were performed in 4 batches of 10 lysates with AMPK activity determined as multiples of a protein of previously documented AMPK activity.

### **2.2.2.5 Statistical analysis**

Analysis was performed using the Minitab statistical package. All data are expressed as standard error of the mean unless stated otherwise. Equivalent values from each study phase were compared using paired student t tests. In all cases, p<0.05 was taken as signifying conventional levels of statistical significance. Power calculations based on previous wire myography studies suggest that 16 patients will be required to demonstrate a 10% change in pD<sub>2</sub> (change of sensitivity of ACh dose-response curve) with 90% power (p<0.05, two-tailed). We planned to recruit 20 patients to allow for potential drop-out and technical difficulties with myography protocols. The crossover

study design allows us to use paired t-tests for comparison of the primary outcome measures, namely *ex vivo* resistance artery endothelial function ( $pD_2$  for ACh curves).

## **Chapter 3**

***Studies on vascular function and plasma asymmetric dimethylarginine (ADMA) concentrations after metformin and gliclazide therapy in patients with Type 2 diabetes.***

### **3.1 Introduction**

Metformin is a widely used and useful hypoglycaemic agent. In the United Kingdom Prospective Diabetes Study (UKPDS, 1998), metformin reduced the incidence of myocardial infarction by 39% in comparison with conventional treatment. In addition, when the metformin group was compared with a group treated with sulphonylurea or insulin, there was a significant reduction in the incidence of stroke. These results are supported by a recent cross-sectional study in which mortality rates were significantly reduced in patients on metformin monotherapy compared with sulphonylurea monotherapy (Johnson *et al* 2002).

Despite these impressive effects, the mechanism of this vascular protective action of metformin remains unclear and the question that remains largely unanswered from UKPDS is why metformin has beneficial cardiovascular effects in overweight diabetic patients? In some animal models, metformin appears to have a vascular action. In spontaneously hypertensive rats, metformin reduced blood pressure possibly via modulation of adrenergic tone (Peuler *et al* 1999). In addition, in streptozocin-induced diabetic rats, metformin lowers blood pressure and improves vascular endothelial function (Katakam *et al* 2000). In man, metformin does not alter BP although BP responses to intravenous infusions of norepinephrine and angiotensin II were blunted supporting the concept that metformin might modulate vascular responsiveness (McAuley *et al* 1997). Furthermore, vascular studies using metformin in T2DM have yielded conflicting data with one study demonstrating a modest improvement while another reporting no change in vascular

endothelial function after 3 and 4 months respectively of metformin therapy versus placebo (Mather *et al* 2001, Natali *et al* 2004). In this chapter, we study of the effect of metformin versus gliclazide on vascular function in subjects with T2DM using a randomised, double-blind, glycaemia controlled crossover design.

Recent work on the cellular mechanism of metformin action has led to the suggestion that it could potentially have beneficial vascular actions, possibly by indirect or by direct stimulation of endothelial nitric oxide synthase (eNOS) activity (Davis *et al* 2006). This chapter investigates a potential mediator of metformin's vascular action; ADMA. One way in which this metformin might exert beneficial effects is by the reduction of asymmetrical dimethylarginine (ADMA) concentrations. ADMA, a guanidino-substituted analogue of L-arginine, is a potent endogenous competitive inhibitor of endothelial nitric oxide (NO) synthase (Palm *et al* 2007). ADMA is produced by methylation of protein bound L-arginine which is catalysed by protein arginine methyltransferases (Palm *et al* 2007). The predominant mode of elimination of ADMA occurs via enzymatic degradation by dimethylarginine dimethylaminohydrolase (DDAH) to citrulline and dimethylamine (Achan *et al* 2003). Increased levels of ADMA reduce NO formation and are associated with endothelial dysfunction. Plasma levels of ADMA have been found to be increased in hypercholesterolaemia (Boger *et al* 1998), hypertriglyceridaemia (Lundman *et al* 2001), hyperhomocyst(e)inemia (Stuhlinger *et al* 2003), diabetes mellitus (Abbasi *et al* 2001), gestational diabetes (Mittlemayer *et al* 2002) and, most recently, polycystic ovary syndrome (Heutling *et al* 2008). There is a close relationship between concentrations of ADMA and insulin resistance (Stuhlinger *et al* 2002). Indeed, in several prospective studies, ADMA has been noted to be an independent predictor of cardiovascular events (Mittlemayer *et al* 2002, Valkonen *et al* 2001, Krzyzanowska *et al* 2007). Metformin was reported to decrease the plasma ADMA concentrations in poorly controlled subjects with Type 2 diabetes in an open labelled, uncontrolled study without a

placebo arm; the fall in ADMA concentration was associated with improved fasting plasma glucose, although the study failed to demonstrate a relationship between the decrease in ADMA concentrations and improvement in glycaemic control (Asagami *et al* 2002). Importantly, no assessment of vascular function was made in this study. Furthermore, in a recent interesting study, a subgroup of women with PCOS who received metformin demonstrated reductions in ADMA concentration independent of a reduction in BMI and fasting blood glucose (Heutling *et al* 2008). There was a marginal improvement in carotid intima media thickness in favour of metformin therapy. However, once again, this was also an uncontrolled study involving a small subset of patients without the benefit of a placebo or control arm and it is clear that more data would be beneficial to help confirm any metformin-induced reduction in ADMA concentration and, if so, whether such a change would be in association with beneficial effects on vascular function in overweight subjects with Type 2 diabetes.

I hypothesized therefore that a short duration of metformin therapy would improve vascular endothelial function in association with a change in the circulating concentration of ADMA in comparison with gliclazide therapy.

### ***3.2 Aims of this chapter***

This chapter was initiated to extend the observations discussed above and addressed the following issues.

- i.** Does metformin confer benefit in vascular function when compared with gliclazide therapy?
- ii.** Are the effects of metformin on ADMA reproducible in a randomised controlled double-blind crossover trial?
- iii.** Does metformin have a beneficial effect on ADMA concentrations in subjects with Type 2 diabetes independent of changes in glycaemic control?

Accordingly, this chapter compares the effect of metformin and gliclazide on plasma ADMA and vascular function in subjects with Type 2 diabetes using a randomised, double-blind, glycaemia controlled crossover design.

### **3.3 Results**

#### **3.3.1 Clinical characteristics at recruitment**

At the time of recruitment; 12 subjects were on metformin monotherapy, 6 were diet controlled and 2 were on gliclazide monotherapy. 19 subjects were on statin therapy. 17 subjects were on ACE inhibitor therapy. No subjects dropped out of the study. There were no reports of changes to drug therapy during the study period. The clinical characteristics of the 20 subjects at recruitment are summarised in Table 3.1. The mean (SD) age of study participants was 56.5 (3.9) years; mean (SD) BMI was 31.4 (4.3) kg/m<sup>2</sup>. The mean (SD) HbA1c at recruitment was 8.3 (1.2) %. The mean (SD) creatinine was 105.3 (18.8) µmol/l. The mean (SD) duration of diabetes was 3.5 (2.6) years (minimum 6 months).

**Table 3.1: Clinical characteristics at recruitment**

Parameter	Mean	SD
Age	56.5yrs	3.9
HbA1c	8.3%	1.2
Creat	105.3	18.8
BMI	31.4kg/m <sup>2</sup>	4.3
Duration of diabetes	3.5yrs	2.6

### **3.3.2 Body mass index and blood pressure**

Mean BMI was not different after each period of therapy ( $p=0.80$ ), (Table 3.2). There was also no significant difference in either systolic blood pressure ( $p=0.906$ ) or diastolic blood pressure ( $p=0.193$ , Table 3.2).

**Table 3.2: Clinical measures**

Outcome	Raw outcomes by treatment		Treatment Effect		
	Metformin	Gliclazide	Estimate	95% CI	p-value
BMI (Kg/m <sup>2</sup> )	31.0 (4.3)	31.0 (4.5)	-0.1	(-0.5, 0.4)	p=0.80
SBP (mmHg)	137.2 (19.7)	137.0 (21.0)	-0.3	(-4.3, 3.8)	p=0.91
DBP (mmHg)	72.3 (9.5)	75.0 (11.8)	2.8	(-1.2, 6.7)	p=0.19

### **3.3.3 Metabolic parameters**

Gliclazide therapy was more effective at lowering HbA1c than metformin [7.8% +/- 1.74 versus 8.3% +/- 1.7, p<0.001]. This was complemented by significantly lower fasting blood glucose with gliclazide versus metformin therapy [10.3mM +/- 3.2 versus 12.1mM +/-, p=0.005]. Furthermore, average LDL cholesterol concentration was significantly lower after gliclazide therapy compared with metformin therapy [2.42 +/-0.74 versus 2.79 +/- 0.74, p=0.022]. There was, however, no significant difference in total cholesterol, HDL cholesterol or triglyceride between the two stages of treatment (Table 3.3).

**Table 3.3: Metabolic parameters**

Outcome	Raw outcomes by treatment		Treatment Effect		
	Metformin	Gliclazide	Estimate	95% CI	p-value
HbA1c (%)	8.34 (1.70)	7.82 (1.74)	0.53	(0.29, 0.76)	p<0.001
Glucose (3.5-5.5) mM	12.1 (4.7)	10.3 (3.2)	1.8	(0.7, 2.9)	p=0.005
Insulin (U/L)	15.6 (13.8)	14.3 (6.0)	1.3	(-4.9, 7.5)	p=0.69
Cholesterol (mmol/l)	5.16 (1.16)	4.75 (1.59)	0.40	(-0.02, 0.78)	p=0.054
HDL-C (mmol/l)	1.25 (0.24)	1.20 (0.24)	0.05	(-0.01, 0.11)	p=0.13
LDL-C (mmol/l)	2.79 (0.74)	2.42 (0.73)	0.40	(0.09, 0.70)	p=0.022
Triglycerides (mmol/l)	2.78 (3.76)	2.94 (4.80)	-0.16	(-0.70, 0.38)	p=0.58

### **3.3.4 ADMA and related metabolites**

ADMA was lower following metformin therapy [0.48 $\mu\text{mol/l}$  +/- 0.08 versus 0.50 $\mu\text{mol/l}$  +/- 0.07, p=0.019]. The raw data plot (Figure 3.1) demonstrates that 13 out of the 20 subjects had lower values of ADMA concentrations after metformin, five had lower ADMA concentrations after gliclazide, and two subjects showed no difference. There was no significant difference in SDMA or arginine concentrations between the two treatment groups (Table 3.4). Moreover, there was no difference in the arginine/ADMA ratio [127.1 +/- 24.4 versus 129.6 +/- 23.94, p=0.74].

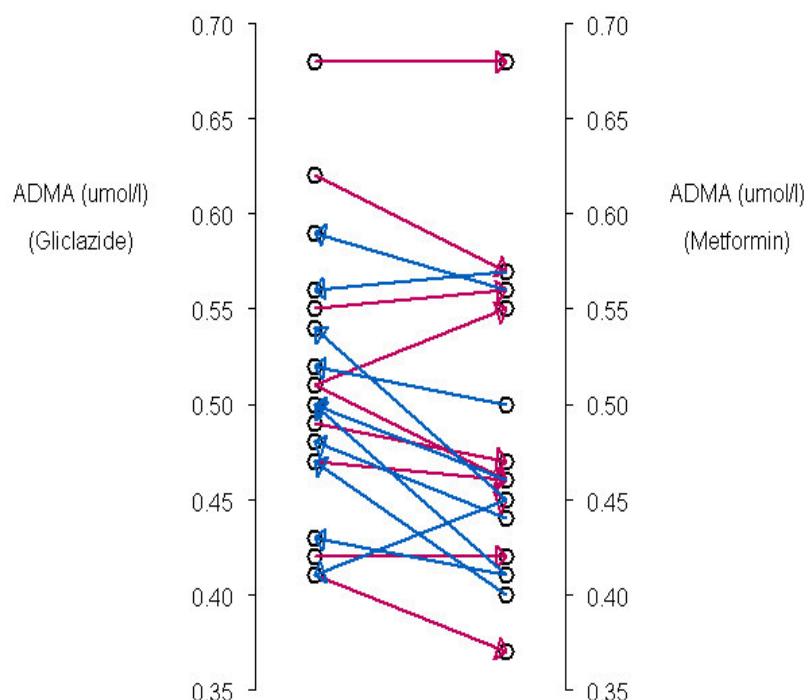
### **3.3.5 Pulse wave velocity**

A satisfactory number of acceptable tracings in both periods of treatment could not be obtained for four subjects and these data were discarded. Mean PWV for the remaining 16 subjects was not different after each period of therapy (p=0.40, Table 3.4). Finally, there was also no correlation between metformin to gliclazide differences in ADMA and PWV, respectively (r=0.069, p=0.80), (Figure 3.2).

**Table 3.4: PWV, ADMA and related metabolites**

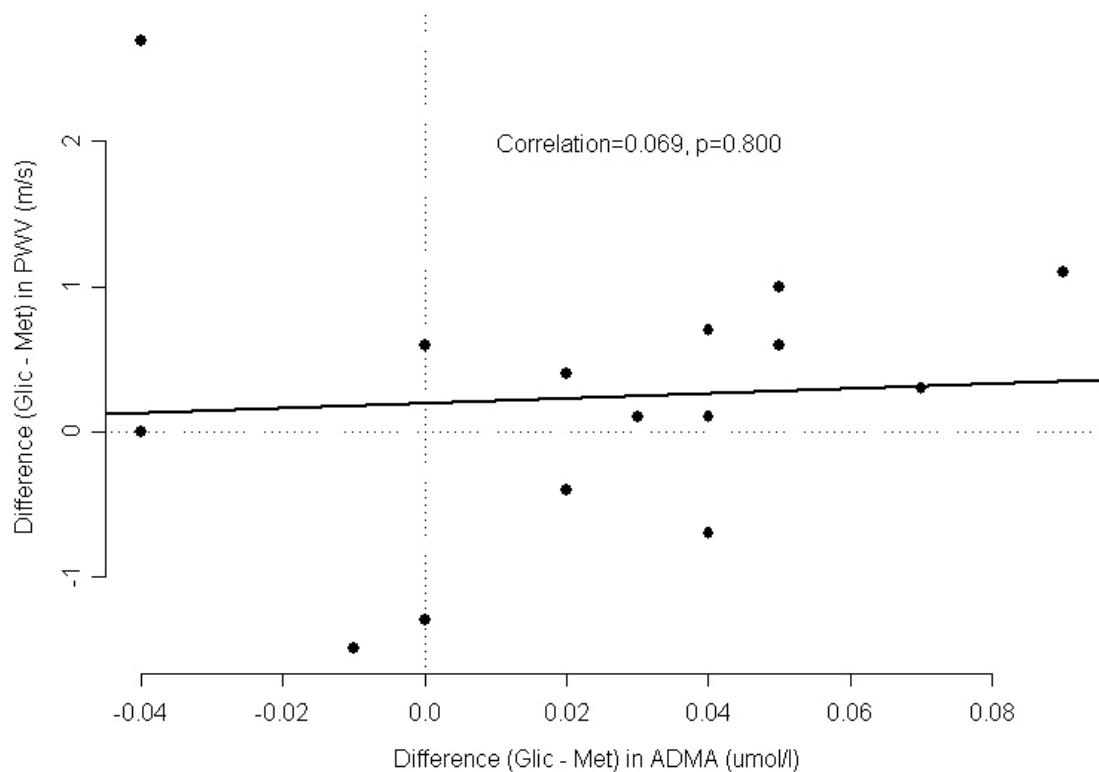
Outcome	Raw outcomes by treatment		Treatment Effect		
	Metformin	Gliclazide	Estimate	95% CI	p-value
PWV (m/s)	7.89 (1.64)	8.12 (1.49)	-0.22	(-0.73, 0.28)	p=0.40
ADMA ( $\mu\text{mol/l}$ )	0.48 (0.08)	0.50 (0.07)	-0.02	(-0.01, -0.04)	p=0.019
SDMA ( $\mu\text{mol/l}$ )	0.41 (0.08)	0.42 (0.09)	-0.01	(-0.04, 0.01)	p=0.36
HARG ( $\mu\text{mol/l}$ )	2.09 (0.72)	2.18 (0.62)	-0.09	(-0.34, 0.15)	p=0.46
ARG ( $\mu\text{mol/l}$ )	61.6 (17.0)	65.6 (16.7)	-4.0	(-10.2, 2.2)	p=0.22

**Figure 3.1: Comparison of ADMA concentrations for individual subjects ( $n=20$ ) after gliclazide and metformin.**



The raw data plot demonstrating that 13 out of the 20 subjects had lower values of ADMA concentrations after metformin, five had lower ADMA concentrations after gliclazide, and two subjects showed no difference. Red arrows indicate those patients randomised to gliclazide first and blue arrows indicate those patients randomised to metformin first.

**Figure 3.2: Correlation of metformin to gliclazide differences in ADMA and PWV.**



### **3.3.6 Wire myography**

In total, forty biopsies (twenty subjects x 2) were performed. Twenty two biopsies successfully harvested vessels of a suitable diameter. Eighteen subjects had at least one successful biopsy with four subjects having full paired myography data. 33 vessels were successfully harvested from the 11 subjects in the metformin phase versus 29 vessels from 7 subjects in the gliclazide phase. Four vessels were not successfully isolated in all cases so responses have been analysed in a paired and unpaired manner.

#### ***3.3.6.1 Comparison of the effect of metformin and gliclazide on norepinephrine-induced vasoconstriction.***

For this analysis, results were obtained from 18 subjects, with 4 subjects having data from both metformin and gliclazide phases. Each subject had data from between one and four arteries available for analysis, the mean of which was used as the CRC for that volunteer. Vessels from both groups displayed the characteristic sigmoid relationship to increasing concentrations of NE. There was no evidence of tachyphylaxis in response to NE in vessels used as controls for time of study, which were exposed to NE. To compare NE-induced vasoconstriction during metformin and gliclazide phases, data were analyzed as paired [*i.e.* subjects with completed myography studies from both phases: ( $n = 4$ )] or unpaired (*i.e.* including data from subjects with only one successful biopsy;  $n = 18$ ). After paired analysis, there was a borderline statistically significant difference in the  $pD_2$  between phases [mean  $pD_2$  during metformin,  $6.72 \pm 0.17$ ;  $pD_2$  during gliclazide,  $6.35 \pm 0.22$  ( $P = 0.05$ )]. There was, however, no statistical difference in the  $pD_2$  between phases [mean  $pD_2$  during metformin,  $6.49 \pm 0.16$ ;  $pD_2$  during gliclazide,  $6.16 \pm 0.14$  ( $P = 0.12$ )] following unpaired analysis.

### ***3.3.6.2 Acetylcholine-mediated (endothelial-dependent) vasodilation***

Both groups also exhibited similar endothelium-dependent relaxation to Ach: unpaired data, maximum relaxation, metformin  $64.9 \pm 5.0\%$  ( $n = 8$ ) vs. gliclazide  $69.6 \pm 7.2\%$  ( $n = 7$ ); ( $P = 0.59$ ). Paired data were only available for one subject.

### ***3.3.6.3 Insulin-mediated attenuation of NE-induced vasoconstriction***

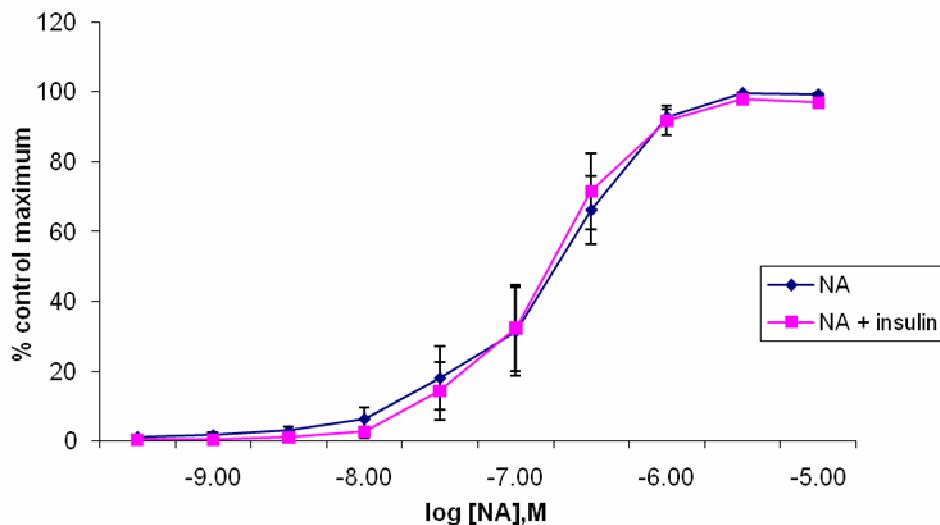
#### ***3.3.6.3.1 Metformin phase***

Figure 3.2 demonstrates the effect of preincubation of vessels with insulin on NE-mediated vasoconstriction during the metformin phase. Vessels were preincubated with insulin [1 nM]. Insulin-mediated attenuation of NE-induced vasoconstriction (a right shift in the CRC) or in maximal tension developed in response to NE was not demonstrated:  $pD_2$  control,  $6.48 \pm 0.13$ ;  $pD_2$  insulin,  $6.48 \pm 0.15$  ( $P = 0.99$ ); maximum contraction,  $97 \pm 1.5\%$  ( $P = 0.10$  vs. control).

#### ***3.3.6.3.2 Gliclazide phase***

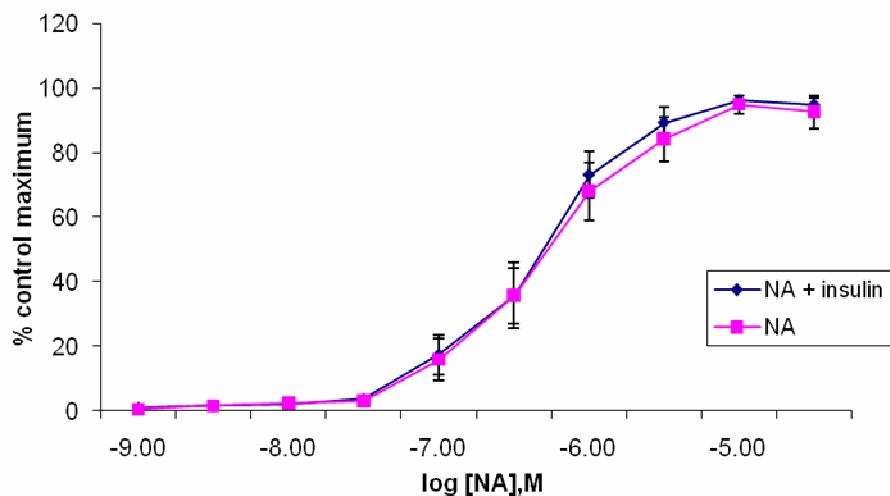
Figure 3.3 demonstrates the effect of preincubation of vessels with insulin on NE-mediated vasoconstriction during the gliclazide phase. Vessels were preincubated with insulin [1 nM]. Insulin-mediated attenuation of NE-induced vasoconstriction (a right shift in the CRC) or in maximal tension developed in response to NE was not demonstrated:  $pD_2$  control,  $6.14 \pm 0.12$ ;  $pD_2$  insulin,  $6.14 \pm 0.13$  ( $P = 0.94$ ); maximum contraction:  $95 \pm 1.5\%$  ( $P = 0.19$  vs. control).

**Figure 3.3: NE-induced vasoconstriction in Metformin phase**



The effect of pre-incubation with insulin 1nM on NE induced vasoconstriction in human resistance vessels dissected during the metformin phase was examined ( $n=11$ ). Insulin-mediated attenuation of NE-induced vasoconstriction (a right shift in the CRC) or in maximal tension developed in response to NE was not demonstrated. Error bars represent standard errors.

**Figure 3.4: NE-induced vasoconstriction in Gliclazide phase**

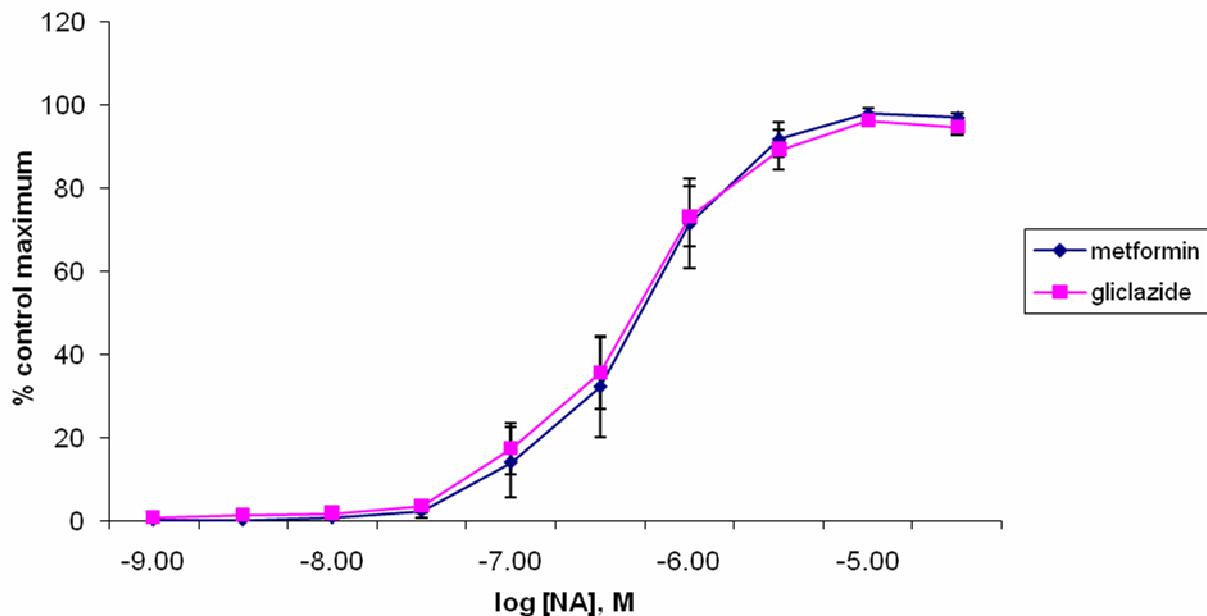


The effect of pre-incubation with insulin 1nM on NE induced vasoconstriction in human resistance vessels dissected during the gliclazide phase was examined (n=7). Insulin-mediated attenuation of NE-induced vasoconstriction (a right shift in the CRC) or in maximal tension developed in response to NE was not demonstrated. Error bars represent standard errors.

### ***3.3.6.3.3 Comparison of insulin action during metformin and gliclazide phases***

To compare insulin-mediated relaxation of NE-induced vasoconstriction during metformin and gliclazide phases (Figure 3.4) data were analyzed as paired [*i.e.* subjects with completed myography studies from both phases: 1 nM insulin ( $n = 4$ ) or unpaired (*i.e.* including data from subjects with only one successful biopsy;  $n = 18$ ). pD<sub>2</sub> and maximum contraction were compared between phases. On analysis of paired data, there was no significant difference in any of these variables at 1 nM insulin [pD<sub>2</sub>: metformin,  $6.68 \pm 0.2$ ; gliclazide,  $6.39 \pm 0.21$  ( $P = 0.42$ ); maximal contraction: metformin,  $99.5 \pm 1.3\%$ ; gliclazide,  $96.9 \pm 1.2\%$  ( $P = 0.26$ )]. On analysis of unpaired data, there was no significant difference in any of these variables at 1 nM insulin [pD<sub>2</sub>: metformin,  $6.48 \pm 0.13$ ; gliclazide,  $6.12 \pm 0.13$  ( $P = 0.07$ ); maximal contraction: metformin,  $97.3 \pm 1.5\%$ ; gliclazide,  $95.9 \pm 1.4\%$  ( $P = 0.49$ )].

**Figure 3.5: Comparison of metformin and gliclazide phases**



Insulin action, measured as pD<sub>2</sub> in isolated human vessels, was compared between metformin and gliclazide phases (n=18). On analysis of unpaired data, there was no significant difference in pD<sub>2</sub> and maximum contraction between phases at 1 nM insulin. Error bar represent standard errors.

### 3.4 Discussion

This chapter compared the effects of metformin versus gliclazide on large artery stiffness, resistance artery function and circulating concentrations of ADMA using a randomised, double-blind crossover design in subjects with Type 2 diabetes. One of the main aims was to confirm or refute a beneficial effect of metformin on circulating ADMA concentrations in view of recent supportive data to this effect from uncontrolled trials. It was also hypothesised that ADMA might not only be a predictive marker but also a causative risk factor for cardiovascular disease and thus any metformin-induced reduction in ADMA concentration would be accompanied by an improvement in vascular function. The data demonstrate that metformin does indeed lower ADMA compared with gliclazide despite better glycaemia changes with the latter, supporting a glucose-independent mechanism for this metformin effect. That said, the reduction in ADMA was modest and the observed magnitude of difference contrasts with reports of much larger reductions in ADMA concentration in uncontrolled studies of metformin therapy in women with PCOS as well as poorly controlled subjects with Type 2 diabetes, where the drug was added to diet control or sulphonylurea therapy (Abbasi *et al* 2001, Heutling *et al* 2008). Finally, the data shows that the change in ADMA concentration was not associated with any difference in large artery stiffness as measured by pulse wave velocity or resistance artery function as measured by wire myography.

A major stimulus for this study was the notion that metformin therapy confers cardiovascular benefit in overweight diabetic subjects when compared with sulphonylurea therapy or insulin. It was hypothesised that this would be reflected in a change in pulse wave velocity, an arterial stiffness measure which also has a vascular function component. Abnormal large artery function plays an important role in the development of cardiovascular disease. In the normal arterial system, there is a steep gradient of increasing arterial stiffness moving outward from the heart. In a young adult, pulse wave velocity (PWV), a close correlate of arterial wall stiffness, is only 4 to 6 m/s in

the highly compliant proximal aorta and increases to 8 to 10 m/s in the stiffer peripheral muscular arteries (Yasmin *et al* 2004). Carotid-femoral PWV (cfPWV) is a measure of aortic distensibility. It is known to predict all-cause and cardiovascular mortality in patients with end-stage renal failure, hypertension, diabetes and the elderly, independently of known confounding factors (Blacher *et al* 1999, Laurent *et al* 2001, Meaume *et al* 2001, Cruickshank *et al* 2002). It was hypothesised that metformin therapy would lead to reduced PWV in subjects with Type 2 diabetes when compared with gliclazide therapy.

Traditionally, information about the properties of small arteries had to be inferred from haemodynamic studies or visual examination of vessels of readily accessible vascular beds. Within the last decade techniques have become available that allow routine *in vitro* investigation of small arteries. One technique popularised through the work of Mulvany is wire myography (Mulvany *et al* 1990). This involves the harvesting of small resistance arteries from adipose tissue (the buttock) and their mounting over two fine wires within small chambers filled with physiological salts solution (PSS). This allows ex vivo investigation of arterial tissue. Various agents may be added to the chambers to investigate vascular responses and these are measured using computer software specially calibrated to detect small changes in vessel tone.

The hypothesis tested was that metformin has similar beneficial effects on different parts of the vasculature; aortic compliance measured by cfPWV and endothelial function by wire myography of resistance arteries. The results here have demonstrated that in subjects with Type 2 diabetes, insulin-mediated attenuation of NE-induced vasoconstriction (a right shift in the CRC) is not demonstrated. The data are insufficiently powered to detect any small changes in vascular function in association with this dose and duration of treatment. With this in mind, it is particularly important to consider the implication of the missing data set in the wire myography data; eighteen subjects had

at least one successful biopsy with myography data with only four subjects having paired myography results. An absence of vessels of suitable diameter was the reason for failure to obtain vessels from biopsies with no myography data with the vessel yield in this study being lower than other wire myography studies completed by our group.

The study also failed to show a significant benefit in pulse wave velocity and I need to consider why. In the present study subjects were not randomised to the maximum dose of each drug and the duration of treatment was relatively short at 10 weeks. Whether a longer duration of each treatment in higher dose would have been associated with a larger change in ADMA concentration with measurable improvements in vascular function in favour of metformin is unknown and could be the subject of further study. Additionally, it is important to consider whether some of the other changes noted may have accounted for the variable alteration in vascular function that we noted. Recently it has been demonstrated that weight loss in morbidly obese patients can decrease circulating ADMA concentrations (Krzyanowska *et al* 2004). There was no significant difference in weight after each therapy excluding this as a confounding factor. While metformin was associated with a fall in ADMA, the gliclazide arm was associated with improved glycaemic control as assessed by fasting blood glucose and haemoglobin A1<sub>c</sub>. The study design aimed to match glycaemic control at the end of each phase but this, in the event, proved not to be possible. Associated with these changes was a small improvement in plasma LDL cholesterol. Thus, it is reasonable to speculate that confounding effects related to better short term glycaemia and LDL-cholesterol improvements with gliclazide may have attenuated any metformin-induced change in ADMA being associated with an associated beneficial improvement in vascular function. Nevertheless, despite these changes, the data still recorded a significant reduction in ADMA with metformin, which adds some confidence to the validity of this latter observation. This finding is important since increasing evidence indicates that

higher levels of ADMA are predictive of cardiovascular events independently of other risk factors (Krzyszewska *et al* 2007, Asagami *et al* 2002, Heutling *et al* 2008).

In conclusion, this chapter provides the strongest evidence thus far to support a beneficial effect of metformin therapy on circulating ADMA concentrations in patients with type 2 diabetes, although we could not confirm whether this improvement confers benefit on vascular function in subjects with type 2 diabetes. Future studies of metformin at higher doses are required to determine whether ADMA lowering by metformin improves vascular function and more importantly, whether such a reduction helps explain the vascular end-point benefits of this treatment in prospective clinical trials.

## **Chapter 4**

***Studies on adipose AMP-activated protein kinase and plasma adiponectin after metformin and gliclazide therapy in patients with Type 2 diabetes.***

### **4.1 Introduction**

Activation of AMPK triggers catabolic pathways that produce energy, while turning off anabolic pathways that consume energy thereby maintaining cellular energy stores. For example, AMPK inhibits lipid and triglyceride synthesis in both liver and adipose tissue and stimulates skeletal muscle glucose uptake, glycolysis and fatty acid oxidation (Towler and Hardie 2007). Stimulation of fatty acid oxidation occurs when AMPK directly phosphorylates and inactivates the downstream target, acetyl-coA carboxylase (ACC). ACC synthesizes malonyl-CoA which inhibits the rate-controlling step of fatty oxidation involving the transfer of fatty acids into the mitochondria by the enzyme carnitine palmitoyltransferase 1 (CPT1) (Trumble *et al* 1995, Ruderman *et al* 1999). The ability of AMPK to induce lipid oxidation and thus the amount of fat deposited in skeletal muscle and liver is considered an important feature of AMPK activation. Therefore it may be that AMPK plays an important role in controlling the whole body energy homoeostasis including the regulation of plasma glucose levels and body weight. The control of AMPK activity provides an attractive target for therapeutic intervention in metabolic disorders such as T2DM.

AMPK is thought have important role in adipose tissue by inhibiting lipolysis and lipogenesis. Indeed, AMPK has been implicated in the regulation of lipolysis through direct phosphorylation of hormone-sensitive lipase (Garton *et al* 1989). Lipolysis and lipogenesis are inhibited in rat adipocytes when using the AMPK activator AICAR, (Sullivan *et al* 1994). It is also suggested that when the regulation of lipid metabolism by AMPK is absent, then weight gain and insulin resistance prevail. Indeed when AMPK  $\alpha 2$  knockout mice were fed with a high-fat diet they have

been shown to exhibit increased body weight, increased adiposity and a degree of insulin resistance when compared with wild-type mice (Viollet *et al* 2003, Jorgensen *et al* 2004, Villena *et al* 2004). It has also been recently shown that glucocorticoids inhibit AMPK activity in adipose tissue of insulin resistant patients with Cushing's syndrome (Kola *et al* 2008). Moreover, metformin has been found to prevent the glucocorticoid effects on *ex vivo* cultured human adipocytes (Christ-Crain *et al* 2008). The effect of insulin sensitizers such as metformin or the thiazolidinediones on AMPK activity in human adipose tissue has not yet been investigated. Further work is required to examine whether metformin exerts its beneficial metabolic effects in patients with T2DM in part by increasing AMPK activity in human adipose.

Plasma levels of adiponectin negatively correlate to insulin resistance, obesity and cardiovascular disease. Interestingly, studies of the Pima Indian population in the USA have shown that in normal healthy individuals the concentration of adiponectin can be used to predict the likelihood of developing T2DM later in life, with lower levels resulting in increased risk (Lindsay *et al* 2002). The effects of the adiponectin are partly accounted for by adiponectin induced AMPK activation which results in stimulation skeletal muscle fatty acid oxidation and glucose uptake (Tomas *et al* 2002, Yamauchi *et al* 2002). Furthermore, activation of AMPK by adiponectin suppresses hepatic gluconeogenesis. (Yamauchi *et al* 2002). Adiponectin has also been demonstrated to increase nitric oxide production (Hattori *et al* 2003), by a pathway proposed to involve the direct phosphorylation of eNOS by AMP-activated protein kinase (AMPK), and also by a parallel pathway involving PI3K and PKB activation by AMPK (Chen *et al* 2003, Tan *et al* 2004, Ouchi *et al* 2004, Chen *et al* 2005). Furthermore, adiponectin has been shown to reduce infarct size and improved left ventricular function and coronary blood flow following ischaemia and reperfusion of isolated rat hearts by a mechanism involving AMPK and NO release (Gonon *et al* 2008). Moreover, AMPK may be necessary for adiponectin to exert its cardio protective effect as ischaemia and reperfusion

caused a larger myocardial infarct and enhanced myocardial apoptosis in adiponectin knockout mice when compared with wild type mice (Shibata *et al* 2005). Adiponectin knockout mice have been shown to have progressive cardiac remodelling in a pressure-overloaded condition due to reduced AMPK signalling and increased insulin resistance when compared to wild type mice (Liao *et al* 2005). Therefore, AMPK appears to be important in mediating both the metabolic and the vascular effects of adiponectin but as yet the exact signalling mechanisms have not been clarified.

Whilst adiponectin would appear to have a preventative role in atherosclerosis, its role as a predictive marker of vascular risk is less clear (Chen, Tsai *et al* 2005, Lindsay *et al* 2005, Schulze *et al* 2005, Sattar *et al* 2006, Frystyk *et al* 2007). Indeed, the addition of metformin to sulphonylurea therapy has been shown to have no effect on circulating concentrations of adiponectin in subjects with Type 2 diabetes (Phillips *et al* 2003). Data concerning the effects of sulphonylureas on adiponectin is less robust with some evidence of an increase in adiponectin concentration in both rat adipocytes (Kanda *et al* 2008) and subjects with Type 2 diabetes (Drzewoski *et al* 2006). It is nevertheless tempting to hypothesise that any increase in adipose AMPK may be mediated by an increase in plasma adiponectin concentration

Metformin has been previously shown to increase AMPK activity in hepatocytes (Zhou *et al* 2001) and human muscle of subjects with Type 2 diabetes (Musi *et al* 2002), potentially leading to improvements in both insulin sensitivity and glycaemia. In hepatocytes, AMPK activation leads to increased fatty acid oxidation and attenuated gluconeogenesis, fatty acid and cholesterol synthesis (Zhou *et al* 2001). In muscle, AMPK activation promotes fatty acid oxidation and glucose transport (Musi *et al* 2002). Conversely, in rat adipocytes AMPK activation has been proposed to attenuate both lipolysis and fatty acid synthesis (Sullivan *et al* 1994). Indeed incubation of 3T3-L1 adipocytes with AICAR, a pharmacological activator of AMPK, has been demonstrated to attenuate

insulin-stimulated glucose uptake, the *opposite* of what has been observed in skeletal muscle (Salt *et al* 2000). Moreover, metformin has been shown to stimulate AMPK activity, decrease lipolysis and inhibit fatty acid synthesis in 3T3L1 adipocytes (An *et al* 2007). Metformin and AICAR have been shown to stimulate AMPK activity but reduce adiponectin protein expression also in 3T3L1 adipocytes (Huypens *et al* 2005). Previous work on AMPK has concentrated on rat adipocytes with work on human adipocytes or human adipose tissue confined to studies of AMPK activity in adipose tissue of subjects with corticosteroid excess and the prevention of corticosteroid-induced effects on AMPK in human adipocytes with metformin.

If metformin counteracts insulin action in adipose tissue via this mechanism, this may partly explain the common clinical observation of reduced fat mass on this drug (usually ascribed to appetite reduction secondary to gastrointestinal side-effects). Indeed, in many ways, metformin's action parallels that of aerobic exercise, helping to divert calories away from storage tissues into skeletal muscle. It is possible that AMPK activation is a common mechanism utilised by both pharmacological and physiological interventions. Activation of AMPK in human adipose tissue would, therefore, be an attractive target for the treatment of T2DM and may explain some of metformin's therapeutic effects.

To test these hypotheses, it was vital that this thesis explored metformin's action in human adipose tissue and on plasma adiponectin, an area thus far neglected in the literature.

#### ***4.2 Aims of this chapter***

This chapter was initiated to extend the observations discussed above and addressed the following issues.

- i. Does metformin increase AMPK activity in adipose tissue of subjects with Type 2 diabetes?
- ii. Is any increase in AMPK activity associated with a change in circulating levels of adiponectin?

Accordingly, this chapter compares the effect of metformin and gliclazide on adipose AMPK and plasma adiponectin levels in subjects with Type 2 diabetes using a randomised, double-blind, glycaemia controlled crossover design.

## 4.3 Results

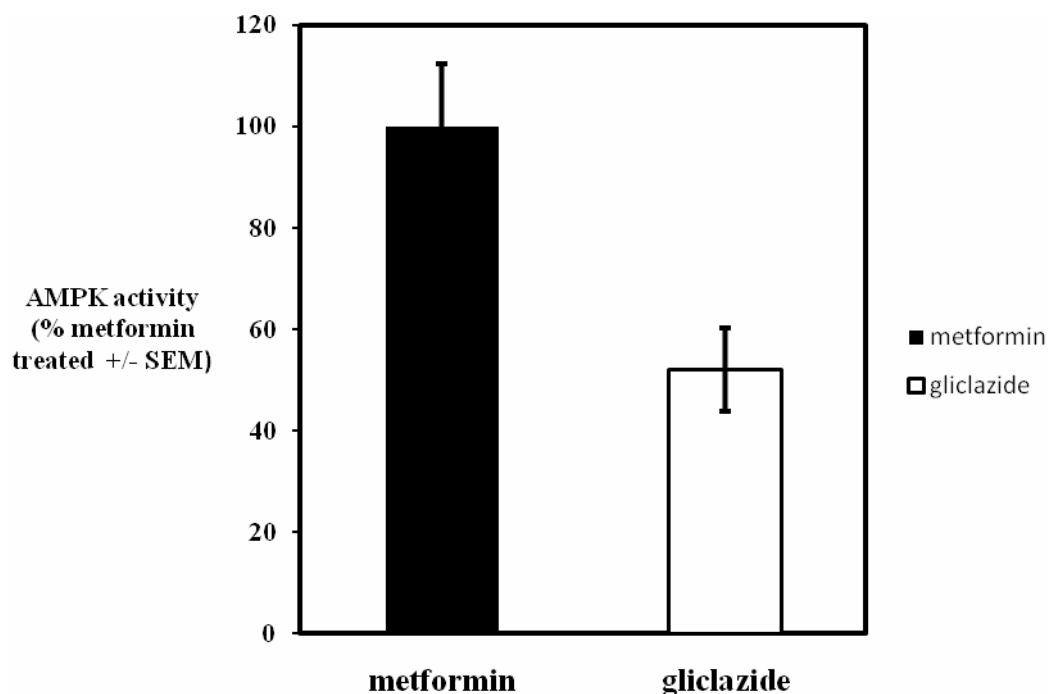
### 4.3.1 Adipose AMPK activity

There was a significant increase in AMPK activity expressed as a multiple of an internal standard of known AMPK activity (K) with metformin therapy when compared with gliclazide therapy [0.085+/-0.047 versus 0.044+/-0.035, p=0.002], (Table 4.1, Figure 4.1).

**Table 4.1: Adipose and AMPK**

Outcome	Raw outcomes by treatment		Treatment Effect		
	Metformin	Gliclazide	Estimate	95% CI	p-value
AMPK	0.085 (0.047)	0.044 (0.035)	-0.041	(-0.063, -0.019)	p=0.002

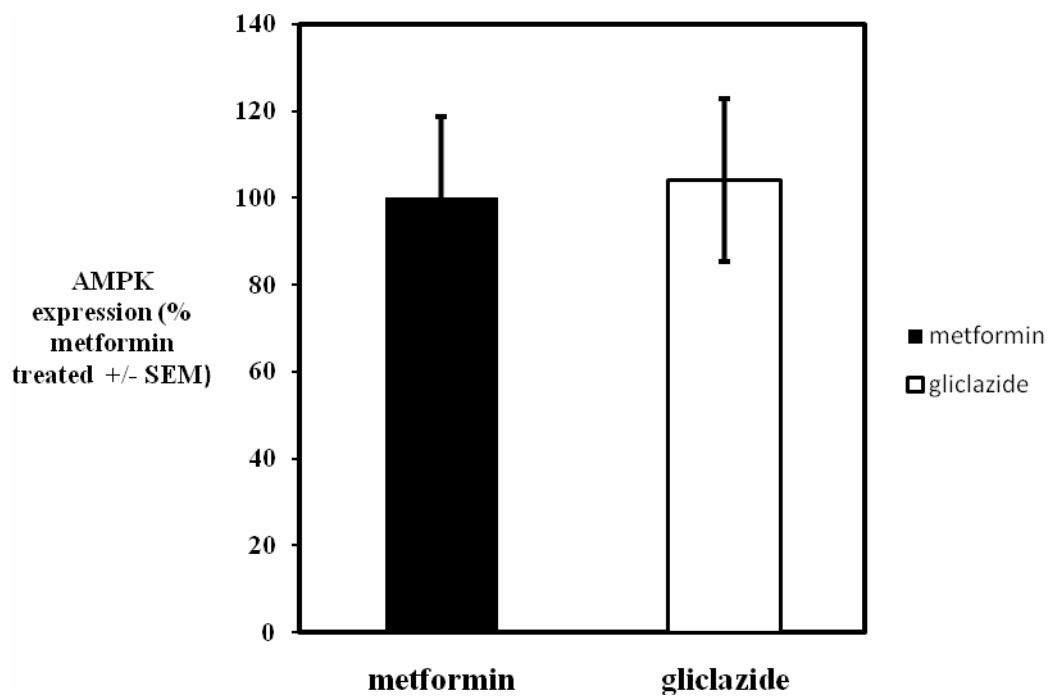
**Figure 4.1: Gliclazide adipose AMPK activity expressed as a % of metformin treated adipose AMPK activity.** There was a significant increase in AMPK activity expressed as a multiple of an internal standard of known AMPK activity (K) with metformin therapy when compared with gliclazide therapy (n=20).



#### 4.3.2 Adipose AMPK protein expression

There was no significant difference in protein expression between the two phases of treatment activity (n=19, p=0.98). (See figure 4.2 where experiment was performed by Dr Ian Salt, University of Glasgow)

**Figure 4.2: Gliclazide adipose AMPK protein expression expressed as a % of metformin treated adipose AMPK protein expression.** There was no significant difference in protein expression between the two phases of treatment activity (n=19 due to insufficient lysate in one sample).



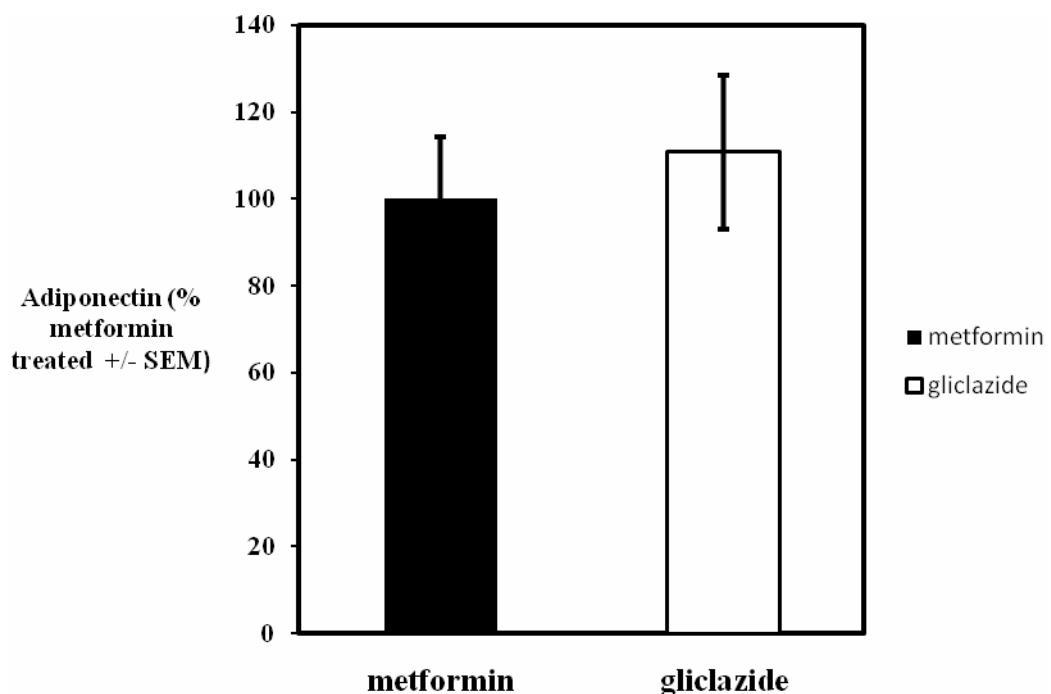
### 4.3.3 Serum total adiponectin concentration

There was no significant difference in total adiponectin levels after each phase of therapy [4193 +/- 2696 versus 4648ng/ml +/- 3291ng/ml, p=0.054]. See Table 4.2, Figure 4.3.

**Table 4.2: Serum total adiponectin concentration**

Outcome	Raw outcomes by treatment		Treatment Effect		
	Metformin	Gliclazide	Estimate	95% CI	p-value
Adiponectin	4648 (3291)	4193 (2696)	456	(22, 889)	p=0.054

**Figure 4.3: Gliclazide plasma adiponectin concentration expressed as a % of metformin adiponectin concentration.** There was no significant difference in total adiponectin levels after each phase of therapy (n=20).



#### 4.4 Discussion

This chapter compared the effects of metformin versus gliclazide on adipose AMPK activity and plasma adiponectin using a randomised, double-blind crossover design in subjects with Type 2 diabetes. The main aim was to confirm or refute a beneficial effect of metformin on adipose AMPK activity. It was also hypothesised that any increase in AMPK activity may be associated with an increase in plasma adiponectin. The data demonstrates that metformin does indeed increase adipose AMPK compared with gliclazide. The increase in adipose AMPK activity was not associated with an increase in AMPK protein expression. This finding suggests that the change in AMPK activity is an increase in the specific activity of AMPK rather than simply a difference in the total amount in AMPK between phases. The study design aimed to match glycaemic control (*see 3.2.3*) at the end of each phase but this, in the event, proved not to be possible. Metformin increased AMPK activity when compared with gliclazide despite better glycaemia changes with the latter, supporting a glucose-independent mechanism for this metformin effect. Associated with these changes was a small improvement in plasma LDL cholesterol (*see 3.2.3*). Somewhat surprisingly, there was no significant difference in BMI (*see 3.2.2*) after each treatment although measurement of skin fold thickness or more sophisticated measurement of body composition may have provided a more specific marker of fat mass. Finally, the data show that the change in adipose AMPK activity was not associated with any difference in plasma adiponectin.

In animal studies activation of AMPK has been proposed to attenuate lipolysis, fatty acid synthesis and insulin-stimulated glucose uptake in adipocytes (Towler and Hardie 2007). These data support the notion that metformin action parallels that of aerobic exercise, helping to divert calories away from storage tissues into skeletal muscle and it is possible that AMPK activation is a common mechanism utilised by both pharmacological and physiological interventions. This may explain some of the beneficial effects of metformin in patients with Type 2 diabetes.

It is important to consider the limitations of the data presented here. Firstly, while a robust and significant difference in AMPK activity with metformin when compared with gliclazide is demonstrated it is important to note that the effect of gliclazide on AMPK activity has not been widely studied. The principal mechanism of action of the sulphonylurea class of drugs is to improve insulin secretion by the beta cells of the endocrine pancreas, and data concerning the effects of sulphonylurea on AMPK are limited. Indeed, glimepiride treatment has been shown to have no effect on AMPK activity in cultured myocytes as expressed by AMPK phosphorylation while gliclazide has no effect (up to 1 hour incubation) in 3T3-LI adipocytes on AMPK activity as assessed by kinase assay (Inukai *et al* 2005, P J Logan, personal communication). Secondly, there are no functional data examining downstream targets of AMPK, which is now the subject of further work out with the scope of this thesis. Indeed, in retrospect it may have been of interest to measure plasma FFA concentrations although these results may again have been confounded by the imperfect matching for glycaemia. Finally, while the vast majority of the tissue biopsy will constitute human adipose only approximately 50% of the cells are adipocytes and therefore it is impossible to quantify whether any presence of blood or macrophages could confound the results.

Despite the chapter demonstrating a change in adipose AMPK it failed to demonstrate a change in plasma adiponectin. This is agreement with a previous study which demonstrated the addition of metformin to sulphonylurea therapy has been shown to have no effect on circulating concentrations of adiponectin in subjects with Type 2 diabetes (Phillips *et al* 2003). The impact of metformin on plasma adiponectin may also be difficult to interpret in this chapter as there is some evidence supporting an increase in adiponectin concentration with gliclazide (Kanda *et al* 2008, Drzewoski *et al* 2006). It is also important to consider the limitations of the plasma adiponectin assay which is a crude measurement of only total adiponectin using ELISA. The exact physiological role of adiponectin is incompletely understood but it is known that adiponectin exists in 3 or more different

higher order complexes: high molecular weight form), low molecular weight form and trimeric form. Adiponectin has also been shown to operate at a molecular level by increasing nitric oxide production (Hattori *et al* 2003) by pathways proposed to involve AMPK. Phosphorylation of eNOS by AMP-activated protein kinase (AMPK) has been proposed but the exact signalling mechanisms have not been clarified (Chen *et al* 2003, Tan *et al* 2004, Ouchi *et al* 2004, Chen *et al* 2005). It is therefore clear that further work is required to determine whether there is a relationship between AMPK and adiponectin in the action of metformin.

Interestingly, it has been recently shown that glucocorticoids inhibit AMPK activity in adipose tissue of insulin resistant patients with Cushing's (Kola *et al* 2008). Moreover, metformin has been found to prevent the glucocorticoid effects on ex vivo cultured human adipocytes (Christ-Crain *et al* 2008). The work in this thesis extends these observations by demonstrating that metformin is capable of activating AMPK in adipose tissue in patients who also have insulin resistance.

In conclusion, this chapter provides the strongest evidence thus far to support a beneficial effect of metformin therapy on adipose AMPK concentrations in patients with type 2 diabetes, although there was no demonstrable change in plasma adiponectin concentration. Further work elucidating the molecular mechanisms by which metformin activate AMPK in adipose tissue may identify novel and tissue specific therapeutic targets in subjects with Type 2 diabetes.

## ***Chapter 5***

***Studies on biguanide effects on AMP-activated protein kinase and nitric oxide synthesis in human aortic endothelial cells.***

### ***5.1 Introduction***

Recent clinical studies have shown that the effects of metformin may go beyond improving HbA1c and may include reductions in cardiovascular end points in T2DM. There is a growing body of evidence to suggest that the biguanide class of anti-diabetic drugs improve endothelial function. The mechanism of metformin action in target tissues that results in altered glucose homeostasis and potentiation of vascular endothelial function remains uncertain. Perhaps the most intriguing development in elucidating metformin's cellular mechanism of action in recent years is the proposed involvement of AMPK. The metabolic and cardio-protective effects of metformin may be attributable to the activation of AMPK and its downstream pathways.

AMPK has an important role in the release of NO from the vascular endothelium. AICAR stimulates eNOS Ser1177 phosphorylation and NO production in human aortic endothelial cells (HAECs) (Morrow *et al* 2003). Indeed, AMPK activation has been demonstrated to stimulate muscle glucose uptake in a NO-dependent manner and NO production has been proposed to be largely responsible for insulin-independent glucose uptake during exercise (Chen *et al* 2000, Fryer *et al* 2000, Hawley *et al* 2002). AMPK also has a role in angiogenesis. Vascular endothelial growth factor (VEGF) is a key regulator of angiogenesis and stimulates differentiation, survival, migration, proliferation and vascular permeability (Ferrara *et al* 2003). VEGF rapidly stimulates endothelial NO synthesis and does so in an AMPK dependent manner (Reihill *et al* 2007).

It has recently been reported that metformin stimulates NO synthesis in an AMPK-dependent manner in BAECs (Zou *et al* 2004). This is in contrast to data that suggests that the incubation of BAECs with phenformin activates AMPK without altering Ser1177 phosphorylation (Mount *et al* 2005). Further work has demonstrated that metformin acts to phosphorylate AMPK and ACC in HUVECs (Hattori *et al* 2006). However in HAECs, metformin failed to show any effect on AMPK activity within 2 hours (Morrow *et al* 2003). The reasons for these conflicting data remain elusive but may be explained by species- and tissue-specific factors. Moreover, the neutral results with metformin may reflect the reduced lipophilicity compared with phenformin, such that phenformin rapidly accumulates in cells and subsequently has a more rapid effect on metabolism compared with metformin. Therefore, a longer incubation period with or higher concentration of metformin may be required to stimulate AMPK and NO synthesis in HAECs. Furthermore, the neutral results with metformin may also be explained by the possibility of cells either not expressing OCT-1 or carrying reduced function polymorphisms of OCT-1.

Exactly how metformin improves insulin action, lowers body weight, improves the lipid profile, improves endothelial function and reduces the incidence of myocardial infarction remains incompletely understood. Further work is required to examine if biguanides mediate these effects by stimulating AMPK in vascular endothelial cells and in this chapter I investigate the effect of both metformin and phenformin in HAECs.

## ***5.2 Aims of this chapter***

This chapter was initiated to extend the observations discussed above and addressed the following issues.

- i.** Does metformin or phenformin stimulate nitric oxide production in cultured HAECs?
- ii.** Does metformin or phenformin stimulate eNOS phosphorylation at Ser1177 in cultured HAECs?
- iii.** Does metformin or phenformin rapidly stimulate eNOS phosphorylation at Ser1177 and NO synthesis in an AMPK-dependent manner?

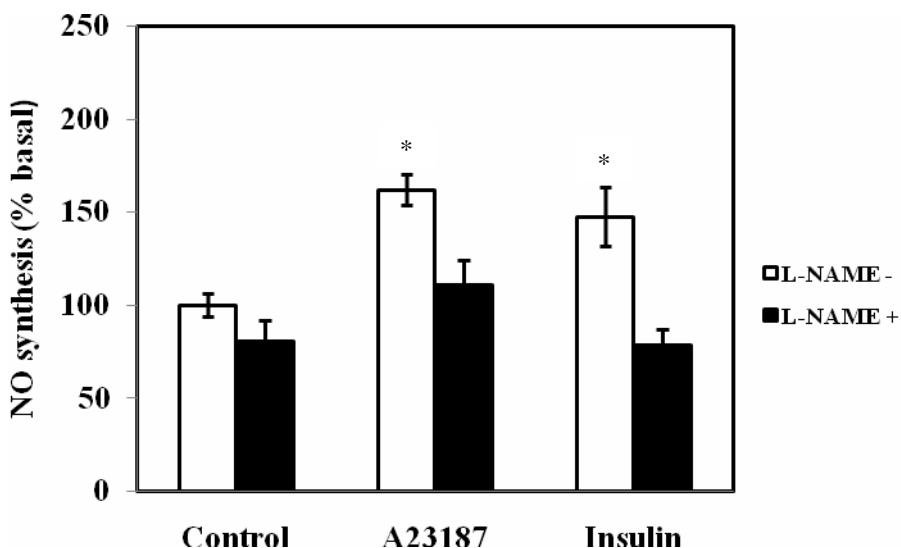
### 5.3 Results

#### 5.3.1 Effect of A23187, insulin and L-NAME on nitric oxide (NO) production in HAECS.

In HAECS, both insulin and A23187 independently stimulated NO production when compared to control (Figure 5.1). This effect was inhibited by pre-treatment with L-NAME.

**Figure 5.1 Stimulation of NO synthesis by A23187 and insulin in HAECS.**

Cells were incubated in KRH buffer in the presence or absence of L-NAME (black bars) for 1 hour. Cells were stimulated with insulin (1 $\mu$ M) or A23187 (3 $\mu$ M) for 15 minutes. After the times indicated, media was removed and assayed for  $\text{NO}_2^-$  content as described in methods. The data shown represents the mean % basal  $\pm$  SEM NO synthesis from 3 independent experiments performed in sextuplicate. \* p<0.05 relative to insulin or A23187 in the absence of L-NAME.



### ***5.3.2 Effect of Metformin and Phenformin on nitric oxide (NO) production.***

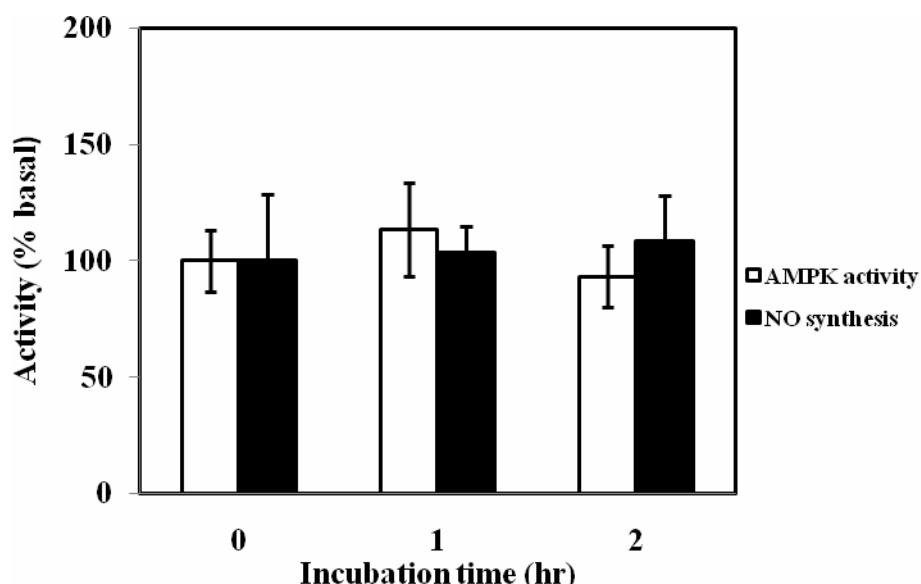
This chapter examined the ability of the anti-diabetic biguanide drug metformin and its derivative, phenformin to modulate NO production in HAECS. Stimulation of HAECS with metformin (200  $\mu$ M) had no effect on the rate of NO synthesis after incubation for either 60 or 120 minutes (Figure 5.2). Stimulation of HAECS with phenformin (200  $\mu$ M) stimulated the rate of NO synthesis within 60 minutes and reached a maximum 3.6-fold increase at 120 minutes (Figure 5.3). Under identical conditions, stimulation of NO by phenformin was dose dependent (Figure 5.4) such that NO synthesis was significantly stimulated by 200  $\mu$ M phenformin reaching a maximum 2.0-fold at 60 minutes.

### ***5.3.3 Effect of Metformin and Phenformin on AMPK activity***

Phosphorylation of eNOS at Ser1177 has been reported to be mediated by several protein kinases, including AMPK. We next examined the ability of metformin to modulate AMPK activity in HAECS as assessed by incorporation of  $^{32}$ P from of [ $\gamma$ - $^{32}$ P] ATP into the AMPK substrate peptide SAMS. Stimulation of HAECS with metformin (200  $\mu$ M) had no effect on AMPK activity after incubation for 60 or 120 minutes (Figure 5.2). Stimulation of HAECS with phenformin (200  $\mu$ M) stimulated AMPK activity within 60 minutes and reached a maximum 3.3-fold increase at 120 minutes (Figure 5.3). Under identical conditions, stimulation of AMPK activity was also dose-dependent, such that 20  $\mu$ M phenformin significantly stimulated AMPK activity, reaching a maximum 3.1-fold increase (Figure 5.4).

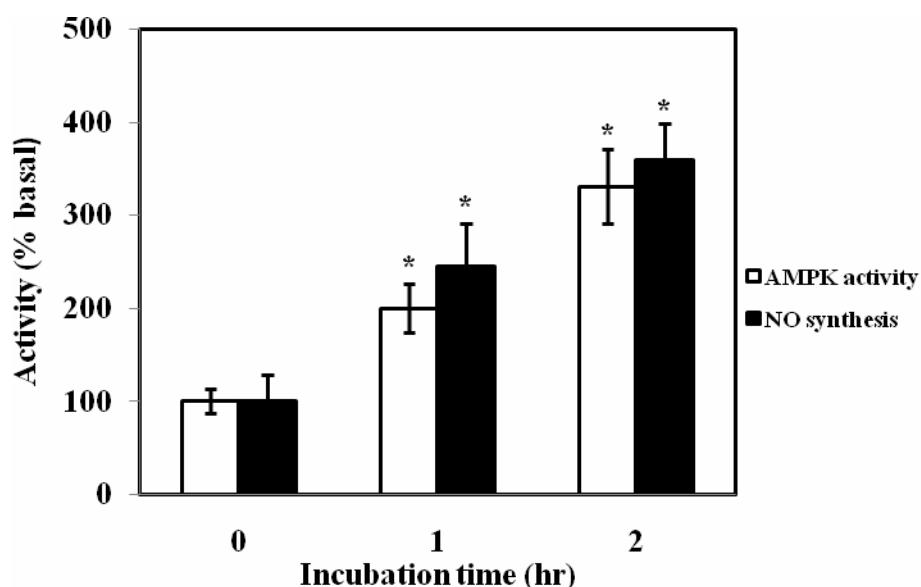
**Figure 5.2 Metformin fails to stimulate AMPK and NO synthesis in HAECs.**

HAEC AMPK activity and the rate of L-NAME-sensitive  $\text{NO}_2^-$  synthesis in media were determined after incubation in 200  $\mu\text{mol/l}$  metformin for the indicated durations. Data shown represents the mean % basal  $\pm$  S.E. NO synthesis or AMPK activity from 3 independent experiments. \* p<0.05 relative to value in absence of metformin.



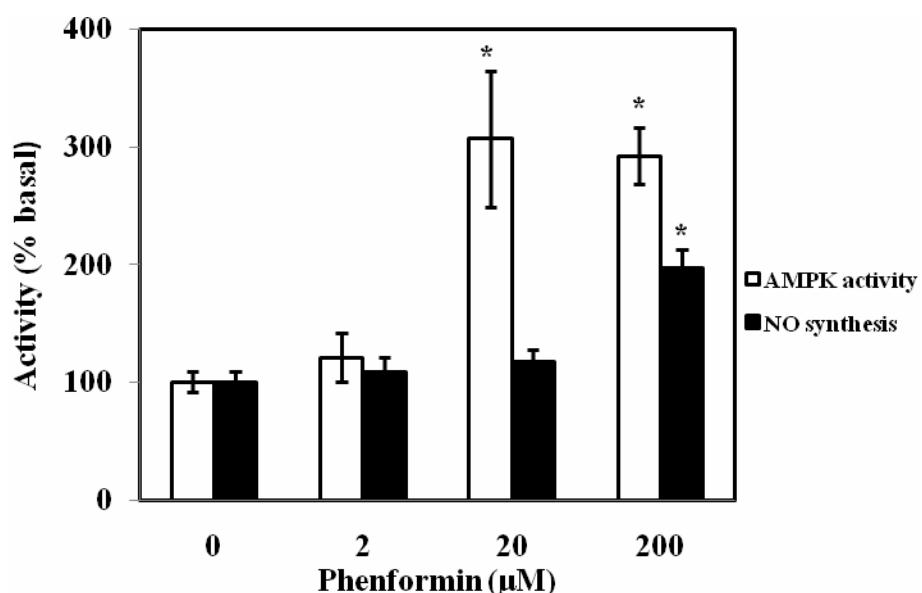
**Figure 5.3 Phenformin stimulates AMPK and NO synthesis in HAEC in a time-dependent manner.**

HAEC AMPK activity and the rate of L-NAME-sensitive  $\text{NO}_2^-$  synthesis in media were determined after incubation in 200  $\mu\text{M}$  phenformin for the indicated durations. Data shown represents the mean % basal  $\pm$  S.E. NO synthesis or AMPK activity from 3 independent experiments. \*  $p<0.05$  relative to value in absence of phenformin.



**Figure 5.4 Phenformin stimulates AMPK and NO synthesis in HAEC in a dose-dependent manner.**

HAEC AMPK activity and the rate of L-NAME-sensitive  $\text{NO}_2^-$  synthesis in media were determined after incubation for the indicated concentrations of phenformin for 1 hour. Data shown represents the mean % basal  $\pm$  S.E. NO synthesis or AMPK activity from 3 independent experiments. \* p<0.05 relative to value in absence of phenformin.

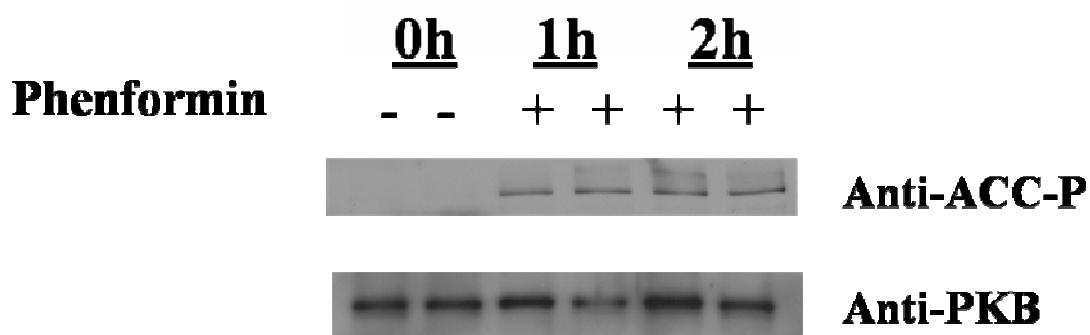


### **5.3.4 Effect on Phenformin on phosphorylation of ACC.**

To further characterise the effect of biguanides on HAEC AMPK activity I determined the phosphorylation status of ACC, which is phosphorylated by AMPK at Ser80 in ACC1 and Ser 220 in ACC2 (human sequence). Using an antibody that recognises both phosphorylated species, phenformin (200 µM) significantly stimulated phosphorylation of ACC in a time-dependent manner (Figure 5.5).

**Figure 5.5 Phenformin stimulates phosphorylation of ACC in HAECS.**

HAEC lysates, prepared from cells incubated with phenformin (200 µM) for 60 minutes and 120 minutes were resolved by SDS PAGE, transferred to nitrocellulose and probed with the antibodies indicated. Representative immunoblots are shown, repeated with similar results on 3 different samples of lysates.

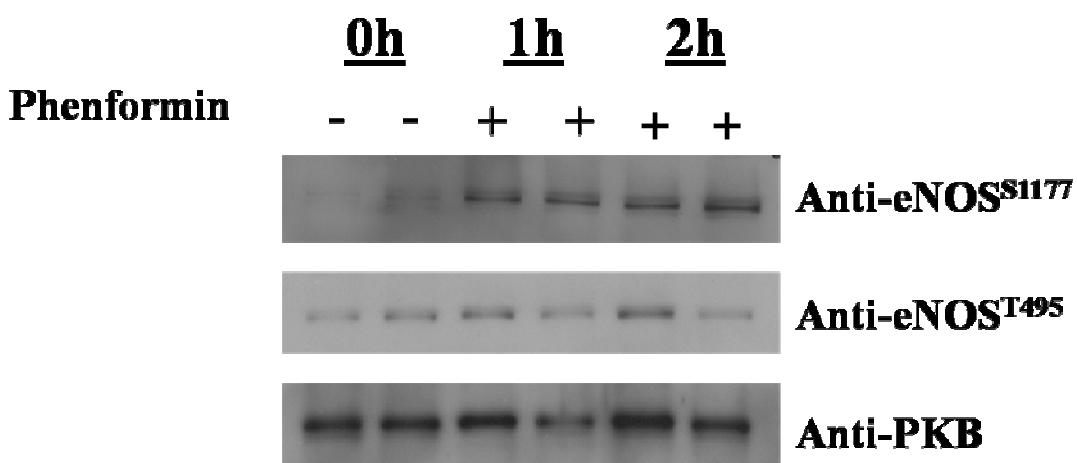


### **5.3.5 Effect of phenformin phosphorylation of eNOS at Ser1177 and Thr495 in HAECs.**

Phosphorylation of eNOS has been demonstrated to be an important determinant of NO production. In particular, phosphorylation at Ser1177 and dephosphorylation of Thr495 has been demonstrated to increase eNOS activity (Lin *et al* 2003). I therefore determined the effects of phenformin on eNOS phosphorylation at these sites in HAECs using phosphorylation site-specific antibodies. Stimulation of HAECs with 200 µM phenformin significantly stimulated phosphorylation of eNOS at Ser1177 (Figure 5.6). In contrast, stimulation of HAECs with phenformin was without any significant effect on phosphorylation of eNOS at Thr495 (Figure 5.6).

**Figure 5.6 Effect of phenformin on eNOS phosphorylation at Ser1177 and Thr495 in HAECs.**

HAEC lysates, prepared from cells incubated with phenformin (200 µM) for 60 min and 120 min were resolved by SDS PAGE, transferred to nitrocellulose and probed with the antibodies indicated. Representative immunoblots are shown, repeated with similar results on 3 different samples of lysates.

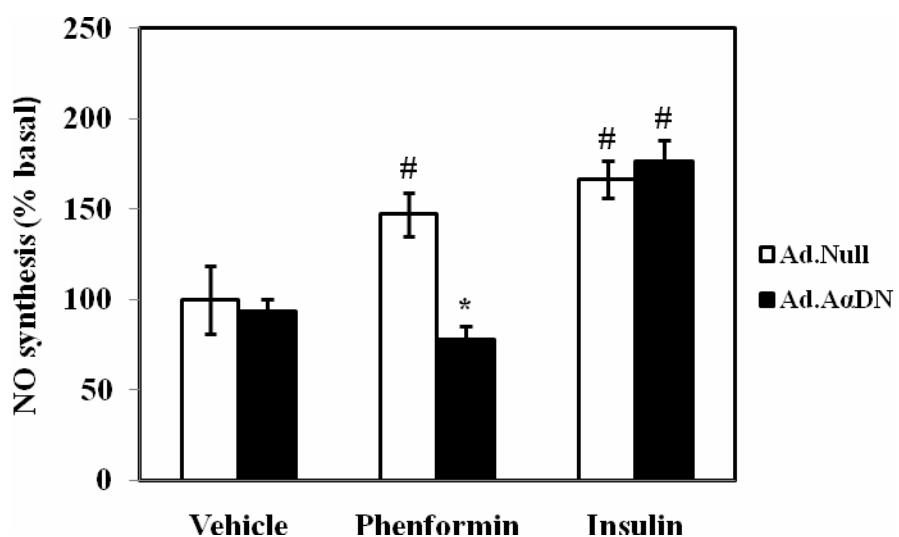


### ***5.3.6 Effect of the use of adenovirus expressing dominant negative AMPK on phenformin stimulated AMPK activation and NO stimulation***

To determine whether AMPK activation was required for phenformin-stimulated NO synthesis, HAECS were infected with control (Ad.Null) adenoviruses or adenoviruses expressing dominant negative AMPK (Ad.alpha1DN) prior to incubation with phenformin (200 µM) for 1h and NO production assessed. HAECS infected with Ad.alpha1DN exhibited significantly attenuated phenformin stimulated NO production at 1 hour compared to control virus-infected cells (Figure 5.4). Insulin has previously been demonstrated to stimulate NO synthesis by PKB-mediated phosphorylation and activation of eNOS, independent of AMPK. Infection of HAECS had no effect on insulin-stimulated (1µM, 15min) NO synthesis indicating that the effect of infection with Ad.alpha1DN did not result in the non-specific downregulation of NO synthesis in an AMPK-independent manner. The effect of infection with Ad.alpha1DN on phosphorylation of eNOS or ACC was not examined.

**Figure 5.7 Phenformin-stimulated NO synthesis is AMPK-dependent.**

The rate of L-NAME-sensitive  $\text{NO}_2^-$  synthesis was determined in media of HAECS infected with Ad.Null or Ad. $\alpha$ 1DN (10 Pfu/cell) after incubation in the presence or absence of phenformin (200  $\mu\text{M}$ , 1h) or insulin (1  $\mu\text{M}$ , 15min). Data shown represents the mean % basal  $\pm$  S.E. NO synthesis from 4 independent experiments performed in sextuplicate. \* p<0.05 relative to value in Ad.Null-infected cells. # p<0.05 relative to vehicle-treated cells.



### 5.3.4 Discussion

The central finding of this chapter is that phenformin rapidly stimulates NO production in an AMPK-dependent manner in HAECS. The stimulation of AMPK by phenformin is associated with increased phosphorylation of eNOS-Ser1177. The stimulation of NO production is attenuated by infection of HAECS with Ad.alpha1DN. These data support the hypothesis that biguanides increase NO production by an AMPK-dependent mechanism. As AMPK has previously been demonstrated to stimulate NO synthesis via phosphorylation of eNOS at Ser1177, the complete inhibition of phenformin-stimulated NO production by infection with Ad.alpha1DN suggests that AMPK-mediated phosphorylation and activation of eNOS underlies phenformin-stimulated NO synthesis.

A number of studies have demonstrated that biguanides have the capacity to improve endothelial function in non-diabetic subjects and subjects with T2DM. The beneficial effects of biguanides have been reported to be independent of glycaemia inferring a direct effect on vascular tissues. The endothelial effects of biguanides may be relatively rapid and stimulation of AMPK activity and NO synthesis by phenformin was achieved at concentration of 200 $\mu$ M. It is difficult to explain the neutral results with metformin. Phenformin is a more lipophilic and potent biguanide which was removed from clinical practice due to the incidence of lactic acidosis and is often used in *in vitro* experimentation as the biguanide of choice. It is possible that metformin, being less lipophilic, was unable to reach a sufficiently high intracellular concentration to exhibit effects during the short course of my experiments.

It has recently been reported that metformin stimulates NO synthesis in an AMPK-dependent manner in BAECs and has been demonstrated to phosphorylate AMPK and ACC in HUVECs (Zou *et al* 2004, Hattori *et al* 2006). In the current study, I was unable to show any effect with metformin within 2 hours on either AMPK activity or NO production, in agreement with our laboratory's

previous findings (Morrow *et al* 2003). In contrast, the related biguanide, phenformin, robustly stimulated AMPK activity, ACC phosphorylation, eNOS Ser1177 phosphorylation and NO production in HAECs. Furthermore, infection of HAECs with Ad.alpha1DN indicates that AMPK activation is required for phenformin-stimulated NO synthesis. It has been previously demonstrated that attenuation of AMPK signalling in this way does not alter NO synthesis in response to stimuli such as insulin. Indeed, insulin has previously been demonstrated to stimulate NO synthesis by PKB-mediated phosphorylation and activation of eNOS, independent of AMPK (Morrow *et al* 2003). Infection of HAECs had no effect on insulin-stimulated (1 $\mu$ M, 10 min) NO synthesis indicating that Ad.alpha1DN solely attenuates AMPK-dependent stimulation of NO synthesis and does not result in the non-specific downregulation of NO synthesis in an AMPK-independent manner.

In contrast to the current study, incubation of BAECs with phenformin was recently reported to activate AMPK without altering Ser1177 phosphorylation (Mount *et al* 2005). The reasons for these conflicting data remain elusive, but may reflect species-specific differences with respect to phenformin action. The neutral results with metformin may reflect the reduced lipophilicity compared with phenformin, such that phenformin rapidly accumulates in cells and subsequently has a more rapid effect on metabolism compared with metformin. Furthermore, the neutral results with metformin may also be explained by the possibility of certain cells carrying reduced function polymorphisms of OCT-1.

It is also important to consider if a longer incubation period or a higher concentration of metformin may be required to stimulate AMPK and NO synthesis in HAECs. The plasma concentrations of metformin in patients treated with the drug are estimated to be 10–20  $\mu$ M (Bailey *et al* 1996). It is important to consider that in many experiments involving cultured cells the doses used to activate

metformin are consistently higher in a range between 1 and 10 mM (Yang *et al* 2006, Zhang *et al* 2007). This can be explained in part by the fact that OCT-1 predominantly expressed in the liver and is not expressed in many cultured cells. Indeed, 25% of the total dose of metformin is found in the liver 10 minutes after intravenous administration to wild type mice compared with <1% with OCT-1 knockout mice (Wang *et al* 2002). Higher doses of metformin are not used in patients with T2DM due to gastrointestinal side effects and also because of the risk of lactic acidosis, both of which might be caused by the ability of the compound to act as an inhibitor of the respiratory chain at high concentrations (Owen *et al* 2000). Further work is required to determine the time and concentration of metformin which will result in the maximal beneficial effects of AMPK activation without the development of gastrointestinal side effects and lactic acidosis. Indeed, it has been shown that an increase in the phosphorylation of ACC is evident when the degree of phosphorylation and activation of AMPK is very small (Hawley *et al* 2002). This suggests that to obtain beneficial therapeutic effects of metformin, maximal activation of AMPK may therefore not be necessary. However, with that in mind it may now be possible to develop a new generation of drugs that are more precisely targeted at AMPK. If new drugs could activate AMPK at lower concentrations they may have fewer unwanted side effects.

Taken together with these previous studies, this thesis postulates that phenformin is able to stimulate endothelial NO synthesis in an AMPK-dependent manner, but species- and tissue-specific factors may underlie the seemingly conflicting data concerning the effects of metformin obtained to date.

## **Chapter 6**

### ***Studies on thiazolidinedione effects on AMP-activated protein kinase and nitric oxide synthesis in human aortic endothelial cells.***

#### ***6.1 Introduction***

There is a growing body of evidence to suggest that the TZD class of anti-diabetic drugs improve endothelial function, as assessed by endothelium-dependent vasodilatation in patients with T2DM or insulin resistance (Pitrosch *et al* 2004, Natali *et al* 2004, Semple *et al* 2006). The hypoglycaemic effects of TZDs are mediated by the transcription factor PPARgamma, but recent work suggests that the improvement of endothelial function by TZDs is independent of the effect on glycaemia (Satoh *et al* 2003, Pritosch *et al* 2004, Semple *et al* 2006). Therefore, the mechanism of action by which TZDs improve vascular endothelial function remains uncertain.

Recent studies have suggested that prolonged exposure to TZDs directly improves NO bioavailability in endothelial cells and increases phosphorylation of eNOS at Ser1177 (Calnek *et al* 2003, Cho *et al* 2004, Polikandriotis *et al* 2005, Kim *et al* 2006). Several protein kinases have been demonstrated to phosphorylate eNOS Ser1177 in endothelial cells, including PKB and AMPK, but the protein kinase and signalling mechanism responsible for phosphorylation of eNOS in response to TZDs is as yet undetermined (Fulton *et al* 1999, Dimmeler *et al* 1999, Chen *et al* 1999, Fryer *et al* 2002, Morrow *et al* 2003). As TZDs have been demonstrated to rapidly and chronically activate AMPK in muscle, liver and adipose, it is possible that activation of AMPK may mediate eNOS phosphorylation and NO synthesis in response to TZDs in endothelial cells (Fryer *et al* 2002, Saha *et al* 2004, Konrad *et al* 2005, Lebrasseur *et al* 2006).

## ***6.2 Aims of this chapter***

This chapter was initiated to extend the observations discussed above and addressed the following issues.

- i.** Does rosiglitazone stimulate nitric oxide production in HAECS?
- ii.** Does rosiglitazone stimulate eNOS phosphorylation at Ser1177 in HAECS?
- iii.** Does rosiglitazone rapidly stimulate eNOS phosphorylation at Ser1177 and NO synthesis in an AMPK-dependent manner and/or PPAR gamma-independent manner?

### **6.3 Results**

#### ***6.3.1 Effect of Rosiglitazone on nitric oxide (NO) production***

Stimulation of HAECs with rosiglitazone (200  $\mu$ M) stimulated the rate of NO synthesis within 30 minutes and reached a maximum 2.1 fold increase at 60 min (Fig. 6.1a). The increase in the rate of NO synthesis was sustained for 24h. Under identical conditions, Stimulation of NO by rosiglitazone was dose dependent (Fig. 6.1b) such that NO synthesis was significantly stimulated by 2  $\mu$ M rosiglitazone and was stimulated maximally by 20  $\mu$ M rosiglitazone (2.1 fold).

#### ***6.3.2 Effect of Rosiglitazone on AMPK activity***

Stimulation of HAECs with rosiglitazone (200  $\mu$ M) maximally stimulated AMPK activity 3.5 fold within 30 minutes and activation was sustained for 24 hours (Figure 6.1a). Under identical conditions, stimulation of AMPK activity was also dose-dependent, such that 2  $\mu$ M rosiglitazone significantly stimulated AMPK activity, reaching a maximum 2.4 fold increase in AMPK activity at 200  $\mu$ M (Figure 6.1b).

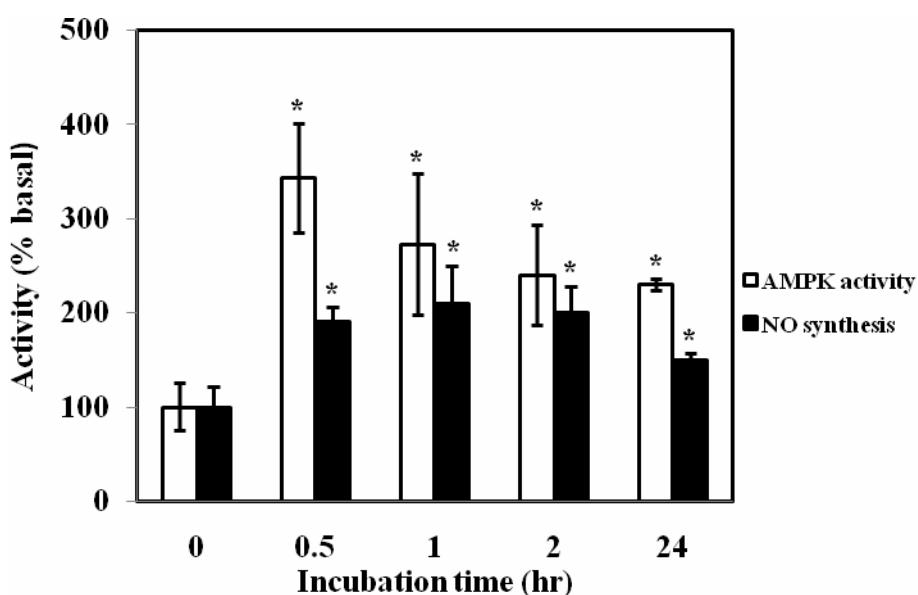
#### ***6.3.3 Effect of a PPARgamma inhibitor on Rosiglitazone stimulated NO production and AMPK activity***

The principal therapeutic actions of the TZDs are thought to be alterations in gene expression mediated by PPARgamma. Preincubation of HAECs with the PPARgamma inhibitor, GW9662 (5  $\mu$ M) was without effect on rosiglitazone-stimulated AMPK activity and NO synthesis (Figure 6.1b).

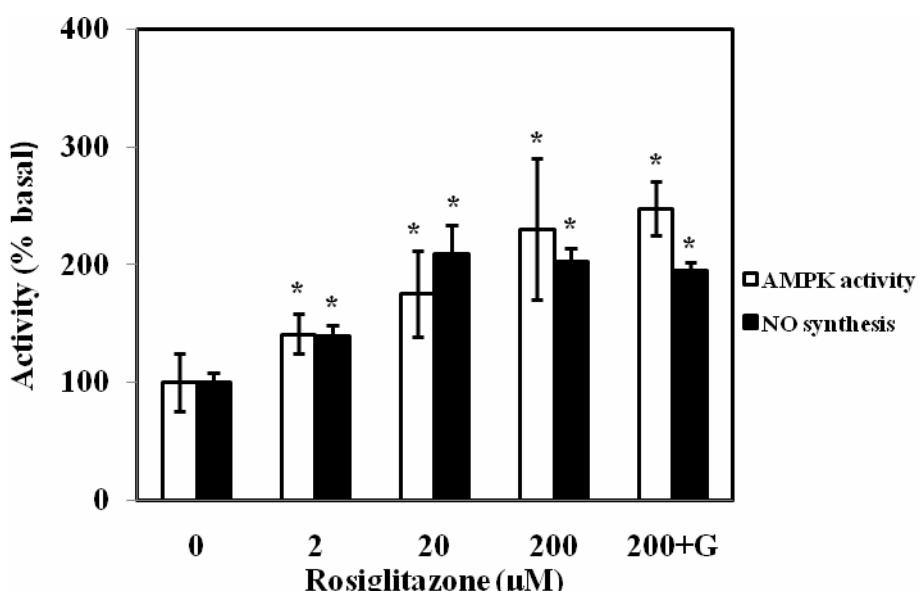
**Figure 6.1 Rosiglitazone stimulates AMPK and NO synthesis in HAECS.**

HAEC AMPK activity and the rate of L-NAME-sensitive  $\text{NO}_2^-$  synthesis in media were determined after incubation **A**) in 200  $\mu\text{M}$  rosiglitazone for the indicated durations and **B**) for the indicated concentrations of rosiglitazone for 1h after preincubation with GW9662 (G, 5  $\mu\text{M}$ ) for a further 1h. Data shown represents the mean % basal  $\pm$  S.E. NO synthesis or AMPK activity from 3 independent experiments. \*  $p<0.05$  relative to value in absence of rosiglitazone.

**A**



**B**

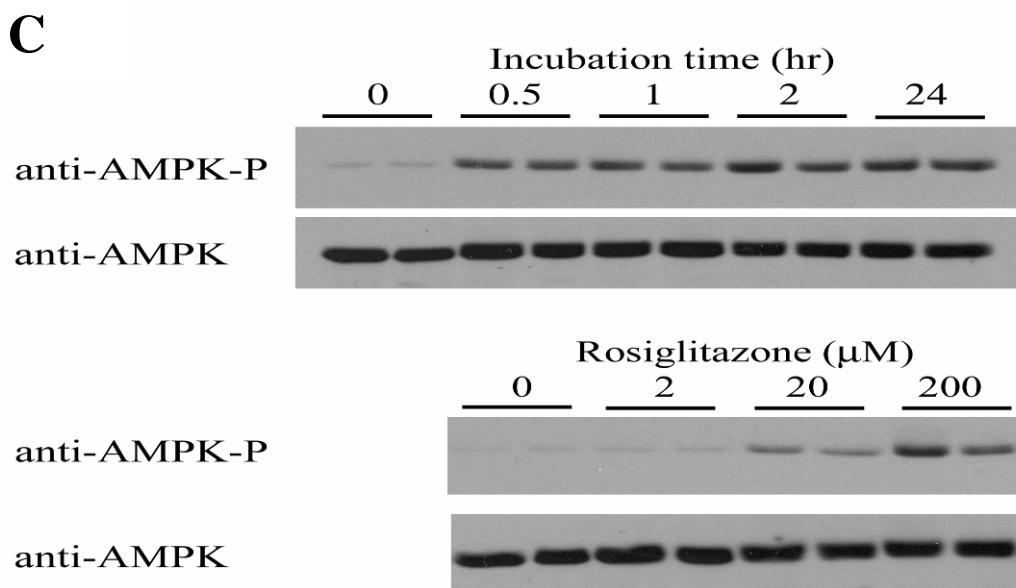


### 6.3.4 Effect on Rosiglitazone on phosphorylation of AMPK

Activation of AMPK requires phosphorylation at Thr172 by an AMPK kinase. Using a phospho-Thr172-specific anti-AMPK antibody, it was observed that that rosiglitazone also stimulates phosphorylation of AMPK at Thr172 in a time and concentration-dependent manner (Figures 6.1c); in close agreement with the AMPK assay data (Figures 6.1a & 6.1b).

**Figure 6.1 Rosiglitazone stimulates AMPK and NO synthesis in HAECs.**

C) Under identical conditions, AMPK Thr172 phosphorylation was determined in HAEC lysates. Representative immunoblots are shown from 3 independent experiments.



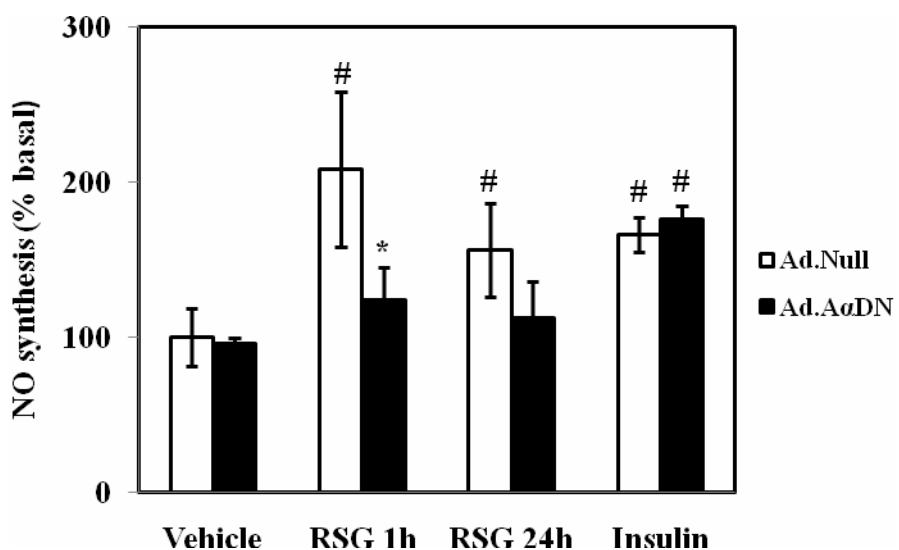
### ***6.3.5 Effect of the use of adenovirus expressing dominant negative AMPK on Rosiglitazone stimulated AMPK activation and NO stimulation***

To determine whether AMPK activation was required for rosiglitazone-stimulated NO synthesis, HAECS were infected with control (Ad.Null) adenoviruses or adenoviruses expressing dominant negative AMPK (Ad.alpha1DN) prior to incubation with rosiglitazone (200 µM) for 1 hours or 24 hours and NO production assessed. HAECS infected with Ad.alpha1DN exhibited significantly attenuated rosiglitazone (1 hour) stimulated NO production (Fig. 6.2a) compared with control virus-infected cells. In HAECS incubated with rosiglitazone for 24 hours there was a non-significant reduction in NO synthesis in cells infected with Ad.alpha1DN compared with control virus-infected cells. Insulin has previously been demonstrated to stimulate NO synthesis by PKB-mediated phosphorylation and activation of eNOS, independent of AMPK. Infection of HAECS had no effect on insulin-stimulated (1µM, 15min) NO synthesis indicating that the effect of infection with Ad.alpha1DN did not result in the non-specific downregulation of NO synthesis in an AMPK-independent manner (Fig. 6.2a). Infection with Ad.alpha1DN markedly attenuated rosiglitazone-stimulated AMPK activity, as assessed by ACC phosphorylation and was without effect on eNOS expression (Fig. 6.2b)

**Figure 6.2. Rosiglitazone-stimulated NO synthesis is AMPK-dependent.**

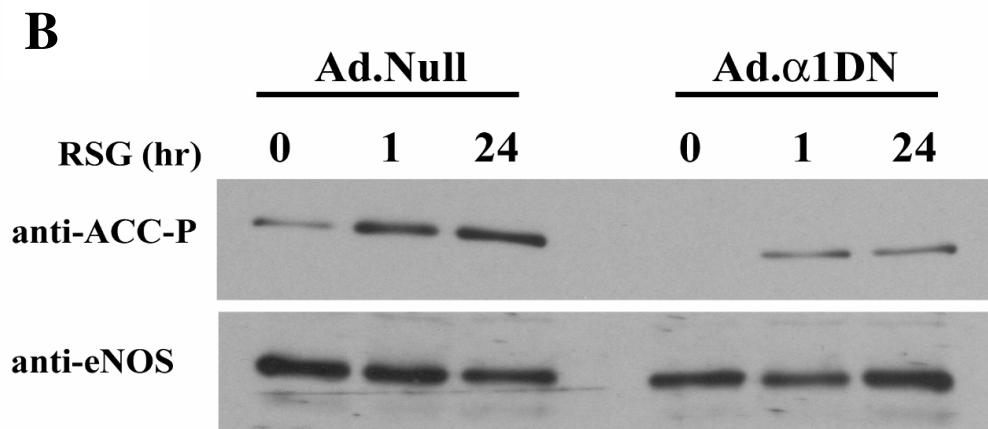
**A)** The rate of L-NAME-sensitive  $\text{NO}_2^-$  synthesis was determined in media of HAECS infected with Ad.Null or Ad. $\alpha$ 1DN after incubation in the presence or absence of rosiglitazone (RSG, 200  $\mu\text{M}$ , 1h or 24h) or insulin (1  $\mu\text{M}$ , 15min). Data shown represents the mean % basal  $\pm$  S.E. NO synthesis from 4 independent experiments. \* p<0.05 relative to value in Ad.Null-infected cells. # p<0.05 relative to vehicle-treated cells.

**A**



**Figure 6.2. Rosiglitazone-stimulated NO synthesis is AMPK-dependent.**

**B)** Under identical conditions, ACC phosphorylation and eNOS expression were determined in HAEc lysates. Representative immunoblots are shown from 3 independent experiments.

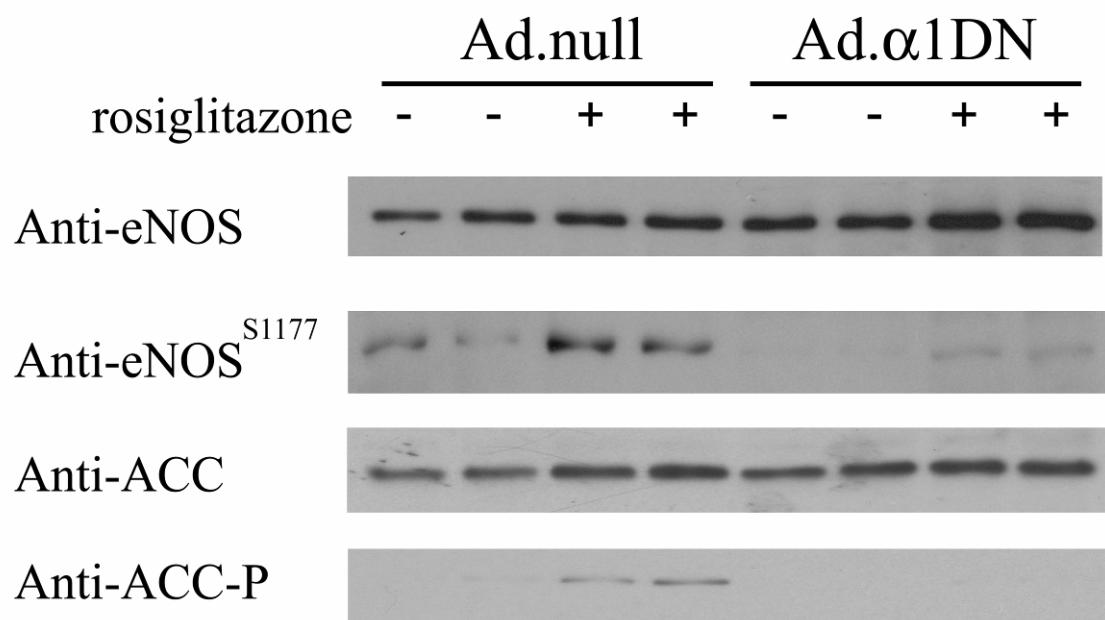


### ***6.3.6 Effect on Rosiglitazone on eNOS phosphorylation***

Phosphorylation of eNOS has been demonstrated to be an important determinant of NO production. In particular, phosphorylation at Ser1177 has been demonstrated to increase eNOS activity. I therefore determined the effects of thiazolidinediones on eNOS phosphorylation at these sites in HAECs using phosphorylation site-specific antibodies. Incubation of HAECs with rosiglitazone (20 $\mu$ M) stimulated phosphorylation of eNOS at Ser1177 (Fig. 6.3). Furthermore, rosiglitazone-stimulated eNOS Ser1177 phosphorylation was completely inhibited in HAECs infected with Ad.alpha1DN compared to cells infected with control Ad.null viruses. In the same lysates, infection with Ad.alpha1DN abrogated rosiglitazone-stimulated AMPK activity, as assessed by ACC phosphorylation (Fig. 6.3).

**Figure 6.3. Rosiglitazone-stimulated eNOS Ser1177 phosphorylation is AMPK-dependent.**

Phosphorylation of eNOS Ser1177 and ACC was determined in lysates prepared from HAECS infected with Ad.Null or Ad. $\alpha$ 1DN after incubation with rosiglitazone (20  $\mu$ M) for 1h. Representative immunoblots are shown, repeated with similar results on 3 different samples of lysates.



#### **6.4 Discussion**

The central finding of this chapter is that rosiglitazone rapidly stimulates NO production and eNOS Ser1177 phosphorylation in an AMPK-dependent manner in HAECS. The stimulation of AMPK by rosiglitazone is associated with increased phosphorylation of eNOS-Ser1177. The stimulation of NO production and phosphorylation of eNOS at Ser1177 is attenuated by infection of HAECS with Ad.alpha1DN. These data support the hypothesis that thiazolidinediones increase NO production by an AMPK-dependent mechanism. As AMPK has previously been demonstrated to stimulate NO synthesis via phosphorylation of eNOS at Ser1177, the complete inhibition of rosiglitazone-stimulated eNOS Ser1177 phosphorylation by infection with Ad.alpha1DN provides strong evidence that AMPK-mediated phosphorylation and activation of eNOS underlies rosiglitazone-stimulated NO synthesis.

Previous studies have demonstrated that incubation of human umbilical vein endothelial cells (HUVECs) with rosiglitazone for 48h stimulated NO synthesis and phosphorylation of eNOS at Ser1177 (Cho *et al* 2004). Similarly, troglitazone has been reported to stimulate NO synthesis within 3h and eNOS phosphorylation at the equivalent site (Ser1179) in bovine aortic endothelial cells (BAECs), (Polikandriotis *et al* 2005). In this chapter, I show for the first time that rosiglitazone stimulates NO synthesis and eNOS Ser1177 phosphorylation in HAECS, in agreement with the previous studies in HUVECs & BAECs. Furthermore, I demonstrate a mechanism by which the rapid stimulation of NO synthesis and eNOS Ser1177 phosphorylation is achieved.

In HUVECs, rosiglitazone-stimulated NO synthesis and eNOS Ser1177 phosphorylation were reported to be completely inhibited in the presence of the PPARgamma inhibitor, GW9662 (Polikandriotis *et al* 2005). In BAECs, however, troglitazone-stimulated NO synthesis and eNOS

Ser1179 phosphorylation was only partially inhibited in the presence of the PPARgamma inhibitor, BADGE (Cho *et al* 2004). In contrast, I demonstrated that rosiglitazone-stimulated NO synthesis was unaffected by the PPARgamma inhibitor, GW9662. The likely reason for the differences observed between the findings of this chapter and those conducted previously may reflect the different cell types utilized, but is more likely to be a result of the markedly different durations of rosiglitazone stimulation. I incubated cells with rosiglitazone for 1-2 hours, whereas Polikandriotis and colleagues stimulated HUVECs with rosiglitazone for 24 hours and Cho and co-workers stimulated with troglitazone for up to 24 hours (Cho *et al* 2004, Polikandriotis *et al* 2005).

As the principal effect of PPARgamma is the regulation of gene transcription, it seems likely that the acute effects observed in the current study are too rapid to be the result of PPARgamma-mediated alterations in gene transcription. I cannot, however, rule out the later (12-24 hours) effects of rosiglitazone and other thiazolidinediones being the result of altered gene transcription.

A number of studies have demonstrated that thiazolidinediones have the capacity to improve endothelial function in non-diabetic subjects and subjects with type 2 diabetes. Despite the TZD related improvements in vascular biology and endothelial function described above, it is important to note that metformin remains the only oral hypoglycaemic drug to demonstrate good evidence for cardiovascular protection in a randomised trial. Indeed, in both the large scale clinical trials involving thiazolidinediones, PROactive and RECORD, pioglitazone and rosiglitazone failed to confer cardiovascular protection in patient with T2DM. It is therefore clear that the molecular mechanisms by which rosiglitazone and other thiazolidinediones have their effects on the cardiovascular system are complex.

The beneficial effects of TZDs have been reported to be independent of glycaemia, inferring a direct effect on vascular tissues (Satoh *et al* 2003, Pitrosch *et al* 2004). The endothelial effects of TZDs may be relatively rapid; indeed rosiglitazone has been reported to improve endothelial function within 24 hours in healthy human subjects and it is increasingly clear that the TZDs have non-genomic, PPARgamma-independent effects (Hetzl *et al* 2005). Furthermore, stimulation of NO synthesis and AMPK activity by rosiglitazone was achieved at concentrations as low as 2  $\mu$ M. These concentrations are close to those achieved clinically; healthy subjects given 8 mg of rosiglitazone have been demonstrated to exhibit a peak plasma rosiglitazone concentration of 0.7 - 0.8  $\mu$ M after 1 hour (Kim *et al* 2004). The findings of this final chapter of results provide evidence for an AMPK-dependent mechanism by which TZDs rapidly contribute to increase NO bioavailability. This mechanism may underlie the rapid effects of TZDs on endothelial function that are independent of PPARgamma-mediated alterations in gene transcription.

The work in this final chapter has been complemented and significantly extended by work carried out by my laboratory colleagues.

I demonstrated that preincubation of HAECS with GW9662 had no effect was on rosiglitazone-stimulated AMPK activity and NO synthesis, suggesting that this was a PPARgamma independent effect of rosiglitazone. Importantly, this observation has been extended with data confirming that preincubation of HAECS with GW9662 prevents rosiglitazone stimulated expression of CD36 mRNA, indicating that GW9662 does effectively inhibit PPARgamma-mediated transcription in HAECS under these conditions. This finding adds weight to the suggestion that that PPARgamma is not an upstream component of rapid thiazolidinedione-stimulated, AMPK-dependent NO synthesis (Boyle *et al* 2008).

Two upstream AMPK kinases have been isolated to date, LKB1 and Ca<sup>2+</sup>/calmodulin-dependent kinase kinase (CaMKK), (Hawley *et al* 2003, Woods *et al* 2003, Hawley *et al* 2005).

It has been proposed that LKB1 activity is constitutive, such that AMP binding to AMPK inhibits dephosphorylation at Thr172 permitting phosphorylation and activation by LKB1 (Woods *et al* 2003). Further work by laboratory colleagues has determined that LKB1 activity is necessary for rosiglitazone-stimulated AMPK activity. HeLa cells do not express endogenous LKB1, and therefore the effect of rosiglitazone on AMPK activity and AMPK Thr172 phosphorylation in HeLa cells stably expressing wild type (LKB1-WT) or kinase inactive mutant (LKB1-KD) LKB1 was investigated (Sapkota *et al* 2002). Rosiglitazone stimulated AMPK activity and AMPK Thr172 phosphorylation in cells expressing LKB1-WT, but no effect of rosiglitazone was apparent in HeLa cells expressing LKB1-KD (Boyle *et al* 2008). Moreover, Rosiglitazone had no significant effect on LKB1 activity as assessed by its ability to activate recombinant AMPK kinase domain. These data provides evidence that rosiglitazone stimulates AMPK activity utilizing a LKB1-dependent mechanism and is in agreement with previous studies showing no changes in LKB1 activity with phenformin, AICAR, muscle contraction and extreme ischemia in a variety of tissues (Lizcano *et al* 2004, Sakamoto *et al* 2004, Altarejos *et al* 2005).

It is now apparent that CaMKK can act as an alternate upstream kinase to LKB1 that activates AMPK in a Ca<sup>2+</sup>-dependent and AMP-independent manner (Hawley *et al* 2005, Woods *et al* 2005). It has been demonstrated that HAECS express CaMKK and that CaMKK mediates VEGF-stimulated AMPK activation in HAECS (Reihill *et al* 2007). Further work has demonstrated that preincubation of cells with the CaMKK inhibitor STO-609 has no significant effect on rosiglitazone-stimulated AMPK activity, AMPK Thr172 phosphorylation or phosphorylation of the AMPK substrate, acetyl CoA carboxylase (ACC), yet completely inhibits VEGF-stimulated

AMPK activity (Boyle *et al* 2008). This observation suggests that CaMKK is not an upstream kinase responsible for AMPK activation in response to rosiglitazone.

It has also been determined that other thiazolidinediones activate AMPK in HAECS. Stimulation of HAECS with troglitazone or pioglitazone (20  $\mu$ M) for one hour stimulated AMPK activity, AMPK Thr172 phosphorylation and ACC phosphorylation and preincubation with GW9662 was without effect (Boyle *et al* 2008). This observation suggests that activation of AMPK is a common mechanism utilized by thiazolidinediones.

Finally, endothelial NO synthesis has been demonstrated to inhibit monocyte adhesion to the endothelium, an early, key step in atherogenesis. A previous study has demonstrated a modest reduction in TNF- $\alpha$ -stimulated adhesion of U937 cells to HAECS in response to incubation with pioglitazone for 24h (Kurebayashi *et al* 2005). Further work in our laboratory demonstrated that acute (1 hour) stimulation with rosiglitazone was sufficient to reduce TNF- $\alpha$ -stimulated adhesion of U937 cells to HAECS in an L-NAME-sensitive manner (Boyle *et al* 2008). These data suggest that AMPK-mediated rosiglitazone-stimulated NO synthesis has rapid functional effects.

In summary, the findings of this chapter provide evidence for an AMPK-dependent mechanism by which thiazolidinediones rapidly contribute to increased NO bioavailability. This mechanism may underlie the rapid effects of thiazolidinediones on endothelial function that are independent of PPARgamma-mediated alterations in gene transcription.

*Chapter 7 Discussion*

### **7.1 Background**

Cardiovascular risk assessment in patients with T2DM shows that even when traditional cardiovascular risk factors (smoking, hypertension and hypercholesterolaemia) are corrected for, insulin resistance remains an independent risk factor for vascular disease (Laakso 1999). Moreover, despite aggressive treatment of the individual risk factors, death from T2DM remains unacceptably high (Ford 2005). Insulin sensitizers such as the biguanides and thiazolidinediones are widely used in the treatment of T2DM but the site and precise mode of their action remain uncertain. The prevalence of T2DM is reaching epidemic proportions and there is a growing need for new therapeutic targets. AMPK is a novel therapeutic target because it has been demonstrated to mediate, at least in part, the effects of a number of physiological and pharmacological factors that exert beneficial effects on metabolism and the vasculature. Thus, the development of specific tissue specific AMPK activators is an attractive prospect for the future.

### **7.2 Aims**

The aims of this thesis were as follows:

- i. To determine if metformin improves vascular endothelial function in association with a lower circulating concentration of ADMA.
- ii. To determine if metformin exerts beneficial metabolic effects in patients with T2DM in association with altered AMPK activity in human adipose.
- iii. To determine if insulin sensitizers such as metformin and the thiazolidinediones, acting directly on vascular endothelial cells, increase NO production by increasing AMPK activity thus accounting for beneficial effects on endothelial function and, in metformin's, case cardiovascular outcome.

***7.3 Metformin fails to improve vascular function but is associated with a small fall in circulating concentrations of ADMA when compared with Gliclazide therapy.***

I hypothesized that a short duration of metformin therapy would improve vascular function in association with lower circulating concentrations of ADMA in comparison with gliclazide therapy. To test this hypothesis I compared the effects of metformin versus gliclazide on large artery stiffness, resistance artery function and circulating concentrations of ADMA using a randomised, double-blind crossover design in subjects with Type 2 diabetes. One of the main aims was to confirm or refute a beneficial effect of metformin on circulating ADMA concentrations in view of recent supportive data to this effect from uncontrolled trials. It was also hypothesised that ADMA might not only be a predictive marker but also a causative risk factor for cardiovascular disease and thus any metformin-induced reduction in ADMA concentration would be accompanied by an improvement in vascular function. The data demonstrates that metformin does indeed lower ADMA compared with gliclazide despite better glycaemia changes with the latter, supporting a glucose-independent mechanism for this metformin effect. Finally, the data show that the change in ADMA concentration was not associated with any difference in large artery stiffness as measured by pulse wave velocity or resistance artery function as measured by wire myography.

There are certain limitations to this work. The reduction in ADMA was modest and the observed magnitude of difference contrasts with reports of much larger reductions in ADMA concentration in uncontrolled studies of metformin therapy in women with PCOS as well as poorly controlled subjects with Type 2 diabetes, where the drug was added to diet control or sulphonylurea therapy (Abbasi *et al* 2001, Heutling *et al* 2008). Thus, while of interest, the difference in ADMA could be deemed as biologically insignificant and clinically irrelevant. It is also particularly important to consider the implication of the missing data set in the wire myography data; eighteen subjects had at least one successful biopsy with myography data with only four subjects having paired

myography results. An absence of vessels of suitable diameter was the reason for failure to obtain vessels from biopsies with no myography data although the vessel yield in this study is consistent with other wire myography studies completed by our group and others. The data are therefore insufficiently powered to detect any small changes in vascular function in association with this dose and duration of treatment. The data also failed to show that metformin conferred a benefit in pulse wave velocity and it was important to consider why. In the present study subjects were not randomised to the maximum dose of each drug and the duration of treatment was relatively short at 10 weeks. Whether a longer duration of each treatment in higher dose would have been associated with a larger change in ADMA concentration with measurable improvements in vascular function in favour of metformin is unknown and could be the subject of further study. The study design aimed to match glycaemic control at the end of each phase but this, in the event, proved not to be possible. Associated with these changes was a small improvement in plasma LDL cholesterol. Thus, it is reasonable to speculate that confounding effects related to better short term glycaemia and LDL-cholesterol improvements with gliclazide may have attenuated any metformin-induced change in ADMA being associated with an associated beneficial improvement in vascular function. Finally, the decision at the design stage to not make baseline measurements of both PWV and ADMA concentration at the beginning of each phase may have made strict interpretation of the results more difficult. From this we could truly know if metformin treatment lowered ADMA concentration as it is impossible to distinguish if the final results represent an effect of gliclazide to raise ADMA concentration or PWV. The protocol for each patient was already fairly rigorous and therefore an assessment of resistance artery endothelial function would have been difficult to justify (two additional buttock biopsies). Future studies of metformin at higher doses for a longer duration are required to determine whether ADMA lowering by metformin improves vascular function and more importantly, whether such a reduction helps explain the vascular end-point benefits of this treatment in prospective clinical trials.

#### ***7.4 Metformin increases AMPK activity in adipose tissue in patients with T2DM when compared with Gliclazide therapy.***

I hypothesized that a short duration of metformin therapy exerts beneficial metabolic effects in patients with T2DM in association with increased AMPK activity in human adipose and that this may be associated with changes in circulating levels of plasma adiponectin concentration. To test these hypotheses, this thesis explored metformin's action in human adipose tissue and plasma adiponectin, an area thus far neglected in the literature. This chapter compared the effects of metformin versus gliclazide on adipose AMPK activity and plasma adiponectin using a randomised, double-blind crossover design in subjects with Type 2 diabetes. The main aim was to confirm or refute a beneficial effect of metformin on adipose AMPK activity. It was also hypothesised that any increase in AMPK activity may be associated with an increase in plasma adiponectin concentration. The data demonstrate that metformin does indeed increase adipose AMPK compared with gliclazide. The increase in adipose AMPK activity was not associated with an increase in AMPK protein expression. This finding suggests that the change in AMPK activity is an increase in the specific activity of AMPK rather than simply a difference in the total amount in AMPK between phases. The study design aimed to match glycaemic control at the end of each phase but this, in the event, proved not to be possible. Metformin increased AMPK activity when compared with gliclazide despite better glycaemia changes with the latter, supporting a glucose-independent mechanism for this metformin effect. Associated with these changes was a small improvement in plasma LDL cholesterol with gliclazide. Somewhat surprisingly, there was no significant difference in BMI after each treatment although measurement of skin fold thickness or more sophisticated measurement of body composition may have provided a more specific marker of fat mass. Finally, the data show that the change in adipose AMPK activity was not associated with any difference in plasma adiponectin.

There are certain limitations to this work. Firstly, while a robust and significant difference in AMPK activity with metformin when compared with gliclazide is demonstrated it is important to note that the effect of gliclazide on AMPK activity has not been widely studied. The principal mechanism of action of the sulphonylurea class of drugs is to improve insulin secretion by the beta cells of the endocrine pancreas, and the data concerning the effects of sulphonylurea on AMPK are limited. Indeed, glimepiride treatment has been shown to have no effect on AMPK activity in cultured myocytes as expressed by AMPK phosphorylation while gliclazide has no effect (up to 1 hour incubation) in 3T3-L1 adipocytes on AMPK activity as assessed by kinase assay (Inukai *et al* 2005, P J Logan, personal communication). Secondly, there is also no functional data examining downstream targets of AMPK. Without evidence of downstream effects any beneficial metabolic effects associated with increased AMPK activity with metformin therapy can only be implied. Finally, while the vast majority of the tissue biopsy will constitute human adipose only approximately 50% of the cells are adipocytes and therefore it is impossible to quantify whether any presence of blood or macrophages could confound the results and that the increase in AMPK activity is in the circulating plasma or in vascular cells. Finally, again the decision at the design stage to not make baseline measurements of plasma adiponectin at the beginning of each phase may have made strict interpretation of the results more difficult. From this we could truly know if metformin treatment increased plasma adiponectin as it is impossible to distinguish if the final results represent an effect of gliclazide to increase adiponectin concentration. It is also important to consider the limitations of the plasma adiponectin assay which is a crude measurement of only total adiponectin using ELISA. The exact physiological role of adiponectin is incompletely understood and further work is required to determine whether there is a relationship between AMPK and adiponectin in the action of metformin. Further work elucidating the molecular mechanisms by which metformin activates AMPK in adipose tissue may identify novel and tissue specific therapeutic targets in subjects with Type 2 diabetes.

### **7.5 Phenformin, but not Metformin stimulates NO in an AMPK dependant manner.**

I hypothesized that biguanide therapy would stimulate NO production in an AMPK dependant manner in HAECS. Metformin failed to stimulate NO production or AMPK activity in HAECS. In contrast, phenformin rapidly stimulated NO production in an AMPK-dependent manner in HAECS. The stimulation of AMPK by phenformin is associated with increased phosphorylation of eNOS-Ser1177. The stimulation of NO production is attenuated by infection of HAECS with Ad.alpha1DN. These data support the hypothesis that phenformin increases NO production by an AMPK-dependent mechanism. As AMPK has previously been demonstrated to stimulate NO synthesis via phosphorylation of eNOS at Ser1177, the inhibition of phenformin-stimulated NO production by infection with Ad.alpha1DN suggests that AMPK-mediated phosphorylation and activation of eNOS underlies phenformin-stimulated NO synthesis.

There are certain limitations to this work. In retrospect, the hypothesis that AMPK-mediated phosphorylation and activation of eNOS underlies phenformin-stimulated NO synthesis could have been further strengthened if I had demonstrated that phosphorylation of eNOS at Ser1177 and ACC was attenuated by infection with Ad.alpha1DN. It is also important to consider the neutral data with metformin. It has recently been reported that metformin stimulates NO synthesis in an AMPK-dependent manner in BAECs and has been demonstrated to phosphorylate AMPK and ACC in HUVECs (Zou *et al* 2004, Hattori *et al* 2006). I was unable to show any effect of metformin within 2 hours on either AMPK activity or NO production, in agreement with previous findings (Morrow *et al* 2003). Phenformin, however, robustly stimulated AMPK activity, ACC phosphorylation, eNOS Ser1177 phosphorylation and NO production in HAECS. The contrasting results make interpretation of the findings with phenformin more difficult. Phenformin is a more lipophilic and potent biguanide which was removed from clinical practice due to the incidence of lactic acidosis and is often used in *in vitro* experimentation as the biguanide of choice. The dose of phenformin

most commonly used in the experiments described was 200 $\mu$ M, which is supra-physiological. It is possible that metformin, being less lipophilic, was unable to reach a sufficiently high intracellular concentration to exhibit effects during this short course of my experiments. It is also important to consider if a longer incubation period or a higher concentration of metformin may be required to stimulate AMPK and NO synthesis in HAECs. The plasma concentrations of metformin in patients treated with the drug are estimated to be 10–20  $\mu$ M (Bailey *et al* 1996). It is important to consider that in many experiments the doses used to activate metformin are consistently higher in a range between 1 and 10 mM (Yang *et al* 2006, Zhang *et al* 2007). Higher doses of metformin are not used in patients with T2DM due to gastrointestinal side effects and also because of the risk of lactic acidosis, both of which might be caused by the ability of the compound to act as an inhibitor of the respiratory chain at high concentrations (Owen *et al* 2000). Further work is required to determine the time and concentration of metformin which will result in the maximal beneficial effects of AMPK activation without the development of gastrointestinal side effects and lactic acidosis. Furthermore, it is impossible to exclude if the neutral results with metformin may also be explained by the possibility of certain HAECs either not expressing OCT-1 which is expressed particularly in the liver or carrying reduced function polymorphisms of OCT-1 (Wang *et al* 2002). Finally, it is important to state that while phenformin has been shown to stimulate NO production and AMPK activity in HAECs, further work is required to confirm if this would translate into beneficial metabolic and vascular effects in patients with T2DM.

### **7.6 Rosiglitazone stimulates NO in an AMPK dependent manner.**

I hypothesized that rosiglitazone therapy would stimulate NO production in an AMPK dependant manner in HAECs. Rosiglitazone rapidly stimulates NO production and eNOS Ser1177 phosphorylation in an AMPK-dependent manner in HAECs. The stimulation of AMPK by rosiglitazone is associated with increased phosphorylation of eNOS-Ser1177. The stimulation of

NO production and phosphorylation of eNOS at Ser1177 is attenuated by infection of HAECS with Ad.alpha1DN. These data support the hypothesis that thiazolidinediones increase NO production by an AMPK-dependent mechanism. As AMPK has previously been demonstrated to stimulate NO synthesis via phosphorylation of eNOS at Ser1177, the complete inhibition of rosiglitazone-stimulated eNOS Ser1177 phosphorylation by infection with Ad.alpha1DN provides strong evidence that AMPK-mediated phosphorylation and activation of eNOS underlies rosiglitazone-stimulated NO synthesis. Moreover, rosiglitazone-stimulated NO synthesis was unaffected by the PPARgamma inhibitor, GW9662.

There are certain limitations to this work. I demonstrated that rosiglitazone-stimulated NO synthesis was unaffected by the PPARgamma inhibitor, GW9662. As the principal effect of PPARgamma is the regulation of gene transcription, it seems likely that the acute effects observed in the current study are too rapid to be the result of PPARgamma-mediated alterations in gene transcription. This is in disagreement with work in other cell lines. Indeed, in HUVECs, rosiglitazone-stimulated NO synthesis and eNOS Ser1177 phosphorylation were reported to be completely inhibited in the presence of the PPARgamma inhibitor, GW9662 (Polikandriotis *et al* 2005). Moreover, in BAECs, however, troglitazone-stimulated NO synthesis and eNOS Ser1179 phosphorylation was only partially inhibited in the presence of the PPARgamma inhibitor, BADGE (Cho *et al* 2004). The likely reason for the differences observed between the findings in this thesis and those conducted previously may reflect the different cell types utilized, but is more likely to be a result of the markedly different durations of rosiglitazone stimulation. I incubated cells with rosiglitazone for 1-2 hours, whereas Polikandriotis and colleagues stimulated HUVECs with rosiglitazone for 24 hours and Cho and co-workers stimulated with troglitazone for up to 24 hours (Cho *et al* 2004, Polikandriotis *et al* 2005). I cannot, however, rule out the later (12-24 hours) effects of rosiglitazone and other thiazolidinediones being the result of altered gene transcription and it would

have been of value to compare the effect of GW9962 on NO production and AMPK activity at both the 1 and 24 hour time points. Finally, it is important to state that while rosiglitazone has been shown to stimulate NO production and AMPK activity in HAECS, further work is required to confirm if this would translate into beneficial metabolic and vascular effects in patients with T2DM. It is also important to consider that despite the TZD related improvements in vascular biology and endothelial function described above metformin remains the only oral hypoglycaemic drug to demonstrate good evidence for cardiovascular protection in a randomised trial. Indeed, in both the large scale clinical trials involving thiazolidinediones, PROactive and RECORD, pioglitazone and rosiglitazone failed to confer cardiovascular protection in patients with T2DM. It is therefore clear that the molecular mechanisms by which rosiglitazone and other thiazolidinediones have their effects on the cardiovascular system are complex.

### ***7.7 Future work and summary***

AMPK is an attractive goal for therapeutic intervention in T2DM. As demonstrated in the data presented in this thesis in both adipose tissue of patients with T2DM and cultured human vascular endothelial cells, two of the most commonly used treatments for diabetes, biguanides and thiazolidinediones, may well exert a proportion of their beneficial effects through the activation of AMPK. The generation of more specific and potent activators of AMPK, however, could have additional metabolic and vascular benefits for patients with T2DM.

At this point in time, it is still uncertain whether direct activation of the AMPK pathway in patients with T2DM will be safe. It is unknown whether directly activating AMPK in the absence of depleted energy stress or physiological stress would be detrimental. It is certain that the effects of AMPK activation in other organs or tissues need to be carefully evaluated as the widespread cellular functions of AMPK will make selective targeting challenging for the pharmaceutical

industry. A greater understanding of specific AMPK signalling pathways and their regulation will be crucial for the development of such drug therapy.

Three target tissues have caused concern in the literature to date; hypothalamus, pancreas and the heart. The hypothalamus plays a key role in the regulation of energy balance. It does so by using AMPK as a fuel gauge that can respond to both hormones, anorexia or an excess of food. (Schwartz *et al* 2000, Andersson *et al* 2004). Injection of leptin into rodents inhibits AMPK activity in the hypothalamus whereas administration of ghrelin stimulates AMPK activity (Andersson *et al* 2004). Moreover, fasting stimulates AMPK activity whereas hyperglycaemia and refeeding inhibits AMPK activity in the hypothalamus (Minokowski *et al* 2004). Therefore, it appears that activating AMPK can stimulate food intake through its action in the hypothalamus. While this effect probably represents a physiological response in glucose homeostasis, the prospect of patients gaining weight through the pharmacological targeting of AMPK for the treatment of T2DM is unattractive and presents a significant challenge to the pharmaceutical industry. It is important to note, however, that this theoretical side effect of AMPK activators remains to be established.

Another area of concern is AMPK's role in the pancreas. It has been demonstrated that an increase in glucose levels represses AMPK activity in  $\beta$  cell lines (Salt *et al* 1998, Le Clerc *et al* 2004), whereas when AMPK is activated by AICAR, insulin release in response to high glucose is attenuated (Zhang *et al* 1995). Furthermore, overexpression of a constitutively active form of AMPK results in a reduction in insulin release from  $\beta$  cell lines while overexpression of a dominant negative form of AMPK leads to an increase in insulin release (Da Silva Xavier *et al* 2003). Moreover, when metformin is added to  $\beta$  cell lines, AMPK is activated and insulin secretion in response to insulin is attenuated (Leclerc *et al* 2004). The activation of pancreatic AMPK is therefore associated with decreased insulin secretion which could have a negative effect on

glycaemic control in patients with T2DM. This effect may, however, be a protective effect during periods of hypoglycaemia, where AMPK is activated and insulin secretion falls to maintain euglycaemia. Nevertheless, the exact role of AMPK in insulin release remains incompletely understood and further work is required before AMPK can be targeted by drug therapy in patients with T2DM.

Finally, alterations in cardiac AMPK activity are associated with a number of cardiovascular-related diseases including glycogen storage cardiomyopathy (Arrad *et al* 2003) and Wolff–Parkinson–White syndrome (Gollob *et al* 2001) suggesting that the manipulation of AMPK activity in cardiac tissue may not be without risks.

All of these uncertainties will need to be clarified by further work defining the effects of AMPK activation in the various target tissues described above. An ideal AMPK activator would be administrated by the oral route, activate AMPK at low concentration and be effective in specific target organs, such as the liver, skeletal muscle and adipose tissue but not the hypothalamus or pancreas. Tissue-specific pharmacological activation of AMPK is, therefore, essential and could potentially be achieved through isoform-specific activation or targeting of downstream substrates of AMPK. The first validation of the beneficial effects of a direct AMPK activator were recently reported (Cool *et al* 2006). The small molecule, compound A-769662 has been shown to directly activate AMPK both *in vitro* and *in vivo*. In cell culture studies A-769662 has been shown to reduce fatty acid synthesis in primary rat hepatocytes (Cool *et al* 2006). Moreover, acute intraperitoneal injection of A-769662 in Sprague–Dawley rats rapidly decreased hepatic malonyl CoA levels and stimulated fatty acid oxidation. Furthermore, treatment of ob/ob mice with A-769662 for five days, again by injection, decreased plasma glucose and triglyceride concentrations, lowered hepatic triglyceride content and glucose production and reduce body weight. These results

demonstrate that small-molecule-mediated activation of AMPK *in vivo* is feasible and, therefore may represent a promising approach for the treatment of patients with T2DM. It is important to note that the compound A-769662 has poor oral bioavailability which at the present time limits its use in a clinical setting. This study does however pave the way for the development of an alternative small-molecule compound that if safe, potent, acted directly on AMPK and had good oral bioavailability, would be an attractive candidate to progress towards clinical study.

*Bibliography*

Abate N, Garg A, Peshock RM, Stray-Gundersen J, Adams-Huet B, Grundy SM: Relationship of generalized and regional adiposity to insulin sensitivity in men with NIDDM. *Diabetes* 45:1684-1693, 1996.

Abbasi F, Carantoni M, Chen YD, Reaven GM: Further evidence for a central role of adipose tissue in the antihyperglycemic effect of metformin. *Diabetes Care* 21:1301-1305, 1998.

Abbasi F, Asagami T, Cooke JP: Plasma concentrations of asymmetric dimethylarginine are increased in patients with type 2 diabetes mellitus. *Diabetes* 50:147 (suppl 2), 2001.

Abe H, Yamada N, Kamata K, Kuwaki T, Shimada M, Osuga J, Shionoiri F, Yahagi N, Kadokawa T, Tamemoto H, Ishibashi S, Yazaki Y, Makuuchi M: Hypertension, hypertriglyceridemia, and impaired endothelium-dependent vascular relaxation in mice lacking insulin receptor substrate-1. *J.Clin.Invest.* 101:1784-1788, 1998.

Achan V, Broadhead M, Malaki M, Whitley G, Leiper J, MacAllister R, Vallance P: Asymmetric dimethylarginine causes hypertension and cardiac dysfunction in humans and is actively metabolized by dimethylarginine dimethylaminohydrolase. *Arterioscler.Thromb.Vasc.Biol.* 23: 1455-1459, 2003.

Alberti KGMM, Zimmet P, Shaw J: for the IDF Epidemiology Task Force Consensus Group: The metabolic syndrome – a new worldwide definition. *Lancet* 366:1059 -1062, 2005.

Altarejos JY, Taniguchi M, Clanachan AS, Lopaschuk GD: Myocardial ischemia differentially regulates LKB1 and an alternate 5'-AMP-activated protein kinase kinase. *J.Biol.Chem.* 280:183-190, 2005.

Altinova AE, Arslan M, Sepici-Dincel A, Akturk M, Altan N, Toruner FB: Uncomplicated type 1 diabetes is associated with increased asymmetric dimethylarginine concentrations. *J.Clin.Endocrinol.Metab.* 92:1881-1885, 2007.

An Z, Wang H, Song P, Zhang M, Geng X, Zou MH: Nicotine-induced activation of AMP-activated protein kinase inhibits fatty acid synthase in 3T3L1 adipocytes: a role for oxidant stress. *J.Biol.Chem.* 282:26793-26801, 2007.

Andersson U, Filipsson K, Abbott CR, Woods A, Smith K, Bloom SR, Carling D, Small CJ: AMP-activated protein kinase plays a role in the control of food intake. *J.Biol.Chem.* 279:12005–12008, 2004.

Andreelli F, Foretz M, Knauf C, Cani PD, Perrin C, Iglesias MA, Pillot B, Bado A, Tronche F, Mithieux G, Vaulont S, Burcelin R, Viollet B: Liver adenosine monophosphate-activated kinase-alpha2 catalytic subunit is a key target for the control of hepatic glucose production by adiponectin and leptin but not insulin. *Endocrinology* 147:2432–2441, 2006.

Andreozzi F, Laratta E, Procopio C, Hribal ML, Sciacqua A, Perticone M, Miele C, Perticone F, Sesti G: Interleukin-6 impairs the insulin signaling pathway, promoting production of nitric oxide in human umbilical vein endothelial cells. *Mol. Cell Biol.* 27:2372-2383, 2007.

Angus JA, Wright CE: Techniques to study the pharmacodynamics of isolated large and small blood vessels. *J.Pharmacol.Toxicol.Methods* 44:395-407, 2000.

Arad M, Benson DW, Perez-Atayde AR, McKenna WJ, Sparks EA, Kanter RJ, McGarry K, Seidman JG, Seidman CE: Constitutively active AMP kinase mutations cause glycogen storage disease mimicking hypertrophic cardiomyopathy. *J.Clin.Invest.* 109:357–362, 2002.

Arita Y, Kihara S, Ouchi N, Takahashi M, Maeda K, Miyagawa J, Hotta K, Shimomura I, Nakamura T, Miyaoka K, Kuriyama H, Nishida M, Yamashita S, Okubo K, Matsubara K, Muraguchi M, Ohmoto Y, Funahashi T, Matsuzawa Y: Paradoxical decrease of an adipose-specific protein, adiponectin, in obesity. *Biochem.Biophys.Res.Commun.* 257:79-83, 1999.

Asagami T, Abbasi F, Stuelinger M, Lamendola C, McLaughlin T, Cooke JP, Reaven GM, Tsao PS: Metformin treatment lowers asymmetric dimethylarginine concentrations in patients with type 2 diabetes *Metabolism* 51:843-846, 2002.

Asmar R, Benetos A, Topouchian J, Laurent P, Pannier B, Brisac AM, Target R, Levy BI: Assessment of arterial distensibility by automatic pulse wave velocity measurement. Validation and clinical application studies. *Hypertension* 26:485-490, 1995.

Asmar R, Rudnichi A, Blacher J, London GM, Safar ME: Pulse pressure and aortic pulse wave are markers of cardiovascular risk in hypertensive populations. *Am.J.Hypertens.* 14:91-97, 2001.

Bailey CJ, Mynett KJ, Page T: Importance of the intestine as a site of metformin-stimulated glucose utilization. *Br.J.Pharmacol.* 112:671-675, 1994.

Bailey CJ, Turner RC: Metformin. *N Engl J Med.* 334, 574–579, 1996.

Bailey CJ, Day C: Metformin: its botanical background. *Practical Diabetes International* 21:115–117, 2004.

Banerji M, Chaiken R, Gordon D, Lebowitz H: Does intra-abdominal adipose tissue in black men determine whether NIDDM is insulin resistant or insulin sensitive? *Diabetes* 44:141-146, 1995.

Basu A, Basu R, Shah P, Vella A, Johnson CM, Nair KS, Jensen MD, Schwenk WF, Rizza RA: Effects of type 2 diabetes on the ability of insulin and glucose to regulate splanchnic and muscle glucose metabolism: evidence for a defect in hepatic glucokinase activity. *Diabetes* 49:272-283, 2000.

Behrendt D, Ganz P: Endothelial Function: From vascular biology to clinical applications. *Am. J. Cardiol.* 90(suppl):40-48, 2002

Bevan JA, Osher JV: A direct method for recording tension changes in the wall of small blood vessels in vitro. *Agents Actions* 2:257-260, 1972.

Bevan P: Insulin signalling. *J. Cell Sci.* 114:1429-1430, 2001.

Birk JB, Wojtaszewski JF: Predominant alpha2/beta2/gamma3 AMPK activation during exercise in human skeletal muscle. *J Physiol.* 577:1021–1032, 2006.

Blacher J, Asmar R, Djane S, London GM, Safar ME: Aortic pulse wave velocity as a marker of cardiovascular risk in hypertensive patients. *Hypertension* 33:1111-1117, 1999.

Blackwell S, O'Reilly DS, Talwar D: Biological variation of asymmetric dimethylarginine and related arginine metabolites and analytical performance goals for their measurement in human plasma. *Eur.J. Clin.Invest.* 37:364-371, 2007.

Boden G: Role of fatty acids in the pathogenesis of insulin resistance and NIDDM. *Diabetes* 46:3-10, 1997.

Boger RH, Bode-Boger SM, Szuba A, Tsao PS, Chan JR, Tangphao O, Blaschke TF, Cooke JP: Asymmetric dimethylarginine (ADMA): a novel risk factor for endothelial dysfunction: its role in hypercholesterolemia. *Circulation* 98:1842-1847, 1998.

Bohlen HG: Localization of vascular resistance changes during hypertension. *Hypertension* 8:181-183, 1986.

Boisvert WA, Santiago R, Curtiss LK, Terkeltaub RA: A leukocyte homologue of the IL-8 receptor CXCR-2 mediates the accumulation of macrophages in atherosclerotic lesions of LDL receptor-deficient mice. *J.Clin.Invest.* 101:353-363, 1998.

Bonetti PO, Lerman LO, Lerman A: Endothelial Dysfunction. A marker of atherosclerotic risk. *Arterioscler.Thromb.Vasc.Biol.* 23:168-175, 2003.

Bonow RO, Smaha LA, Smith SC, Mensah GA, Lenfant C: World Heart Day 2002: the international burden of cardiovascular disease: responding to the emerging global epidemic. *Circulation* 106:1602-1605, 2002.

Borge PD, Moibi J, Greene SR, Trucco M, Young RA, Gao Z Wolf BA: Insulin receptor signalling and sarco/endoplasmic reticulum calcium ATPase in beta cells. *Diabetes* 51 (Suppl) 3:427-433, 2002.

Boyle JG, Logan PJ, Ewart MA, Reihill JA, Ritchie SA, Connell JM, Cleland SJ, Salt IP: Rosiglitazone stimulates nitric oxide synthesis in human aortic endothelial cells via AMP activated protein kinase. *J.Biol.Chem.* 283: 11210-11217, 2008.

Bradford MM: A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal.Biochem.* 72:248-254, 1976.

Bramwell JA, Hill AV: Velocity of transmission of the pulse wave and elasticity of arteries. *Lancet* 1:891-892, 1922.

Brown EJ, Albers MW, Shin TB, Ichikawa K, Keith CT, Lane WS, Schreiber SL: A mammalian protein targeted by G1-arresting rapamycin-receptor complex. *Nature* 369:756–758, 1994.

Brunmair B, Staniek K, Gras F, Scharf N, Althaym A, Clara R, Roden M, Gnaiger E, Nohl H, Waldhäusl W, Fürnsinn C: Thiazolidinediones, like metformin, inhibit respiratory complex I: a common mechanism contributing to their antidiabetic actions? *Diabetes* 53:1052-1059, 2004.

Caballero AE, Delgado A, Aguilar-Salinas CA, Herrera AN, Castillo JL, Cabrera T, Gomez-Perez FJ, Rull JA: The differential effects of metformin on markers of endothelial activation and inflammation in subjects with impaired glucose tolerance: a placebo-controlled, randomized clinical trial. *J.Clin.Endocrinol.Metab.* 89:3943-3948, 2004.

Calnek D, Mazzella L, Roser S, Roman J, Hart CM: Peroxisome proliferator activated receptor gamma ligands increase release of nitric oxide from endothelial cells. *Arterioscler.Thromb.Vasc.Biol.* 23:52-57, 2003.

Calvert, JW, Gundewar S, Jha S, Greer JJ, Bestermann, WH, Tian R, Lefer DJ: Acute metformin therapy confers cardioprotection against myocardial infarction via AMPK-eNOS-mediated signaling. *Diabetes* 57:696–705, 2008.

Campbell P, Carlson M: Impact of obesity on insulin action in NIDDM. *Diabetes* 42:405-410, 1993.

Carling D: The AMP-activated protein kinase cascade: a unifying system for energy control. *Trends Biochem.Sci.* 29:18–24, 2004.

Chan JC, Tomlinson B, Critchley JA, Cockram CS, Walden RJ: Metabolic and hemodynamic effects of metformin and glibenclamide in normotensive NIDDM patients. *Diabetes Care* 16:1035-1038, 1993.

Chen H, Montagnani M, Funahashi T, Shimomura I, Quon MJ: Adiponectin stimulates production of nitric oxide in vascular endothelial cells. *J.Biol.Chem.* 278:45021-45026, 2003.

Chen MB, McAinch AJ, Macaulay SL, Castelli LA, O'Brien PE, Dixon JB, Cameron-Smith D, Kemp BE, Steinberg GR: Impaired activation of AMP-kinase and fatty acid oxidation by globular adiponectin in cultured human skeletal muscle of obese type 2 diabetics. *J.Clin.Endocrinol.Metab.* 90:3665-3672, 2005.

Chen MP, Tsai JC, Chung FM, Yang SS, Hsing LL, Shin SJ, Lee YJ: Hypoadiponectinemia is associated with ischemic cerebrovascular disease. *Arterioscler.Thromb.Vasc.Biol.* 25:821-826, 2005.

Chen ZP, Mitchelhill KI, Michell BJ, Stapleton D, Rodriguez-Crespo I, Witters LA, Power DA, Ortiz de Montellano PR, Kemp BE: AMP-activated protein kinase phosphorylation of endothelial NO synthase. *FEBS Lett.* 1443: 285-289, 1999.

Chen ZP, McConell GK, Michell BJ, Snow RJ, Canny BJ, Kemp BE: AMPK signaling in contracting human skeletal muscle: acetyl-CoA carboxylase and NO synthase phosphorylation. *Am.J.Physiol.Endocrinol.Metab.* 279:1202-1206, 2000.

Chinen I, Shimabukuro M, Yamakawa K, Higa N, Matsuzaki T, Noguchi K, Ueda S, Sakanashi M, Takasu N: Vascular lipotoxicity: endothelial dysfunction via fatty-acid induced reactive oxygen species overproduction in obese Zucker diabetic fatty rats. *Endocrinology* 148:160-165, 2007.

Cho DH, Choi YJ, Jo SA, Jo I: Nitric oxide production and regulation of endothelial nitric-oxide synthase phosphorylation by prolonged treatment with troglitazone: evidence for involvement of peroxisome proliferator-activated receptor (PPAR) gamma-dependent and PPARgamma-independent signalling pathways. *J.Biol.Chem.* 279: 2499-2506, 2004.

Chowienczyk PJ, Watts GF, Cockcroft JR, Ritter JM: Impaired endothelium dependent vasodilation of forearm resistance vessels in hypercholesterolaemia. *Lancet* 340:1430-1432, 1992.

Christ-Crain M, Kola B, Lolli F, Fekete C, Seboek D, Wittmann G, Feltrin D, Igreja SC, Ajodha S, Harvey-White J, Kunos G, Müller B, Pralong F, Aubert G, Arnaldi G, Giacchetti G, Boscaro M, Grossman AB, Korbonits M: AMP-activated protein kinase mediates glucocorticoid-induced metabolic changes: a novel mechanism in Cushing's syndrome. *FASEB J.* 22:1672-1683, 2008.

Cleland SJ, Petrie JR, Small M, Elliott HL, Connell JM: Insulin action is associated with endothelial dysfunction in hypertension and type 2 diabetes. *Hypertension* 35: 500-507, 2000.

Coggins M, Lindner J, Rattigan S, Jahn L, Fasy E, Kaul S, Barrett E: Physiologic hyperinsulinemia enhances human skeletal muscle perfusion by capillary recruitment. *Diabetes* 50:2682-2690, 2001.

Considine RV, Sinha MK, Heiman ML, Kriauciunas A, Stephens TW, Nyce MR, Ohannesian JP, Marco CC, McKee LJ, Bauer TL: Serum immunoreactive-leptin concentrations in normal-weight and obese humans. *N Engl J Med.* 334:292-295, 1996.

Cool B, Zinker B, Chiou W, Kifle L, Cao N, Perham M, Dickinson R, Adler A, Gagne G, Iyengar R, Zhao G, Marsh K, Kym P, Jung P, Camp HS, Frevert E: Identification and characterization of a small molecule AMPK activator that treats key components of type 2 diabetes and the metabolic syndrome. *Cell metabolism* 3:403–416, 2006.

Cross DA, Alessi DR, Cohen P, Andjelkovich M, Hemmings BA: Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. *Nature* 378:785-789, 1995.

Cruickshank K, Riste L, Anderson SG, Wright JS, Dunn G, Gosling RG: Aortic pulse-wave velocity and its relationship to mortality in diabetes and glucose intolerance: an integrated index of vascular function? *Circulation* 106:2085-2090, 2002.

Currie CJ, Kraus D, Morgan CL, Gill L, Stott NC, Peters JR: NHS acute sector expenditure for diabetes: the present, future, and excess in-patient cost of care. *Diabet.Med.* 14:686-692, 1997.

Da Silva Xavier G, Leclerc I, Varadi A, Tsuboi T, Moule SK, Rutter GA: Role for AMP-activated protein kinase in glucose-stimulated insulin secretion and proinsulin gene expression. *Biochem.J.* 371(Pt 3):761-774, 2003.

Danielsson A, Ost A, Nystrom FH, Stralfors P: Attenuation of insulin-stimulated insulin receptor substrate-1 serine 307 phosphorylation in insulin resistance of type 2 diabetes. *J.Biol.Chem.* 280:34389-34392, 2005.

Daousi C, Casson IF, Gill GV, MacFarlane IA, Wilding JP, Pinkney JH: Prevalence of obesity in type 2 diabetes in secondary care: association with cardiovascular risk factors. *Postgrad.Med.J.* 82:280-284, 2006.

Davies JK, Wells DJ, Liu K, Whitrow HR, Daniel TD, Grignani R, Lygate CA, Schneider JE, Noel G, Watkins H, Carling D: Characterization of the role of  $\gamma$  2 R531G mutation in AMP-activated protein kinase in cardiac hypertrophy and Wolff-Parkinson-White syndrome. *Am.J.Physiol. Heart Circ.Physiol.* 290:1942-1951, 2006.

Davis BJ, Xie Z, Viollet B, Zou MH: Activation of the AMP-activated kinase by antidiabetes drug metformin stimulates nitric oxide synthesis in vivo by promoting the association of heat shock protein 90 and endothelial nitric oxide synthase. *Diabetes* 55:496-505, 2006.

Davis MJ, Ferrer PN, Gore RW: Vascular anatomy and hydrostatic pressure profile in the hamster cheek pouch. *Am.J.Physiol.* 250:291-303, 1986.

De Aguiar LG, Bahia LR, Villela N, Laflor C, Sicuro F, Wiernsperger N, Bottino D, Bouskela E: Metformin improves endothelial vascular reactivity in first-degree relatives of type 2 diabetic patients with metabolic syndrome and normal glucose tolerance. *Diabetes Care* 29:1083-1089, 2006.

De Jager J, Kooy A, Lehert P, Bets D, Wulffelé MG, Teerlink T, Scheffer PG, Schalkwijk CG, Donker AJ, Stehouwer CD: Effects of short-term treatment with metformin on markers of endothelial function and inflammatory activity in type 2 diabetes mellitus: a randomized, placebo-controlled trial. *J.Intern.Med.* 257:100-109, 2005.

Defronzo R, Tobin JD, Anders R: Glucose clamp technique: a method for quantifying insulin secretion and resistance. *Am.J.Physiol.* 237:214-223, 1979.

Defronzo RA, Ferrannini E, Simonson DC: Fasting hyperglycemia in non-insulin-dependent diabetes mellitus: contributions of excessive hepatic glucose production and impaired tissue glucose uptake. *Metabolism* 38:387-395, 1989.

DeFronzo RA, Bonadonna RC, Ferrannini E: Pathogenesis of NIDDM. A balanced overview. *Diabetes Care* 15:318-368, 1992.

Defronzo RA, Ferrannini E, Keen H, Zimmet P: International Textbook of Diabetes Mellitus. Wiley Press Third Edition, Volume 2. 2004.

Diamanti-Kandarakis E, Alexandraki K, Protogerou A, Piperi C, Papamichael C, Aessopos A, Lekakis J, Mavrikakis M: Metformin administration improves endothelial function in women with polycystic ovary syndrome. *Eur.J.Endocrinol.* 152:749-756, 2005.

Dimmeler S, Fleming I, Fisslthaler B, Hermann C, Busse R, Zeiher AM: Activation of nitric oxide synthase in endothelial cells by Akt-dependent phosphorylation. *Nature* 399: 601-605, 1999.

Dominguez LJ, Davidoff AJ, Srinivas PR, Standley PR, Walsh MF, Sowers JR: Effects of metformin on tyrosine kinase activity, glucose transport, and intracellular calcium in rat vascular smooth muscle. *Endocrinology* 137:113-121, 1996.

Dormandy JA, Charbonnel B, Eckland DJ, Erdmann E, Massi-Benedetti M, Moules IK, Skene AM, Tan MH, Lefèvre PJ, Murray GD, Standl E, Wilcox RG, Wilhelmsen L, Betteridge J, Birkeland K, Golay A, Heine RJ, Korányi L, Laakso M, Mokán M, Norkus A, Pirags V, Podar T, Scheen A, Scherbaum W, Schernthaner G, Schmitz O, Skrha J, Smith U, Taton J; PROactive investigators. Secondary prevention of macrovascular events in patients with type 2 diabetes in the PROactive Study (PROspective pioglitAzone Clinical Trial In macroVascular Events): a randomised controlled trial. *Lancet* 366:1279-1289, 2005.

Dresner A, Laurent D, Marcucci M, Griffin ME, Dufour S, Cline GW, Slezak LA, Andersen DK, Hundal RS, Rothman DL, Petersen KF, Shulman GI: Effects of free fatty acids on glucose transport and IRS-1-associated phosphatidylinositol 3-kinase activity. *J.Clin.Invest.* 103:253-259, 1999

Dresser MJ, Xiao G, Leabman MK, Gray AT, Giacomini KM: Interactions of n-tetraalkylammonium compounds and biguanides with a human renal organic cation transporter (hOCT2). *Pharm.Res.* 19:1244–1247, 2002.

Drzewoski J, Zurawska-Klis M: Effect of gliclazide modified release on adiponectin, interleukin-6, and tumor necrosis factor-alpha plasma levels in individuals with type 2 diabetes mellitus. *Curr.Med.Res.Opin.* 22:1921-1926, 2006.

Duplain H, Burcelin R, Sartori C, Cook S, Egli M, Lepori M, Vollenweider P, Pedrazzini T, Nicod P, Thorens B, Scherrer U: Insulin resistance, hyperlipidemia, and hypertension in mice lacking endothelial nitric oxide synthase. *Circulation* 104:342-345, 2001.

El-Mir MY, Nogueira V, Fontaine E, Averet N, Rigoulet M, Leverve X: Dimethylbiguanide inhibits cell respiration via an indirect effect targeted onthe respiratory chain complex I. *J.Biol.Chem.* 275:223–228, 2000.

European Medicines Agency: EMEA recommends new warnings and contraindications for rosiglitazone. E.M.E.A./42232/2008.

Farooqi IS, Matarese G, Lord GM, Keogh JM, Lawrence E, Agwu C, Sanna V, Jebb SA, Perna F, Fontana S, Lechler RI, DePaoli AM, O'Rahilly S: Beneficial effects of leptin on obesity, T cell

hyporesponsiveness, and neuroendocrine/metabolic dysfunction of human congenital leptin deficiency. *J.Clin.Invest.* 110:1093-1103, 2002.

Feletou M, Vanhoutte PM: Endothelium-derived hyperpolarizing factor: where are we now? *Arterioscler.Thromb.Vasc.Biol.* 26:1215-1225, 2006.

Ferrara N, Gerber HP, LeCouter J: The biology of VEGF and its receptors. *Nat.Med.* 29:669-676, 2003.

Fisher FF, Trujillo ME, Hanif W, Barnett AH, McTernan PG, Scherer PE, Kumar S: Serum high molecular weight complex of adiponectin correlates better with glucose tolerance than total serum adiponectin in Indo-Asian males. *Diabetologia* 48:1084-1087, 2005.

Ford ES: Risks for all-cause mortality, cardiovascular disease, and diabetes associated with the metabolic syndrome: a summary of the evidence. *Diabetes Care* 28:1769-1778, 2005.

Forstermann U, Munzel T: Endothelial nitric oxide synthase in vascular disease: from marvel to menace. *Circulation* 113:1708-1714, 2006.

Fox CS, Coady S, Sorlie PD, D'Agostino RB, Sr., Pencina MJ, Vasan RS, Meigs JB, Levy D, Savage PJ: Increasing cardiovascular disease burden due to diabetes mellitus: the Framingham Heart Study. *Circulation* 115:1544-1550, 2007.

Fryer LG, Hajduch E, Rencurel F, Salt IP, Hundal HS, Hardie DG, Carling D: Activation of glucose transport by AMP-activated protein kinase via stimulation of nitric oxide synthase. *Diabetes* 49:1978-1785, 2000.

Fryer LG, Parbu-Patel A, Carling D: The Anti-diabetic drugs rosiglitazone and metformin stimulate AMP-activated protein kinase through distinct signaling pathways. *J.Biol.Chem.* 277:25226-25232, 2002.

Frystyk J, Berne C, Berglund L, Jensevik K, Flyvbjerg A, Zethelius B: Serum adiponectin is a predictor of coronary heart disease: a population-based 10-year follow-up study in elderly men. *J.Clin.Endocrinol.Metab.* 92:571-576, 2007.

Fulton D, Gratton JP, McCabe TJ, Fontana J, Fujio Y, Walsh K, Franke TF, Papapetropoulos A, Sessa WC. Regulation of endothelium-derived nitric oxide production by the protein kinase Akt. *Nature* 399: 597-601, 1999.

Gao X, Pan D: TSC1 and TSC2 tumor suppressors antagonize insulin signaling in cell growth. *Genes Dev.* 15:1383–1392, 2001.

Garber AJ, Duncan TG, Goodman AM, Mills DJ, Rohlf JL: Efficacy of metformin in type II diabetes: results of a double-blind, placebo-controlled, dose-response trial. *Am.J.Med.* 103:491-7, 1997.

Garland JG, McPherson GA: Evidence that nitric oxide does not mediate the hyperpolarization and relaxation to acetylcholine in the rat small mesenteric artery. *Br.J.Pharmacol.* 105:429-435, 1992.

Garton AJ, Campbell DG, Carling D, Hardie DG, Colbran RJ, Yeaman SJ: Phosphorylation of bovine hormone-sensitive lipase by the AMP-activated protein kinase. A possible antilipolytic mechanism. *Eur.J.Biochem.* 179:249–254, 1989.

Gerich JE: Oral hypoglycemic agents. *N. Engl. J. Med.* 321:1231-1245, 1989.

Goldner MG, Knatterud GL, Prout TE: Effects of hypoglycemic agents on vascular complications in patients with adult-onset diabetes. 3. Clinical implications of UGDP results. *JAMA* 218:1400-1410, 1971.

Goldstein BJ, Scalia R: Adiponectin: A novel adipokine linking adipocytes and vascular function. *J.Clin.Endocrinol.Metab.* 89:2563-2568, 2004.

Gollob MH, Seger JJ, Gollob TN, Tapscott T, Gonzales O, Bachinski L, Roberts, R: Novel PRKAG2 mutation responsible for the genetic syndrome of ventricular preexcitation and conduction system disease with childhood onset and absence of cardiac hypertrophy. *Circulation* 104:3030–3033, 2001.

Gonon AT, Widegren U, Bulhak A, Salehzadeh F, Persson J, Sjöquist PO, Pernow J: Adiponectin protects against myocardial ischaemia-reperfusion injury via AMP-activated protein kinase, Akt, and nitric oxide: *Cardiovasc.Res.* 278:116-122, 2008.

Gorus FK, Schuit FC, In't Veld PA, Gepts W, Pipeleers DG: Interaction of sulfonylureas with pancreatic beta-cells. A study with glyburide. *Diabetes* 37:1090-1095, 1988.

Goumas G, Tentolouris C, Tousoulis D, Stefanadis C, Toutouzas P: Therapeutic modification of the L-arginine-eNOS pathway in cardiovascular diseases. *Atherosclerosis* 154:255-267, 2001.

Grundy SM, Pasternack R, Greenland P, Smith S, Fuster V: Assessment of cardiovascular risk by use of multiple-risk-factor assessment equations: a statement for healthcare professionals from the American Heart Association and the American College of Cardiology. *Circulation* 100:1481-1492, 1999.

Grundy SM, Cleeman JI, Daniels SR, Donato KA, Sacks MS, Stone NJ, Loria PM, Eckel RH, Franklin BA, Smith SC, Sacks FM, Smith SC Jr, Svetkey LP, Devereux RB, Gordon DJ, Krauss RM, Savage PJ, Smith SC, Spertus JA, Costa F: Diagnosis and management of the metabolic syndrome. An American Heart Association/National Heart, Lung and Blood Institute Scientific Statement. *Circulation* 112:2735-2752, 2005.

Gundewar S, Calvert JW, Jha S, Toedt-Pingel I, Ji SY, Nunez D, Ramachandran A, Anaya-Cisneros M, Tian R, Lefer DJ: Activation of AMP-Activated Protein Kinase by Metformin Improves Left Ventricular Function and Survival in Heart Failure. *Circ. Research* 104:403-411, 2009.

Halaas JL: Weight-reducing effects of the plasma protein encoded by the obese gene. *Science* 269:543-546, 1995.

Hansson GK: Inflammation, atherosclerosis, and coronary artery disease. *N Engl J Med*. 352:1685-1695, 2005.

Haque M: Role of the sympathetic nervous system and insulin in enhancing glucose uptake in peripheral tissues after intrahypothalamic injection of leptin in rats. *Diabetes* 48:1706-1712, 1999.

Hardie DG: The AMP-activated protein kinase cascade, the key sensor of cellular energy status. *Endocrinology* 144:5179–5183, 2003.

Hardie DG: The AMP-activated protein kinase pathway: new players upstream and downstream. *J. Cell Sci.* 117:5479-5487, 2004.

Harrison DG: Cellular and molecular mechanisms of endothelial cell dysfunction. *J.Clin.Invest.* 100:2153-2157, 1997.

Hartell NA, Archer HE, Bailey CJ: Insulin-stimulated endothelial nitric oxide release is calcium independent and mediated via protein kinase B. *Biochem.Pharmacol.* 69:781-790, 2005.

Hattori Y, Suzuki M, Hattori S, Kasai K: Globular adiponectin upregulates nitric oxide production in vascular endothelial cells. *Diabetologia* 46:1543-1549, 2003.

Hattori Y, Kasai K, Gross SS: NO suppresses while peroxynitrite sustains NF-kappaB: a paradigm to rationalize cytoprotective and cytotoxic actions attributed to NO. *Cardiovasc.Res.* 63:31-40, 2004.

Hattori Y, Suzuki K, Hattori S, Kasai K: Metformin inhibits cytokine-induced nuclear factor kappaB activation via AMP-activated protein kinase activation in vascular endothelial cells. *Hypertension* 47:1183-1188, 2006.

Haupt E, Köberich W, Beyer J, Schöffling K: Pharmacodynamic aspects of tolbutamide, glibenclamide, glibornuride and glisoxepide. I. Dose response relations and repeated administration in diabetic subjects. *Diabetologia* 7:449-454, 1971.

Hawley SA, Gadalla AE, Olsen GS, Hardie DG: The antidiabetic drug metformin activates the AMP-activated protein kinase cascade via an adenine nucleotide-independent mechanism. *Diabetes* 51:2420-2425, 2002.

Hawley SA, Boudeau J, Reid JL, Mustard, KJ, Udd L, Makela TP, Alessi DR, Hardie DG: Complexes between the LKB1 tumor suppressor, STRAD  $\alpha/\beta$  and MO25  $\alpha/\beta$  are upstream kinases in the AMP-activated protein kinase cascade. *J. Biol.* 2:28, 2003.

Hawley SA, Pan DA, Mustard KJ, Ross L, Bain J, Edelman AM, Frenguelli BG, Hardie DG: Calmodulin-dependent protein kinase kinase-beta is an alternative upstream kinase for AMP-activated protein kinase. *Cell Metab.* 2:9-19, 2005.

Hetzl J, Balletshofer B, Rittig K, Walcher D, Kratzer W, Hombach V, Haring HU, Koenig W, Marx N: Rapid effects of rosiglitazone treatment on endothelial function and inflammatory biomarkers. *Arterioscler.Thromb.Vasc.Biol.* 25:1804-1809, 2005.

Heutling D, Schulz H, Nickel I, Kleinstein J, Kaltwasser P, Westphal S, Mittermayer F, Wolzt M, Krzyzanowska K, Randeva H, Schernthaner G, Lehnert H: Asymmetrical dimethylarginine, inflammatory and metabolic parameters in women with polycystic ovary syndrome before and after metformin treatment. *J.Clin.Endocrinol.Metab.* 93:82-90, 2008.

Home PD, Pocock SJ, Beck-Nielsen H, Gomis R, Hanefeld M, Jones NP, Komajda M, McMurray JJ: RECORD Study Group. Rosiglitazone evaluated for cardiovascular outcomes--an interim analysis. *N Engl J Med.* 357:28-38, 2007.

Home PD, Pocock SJ, Beck-Nielsen H, Curtis PS, Gomis R, Hanefeld M, Jones NP, Komajda M, McMurray JJ: RECORD Study Team. Rosiglitazone evaluated for cardiovascular outcomes in oral agent combination therapy for type 2 diabetes (RECORD): a multicentre, randomised, open-label trial. *Lancet* 373:2125-2135, 2009.

Horike N, Sakoda H, Kushiyama A, Ono H, Fujishiro M, Kamata H, Nishiyama K, Uchijima Y, Kurihara Y, Kurihara H, Asano T: AMP-activated protein kinase activation increases phosphorylation of glycogen synthase kinase 3 beta and thereby reduces cAMP-responsive element transcriptional activity and phosphoenolpyruvate carboxykinase C gene expression in the liver. *J Biol Chem.* 283:33902-33910, 2008.

Hotamisligil GS, Shargill NS, Spiegelman BM: Adipose expression of tumor necrosis factor-alpha: direct role in obesity-linked insulin resistance. *Science* 259:87-91, 1993.

Hotamisligil GS, Arner P, Caro JF, Atkinson RL, Spiegelman BM: Increased adipose tissue expression of tumor necrosis factor-alpha in human obesity and insulin resistance. *J Clin Invest.* 95:2409-2415, 1995.

Hotta K, Funahashi T, Arita Y, Takahashi M, Matsuda M, Okamoto Y, Iwahashi H, Kuriyama H, Ouchi N, Maeda K, Nishida M, Kihara S, Sakai N, Nakajima T, Hasegawa K, Muraguchi M, Ohmoto Y, Nakamura T, Yamashita S, Hanafusa T, Matsuzawa Y: Plasma concentrations of a

novel, adipose-specific protein, adiponectin, in type 2 diabetic patients. *Arterioscler.Thromb.Vasc.Biol.* 20:1595-1599, 2000.

Howlett, H.C.S., Bailey, C.J. Metformin: The Gold Standard. Wiley Press First Edition, 2007.

Hu G, Qiao Q, Tuomilehto J, Balkau B, Borch-Johnsen K, Pyorala K: Prevalence of the metabolic syndrome and its relation to all-cause and cardiovascular mortality in nondiabetic European men and women. *Arch.Intern.Med.* 164:1066-1076, 2004.

Hu S, Wang S, Fanelli B, Bell PA, Dunning BE, Geisse S, Schmitz R, Boettcher BR: Pancreatic beta-cell K(ATP) channel activity and membrane-binding studies with nateglinide: A comparison with sulfonylureas and repaglinide. *J.Pharmacol.Exp.Ther.* 293:444-452, 2000.

Hubbard SR, Wei L, Ellis L, Hendrickson WA: Crystal structure of the tyrosine kinase domain of the human insulin receptor. *Nature* 372:746-754, 1994.

Hubbard SR: Crystal structure of the activated insulin receptor tyrosine kinase in complex with peptide substrate and ATP analog. *EMBO J.* 16:5572-5581, 1997.

Hundal HS, Ahmed A, Gumà A, Mitsumoto Y, Marette A, Rennie MJ, Klip A: Biochemical and immunocytochemical localization of the 'GLUT5 glucose transporter' in human skeletal muscle. *Biochem J.* 286:339-343, 1992.

Hundal RS, Krssak M, Dufour S, Laurent D, Lebon V, Chandramouli V, Inzucchi SE, Schumann WC, Petersen KF, Landau BR, Shulman GI: Mechanism by which metformin reduces glucose production in type 2 diabetes. *Diabetes* 49:2063-2069, 2000.

Hutber CA, Hardie DG, Winder WW: Electrical stimulation inactivates muscle acetyl-CoA carboxylase and increases AMP-activated protein kinase. *Am.J.Physiol.Endocrinol.Metab.* 272:262-266, 1997.

Huygens P, Quartier E, Pipeleers D, Van de Casteele M: Metformin reduces adiponectin protein expression and release in 3T3-L1 adipocytes involving activation of AMP activated protein kinase. *Eur.J.Pharmacol.* 518:90-95, 2005.

Information Centre Quality Outcomes Framework Data 2005/06. Diabetes Indicators: accessible at [http://www.ic.nhs.uk/servicesnew/qof06/spreadsheets/ICfolder\\_view](http://www.ic.nhs.uk/servicesnew/qof06/spreadsheets/ICfolder_view), 2006.

Inukai K, Watanabe M, Nakashima Y, Takata N, Isoyama A, Sawa T, Kurihara S, Awata T, Katayama S: Glimepiride enhances intrinsic peroxisome proliferator-activated receptor-gamma activity in 3T3-L1 adipocytes. *Biochem.Biophys.Res.Commun.* 328:484-490, 2005.

Inzucchi SE: Oral antihyperglycemic therapy for type 2 diabetes: scientific review. *JAMA* 287:360-372, 2002.

Ito N, Rubin GM: Gigas, a Drosophila homolog of tuberous sclerosis gene product-2, regulates the cell cycle. *Cell* 96:529-539, 1999.

Jadhav S, Ferrell W, Greer IA, Petrie JR, Cobbe SM, Sattar N: Effects of metformin on microvascular function and exercise tolerance in women with angina and normal coronary arteries: a randomized, double-blind, placebo-controlled study. *J.Am.Coll.Cardiol.* 48:956-963, 2006.

Johansen K: Efficacy of metformin in the treatment of NIDDM. Meta-analysis. *Diabetes Care* 22:33-37, 1999.

Johnson AB, Webster JM, Sum CF, Heseltine L, Argyraki M, Cooper BG, Taylor R: The impact of metformin therapy on hepatic glucose production and skeletal muscle glycogen synthase activity in overweight type II diabetic patients. *Metabolism* 42:1217-1222, 1993.

Johnson JA, Majumdar SR, Simpson SH, Toth EL: Decreased mortality associated with the use of metformin compared with sulfonylurea monotherapy in type 2 diabetes *Diabetes Care* 25:2244-2248, 2002.

Jonker JW, Schinkel AH: Pharmacological and physiological functions of the polyspecific organic cation transporters, OCT1, 2, and 3 (SLC22A1- SLC22A3). *J.Pharmacol.Exp.Therap.* 308, 2-9, 2004.

Jorgensen SB, Viollet B, Andreelli F, Frosig C, Birk JB, Schjerling P, Vaulont S, Richter EA, Wojtaszewski JF: Knockout of the alpha2 but not alpha1 5'-AMP-activated protein kinase isoform abolishes 5-aminoimidazole-4-carboxamide-1-beta-4-ribofuranosidebut not contraction-induced glucose uptake in skeletal muscle. *J.Biol.Chem.* 279:1070-1079, 2004.

Kahn BB, Flier JS: Obesity and insulin resistance. *J.Clin.Invest.* 106:473-481, 2000.

Kahn BB, Alquier T, Carling D, Hardie, DG: AMP-activated protein kinase: ancient energy gauge provides clues to modern understanding of metabolism. *Cell Metab.* 1:15–25, 2005.

Kanda Y, Matsuda M, Tawaramoto K, Kawasaki F, Hashiramoto M, Matsuki M, Kaku K: Effects of sulfonylurea drugs on adiponectin production from 3T3-L1 adipocytes: implication of different mechanism from pioglitazone. *Diabetes Res.Clin.Pract.* 8113-8118, 2008.

Kang KT, Sullivan JC, Sasser JM, Imig JD, Pollock JS: Novel nitric oxide synthase -- dependent mechanism of vasorelaxation in small arteries from hypertensive rats. *Hypertension* 49:893-901, 2007.

Karttunen P, Uusitupa M, Lamminisivu U: The pharmacokinetics of metformin: a comparison of the properties of a rapid-release and a sustained-release preparation. *Int.J.Clin.Pharmacol.Ther.Toxicol.* 21:31-36, 1983.

Katakam PVG. Ujhelyi MR. Hoenig M. Miller AW: Metformin Improves Vascular Function in Insulin-Resistant Rats. *Hypertension* 35:108, 2000.

Kelley DE, Simoneau JA: Impaired free fatty acid utilization by skeletal muscle in non-insulin-dependent diabetes mellitus. *J.Clin.Invest.* 94:2349-2356, 1994

Kelly AS, Thelen AM, Kaiser DR, Gonzalez-Campoy JM, Bank AJ: Rosiglitazone improves endothelial function and inflammation but not asymmetric dimethylarginine or oxidative stress in patients with type 2 diabetes mellitus. *Vasc.Med.* 12:311-318, 2007.

Kelly CJ, Speirs A, Gould GW, Petrie JR, Lyall H, Connell JM: Altered vascular function in young women with polycystic ovary syndrome. *J.Clin.Endocrinol.Metab.* 87:742-746, 2002.

Kern PA, Saghizadeh M, Ong JM, Bosch RJ, Deem R, Simsolo RB: The expression of tumor necrosis factor in human adipose tissue. Regulation by obesity, weight loss, and relationship to lipoprotein lipase. *J.Clin.Invest.* 95:2111-2119, 1995.

Kido Y, Nakae J, Accili D: Clinical review 125: The insulin receptor and its cellular targets. *J.Clin.Endocrinol.Metab.* 86:972-979, 2001.

Kim F, Gallis B, Corson MA: TNF-alpha inhibits flow and insulin signaling leading to NO production in aortic endothelial cells. *Am.J.Physiol. Cell Physiol.* 280:C1057-C1065, 2001.

Kim HJ, Higashimori T, Park SY, Choi H, Dong J, Kim YJ, Noh HL, Cho YR, Cline G, Kim YB, Kim JK: Differential effects of interleukin-6 and -10 on skeletal muscle and liver insulin action in vivo. *Diabetes* 53:1060-1067, 2004.

Kim KA, Park JY: Simple and extractionless high-performance liquid chromatographic determination of rosiglitazone in human plasma and application to pharmacokinetics in humans. *Biomed.Chromatogr.* 18:613-614, 2004.

Kim KY, Cheon HG: Antiangiogenic effect of rosiglitazone is mediated via peroxisome proliferator-activated-receptor gamma-activated maxi-K channel opening in human umbilical vein endothelial cells. *J.Biol.Chem.* 281: 13503-13512, 2006

Kimura N, Masuda S, Tanihara Y, Ueo H, Okuda M, Katsura T, Inui K: Metformin is a superior substrate for renal organic cation transporter OCT2 rather than hepatic OCT1. *Drug Metab Pharmacokinet.* 20:379-386, 2005.

Kohn AD, Summers SA, Birnbaum MJ, Roth RA: Expression of a constitutively active Akt Ser/Thr kinase in 3T3-L1 adipocytes stimulates glucose uptake and glucose transporter 4 translocation. *J.Biol.Chem.* 271:31372-31378, 1996.

Koistinen HA, Galuska D, Chibalin AV, Yang J, Zierath JR, Holman GD, Wallberg-Henriksson H: 5-amino-imidazole carboxamide riboside increases glucose transport and cell-surface GLUT4 content in skeletal muscle from subjects with type 2 diabetes. *Diabetes* 52:1066-1072, 2003.

Kojda G, Harrison D: Interactions between NO and reactive oxygen species: pathophysiological importance in atherosclerosis, hypertension, diabetes and heart failure. *Cardiovasc.Res.* 43:562-571, 1999.

Kola B, Christ-Crain M, Lolli F, Arnaldi G, Giacchetti G, Boscaro M, Grossman AB, Korbonits M: Changes in adenosine 5'-monophosphate-activated protein kinase as a mechanism of visceral obesity in Cushing's syndrome. *J.Clin.Endocrinol.Metab.* 93:4969-4973, 2008.

Konrad D, Rudich A, Bilan PJ, Patel N, Richardson C, Witters LA, Klip A: Troglitazone causes acute mitochondrial membrane depolarisation and an AMPK-mediated increase in glucose phosphorylation in muscle cells. *Diabetologia.* 48: 954-966, 2005.

Korytkowski M, Thomas A, Reid L, Tedesco MB, Gooding WE, Gerich J: Glimepiride improves both first and second phases of insulin secretion in type 2 diabetes. *Diabetes Care* 25:1607-1611, 2002.

Krishnamurthy M, Sahouria JJ, Desai R, Caguiat J: Buformin-induced lactic acidosis--a symptom of modern healthcare malady. *J Am Geriatr Soc* 52:1785, 2004.

Krzyzanowska K, Mittermayer F, Kopp HP, Wolzt M, Schernthaner G: Weight loss reduces circulating asymmetrical dimethylarginine concentrations in morbidly obese women. *J Clin Endocrinol Metab* 89:6277-6281, 2004.

Krzyzanowska K, Mittermayer F, Wolzt M, Schernthaner G: Asymmetric dimethylarginine predicts cardiovascular events in patients with type 2 diabetes. *Diabetes Care* 30:1834–1839, 2007.

Kubota N, Tobe K, Terauchi Y, Eto K, Yamauchi T, Suzuki R, Tsubamoto Y, Komeda K, Nakano R, Miki H, Satoh S, Sekihara H, Sciacchitano S, Lesniak M, Aizawa S, Nagai R, Kimura S, Akanuma Y, Taylor SI, Kadowaki T: Disruption of insulin receptor substrate 2 causes type 2 diabetes because of liver insulin resistance and lack of compensatory beta-cell hyperplasia. *Diabetes* 49:1880-1889, 2000.

Kurebayashi S, Xu X, Ishii S, Shiraishi M, Kouhara H, Kasayama S: A novel thiazolidinedione MCC-555 down-regulates tumor necrosis factor-alpha-induced expression of vascular cell adhesion molecule-1 in vascular endothelial cells. *Atherosclerosis* 182:71-77, 2005.

Laakso M: Hyperglycemia and cardiovascular disease in type 2 diabetes. *Diabetes* 48:937-942, 1999.

Lago RM, Singh PP, Nesto RW: Congestive heart failure and cardiovascular death in patients with prediabetes and type 2 diabetes given thiazolidinediones: a meta-analysis of randomised clinical trials. *Lancet* 370:1129-36, 2007.

Laurent S, Boutouyrie P, Asmar Gautier I, Laloux B, Guize L, Ducimetiere P, Benetos A: Aortic stiffness is an independent predictor of all-cause and cardiovascular mortality in hypertensive patients. *Hypertension* 37:1236-1241, 2001.

Le Gouill E, Jimenez M, Binnert C, Jayet PY, Thalmann S, Nicod P, Scherrer U, Vollenweider P: Endothelial nitric oxide synthase (eNOS) knockout mice have defective mitochondrial beta-oxidation. *Diabetes* 56:2690-2696, 2007.

Lebovitz HE, Banerji MA: Point: visceral adiposity is causally related to insulin resistance. *Diabetes Care* 28:2322-2325, 2005

Lebrasseur NK, Kelly M, Tsao TS, Farmer SR, Saha AK, Ruderman NB, Tomas E: Thiazolidinediones can rapidly activate AMP-activated protein kinase in mammalian tissues. *Am.J.Physiol.Endocrinol.Metab.* 291: E175-E181, 2006.

Leclerc I, Woltersdorf WW, da Silva Xavier G, Rowe RL, Cross SE, Korbutt GS, Rajotte RV, Smith R, Rutter GA: Metformin, but not leptin, regulates AMP-activated protein kinase in

pancreatic islets: impact on glucose-stimulated insulin secretion. *Am.J.Physiol.Endocrinol.Metab.* 286:1023–1031, 2004.

Lehmann ED, Gosling RG, Sonksen PH: Arterial wall compliance in diabetes. *Diabet. Med.* 9:114–119, 1992.

Lehmann JM, Moore LB, Smith-Oliver TA, Wilkison WO, Willson TM, Kliewer SA. An antidiabetic thiazolidinedione is a high affinity ligand for peroxisome proliferator-activated receptor gamma (PPAR gamma). *J.Biol.Chem.* 270:12953-12956, 1995.

Lewis GF, Carpentier A, Adeli K, Adria G: Disordered Fat Storage and Mobilization in the Pathogenesis of Insulin Resistance and Type 2 Diabetes. *Endocrine Reviews* 23: 201-229, 2002.

Liao Y, Takashima S, Maeda N, Ouchi N, Komamura K, Shimomura I, Hori M, Matsuzawa Y, Funahashi T, Kitakaze M: Exacerbation of heart failure in adiponectin-deficient mice due to impaired regulation of AMPK and glucose metabolism. *Cardiovasc.Res.* 67:705-713, 2005.

Lillioja S, Mott DM, Spraul M, Ferraro R, Foley JE, Ravussin E, Knowler WC, Bennett PH, Bogardus C: Insulin resistance and insulin secretory dysfunction as precursors of non-insulin-dependent diabetes mellitus. *N Engl J Med.* 329:1988–1992, 1993

Lin MI, Fulton D, Babbitt R, Fleming I, Busse R, Pritchard KA Jr, Sessa WC: Phosphorylation of threonine 497 in endothelial nitric-oxide synthase coordinates the coupling of L-arginine metabolism to efficient nitric oxide production. *J.Biol.Chem.* 278:44719-44726, 2003.

Lindsay RS, Funahashi T, Hanson RL, Matsuzawa Y, Tanaka S, Tataranni PA, Knowler WC, Krakoff J: Adiponectin and development of type 2 diabetes in the Pima Indian population. *Lancet* 360:57-58, 2002.

Lindsay RS, Resnick HE, Zhu J, Tun ML, Howard BV, Zhang Y, Yeh J, Best LG: Adiponectin and coronary heart disease: the Strong Heart Study. *Arterioscler.Thromb.Vasc.Biol.* 25:15-16, 2005.

Liu J, Garcia-Cardena G, Sessa WC: Biosynthesis and palmitoylation of endothelial nitric oxide synthase: mutagenesis of palmitoylation sites, cysteines -15 and/or -26, argues against depalmitoylation-induced translocation of the enzyme. *Biochemistry* 34:12333-12340, 1995.

Lizcano JM, Goransson O, Toth R, Deak M, Morrice, NA, Boudeau J, Hawley SA, Udd L, Makela TP, Hardie DG, Alessi DR: LKB1 is a master kinase that activates 13 kinases of the AMPK subfamily, including MARK/PAR-1. *EMBO J.* 23:833-843, 2004.

Lochhead PA, Salt IP, Walker KS, Hardie DG, Sutherland C: 5-Aminoimidazole-4-carboxamide riboside mimics the effects of insulin on the expression of the 2 key gluconeogenic genes PEPCK and glucose-6-phosphatase. *Diabetes* 49:896-903, 2000.

Lochhead PA, Coghlan M.P, Rice SQJ, Sutherland C: Inhibition of GSK3 selectively reduces G6Pase and PEPCK gene expression. *Diabetes* 50:937-947, 2001.

London GM, Marchais SJ, Safar ME, Genest AF, Guerin AP, Metivier F, Chedid K, London AM: Aortic and large artery compliance in end-stage renal failure. *Kidney Int.* 37:137-142, 1990.

Long YC, Zierath JR: AMP-activated protein kinase signaling in metabolic regulation. *J.Clin.Invest.* 116:1776-1783, 2006.

Ludvik B, Nolan J, Baloga J, Sacks D, Olefsky J: Effect of obesity on insulin resistance in normal subjects and patients with NIDDM. *Diabetes* 44:1121-1125, 1995.

Luiken, JJ, Coort SL, Willems J, Coumans WA, Bonen A., van der Vusse GJ, Glatz, JF: Contraction-induced fatty acid translocase/CD36 translocation in rat cardiac myocytes is mediated through AMP-activated protein kinase signaling. *Diabetes* 52:1627–1634, 2003.

Lundman P, Eriksson MJ, Stuhlinger M, Cooke JP, Hamsten A, Tornvall P: Mild-to-moderate hypertriglyceridemia in young men is associated with endothelial dysfunction and increased plasma concentrations of asymmetric dimethylarginine. *J.Am.Coll.Cardiol.* 38:111–116, 2001.

Luo M, Langlais P, Yi Z, Lefort N, De Filippis EA, Hwang H, Christ-Roberts CY, Mandarino LJ: Phosphorylation of human insulin receptor substrate-1 at Serine 629 plays a positive role in insulin signaling. *Endocrinology* 148:4895-4905, 2007.

Macgowan CK, Henkelman RM, Wood ML: Pulse-wave velocity measured in one heartbeat using MR tagging. *Magn.Reson.Med.* 48:115-121, 2002.

MacKenzie IS, Wilkinson IB, Cockcroft JR: Assessment of arterial stiffness in clinical practice. *Q.J.Med.* 95:67-74, 2002.

Maguire SM, Nugent AG, McGurk C, Johnston GD, Nicholls DP: Abnormal vascular responses in human chronic cardiac failure are both endothelium dependent and endothelium independent. *Heart* 80:141-145, 1998.

Mahmud A, Feely J: Effect of Smoking on Arterial Stiffness and Pulse Pressure Amplification. *Hypertension* 41:183-187, 2003.

Mahomed FA: The physiology and clinical use of the sphygmograph. *Med. Times Gazette* 1:62, 1872

Mather KJ, Verma S, Anderson TJ: Improved Endothelial Function with Metformin in Type 2 Diabetes Mellitus. *Journal of the American College of Cardiology* 37: 1344-1350, 2001.

Matthaei S, Reibold JP, Hamann A, Benecke H, Häring HU, Greten H, Klein HH: In vivo metformin treatment ameliorates insulin resistance: evidence for potentiation of insulin-induced translocation and increased functional activity of glucose transporters in obese (fa/fa) Zucker rat adipocytes. *Endocrinology* 133:304-311, 1993.

McAuley D, McGurk C, Nugent AG, Hanratty C, Maguire S, Johnston GD: Forearm endothelium-dependent vascular responses and the potassium-ATP channel. *British Journal of Clinical Pharmacology* 44: 292-294, 1997.

McDonald DA, Taylor MG: The hydrodynamics of the arterial circulation. *Progr.Biophys.Chem.* 9:697-716, 1959.

McLaughlin T, Stühlinger M, Lamendola C, Abbasi F, Bialek J, Reaven GM, Tsao PS: Plasma asymmetric dimethylarginine concentrations are elevated in obese insulin-resistant women and fall with weight loss. *J.Clin.Endocrinol.Metab.* 91:1896-1900, 2006.

McManus EJ, Sakamoto K, Armit LJ, Ronaldson L, Shpiro N, Marquez R, Alessi DR: Role that phosphorylation of GSK3 plays in insulin and Wnt-signalling defined by knockin analysis. *EMBO J.* 24:1571-1583, 2005

McNeil AM, Rosamund WD, Girman CJ, Golden SH, Schmidy MI, East HE, Ballantyne CM, Heiss G: The Metabolic Syndrome and 11-Year Risk of Incident Cardiovascular Disease in the Atherosclerosis Risk in Communities Study. *Diabetes Care* 28:385-390, 2005

Meaume S, Benetos A, Henry OF, Rudnichi A, Safar ME: Aortic pulse wave velocity predicts cardiovascular mortality in subjects >70 years of age. *Arterioscler.Thromb.Vasc.Biol.* 21:2046-2050, 2001.

Meijssen S, Cabezas MC, Ballieux CG, Derkx RJ, Bilecen S, Erkelens DW: Insulin mediated inhibition of hormone sensitive lipase activity in vivo in relation to endogenous catecholamines in healthy subjects. *J.Clin.Endocrinol.Metab.* 86:4193-4197, 2001.

Merril GF, Kurth EJ, Hardie DG, Winder WW: AICA riboside increases AMP-activated protein kinase, fatty acid oxidation, and glucose uptake in rat muscle. *Am.J.Physiol.* 273: 1107-1112, 1997.

Michel T, Feron O: Nitric oxide synthases: which, where, how, and why? *J.Clin.Invest.* 100:2146-2152, 1997.

Miles JM, Jensen MD: Counterpoint: visceral adiposity is not causally related to insulin resistance.

*Diabetes Care* 28:2326-2328, 2005.

Minokoshi Y, Kim YB, Peroni OD, Fryer LG, Muller C, Carling D, Kahn BB: Leptin stimulates fatty-acid oxidation by activating AMP-activated protein kinase. *Nature* 415:339–343, 2002.

Minokoshi Y, Alquier T, Furukawa N, Kim YB, Lee A, Xue B, Mu J, Foufelle F, Ferré P, Birnbaum MJ, Stuck BJ, Kahn BB: AMP-kinase regulates food intake by responding to hormonal and nutrient signals in the hypothalamus. *Nature* 428:569-574, 2004.

Misbin RI: The phantom of lactic acidosis due to metformin in patients with diabetes. *Diabetes Care* 27:1791-1793, 2004.

Mithieux G, Rajas F, Zitoun C: Glucose utilization is suppressed in the gut of insulin-resistant high fat-fed rats and is restored by metformin. *Biochem.Pharmacol.* 72:1757-1762, 2006.

Mittermayer F, Mayer BX, Meyer A, Winzer C, Pacini G, Wagner OF, Wolzt M, Kautzky-Willer A: Circulating concentrations of asymmetrical dimethyl-L-arginine are increased in women with previous gestational diabetes. *Diabetologia* 45:1372-1378, 2002.

Mittermayer F, Krzyzanowska K, Exner M, Mlekusch W, Amighi J, Sabeti S, Minar E, Muller M, Wolzt M, Schillinger M: Asymmetric dimethylarginine predicts major adverse cardiovascular events in patients with advanced peripheral artery disease. *Arterioscler.Thromb.Vasc.Biol.* 26:2536–2540, 2006.

Mizuno TM, Kleopoulos SP, Bergen HT, Roberts JL, Priest CA, Mobbs CV: Hypothalamic pro-opiomelanocortin mRNA is reduced by fasting and [corrected] in ob/ob and db/db mice, but is stimulated by leptin. *Diabetes* 47:294-297, 1998.

Mohamed-Ali V, Goodrick S, Rawesh A, Katz DR, Miles JM, Yudkin JS, Klein S, Coppack SW: Subcutaneous adipose tissue releases interleukin-6, but not tumor necrosis factor-alpha, in vivo. *J.Clin.Endocrinol.Metab.* 82:4196-4200, 1997.

Mohipaddin RH, Firmin DN, Longmore DB: Age-related changes of human aortic flow wave velocity measured noninvasively by magnetic resonance imaging. *J.Appl.Physiol.* 74:492-497, 1993.

Moncada S, Higgs A: The L-arginine -nitric oxide pathway. *N Engl J Med.* 329:2002-2012, 1993.  
Montagnani M, Chen H, Barr VA, Quon MJ: Insulin-stimulated activation of eNOS is independent of Ca<sup>2+</sup> but requires phosphorylation by Akt at Ser(1179). *J.Biol.Chem.* 276:30392-30398, 2001.

Montagnani M, Golovchenko I, Kim I, Koh GY, Goalstone ML, Mundhekar AN, Johansen M, Kucik DF, Quon MJ, Draznin B: Inhibition of phosphatidylinositol 3-kinase enhances mitogenic actions of insulin in endothelial cells. *J.Biol.Chem.* 277:1794-1799, 2002.

Montagnani M, Ravichandran LV, Chen H, Esposito DL, Quon MJ: Insulin receptor substrate-1 and phosphoinositide-dependent kinase-1 are required for insulin-stimulated production of nitric oxide in endothelial cells. *Mol.Endocrinol.* 16:1931-1942, 2002.

Morrow VA, Foufelle F, Connell JM, Petrie JR, Gould GW, Salt IP: Direct activation of AMP-activated protein kinase stimulates nitric oxide synthesis in human aortic endothelial cells. *J.Biol.Chem.* 278:31629-31639, 2003.

Mount PF, Hill RE, Fraser SA, Levidiotis V, Katsis F, Kemp BE, Power DA: Acute renal ischemia rapidly activates the energy sensor AMPK but does not increase phosphorylation of eNOS-Ser1177. *Am.J.Physiol.Renal.Physiol.* 289:1103-1115, 2005.

Mu J, Brozinick JT, Valladares O, Bucan M, Birnbaum MJ: A role for AMP-activated protein kinase in contraction- and hypoxia-regulated glucose transport in skeletal muscle. *Mol.Cell.* 7:1085–1094, 2001.

Mulvany M: Procedures for investigation of small vessels using small vessel myograph. J.P Trading, Aarhus, Denmark, 1999.

Mulvany MJ, Halpern W: Contractile properties of small arterial resistance vessels in spontaneously hypertensive and normotensive rats. *Circ.Res.* 41:19-26, 1977.

Mulvany MJ, Aalkjaer C: Structure and function of small arteries. *Physiol.Rev.* 70:921-961, 1990.

Mulvany MJ: Small Artery Remodeling in Hypertension. *Curr.Hypertens.Rep.* 4:49-55, 2002.

Munday MR, Campbell DG, Carling, D, Hardie DG: Identification by amino acid sequencing of three major regulatory phosphorylation sites on rat acetyl-CoA carboxylase. *Eur.J.Biochem.* 175:331–338, 1998.

Muniyappa R, Montagnani M, Koh KK, Quon MJ: Cardiovascular actions of insulin. *Endocr Rev.* 28:463-491, 2007.

Muoio DM, Dohm GL, Fiedorek FT, Tapscott EB, Coleman RA: Leptin directly alters lipid partitioning in skeletal muscle. *Diabetes* 46:1360–1363, 1997.

Musi N, Fujii N, Hirshman MF, Ekberg I, Froberg S, Ljungqvist O, Thorell A, Goodyear LJ: AMP-activated protein kinase (AMPK) is activated in muscle of subjects with type 2 diabetes during exercise. *Diabetes* 50:921–927, 2001.

Musi, N, Hirshman MF, Nygren J, Svanfeldt M, Bavenholm P, Rooyackers O, Zhou G Williamson JM, Ljunqvist O, Efendic S, Moller DE, Thorell A, Goodyear LJ: Metformin increases AMP-activated protein kinase activity in skeletal muscle of subjects with type 2 diabetes. *Diabetes* 51:2074-2081, 2002.

Natali A, Baldeweg S, Toschi E, Capaldo B, Barbaro D, Gastaldelli A, Yudkin JS, Ferrannini E: Vascular effects of improving metabolic control with metformin or rosiglitazone in type 2 diabetes. *Diabetes Care* 27: 1349–1357, 2004.

Nichols WW, Edwards DG: Arterial elastance and wave reflection augmentation of systolic blood pressure: deleterious effects and implications for therapy. *J.Cardiovasc.Pharmacol.Ther.* 6:5-21, 2001.

Nichols WW, Singh BM: Augmentation index as a measure of peripheral vascular disease state. *Curr.Opin.Cardiol.* 17:543-551, 2002.

Nieto-Vazquez I, Fernandez-Veledo S, de Alvaro C, Lorenzo M: Dual role of interleukin -6 in regulating insulin sensitivity in murine skeletal muscle. *Diabetes* 57:3211-3221, 2008.

Nisoli E, Tonello C, Cardile A, Cozzi V, Bracale R, Tedesco L, Falcone S, Valerio A, Cantoni O, Clementi E, Moncada S, Carruba MO: Calorie restriction promotes mitochondrial biogenesis by inducing the expression of eNOS. *Science* 310:314-317, 2005.

Nissen SE, Wolski K: Effect of rosiglitazone on the risk of myocardial infarction and death from cardiovascular causes. *N Engl J Med.* 356, 2457-2471, 2007.

O'Rourke M, Staessen J, Vlachopoulos C, Duprez D, Plante G: Clinical applications of arterial stiffness; definitions and reference values. *Am.J.Hypertens.* 15:426-444, 2002.

O'Rourke MF: Mechanical principles in arterial disease. *Hypertension* 26:2-9, 2005.

Ofei F, Hurel S, Newkirk J, Sopwith M, Taylor R: Effects of an engineered human anti-TNF-alpha antibody (CDP571) on insulin sensitivity and glycemic control in patients with NIDDM. *Diabetes* 45:881-885, 1996.

Orio F Jr, Palomba S, Cascella T, De Simone B, Manguso F, Savastano S, Russo T, Tolino A, Zullo F, Lombardi G, Azziz R, Colao A: Improvement in endothelial structure and function after

metformin treatment in young normal-weight women with polycystic ovary syndrome: results of a 6-month study. *J.Clin.Endocrinol.Metab.* 90:6072-5076, 2005.

Ouchi N, Kobayashi H, Kihara S, Kumada M, Sato K, Inoue T, Funahashi T, Walsh K: Adiponectin stimulates angiogenesis by promoting cross-talk between AMP-activated protein kinase and Akt signaling in endothelial cells. *J.Biol.Chem.* 279:1304-1309, 2004.

Owen MR, Doran E, Halestrap AP: Evidence that metformin exerts its anti-diabetic effects through inhibition of complex 1 of the mitochondrial respiratory chain. *Biochem J.* 348, 607-614, 2000.

Palm F, Onozato ML, Luo Z, Wilcox CS: Dimethylarginine dimethylaminohydrolase (DDAH): expression, regulation, and function in the cardiovascular and renal systems. *Am.J.Physiol. Heart Circ.Physiol.* 293:3227-3245, 2007.

Paquot N, Castillo MJ, Lefebvre PJ, Scheen AJ: No increased insulin sensitivity after a single intravenous administration of a recombinant human tumor necrosis factor receptor: Fc fusion protein in obese insulin-resistant patients. *J.Clin.Endocrinol.Metab.* 85:1316-1319, 2000.

Perriello G, Misericordia P, Volpi E, Santucci A, Santucci C, Ferrannini E, Ventura MM, Santeusanio F, Brunetti P, Bolli GB: Acute antihyperglycemic mechanisms of metformin in NIDDM. Evidence for suppression of lipid oxidation and hepatic glucose production. *Diabetes* 43:920-928, 1994.

Petersen KF, Dufour S, Savage DB, Bilz S, Solomon G, Yonemitsu S, Cline GW, Befroy D, Zemany L, Kahn BB, Papademetris X, Rothman DL, Shulman GI: The role of skeletal muscle

insulin resistance in the pathogenesis of the metabolic syndrome. *Proc.Natl.Acad.Sci.U.S.A.* 104:12587-12594, 2007.

Peuler JD: Opposing adrenergic actions of intravenous metformin on arterial pressure in female spontaneously hypertensive rats. *Cardiovascular Research* 43: 237-247, 1999.

Phillips SA, Ciaraldi TP, Kong AP, Bandukwala R, Aroda V, Carter L, Baxi S, Mudaliar SR, Henry RR: Modulation of circulating and adipose tissue adiponectin levels by antidiabetic therapy. *Diabetes* 52:667-674, 2003.

Pistrosch F, Passauer J, Fischer S, Fuecker K, Hanefeld M, Gross P: In type 2 diabetes, rosiglitazone therapy for insulin resistance ameliorates endothelial dysfunction independent of glucose control. *Diabetes Care* 27: 484-490, 2004.

Ploug T, Galbo H, Richter EA: Increased muscle glucose uptake during contractions: no need for insulin. *Am.J.Physiol.* 247:726-731, 1984.

Podor TJ, Jirik FR, Loskutoff DJ, Carson DA, Lotz M: Human endothelial cells produce IL-6. Lack of responses to exogenous IL-6. *Ann.N.Y.Acad.Sci.* 557:374-385, 1989.

Polikandriotis JA, Mazzella LJ, Rupnow HL, Hart CM: Peroxisome proliferatoractivated receptor gamma ligands stimulate endothelial nitric oxide production through distinct peroxisome proliferator-activated receptor gamma-dependent mechanisms. *Arterioscler.Thromb.Vasc.Biol.* 25:1810-1816, 2005

Potter CJ, Huang H, Xu T: Drosophila Tsc1 functions with Tsc2 to antagonize insulin signaling in regulating cell growth, cell proliferation, and organ size. *Cell* 105:357–368, 2001.

Potter CJ, Pedraza LG, Xu T: Akt regulates growth by directly phosphorylating Tsc2. *Nat.Cell Biol.* 4:658-665, 2002.

Rajan AS, Aguilar-Bryan L, Nelson DA, Yaney GC, Hsu WH, Kunze DL, Boyd AE: Ion channels and insulin secretion. *Diabetes Care* 13:340-363, 1990.

Raju TN: The Nobel chronicles. 1998: Robert Francis Furchtgott (b 1911), Louis J Ignarro (b 1941), and Ferid Murad (b 1936). *Lancet* 356:346, 2000.

Reihill JA, Ewart MA, Hardie DG, Salt IP AMP-activated protein kinase mediates VEGF-stimulated endothelial NO production. *Biochem.Biophys.Res.Commun.* 354: 1084–1088, 2007.

Riek U, Scholz R, Konarev P, Rufer A, Suter M, Nazabal A, Ringler P, Chami M, Muller SA, Neumann D, Forstner M, Hennig M, Zenobi R, Engel A, Svergun D, Schlattner U, Wallimann T: Structural properties of AMP-activated protein kinase: dimerization, molecular shape, and changes upon ligand binding. *J.Biol.Chem.* 283:18331–18343, 2008.

Ross R: Atherosclerosis is an inflammatory disease. *Am.Heart J.* 138:S419-S420, 1999.

Rotter V, Nagaev I, Smith U: Interleukin-6 (IL-6) induces insulin resistance in 3T3-L1 adipocytes and is, like IL-8 and tumor necrosis factor-alpha, overexpressed in human fat cells from insulin-resistant subjects. *J.Biol.Chem.* 278:45777-45784, 2003.

Ruderman NB, Saha AK, Vavvas D, Witters, LA: Malonyl-CoA, fuel sensing, and insulin resistance. *Am. J.Physiol.Endocrinol.Metab.* 276:1-18, 1999.

Russell RR, Bergeron R, Shulman GI, Young LH: Translocation of myocardial GLUT-4 and increased glucose uptake through activation of AMPK by AICAR. *Am.J.Physiol.* 277: 643–649, 1999.

Russell RR, Li J, Coven DL, Pypaert M, Zechner C, Palmeri M, Giordano FJ, Mu J, Birnbaum MJ, Young, LH: AMP-activated protein kinase mediates ischemic glucose uptake and prevents post ischemic cardiac dysfunction, apoptosis, and injury. *J.Clin.Invest.* 114: 495–503, 2004.

Saenz A, Fernandez-Esteban I, Mataix A, Ausejo M, Roque M, Moher D: Metformin monotherapy for type 2 diabetes mellitus. *Cochrane Database Systematic Reviews*, CD002966, 2005.

Saha AK, Avilucea PR, Ye JM, Assifi MM, Kraegen EW, Ruderman NB: Pioglitazone treatment activates AMP-activated protein kinase in rat liver and adipose tissue *in vivo*. *Biochem.Biophys.Res.Commun.* 314: 580-585, 2004.

Sakamoto K, Goransson O, Hardie DG, Alessi DR: Activity of LKB1 and AMPK-related kinases in skeletal muscle: effects of contraction, phenformin, and AICAR. *Am.J.Physiol.Endocrinol.Metab.* 287:310-317, 2004.

Sakamoto K, McCarthy A, Smith D, Green KA, Hardie DG, Ashworth A, Alessi DR. Deficiency of LKB1 in skeletal muscle prevents AMPK activation and glucose uptake during contraction. *EMBO J.* 24:1810-1820, 2005.

Salt IP, Johnson G, Ashcroft SJ, Hardie DG: AMP-activated protein kinase is activated by low glucose in cell lines derived from pancreatic beta cells, and may regulate insulin release. *Biochem.J.* 335 (Pt 3):533-539, 1998.

Salt IP, Connell JM, Gould GW: 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR) inhibits insulin-stimulated glucose transport in 3T3-L1 adipocytes. *Diabetes* 49:1649-1656, 2000.

Salt IP, Morrow VA, Brandie FM, Connell JM, Petrie JR: High glucose inhibits insulin stimulated nitric oxide production without reducing endothelial nitric-oxide synthase Ser1177 phosphorylation in human aortic endothelial cells. *J.Biol.Chem.* 278:18791-18797, 2003.

Saltiel AR, Kahn CR: Insulin signalling and the regulation of glucose and lipid metabolism. *Nature* 414:799-806, 2001.

Saltiel AR, Pessin JE: Insulin signalling pathways in time and space. *Trends. Cell. Biol.* 12:65-71, 2002.

Sanders MJ, Grondin PO, Hegarty BD, Snowden MA, Carling D: Investigating the mechanism for AMP activation of the AMP-activated protein kinase cascade. *The Biochemical journal.* 403:139-148, 2007.

Sanger F: The early days of DNA sequences. *Nat.Med.* 7:267-268, 2001.

Sano H, Kane S, Sano, E, Miinea CP, Asara JM, Lane WS, Garner CW, Lienhard GE: Insulin-stimulated phosphorylation of a Rab GTPase-activating protein regulates GLUT4 translocation. *J.Biol.Chem.* 278:14599-14602, 2003

Sapkota GP, Deak M, Kieloch A, Morrice N, Goodarzi AA, Smythe C, Shiloh Y, Lees-Miller SP, Alessi DR: Ionizing radiation induces ataxia telangiectasia mutated kinase (ATM)-mediated phosphorylation of LKB1/STK11 at Thr-366. *Biochem.J.* 368:507-516, 2002.

Sarbassov DD, Guertin DA, Ali SM, Sabatini DM: Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. *Science* 307:1098-1101, 2005.

Satoh N, Ogawa Y, Usui T, Tagami T, Kono S, Uesugi H, Sugiyama H, Sugawara A, Yamada K, Shimatsu A, Kuzuya H, Nakao K: Antiatherogenic effect of pioglitazone in type 2 diabetic patients irrespective of the responsiveness to its antidiabetic effect. *Diabetes Care* 26: 2493–2499, 2003.

Sattar N, Gaw A, Scherbakova O, Ford I, O'Reilly DS, Haffner SM, Isles C, Macfarlane PW, Packard CJ, Cobbe SM, Shepherd J Metabolic syndrome with and without C-reactive protein as a predictor of coronary heart disease and diabetes in the West of Scotland Coronary Prevention Study. *Circulation* 108:414-419, 2003.

Sattar N, Wannamethee G, Sarwar N, Tchernova J, Cherry L, Wallace AM, Danesh J, Whincup PH: Adiponectin and coronary heart disease: a prospective study and meta-analysis. *Circulation* 114:623-629, 2006.

Scherer PE, Williams S, Fogliano M, Baldini G, Lodish HF: A novel serum protein similar to C1q, produced exclusively in adipocytes. *J.Biol.Chem.* 270:26746-26749, 1995.

Schiffrin EL Reactivity of small blood vessels in hypertension: relation with structural changes. State of the art lecture. *Hypertension* 19:1-9, 1992.

Schmid-Antomarchi H, De Weille J, Fosset M, Lazdunski M: The receptor for antidiabetic sulfonylureas controls the activity of the ATP-modulated K<sup>+</sup> channel in insulin-secreting cells. *J.Biol.Chem.* 262:15840-15844, 1987.

Schofield I, Malik R, Izzard A, Austin C, Heagerty A: Vascular Structural and Functional Changes in Type 2 Diabetes Mellitus. Evidence for the Roles of Abnormal Myogenic Responsiveness and Dyslipidemia. *Circulation* 10:3037-3043, 2002.

Schwartz MW, Seeley RJ, Campfield LA, Burn P, Baskin DG: Identification of targets of leptin action in rat hypothalamus. *J.Clin.Invest.* 98:1101-1106, 1996.

Schwartz MW, Erickson JC, Baskin DG, Palmiter RD: Effect of fasting and leptin deficiency on hypothalamic neuropeptide Y gene transcription in vivo revealed by expression of a lacZ reporter gene. *Endocrinology* 139:2629-2635, 1998.

Schwartz MW, Woods SC, Porte D, Seeley RJ, Baskin DG: Central nervous system control of food intake. *Nature*. 404:661–671, 2000.

Semple RK, Chatterjee VK, O'Rahilly S: PPAR gamma and human metabolic disease. *J.Clin.Invest.* 116:581-589, 2006.

Senn JJ, Klover PJ, Nowak IA, Mooney RA: Interleukin-6 induces cellular insulin resistance in hepatocytes. *Diabetes* 51:3391-3399, 2002.

Sesti G, Federici M, Hribal ML, Lauro D, Sbraccia P, Lauro R: Defects of the insulin receptor substrate (IRS) system in human metabolic disorders. *FASEB J.* 15:2099-2111, 2001.

Shaw RJ, Lamia KA, Vasquez D, Koo SH, Bardeesy N, Depinho RA, Montminy M, Cantley LC: The kinase LKB1 mediates glucose homeostasis in liver and therapeutic effects of metformin. *Science* 310:1642–1646, 2005.

Shibata R, Sato K, Pimentel DR, Takemura Y, Kihara S, Ohashi K, Funahashi T, Ouchi N, Walsh K: Adiponectin protects against myocardial ischemia-reperfusion injury through AMPK- and COX-2-dependent mechanisms. *Nat.Med.* 11:1096–1103, 2005.

Shimokawa H and Takeshita A: Endothelium-Dependent regulation of the Cardiovascular system. *Int. Med.* 34:939-946, 1995.

Shimokawa H, Yasutake H, Fujii K, Owada MK, Nakaike R, Fukumoto Y, Takayanagi T, Nagao T, Egashira K, Fujishima M, Takeshita A: The importance of the hyperpolarizing mechanism increases as the vessel size decreases in endothelium-dependent relaxations in rat mesenteric circulation. *J.Cardiovasc.Pharmacol.* 5:703-711, 1996.

Shu Y, Leabman MK, Feng B, Mangravite LM, Huang CC, Stryke D, Kawamoto M, Johns SJ, DeYoung J, Carlson E, Ferrin TE, Herskowitz I, Giacomini KM: Pharmacogenetics Of Membrane Transporters Investigators. Evolutionary conservation predicts function of variants of the human organic cation transporter, OCT1. *Proc.Natl.Acad.Sci.U.S.A.* 100:5902-5907, 2003.

Shu Y, Sheardown SA, Brown C, Owen RP, Zhang S, Castro RA, Ianculescu AG, Yue L, Lo JC, Burchard EG, Brett CM, Giacomini KM: Effect of genetic variation in the organic cation transporter 1 (OCT1) on metformin action. *J.Clin.Invest.* 117:1422-1431, 2007.

Stehouwer CD, Lambert J, Donker AJ, van Hinsberg VW: Endothelial dysfunction and pathogenesis of diabetic angiopathy. *Cardiovac.Res.* 34:55-68, 1997.

Steinberg HO, Baron AD: Vascular Function, insulin resistance and fatty acids. *Diabetologia* 45:623-634, 2002.

Sterne J: The present state of knowledge on the mode of action of the antidiabetic diguanides. *Metabolism* 13:791-798, 1964.

Sterne J: Aspects of pharmacology and mechanism of action. *Research Clinical Forums* 1:13-20, 1979.

Stühlinger MC, Abbasi F, Chu JW, Lamendola C, McLaughlin TL, Cooke JP, Reaven GM, Tsao PS: Relationship between insulin resistance and an endogenous nitric oxide synthase inhibitor. *JAMA* 287: 1420-1426, 2002.

Stuhlinger MC, Oka RK, Graf EE, Schmolzer I, Upson BM, Kapoor O, Szuba A, Malinow MR, Wascher TC, Pachinger O, Cooke JP: Endothelial dysfunction induced by hyperhomocyst(e)inemia: role of asymmetric dimethylarginine. *Circulation* 108:933–938, 2003.

Sturgess NC, Ashford ML, Cook DL, Hales CN: The sulphonylurea receptor may be an ATP-sensitive potassium channel. *Lancet* 2:474-5, 1985.

Sullivan JE, Brocklehurst KJ, Marley AE, Carey F, Carling D, Beri RK: Inhibition of lipolysis and lipogenesis in isolated rat adipocytes with AICAR, a cell-permeable activator of AMP-activated protein kinase. *FEBS Lett.* 353:33–36, 1994.

Sutherland, C, Leighton, IA and Cohen P: Inactivation of glycogen synthase kinase-3 $\beta$  by phosphorylation; new kinase connections in insulin and growth factor signaling. *Biochem.J.* 296:15-19, 1993.

Taddei S, Salvetti A: Endothelial dysfunction in essential hypertension: clinical implications. *J.Hypertens.* 20:1671-674, 2002.

Tamemoto H, Kadowaki T, Tobe K, Yagi T, Sakura H, Hayakawa T, Terauchi Y, Ueki K, Kaburagi Y, Satoh S, Sekihara H, Yoshioka S, Horikoshi H, Furuta Y, Ikawa Y, Kasuga M, Yazaki Y, Aizawa S: Insulin resistance and growth retardation in mice lacking insulin receptor substrate-1. *Nature* 372:182-186, 1994.

Tan KC, Xu A, Chow WS, Lam MC, Ai VH, Tam SC, Lam KS: Hypoadiponectinemia is associated with impaired endothelium -dependent vasodilation. *J.Clin.Endocrinol.Metab* 89:765-769, 2004.

Tapon N, Ito N, Dickson BJ, Treisman JE, Hariharan IK. The *Drosophila* tuberous sclerosis complex gene homologs restrict cell growth and cell proliferation. *Cell* 105:345–355, 2001.

Teerlink T: Determination of the endogenous nitric oxide synthase inhibitor asymmetric dimethylarginine in biological samples by HPLC. *Meth.Mol.Med.* 108:263–274, 2005.

Tiikkainen M, Häkkinen AM, Korsheninnikova E, Nyman T, Mäkimattila S, Yki-Järvinen H: Effects of rosiglitazone and metformin on liver fat content, hepatic insulin resistance, insulin clearance, and gene expression in adipose tissue in patients with type 2 diabetes. *Diabetes* 53:2169–2176, 2004.

Tomas E, Tsao TS, Saha AK, Murrey HE, Zhang Cc C, Itani SI, Lodish HF, Ruderman NB: Enhanced muscle fat oxidation and glucose transport by ACRP30 globular domain: acetyl-CoA carboxylase inhibition and AMP-activated protein kinase activation. *Proc.Natl.Acad.Sci.U.S.A.* 99:16309–16313, 2002.

Towler MC, Hardie DG: AMP-activated protein kinase in metabolic control and insulin signaling. *Circ.Res.* 100:328–341, 2007.

Treebak JT, Birk JB, Rose AJ, Kiens B, Richter EA, Wojtaszewski JF: AS160 phosphorylation is associated with activation of alpha2beta2gamma1- but not alpha2beta2gamma3-AMPK trimeric complex in skeletal muscle during exercise in humans. *Am.J.Physiol.Endocrinol.Metab.* 292:715–722, 2007.

Trumble G, Smith M, Winder W: Purification and characterization of rat skeletal muscle acetyl-CoA carboxylase. *Eur.J.Biochem.* 231:192-198, 1995

UK Prospective Diabetes Study (UKPDS) Group: Effect of intensive blood-glucose control with metformin on complications in overweight patients with type 2 diabetes (UKPDS 34). *Lancet* 352:854-865, 1998.

Unger RH, Madison LL, Carter NW: Tolbutamide-phenformin in ketoacidosis-resistant patients. *JAMA* 174:2132-2136, 1960.

Uysal KT, Wiesbrock SM, Marino MW, Hotamisligil GS: Protection from obesity-induced insulin resistance in mice lacking TNF-alpha function. *Nature* 389:610-614, 1997.

Valkonen VP, Paiva H, Salonen JT, Lakka TA, Lehtimaki T, Laakso J, Laaksonen R: Risk of acute coronary events and serum concentration of asymmetrical dimethylarginine. *Lancet* 358:2127-2128, 2001.

Vallance P, Collier J, Moncada S: Effects of endothelium-derived nitric oxide on peripheral arterial tone in man. *Lancet* 2:997-1000, 1989.

Vallance P, Chan N: Endothelial function and nitric oxide: clinical relevance. *Heart* 85:342-350, 2001.

Van Weeren PC, De Bruyn KM, De Vries-Smits AM, Van Lint J, Burgering BM: Essential role for protein kinase B (PKB) in insulin-induced glycogen synthase kinase 3 inactivation. Characterization of dominant-negative mutant of PKB. *J.Biol.Chem.* 273:13150-13156, 1998.

Vander Haar E, Lee SI, Bandhakavi S, Griffin TJ, Kim DH: Insulin signalling to mTOR mediated by the Akt/PKB substrate PRAS40. *Nat. Cell Biol.* 9:316-323, 2007.

Villena JA: Induced adiposity and adipocyte hypertrophy in mice lacking the AMP-activated protein kinase-alpha2 subunit. *Diabetes* 53:2242-2249, 2004.

Viollet B: The AMP-activated protein kinase  $\alpha 2$  catalytic subunit controls whole-body insulin sensitivity. *J.Clin.Invest.* 111:91-98, 2003.

Vitale C, Mercuro G, Cornoldi A, Fini M, Volterrani M, Rosano GM: Metformin improves endothelial function in patients with metabolic syndrome. *J.Intern.Med.* 258:250-256, 2005.

Wada T, Kodaira K, Fujishiro K, Maie K, Tsukiyama E, Fukumoto T, Uchida T, Yamazaki S: Correlation of ultrasound-measured common carotid artery stiffness with pathological findings. *Arterioscler.Thromb.Vasc.Biol.* 14:479-482, 1994.

Wajant H, Pfizenmaier K, Scheurich P: Tumor necrosis factor signaling. *Cell Death Differ.* 10:45-65, 2003.

Walker RS, Linton AL: Phenethyldiguanide: a dangerous side-effect. *Br.Med.J.* 2:1005-1006, 1959.

Wallace T, Levy J, Matthews D: Use and abuse of HOMA modeling. *Diabetes Care* 27:1487-1495, 2004

Wang DS, Jonker JW, Kato Y, Kusuvara H, Schinkel AH, Sugiyama Y: Involvement of organic cation transporter 1 in hepatic and intestinal distribution of metformin. *J.Pharmacol.Exp.Ther.* 302:510–515, 2002.

Wang DS, Kusuvara H, Kato Y, Jonker JW, Schinkel AH, Sugiyama Y: Involvement of organic cation transporter 1 in the lactic acidosis caused by metformin. *Mol.Pharmacol.* 63:844-848, 2003.

Wang TD, Chen WJ, Lin JW, Chen MF, Lee, YT: Effects of rosiglitazone on endothelial function, C-reactive protein, and components of the metabolic syndrome in nondiabetic patients with the metabolic syndrome. *Am.J.Cardiol.* 93: 362–365, 2004.

Wang ZV, Scherer PE: Adiponectin, cardiovascular function, and hypertension. *Hypertension* 51:8-14, 2007.

Warram JH, Martin BC, Krolewski AS, Soeldner JS, Kahn CR: Slow glucose removal rate and hyperinsulinemia precede the development of type II diabetes in the offspring of the diabetic parents. *Ann.Intern.Med.* 113:909-912, 1990

Weigert C, Hennige AM, Brischmann T, Beck A, Moeschel K, Schauble M, Brodbeck K, Haring HU, Schleicher ED, Lehmann R: The phosphorylation of Ser318 of insulin receptor substrate 1 is not per se inhibitory in skeletal muscle cells but is necessary to trigger the attenuation of the insulin-stimulated signal. *J.Biol.Chem.* 280:37393-37399, 2005.

Weyer C, Funahashi T, Tanaka S, Hotta K, Matsuzawa Y, Pratley RE, Tataranni PA: Hypoadiponectinemia in obesity and type 2 diabetes: close association with insulin resistance and hyperinsulinemia. *J.Clin.Endocrinol.Metab.* 86:1930-1935, 2001.

Wheatcroft SB, Williams IL, Shah AM, Kearney MT: Pathophysiological implications of insulin resistance on vascular endothelial function. *Diabet.Med.* 20:255-268, 2003.

White MF, Maron R, Kahn CR: Insulin rapidly stimulates tyrosine phosphorylation of a Mr-185,000 protein in intact cells. *Nature* 318:183-186, 1985.

White MF, Takayama S, Kahn CR: Differences in the sites of phosphorylation of the insulin receptor in vivo and in vitro. *J.Biol.Chem.* 260:9470-9478, 1985.

White MF: The IRS-signalling system: a network of docking proteins that mediate insulin action. *Mol. Cell Biochem.* 182:3-11, 1998.

White MF: IRS proteins and the common path to diabetes. *Am.J.Physiol.Endocrinol.Metab.* 283:E413-E422, 2002.

Winder WW, Hardie DG: Inactivation of acetyl-CoA carboxylase and activation of AMP-activated protein kinase in muscle during exercise. *Am.J.Physiol.Endocrinol.Metab.* 270:299-304, 1996.

Winder WW, Hardie DG: AMP-activated protein kinase, a metabolic master switch: possible roles in type 2 diabetes. *Am.J.Physiol.* 277:1-10, 1999.

Withers DJ, Gutierrez JS, Towery H, Burks DJ, Ren JM, Previs S, Zhang Y, Bernal D, Pons S, Shulman GI, Bonner-Weir S, White MF: Disruption of IRS-2 causes type 2 diabetes in mice. *Nature* 391:900-904, 1998.

Withers DJ, Burks DJ, Towery HH, Altamuro SL, Flint CL, White MF: Irs-2 coordinates Igf-1 receptor-mediated beta-cell development and peripheral insulin signalling. *Nature* 23:32-40, 1999

Witters LA: The blooming of the French lilac. *Journal of Clinical Investigation* 108:1105–1107, 2001.

Woods A, Johnstone SR, Dickerson K, Leiper FC, Fryer LG, Neumann D, Schlattner U, Wallimann T, Carlson M, Carling D: LKB1 is the upstream kinase in the AMP-activated protein kinase cascade. *Curr.Biol.* 13, 2004–2008, 2003.

Woods A, Dickerson K, Heath R, Hong SP, Momcilovic M, Johnstone SR, Carlson M, Carling D: Ca<sup>2+</sup>/calmodulin-dependent protein kinase kinase-beta acts upstream of AMP-activated protein kinase in mammalian cells. *Cell Metab.* 2:21-33, 2005.

Wulffelé MG, Kooy A, de Zeeuw D, Stehouwer CD, Gansevoort RT: The effect of metformin on blood pressure, plasma cholesterol and triglycerides in type 2 diabetes mellitus: a systematic review. *J.Intern.Med.* 256:1-14, 2004.

Xing Y, Musi N, Fuji N, Zou L, Luptak I, Hirshman MF, Goodyear LJ, Tian R: Glucose metabolism and energy homeostasis in mouse hearts overexpressing dominant negative  $\alpha 2$  subunit of AMP-activated protein kinase. *J.Biol.Chem.* 278: 28372–28377, 2003.

Yamauchi T, Tobe K, Tamemoto H, Ueki K, Kaburagi Y, Yamamoto-Honda R, Takahashi Y, Yoshizawa F, Aizawa S, Akanuma Y, Sonenberg N, Yazaki Y, Kadowaki T: Insulin signalling and insulin actions in the muscles and livers of insulin-resistant, insulin receptor substrate 1-deficient mice. *Mol. Cell Biol.* 16:3074-3084, 1996.

Yamauchi T, Kamon J, Minokoshi Y, Ito Y, Waki H, Uchida S, Yamashita S, Noda M, Kita S, Ueki K, Eto K, Akanuma Y, Froguel P, Foufelle F, Ferre P, Carling D, Kimura S, Nagai R, Kahn BB, Kadowaki T: Adiponectin stimulates glucose utilization and fatty-acid oxidation by activating AMP-activated protein kinase. *Nat.Med.* 8:1288–1295, 2002.

Yang J, Holman GD: Long-term metformin treatment stimulates cardiomyocyte glucose transport through an AMP-activated protein kinase-dependent reduction in GLUT4 endocytosis. *Endocrinology* 147:2728–2736, 2006.

Yasmin, McEnery CM, Wallace S, Mackenzie IS, Cockcroft JR, Wilkinson IB: C-Reactive Protein Is Associated With Arterial Stiffness in Apparently Healthy Individuals. *Arterioscler.Thromb.Vasc.Biol.* 24:969-974, 2004.

Yeni-Komshian H, Carantoni M, Abbasi F and Reaven GM: Relationship between several surrogate estimates of insulin resistance and quantification of insulin-mediated glucose disposal in 490 healthy, nondiabetic volunteers. *Diabetes Care* 23:171–175, 2000.

Yki-Järvinen H: Role of insulin resistance in the pathogenesis of NIDDM. *Diabetologia* 38:1378-1388, 1995.

Yki-Jäarvinen H, Utriainen T: Insulin-induced vasodilatation: physiology or pharmacology? *Diabetologia* 41:369-379, 1998.

Yudkin JS, Stehouwer CD, Emeis JJ, Coppack SW: C-reactive protein in healthy subjects: associations with obesity, insulin resistance, and endothelial dysfunction: a potential role for cytokines originating from adipose tissue? *Arterioscler.Thromb.Vasc.Biol.* 19:972-978, 1999.

Zang M, Zuccollo A, Hou X, Nagata D, Walsh K, Herscovitz H, Brecher P, Ruderman NB, Cohen RA: AMP-activated protein kinase is required for the lipid-lowering effect of metformin in insulin-resistant human HepG2 cells. *J.Biol.Chem.* 279:47898-4790, 2004.

Zeng G, Quon MJ: Insulin-stimulated production of nitric oxide is inhibited by wortmannin. Direct measurement in vascular endothelial cells. *J.Clin.Invest.* 98:894-898, 1996.

Zhang J, Gao Z, Yin J, Quon MJ, Ye J: S6K directly phosphorylates IRS-1 on Ser270 to promote insulin resistance in response to TNF-alpha signaling through IKK2. *J.Biol.Chem.* 283:35375-35382, 2008.

Zhang L, He H, Balschi JA: Metformin and phenformin activate AMP-activated protein kinase in the heart by increasing cytosolic AMP concentration. *Am.J.Physiol. Heart Circ.Physiol.* 293:457-466, 2007.

Zhang S, Kim, KH: Glucose activation of acetyl-CoA carboxylase in association with insulin secretion in a pancreatic beta-cell line. *J.Endocrinol.* 147:33-41, 1995.

Zhou G, Myers R, Li Y, Chen Y, Shen X, Fenyk-Melody J, Wu M, Ventre J, Doepper T, Fujii N, Musi N, Hirshman MF, Goodyear LJ, Moller DE: Role of AMP-activated protein kinase in mechanism of metformin action. *J.Clin.Invest.* 108:1167–74, 2001.

Zhou K, Donnelly LA, Kimber CH, Donnan PT, Doney AS, Leese G, Hattersley AT, McCarthy MI, Morris AD, Palmer CN, Pearson ER: Reduced-function SLC22A1 polymorphisms encoding organic cation transporter 1 and glycemic response to metformin: a GoDARTS study. *Diabetes* 58:1434-1439, 2009.

Zimmet PZ, Alberti KG: The changing face of macrovascular disease in non-insulin dependent diabetes mellitus: an epidemic in progress. *Lancet* 350 (Suppl) 1:SI1-SI4, 1997.

Zou MH, Hou XY, Shi CM, Nagata D, Walsh K, Cohen RA: Modulation by peroxynitrite of Akt- and AMP-activated kinase-dependent Ser1179 phosphorylation of endothelial nitric oxide synthase. *J.Biol.Chem.* 277:32552-32557, 2002.

Zou MH, Kirkpatrick SS, Davis BJ, Nelson JS, Wiles WG 4th, Schlattner U, Neumann D, Brownlee M, Freeman MB, Goldman MH: Activation of the AMP-activated protein kinase by the anti-diabetic drug metformin in vivo. Role of mitochondrial reactive nitrogen species. *J.Biol.Chem.* 279:43940-43951, 2004.