Role of NADPH oxidase (Nox) 1/4 in adipocyte-derived aldosterone production and in inflammation and fibrosis in obesity.

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Abstract

Obesity plays a key role in the development of metabolic syndrome, but the underlying mechanisms still remain elusive. Factors that have been implicated include activation of the renin angiotensin aldosterone system (RAAS) and increased bioavailability of reactive oxygen species (ROS) (termed oxidative stress), which impact on inflammation in adipose and other tissues. Inflammatory processes in fat appear to be an important initiator of metabolic syndrome and associated cardiovascular disease. Oxidative stress and aldosterone levels are positively associated with adiposity. Exact mechanisms contributing to high aldosterone levels in obesity are unclear, but adipocytes can produce aldosterone, as well as ROS, phenomena that are amplified in obesity.

In the present studies, we examined the relationship between ROS, aldosterone and adipocytes in the context of obesity. In particular we questioned whether ROS influence aldosterone production by adipocytes and whether these processes influence adipocyte function related to cell maturation, inflammation and fibrosis. We also explored the enzymatic source of ROS in adipocytes, focusing specifically on Nox isoforms, Nox1 and Nox4.

To address these questions we performed *in vivo* and *in vitro* studies. Adipose tissue and mature adipocytes from obese *db/db* mice were studied. To translate our findings to humans, we also studied human mature adipocytes. To interrogate Nox1 and Nox4, we used a dual Nox1/4 inhibitor, GKT137831 (Genkyotex S.A, Geneva, Switzerland) *in vivo* and *in vitro* studies.

Aldosterone and corticosterone levels were measured by ELISA while plasma electrolyte, lipid and metabolic profile were assessed with an automated analyser. Gene expression was assessed by qPCR. Fibrosis was determined by picro Sirius red staining and polarized light microscopy. ROS production was determined by chemiluminescence, amplex Red and TBARS.

Our results demonstrate that aldosterone levels were increased by 4 fold in plasma and 4 fold in epididymal fat culture media from *db/db* mice compared to lean mice. These effects were decreased by high dose Nox1/4 inhibitor (GKT).

Visceral fat from *db*/*db* mice had increased levels of oxidative stress and mRNA levels of inflammatory marker such as, *CD206* (150%), *F4/80* (600%), *TNFa* (230%), *iNOS* (50%), as well as collagen 6a (160%). Treatment of obese *db*/*db* mice with GKT did not significantly decrease fibrosis in epididymal fat but it did show a tendency to decrease with low dose treatment. In the perivascular fat from *db*/*db* mice GKT decreased the mRNA levels of pro-inflammatory markers, followed by an increase in mRNA levels of anti-inflammatory markers. Ang II stimulation of human adipocytes increased aldosterone (5 fold) and cortisol (3 fold) production, as well as superoxide (2 fold) and hydrogen peroxide (0.5 fold) levels. Aldosterone production was Nox1/4-dependent and cortisol was Nox4-dependent. These effects are amplified in obesity and may participate in adipocyte dysfunction as evidenced in adipose tissue from obese *db*/*db* mice. Our data also suggest that oxidative stress is increased in fat from obese animals and may play a role in obesity-associated adipose tissue inflammation and potentially fibrosis.

Based on these findings we conclude that in obesity Nox isoforms play an important role in visceral adipocyte tissue inflammation/fibrosis and aldosterone biosynthesis and that adipocyte Nox1/4 may be a putative therapeutic target in obesity-associated hyperaldosteronism and cardiovascular damage.

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Acknowledgements

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Author's Declaration

I declare that this thesis has been entirely written by myself and has not been previously submitted for a higher degree. The record of research has been carried out by myself under the supervision of Prof Rhian Touyz, Dr Augusto Montezano and Dr Aurélie Nguyen Dinh Cat unless otherwise stated.

I would like to recognize that the *in vivo* mouse experiments and the metabolic/ biochemical and blood pressure measurements were conducted by Dr Montezano and Ying He at the Kidney Research Centre, Ottawa Hospital Research Institute (OHRI)/University of Ottawa. Tayze Antunes provided the EVAT adiposity results while Dr Nguyen Dinh Cat carried out the aldosterone and corticosterone measurements from cultured mature adipocytes and plasma (OHRI/University of Ottawa). I performed the rest of the *in vitro* and cell-based studies presented in this thesis. Iain MacMillan and Dr Francisco Rios helped to establish the polarized light microscope protocol. John McClure helped with the statistical analysis.

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Sarah Even

Definitions/Abbreviations

µ	Micro (prefix)
˚C	Degrees celsius
3T3-L1 cells	Mouse embryonic fibroblast cell line
11ßhsd1	Hydroxysteroid (11-beta) dehydrogenase 1
11ßhsd2	Hydroxysteroid (11-beta) dehydrogenase 2
ACE	Angiotensin converting enzyme
AChE	Acetylcholinesterase conjugate
ADHP	10-acetyl-3,7-dihydroxyphenoxazine
ACS	AcetylCoA synthase
ACTH	Adrenocorticotropin
ADRF	Adipocyte derived relaxation factor
AG	Adrenal gland
AGT	Angiotensinogen
AngII	Angiotensin II
ANOVA	Analysis of variance
Ap2	Adipocyte protein 2
AT	Adipose tissue
AT_1	Angiotensin II receptor type 1
AT_2	Angiotensin II receptor type 2
ATP	Adenosine triphosphate
BAT	Brown adipose tissue
BMI	Body mass index
BSA	Bovine serum albumin
CBS	Calf bovine serum
CCM	Cell culture media
CD206	Mannose receptor C type 1 (MRC1)
cDNA	Complementary DNA
CVD	Cardiovascular disease
Cyp11b1	Cytochrome P450, family 11, subfamily B, polypeptide 1
Cyp11b2	Cytochrome P450, family 11, subfamily B, polypeptide 2
DC	Detergent compatible
dH_2O	Distilled water
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl Sulphoxide
DNA	Deoxyribonucleic acid
ECL	Enhanced chemiluminescence
ECM	Extracellular matrix
EGTA	Ethylene glycol tetraacetic acid

EIA	Enzyme immunoassay
ELISA	Enzyme-linked immunosorbent assay
eNOS	Endothelium nitric oxide synthase
ERK	Extracellular signal-regulated kinases
EVAT	Epididymal visceral adipose tissue
F4/80	EGF-like module containing, mucin-like, hormone receptor-like sequence
FAD	Flavin adenine dinucleotide
Fblx10	F-box and leucine-rich repeat protein-10
FBS	Foetal bovine serum
Fizz-1	Resistin-like molecule alpha1
g	Gram(s)
Gapdh	Glyceraldehyde 3-Phosphate Dehydrogenase
GC	Glucocorticoid
GR	Glucocorticoid receptor
H_2O_2	Hydrogen Peroxide
HDL	High density lipoprotein
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid
HO-1	Heme oxygenase
HPRT	Hypoxanthine guanine-phosphoribosyl-transferase
hr	Hour
HRP	Horse radish peroxidase
IBMX	3-isobutyl-1-methylxanthine
IgG	Immunoglobulin G
IHC	Immunohistochemistry
Il-6	Interleukin-6
Il-12	Interleukin-12
iNOS	Inducible nitric oxide synthase
Ipo8	Importin-8
LDL	Low density lipoprotein
LEC	Lucigenin-enhanced chemiluminescence
M	Molar(s)
m	Milli (prefix)
M1	Macrophage type 1
M2	Macrophage type 2
MAM	Mature adipocyte medium
MAP kinases	Mitogen-activated protein kinase
MCP-1	Monocyte chemotactic protein 1
MDA	Malondialdehyde
min	Minute
mL	Millilitre(s)

MMP	Matrix metalloproteinases
MR	Mineralocorticoid receptor
mRNA	Messenger ribonucleic acid
NAC	N-Acetyl-cysteine
NADPH	Nicotinamide adenine dinucleotide phosphate
Ngal	Neutrophil gelatinase-associated lipocalin
NO	Nitric oxide
NOS	Nitric oxide synthase
Nox	NADPH oxidase
NT	No treatment
O_2^-	Superoxide
OD	Optical density
$p22^{phox}$	Cytochrome b-245, alpha polypeptide (CYBA)
PAI-1	Plasminogen activator inhibitor 1
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
Pen/strep	Penicillin / Streptomycin
PKC	Protein kinase C
Poldip2	Polymerase delta interacting protein 2
Ppar-y	Peroxisome proliferator-activated receptor gamma
PVAT	Perivascular adipose tissue
RAAS	Renin-angiotensin-aldosterone system
RAS	Renin-angiotensin system
ROS	Reactive oxygen species
RT	Room temperature
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
Sgk1	Serum/glucocorticoid regulated kinase 1
SMC	Smooth muscle cell
SOD	Superoxide dismutase
SW872 cells	Pre-adipocytes from a human liposarcoma cell line
TBARS	Thiobarbituric acid reactive substances
TBS	Tris - buffered saline
TBS-T	0.1% Tween-20 in TBS
TIMP	Tissue inhibitor of metalloproteinases
TNF-α	Tumour necrosis factor-alpha
UCP-1	Uncoupling protein 1
WAT	White adipose tissue
WHO	World Health Organisation

1 Introduction

1.1 Obesity, cardiovascular disease and the renin-angiotensin-aldosterone system (RAAS).

Obesity results from an excess in body fat content (or adiposity). Individuals with adiposity are characterized as being overweight (Body mass index (BMI) 25-30) or obese (BMI > 30). However, the amount of excess fat stored, the regional distribution of fat within the body, and the related health consequences differ amongst obese individuals (Kopelman *et al*, 2000). In general, abdominal obesity is associated with greater cardiovascular risk that generalised obesity. Obesity/overweight has become a public health challenge due to the dramatic global increase over the last three decades. According to the World Health Organisation, obesity has nearly doubled worldwide since 1980 and is the 5th leading risk factor for death (Lim *et al*, 2012; WHO, factsheet). This has been attributed to hereditary factors, rapid economic growth and increased availability of high caloric food as well as lifestyle changes. Accumulating evidence suggests that chronic inflammation of the adipose tissue may play a critical role in the development of obesity-related cardiovascular and metabolic diseases (Furukawa *et al*, 2004; Cinti, 2012; Hassan *et al*, 2012). Numerous studies have shown that abdominal obesity is strongly associated with cardiovascular complications including hypertension, endothelial dysfunction, insulin resistance and type 2 diabetes (Caballero, 2003; Cade *et al*, 2008; Balistreri *et al*, 2010; DeMarco *et al*, 2014) (Figure 1.1). Due to its major role in obesity there is an increasing need to better understand the biology of adipose tissue in physiological and pathological conditions. In particular there is growing interest in elucidating the mechanisms that regulate adipose tissue function and the cross-talk with other organs, such as the heart, kidney and blood vessels.

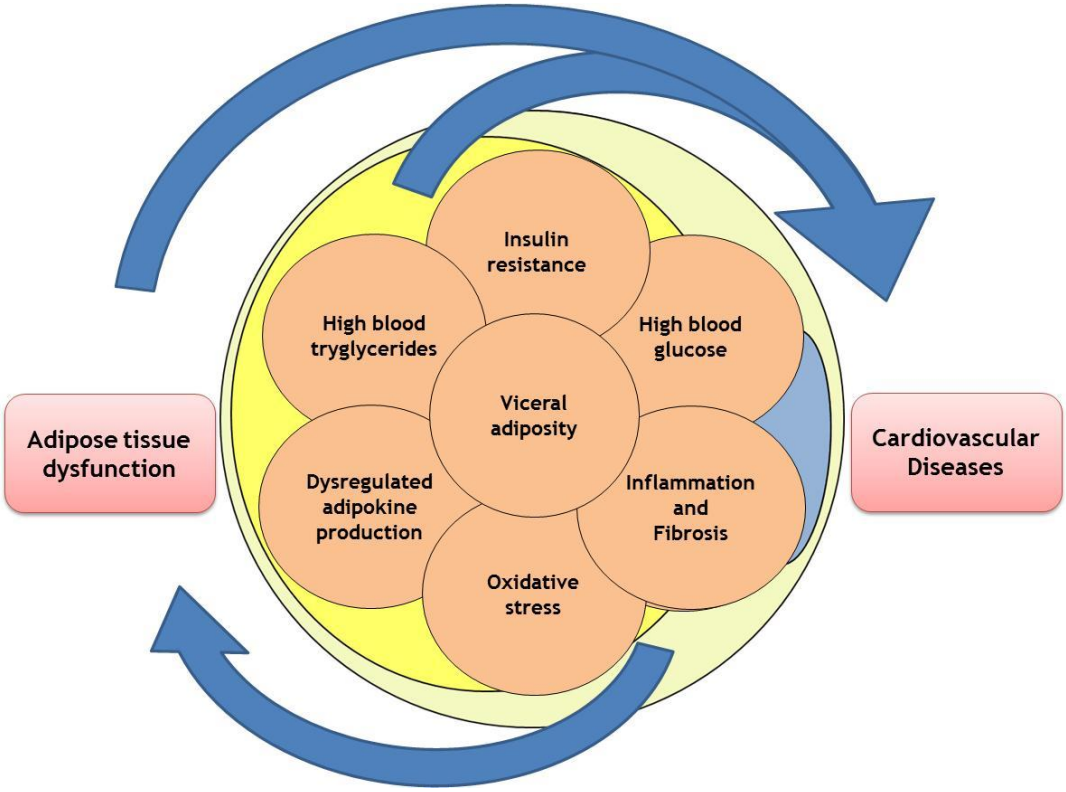


Figure 1.1. Abdominal obesity is associated with cardiovascular diseases. Abdominal obesity is associated with multiple risk factors such as insulin resistance, oxidative stress and increased levels of different (adipo)cytokines and inflammatory markers, all of which ultimately lead to cardiovascular disorders including hypertension, endothelial dysfunction, and atherosclerosis.

In addition of its classical role as a major storage organ for triglycerides, adipose tissue is a highly dynamic endocrine organ and an important metabolic sensor, producing numerous bioactive substances (hormones, cytokines, reactive oxygen species (ROS)), collectively termed adipokines (Kershaw & Flier, 2004; Hauner, 2005; Schaffler *et al*, 2006; Tilg *et al*, 2006; Thalmann *et al*, 2007), that control systemic insulin sensitivity, immune responses and vascular homeostasis (Eringa EC *et al*, 2007). These factors can exert autocrine (on adipocyte function, such as adipogenesis) and paracrine actions (on other organs, including heart, kidney, blood vessels) (Bluher, 2012; Miao *et al*, 2012; Henry *et al*, 2012) (Figure 1.2).

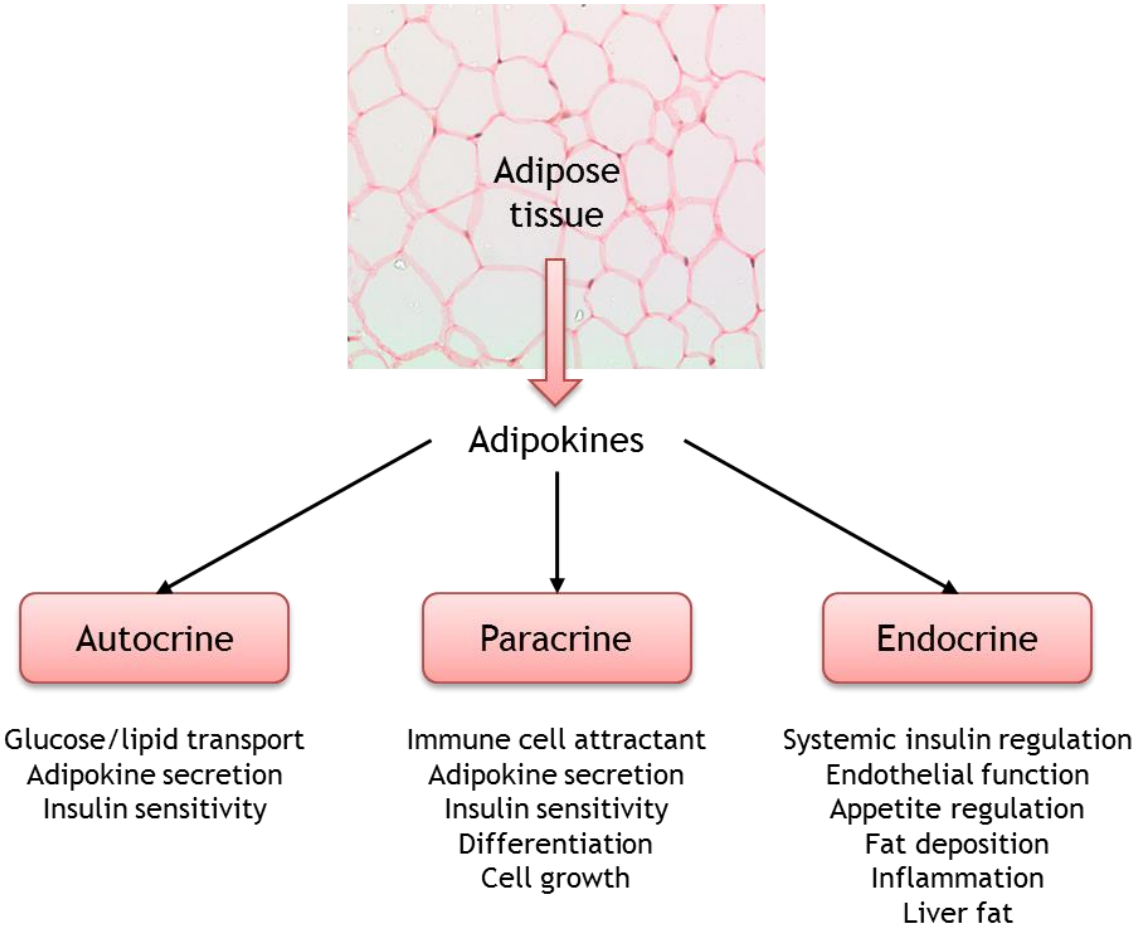


Figure 1.2. Local and systemic effects of adipokines (Adapted from Bluher, 2012). Adipokines are cytokines or bioactive mediators or hormones secreted by adipose tissue. Some—e.g., IL-6 or TNF-α are pro-inflammatory, whilst others—e.g., adiponectin—have anti-inflammatory or insulin-sensitising properties. Adipokines dysregulation appears to play a central role in adiposity, metabolic syndrome, and the co-morbidities of obesity including cardiovascular diseases.

However, the underlying mechanisms that control production of adipokines are still unclear. In obesity, adipocytes undergo hypertrophy and hyperplasia, leading to dysregulation of adipokine expression and secretion, which may promote obesity-linked metabolic disorders such as insulin resistance and alteration of glucose metabolism and cardiovascular diseases such as hypertension. These processes are associated with activation of the renin-angiotensin-aldosterone system (RAAS) as well as oxidative stress. It has been reported that adipocytes possess a local RAAS, thus, producing angiotensinogen (AGT) and angiotensin II (Ang II), which may influence adipocyte function and obesity-related disorders (Engeli *et al*, 2000; Cassis *et al*, 2008). In our laboratory, we recently demonstrated that adipocytes are able to produce

aldosterone (Briones *et al*, 2012) and this adipocyte-derived aldosterone is enhanced in pathological conditions such as in obesity associated with type 2 diabetes. Interestingly, circulating levels of aldosterone are positively correlated with body mass index (Rossi *et al*, 2008), suggesting that adipose tissue may play a role in aldosterone production. In addition, obesity is associated with oxidative stress (Furukawa, 2004). However, the interaction between adipocyte-derived aldosterone and ROS remains unclear.

1.2 Adipose tissue biology and function

Adipocytes are often considered as the 'bad' non-malignant cell type in the body due to their role in obesity. However fat is also important for physiological processes, especially related to normal development and energy metabolism. Moitra *et al* (1998) demonstrated in A-ZIP/F mice, a mouse line engineered to be fat free, that adipose tissue has beneficial effects on growth, reproduction, glucose metabolism, tolerating fasting as well as preventing lipotoxicity. Similar effects have been reported in the human Berardinelli-Seip congenital lipodystrophy syndrome, a rare condition characterised by the lack of adipose tissue (Berardinelli, 1954). This disease is associated with severe insulin resistance, hyperglycaemia, diabetes mellitus, and dyslipidaemia thus underlying that a properly functioning AT is necessary to human health.

1.2.1 Different types of fat

Adipose tissue is composed of 50% mature adipocytes where the remaining 50% consists of pre-adipocytes, immune cells, fibroblasts, endothelial cells, mesenchymal cells, nerve fibres and connective tissues. Adipose tissue (AT) depots are localised in various compartments. Subcutaneous fat, localised under the skin, plays a "cushioning" as well as an insulating role. The different visceral depots such as the perirenal, gonadal, epicardial, retroperitoneal, omental and mesenteric adipose tissues are of major physiological significance. (Roca-Rivada *et al* 2011; Tran *et al*, 2008). Many studies have reported that increased visceral fat mass is associated with metabolic dysfunction, cardiovascular disease risk and inflammation, while increased subcutaneous fat may be protective (Hamdy *et al*, 2006) (Figure 1.3).

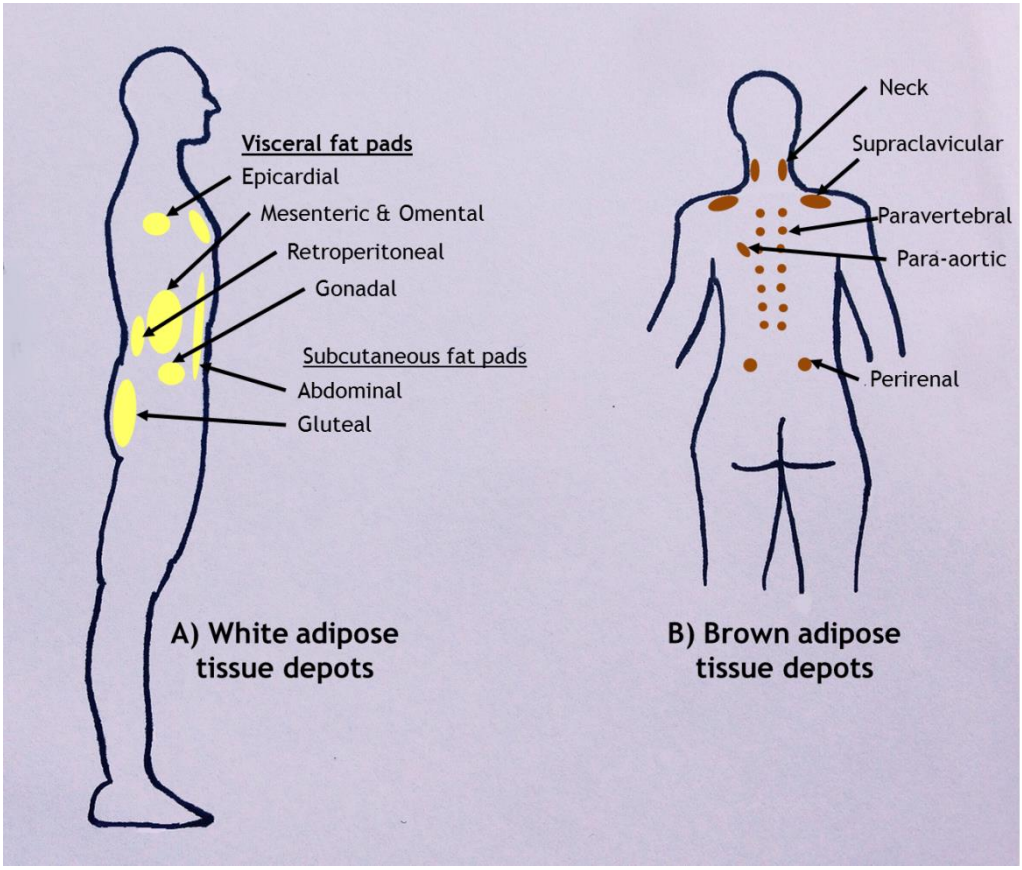


Figure 1.3. Localisation of different fat pads (Adapted from (BAT) Nedergard *et al*, 2007; (WAT) Wronska & Kmeic, 2012). Localisation of different brown (A) and white (B) fat pads in human body.

In mammals, three types of adipocytes are present (white, brown and beige), with different morphologies, precursors and functions.

1) **White adipocytes,** specialized in triglycerides storage, are characterised by a large unilocular lipid droplet which makes up 90% of the cell volume and is surrounded by a thin cytoplasmic rim. The little spare space only allows for a flattened nucleus located on the periphery and few under-developed mitochondria containing short and randomly organised cristae. White adipose tissue (WAT), the most prominent type of AT, is found in subcutaneous, epididymal, perirenal and perivascular adipose tissue depots (Wronska *et al*, 2012).

2) **Brown adipocytes** unlike white adipocytes dissipate stored energy by thermogenesis and are small multilocular cells. They contain several small lipid droplets, a centrally located roundish nucleus and numerous mitochondria with well organised laminar cristae. Brown adipose depots are classically found around the neck, around the aorta, behind the clavicles and surrounding the

vertebrae. The brownish colour of brown adipose tissue (BAT) is due to the high number of mitochondria and vascularisation of the depot required for non-shivering thermogenesis (Cinti *et al*, 2012; Nedergaard *et al*, 2007). Energy expenditure and generation of body heat are facilitated by uncoupling protein 1 (UCP-1), a mitochondrial inner membrane protein. UCP-1 permits thermogenesis via mitochondrial uncoupling and oxidative phosphorylation of free fatty acids. Fuel oxidation is carried out through electron transport leading to an electron gradient that is independent of ATP synthase (Cannon & Nedergaard, 2012/2004). Brown adipocytes demonstrate a protective role in WAT of mice over-expressing UCP-1 with a decrease in adiposity. UCP-1 knockout mice on the other hand are more sensitive to cold and prone to obesity (Enerback, 2010).

3) Beige adipocytes, also known as brown in white or "Brite", demonstrate characteristics found in both white and brown adipocytes. In WAT depots, beige adipocytes produce heat due to their expression of UCP-1. Beige adipocytes resemble white adipocytes when inactive, but upon activation, levels of UCP-1 are increased to levels similar to that of brown adipocytes. Increased BAT mass and activity are associated with improved glucose homeostasis and decreased adiposity, indicating some protective role of beige adipocytes in WAT (Stanford *et al*, 2013; Guerra *et al*, 1998). Browning of AT depots has become an attractive therapeutic target to reduce obesity and its associated CVD (Tran *et al*, 2010; Stanford *et al*, 2013; Qiang *et al*, 2012).

1.2.2 Adipose Tissue as an endocrine organ

The view of adipose tissue as an inert connective tissue and storage organ for lipids started to shift at the establishment of its central role in energy and glucose homeostasis. In 1994, the Friedman laboratory discovered leptin, as the first adipokine, by cloning the *Ob* gene from genetically obese mice and its human homologue, thus clearly establishing the endocrine function of AT (Zhang *et al*, 1994). Since then, AT has been shown to synthesize a vast array of bioactive factors influencing appetite, energy balance/metabolism, insulin sensitivity, inflammation and vascular function (blood pressure, vascular tone).

Adipose tissue produces and releases many pro-inflammatory and anti-inflammatory factors, including the adipokines leptin, adiponectin, resistin, and

visfatin, as well as cytokines and chemokines, such as TNF-α, IL-6, MCP-1, and others (Figure 1.4).

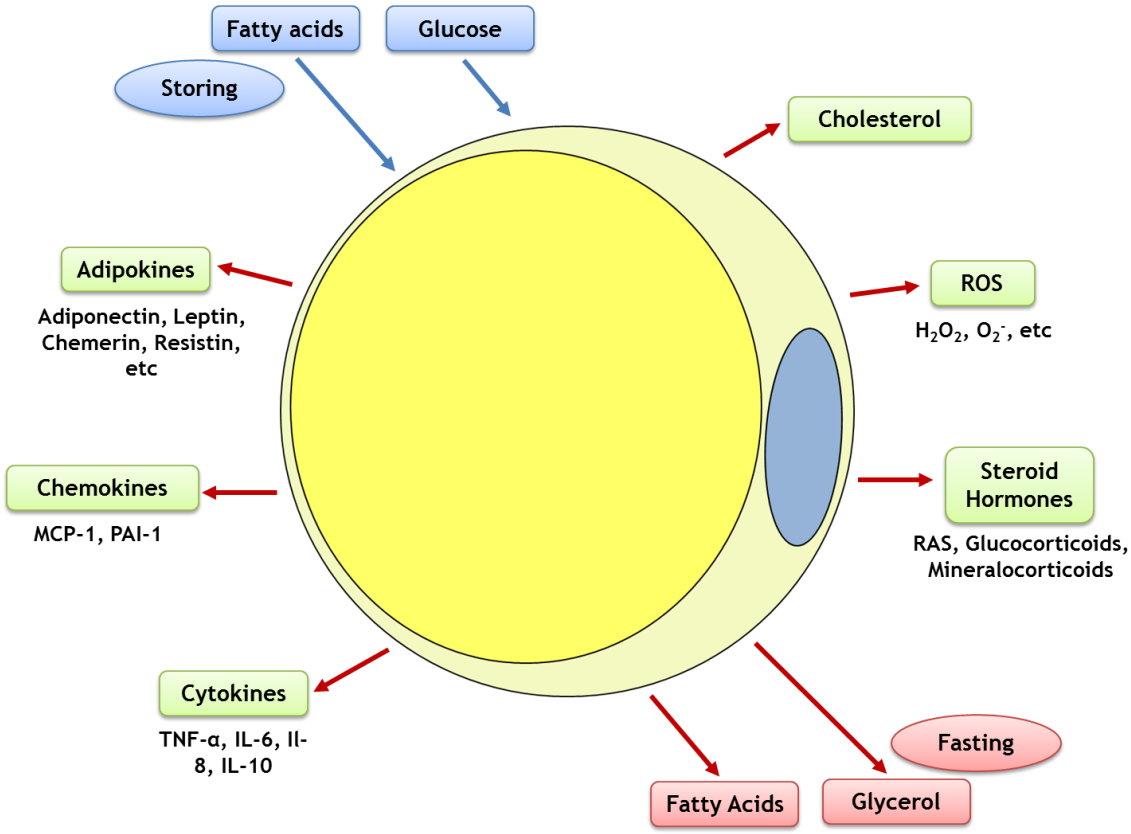


Figure 1.4. Adipocyte as fat storage and endocrine organ. H_2O_2: hydrogen peroxide; IL: interleukin; MCP-1: Monocyte chemoattractant protein 1; O_2^-: superoxide anion; PAI-1: Plasminogen activator inhibitor 1; RAS: renin angiotensin system; ROS: reactive oxygen species; TNF-α: Tumor necrosis factor alpha

Adiponectin is a highly expressed adipokine promoting insulin sensitivity, serum clearance of free fatty acids (FFAs) and glucose. Decreased levels of adiponectin are associated with dysfunctional AT as found in obesity. Leptin also known as the 'satiety hormone' controls energy intake and expenditure (Fenseca-Alaniz *et al*, 2007; Somoza *et al* 2007). By promoting lipolysis and inhibiting further lipid storage it preserves insulin sensitivity. Leptin also known as the satiety hormone binds to its receptor in the hypothalamus and supresses appetite thus permitting the homeostatic control of adipose tissue mass. In obesity, leptin has been shown to have pro-inflammatory properties by promoting phagocytic activity and the release of pro-inflammatory cytokines such as IL-6 and TNF-α by macrophages and other cell types such as adipocytes (Kristiansen *et al*, 2005; Kamimura *et al*, 2003; Harle P & Staub R H, 2006). On the other hand,

adiponectin demonstrates anti-inflammatory properties by promoting insulin sensitivity and inhibiting the expression of chemoattractants such as MCP-1 and TNF-α (Nigro *et al*, 2014). Kamei *et al* and Kanda *et al* both demonstrated that elevated levels of MCP-1 in AT are associated with increased macrophage infiltration and insulin resistance thus promoting adipose tissue dysfunction (Kamei *et al,* 2006; Kanda *et al,* 2006). Neutralization of TNF-α in obese *fa/fa* rats promoted insulin sensitivity and peripheral glucose uptake (Hotamisliglil *et al*, 1993). TNF-α activates inflammatory signalling cascades inhibiting insulin signalling leading to insulin resistance in obesity (Bernstein *et al*, 2006; Lo *et al*, 2007) while plasma IL-6 levels are positively correlated to human obesity and insulin resistance (Bastard *et al,* 2000).

1.2.3 The Renin-Angiotensin-Aldosterone System (RAAS) in Adipocytes

1.2.3.1 The classical RAAS

The RAAS begins with renin release by renal juxtaglomerular cells. Its secretion is triggered by a decrease in blood pressure or plasma salt concentration through tubuloglomerular feedback and by an increase in sympathetic activity. Renin is a key regulator of RAAS activity as it is the initial rate limiting step of converting angiotensinogen (AGT) into angiotensin I (AngI). The inactive angiotensin I is then hydrolysed to angiotensin II (AngII) by angiotensin converting enzyme (ACE) which is predominantly found in the lung capillaries (Figure 1.5). Ang is a potent vasoconstrictor regulating cardiovascular functions via AT1 (vasoconstriction) and the AT2 (vasodilation) receptors (Klingbeil *et al*, 2003; Batenburg *et al*, 2005). Ang II can trigger the release of aldosterone from the adrenal glands leading to sodium reabsorption and water retention. Aldosterone is the classical end product of the RAAS cascade. It is synthesised in the zona glomerulosa of the adrenal glands in response to Ang II, adrenocorticotropin (ACTH) and potassium.

1.2.3.2 The RAAS in adipose tissue

In addition to the classical RAAS, there is a rich tissue RAAS, where Ang II and aldosterone are produced locally, as extensively reviewed by Marcus *et al*

(2013). Growing evidence indicates that adipose tissue also has a dynamic RAAS. The presence of a local RAAS system in AT was first identified by Campbell *et al* in 1987 by demonstrating AGT gene expression in brown AT and mesenteric fat. It is now known that the AT contains all the RAAS components, at least at the mRNA level. The exact function of the RAAS in AT is unclear, but active AT may contribute to local production of Ang II and aldosterone, which could influence adipocyte function in an autocrine manner or regulate surrounding tissue, such as vessels, in a paracrine manner. Briones *et al* recently demonstrated that adipocytes have all the enzymatic machinery to synthesize aldosterone, an effect exacerbated in obesity and diabetes (Briones *et al*, 2012). Moreover adipocytes possess functionally active mineralocorticoid receptors, through which aldosterone signals.

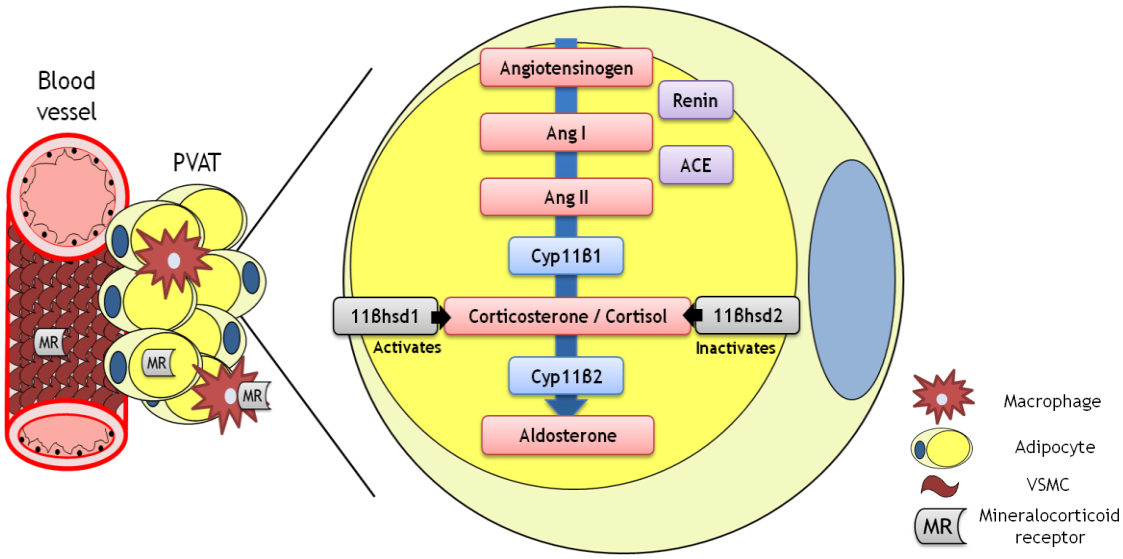


Figure 1.5. Adipose Renin-Angiotensin-Aldosterone System. Adipocytes possess all the components of the renin-angiotensin-aldosterone system (RAAS). Therefore adipocytes can produce angiotensinogen, angiotensin II and aldosterone, which can have autocrine and paracrine effects (on vascular function for example). Ang I: Angiotensin I; Ang II: Angiotensin II; ACE: angiotensin converting enzyme; Cyp11b1/2: Steroid 11ß-hydroxylase or cytochrome P450, family 11, subfamily B, polypeptide 1/2; 11Bhsd1/2: 11ß-hydroxysteroid dehydrogenase type 1/2; MR: mineralocorticoid receptor; PVAT: perivascular adipose tissue.

1.2.3.3 Aldosterone

Aldosterone exerts its effects via the mineralocorticoid receptor (MR). While the well-described “classical” effects of aldosterone on transepithelial Na transport

are mediated by MR present in epithelial cells, MR has been shown to be present in a many other cell types including adipocytes. Activation of MR appears to have important roles in adipose tissue including differentiation of pre-adipocytes into mature adipocytes and promotion of a pro-inflammatory state via induction of cytokines including TNF-α, monocyte chemotactic protein-1 (MCP-1), and IL-6 in WAT, while decreasing the thermogenic activity and lowering uncoupling protein 1 (UCP1) transcription in BAT (Zennaro *et al*, 2009). *MR* mRNA expression was increased in obese *db/db* mice (Hirata *et al*, 2009).

Aldosterone acts via genomic and non-genomic signalling pathways. Classic genomic pathways involve binding to the cytosolic mineralocorticoid receptors (MR) followed by the translocation to the nucleus, and transcription of effector protein involved in regulating of potassium and sodium balance in the kidney epithelial cells (Funder, 2006). Non-genomic pathway occurs within seconds and does not act on sodium-potassium homeostasis. It is involved in rapid activation of tyrosine kinases and subsequent downstream activation of ERK1/2, Rho Kinase, and PKC in association with increased cytosolic calcium and generation of ROS (Grossman & Gekle, 2009). MR is expressed in classical target tissues including kidney, colon and sweat glands. However, studies from the past two decades reported that MR is also expressed in other organs such as the heart, vessels, adipose tissue and macrophages, where the role of the hormone remains unclear, although it may have a pro-fibrotic and pro-inflammatory role (Nguyen Dinh Cat & Jaisser, 2012).

In 1987 Arriza *et al* demonstrated by cloning the human MR that it had the same affinity for both aldosterone and corticosterone/cortisol. These results led to the question as to how aldosterone acts via the MR when the corticosterone/cortisol levels are 100- to 1000-folds greater. This disparity is maintained in hyperaldosteronism. It was found that corticosterone/cortisol could be inactivated and activated by 11ß-hydroxysteroid dehydrogenase types 2 and 1 (11ß-HSD1 and HSD2) respectively (Funder *et al*, 1988; Edwards *et al*, 1988). The selective MR signalling via aldosterone in epithelial cells is due to the high levels and activity of 11ßHSD2. Importantly, aldosterone/MR signalling selectivity is carried out differently in non-epithelial tissues.

1.2.3.4 Effects of aldosterone on adipocyte function

In T37i cells, a brown adipocyte cell line, Zennaro *et al* (1998) first established that aldosterone promotes differentiation of pre-adipocytes towards mature adipocytes with a white adipocyte phenotype. Observed changes include the induction of lipid accumulation and elevated adipocyte differentiation markers: peroxisome proliferator-activated receptor-gamma (PPAR-γ) and adipocyte specific fatty acid binding protein 2 (aP2). Viengchareum *et al* (2001) showed the inhibitory capacity of aldosterone on the expression and function of UCP-1 the protein responsible for non-shivering thermogenesis. Armani *et al* (2014) also showed that aldosterone/MR signalling blocked browning of WAT. Thus, aldosterone promoted brown adipocyte differentiation while shifting towards a white adipocyte phenotype by promoting lipid storage and inhibition of energy expenditure. This could lead to the loss of the beneficial aspect of BAT in chronic MR over activation. Aldosterone/MR signalling promotes differentiation in 3T3-L1, a mouse white adipocyte cell line; however GR signalling by glucocorticoids can also elicit adipocyte differentiation. Hence, the role of MR and GR in adipocyte differentiation is contentious. Caprio *et al* (2007) showed that downregulation of MR and not of GR inhibits adipocyte differentiation while Lee and Fried (2014) argue that GR alone participates in the differentiation process.

1.2.4 Adipose Tissue and reactive oxygen species (ROS)

ROS are by-products of the reduction of oxygen to water. Originally these molecules were thought to be deleterious however they now have been accepted as having beneficial effects under physiological levels. To maintain physiological levels they are controlled by a tight regulatory system also known as the oxidant defence system. ROS are produced by many sources including macrophages, mitochondria, xanthine oxidase, uncoupled nitric oxide synthase and nicotinamide adenine dinucleotide phosphate (NADPH) oxidases (Nox). Interestingly, Noxes are the only source of ROS where they are not formed as a by-product. The physiological role of ROS involves both regulation of transcription factors and induction of host defence mechanisms (Bedard *et al*, 2007) ROS also act as secondary messengers influencing MAP kinases, protein tyrosine kinases, protein phosphatases, and ion channels that regulate cell

proliferation, apoptosis, migration and fibrosis, inflammation, vascular tone and angiogenesis. Adipocytes have been shown to produce ROS, in response to Ang II and aldosterone (Kurata *et al*, 2006). Adipocyte-derived ROS may play a role in promoting inflammation in the context of obesity, commonly associated with oxidative stress (increased ROS bioavailability).

1.2.4.1 Nox isoforms in adipocytes: The Structure of Nox1, 2 and 4

One of the major enzymatic sources of ROS are the NADPH oxidases (Nox) Seven isoforms have been identified: Nox1-5 and Duox1 and Duox2. Here we will focus on the Noxs that have been identified in AT, namely Nox1, 2 and 4 (in mice) and Nox 1, 2, 4 and 5 in humans (Figure 1.6).

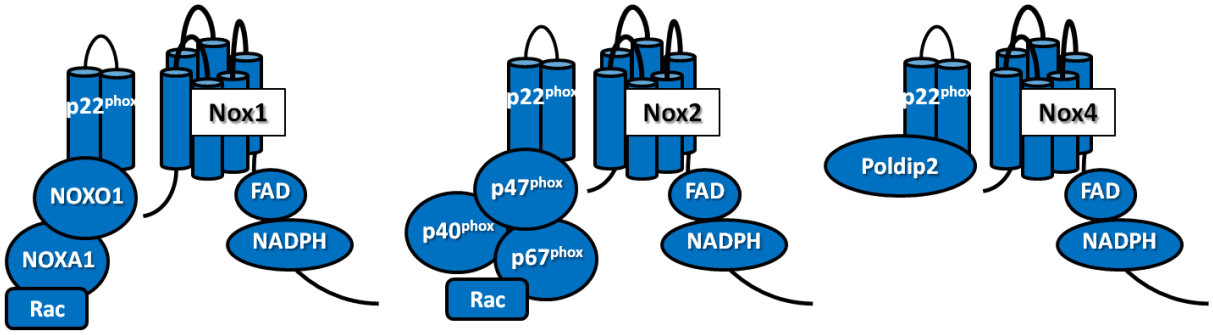


Figure1.6. Structure of NADPH oxidases localized in mouse AT.

The NADPH oxidase (Nox) family shares a core structure consisting of six transmembrane protein domains, four heme histones, a long COOH terminus, FAD binding domain and an NADPH binding site. Each Nox seems to use specific combination subunits required for the activation of the core transmembrane complex. Nox1, 2 and 4 are membrane complexes which require $p22^{phox}$ subunit for their function. The role of $p22^{phox}$ is varied as it can serve to stabilise the Nox subunit as well as being the docking site of regulatory subunits (Ambasta *et al*, 2004). The production of ROS by Nox1 and Nox2 requires the presence of regulatory subunits and of a small RAC GTPase. The regulatory subunits of Nox2 are $p47^{phox}$, $p67^{phox}$ and $p60^{phox}$ that form a complex with small RAC GTPase and the $nox2/p22^{phox}$ heterodimer. Once the complex is formed it is active and results in the translocation of the complex to the plasma membrane. The regulatory subunits of Nox1 are composed of NoxO1 and NoxA1 which are homologues of $p47^{phox}$ and $p67^{phox}$ respectively with a small RAC GTPase. Nox2

also known as the phagocytic Nox for its high expression and vital role in phagocytic cells permits an immune defence response. When activated, phagocytic Nox2 permits the production of large amounts of ROS which are used to kill microbes. Nox2 is also expressed in adipocytes just as is Nox1 but their role in the AT has not yet been established. Unlike Nox1 and 2, Nox4 does not need any regulatory subunit only $p22^{phox}$ is required for ROS production. Poldip2 can bind to the Nox4/$p22^{phox}$ heterodimer to enhance Nox4 activity thus increasing the ROS generation (Lyle *et al*, 2009).

Noxes produce superoxide ions (O_2^-) which are highly reactive and short lived free radicals that are converted to the more stable and membrane permeable hydrogen peroxide (H_2O_2) by superoxide dismutase (SOD). Interestingly, Nox4 is constitutively active and thought to generate the basal ROS levels. Also, it generates H_2O_2 rather than O_2^- but the underlying reasons are unknown. Current theories include that the terminal heme binding site inside the transmembrane allows direct production of H_2O_2 or possibly the SOD is located close to the exit of the six transmembrane protein domains to convert O_2^- to H_2O_2.

1.2.4.2 Redox signalling and physiological role of Noxes in adipocytes

Some studies have underlined the role of ROS in adipocyte differentiation and redox-sensitive mechanisms by modulating adipocyte signalling with physiological ROS levels. Adipocyte differentiation is the transition of pre-adipocyte to mature adipocytes which can store the excess lipid and prevent the present adipocytes from becoming hypertrophic. Adipocytes increase in size as they store lipids however there is a limit as to how much they can store. Several studies propose ROS as a promoter for adipocyte differentiation. 3T3-L1 cells show an increase in ROS production during differentiation but differentiation was inhibited by antioxidants (Furukawa *et al*, 2004). Nox4 is a potential regulator of differentiation as it is a constitutively active enzyme producing H_2O_2 along with its high mRNA gene expression in pre-adipocytes (Mouche *et al*, 2007). Inhibition of Nox4 with siRNA blocked insulin signalling for the terminal differentiation of adipocytes (Schröder *et al*, 2009). These *in vitro* studies suggest that Nox4 plays a role in adipocyte differentiation however this still awaits confirmation.

1.2.5 AT regulation of vascular reactivity

In 1991, Cassis and Soltis demonstrated that "perivascular adipose tissue (PVAT) significantly influences vascular responsiveness'' underlying the role of the adipose tissue on vascular regulation. The vascular contractility of blood vessels pre-incubated in PVAT conditioned media demonstrated the existence of adipocyte derived relaxation factor (ADRF) (Soltis *et al,* 1991). PVAT was shown to promote the release NO and eNOS derived NO thus enhancing vasodilatation an effect lost in obesity. Endothelial nitric oxide synthase (eNOS)-derived nitric oxide (NO) stimulates endothelium-dependent relaxation of the smooth muscle cells by activation of soluble guanylyl cyclase and hyperpolarization (Ortega *et al*, 2010/2009). In addition to ADRF, other PVAT-derived factors may modulate vascular tone including, pro- and anti-inflammatory adipokines, aldosterone and ROS. In obesity, adipocyte- and endothelium- derived relaxing factors are impaired while the adipocyte-derived contractile factors, from the perivascular adipose tissue (PVAT), are enhanced thus promoting endothelial dysfunction (Maenhaut *et al*, 2011; Boyden *et al*, 2012).

1.3 Adipose tissue in Obesity

In obesity, the endocrine function of adipocytes is modified with increased production of leptin, resistin, FFA and pro-inflammatory cytokines while adiponectin production is reduced. Obesity is associated with chronic low grade inflammation of AT where both adipocytes and macrophages contribute to local inflammation and oxidative stress. The increased release of MCP-1 in obesity by adipose tissue leads to a significant increase in inflammatory monocyte infiltration. These monocytes undergo polarization to M1 macrophages, classically activated (pro-inflammatory) instead of M2a macrophages, alternatively activated (anti-inflammatory), which further increases AT inflammation and dysfunction (Lumeng *et al*, 2007, 2008). WAT and BAT are highly vascularized tissues whose physiological function, expansion and adipocyte differentiation require angiogenesis. Effective vascular remodeling and maintained blood flow are crucial to the adipose tissue as it permits the supply of oxygen, nutrients, immune cells, bone-marrow derived stem cells, growth factors and hormones needed for normal functioning. However in

obesity, the AT becomes hypertrophied and hypoxic which further promotes adipocyte dysfunction (Wood *et al*, 2009; Sun *et al* 2011).

Obese individuals have excess collagen I, III and VI deposition in adipose tissue contributing to adipose tissue fibrosis (Henegar *et al* 2008; Divoux *et al* 2010). Fibrosis is defined as an excess of collagen depots promoting pathological conditions and is a hallmark of chronic tissue damage.Nox4 was found to play a role in hepatic and lung fibrosis and this may also occur in the AT (Aoyama *et al*, 2012; Jarman *et al*, 2014). Adipose tissue fibrosis may be a factor linking obesity to cardiovascular diseases along with tissue inflammation.

Due to its role as a major energy storage organ the AT possesses an incredible plasticity and thus undergoes constant remodeling. Along with fibrosis and collagen deposition, the extracellular matrix (ECM) undergoes remodelling. Adipose tissue function is regulated by the interaction between collagen, cells and the ECM (Mariman & Wang, 2010). The ECM is a fibrillar network comprising collagen, fibronectin and proteoglycans. Matrix metalloproteinases (MMP) are enzymes specific for the degradation of ECM components inhibited by tissue inhibitors of metalloproteinases (TIMP). ECM pathological remodelling is due to an imbalance between production and degradation as found in AT fibrosis (Mariman & Wang, 2010).

1.3.1 Role of oxidative stress

Elevated ROS levels and decreased antioxidants induce oxidative stress, which leads to tissue injury and inflammation. Oxidative stress also correlates with fat accumulation in humans and animal models. Oxidative stress, due in part to increased activation of Noxs, in obesity can lead to irreversible oxidative modifications that induce adipocyte dysfunction. Nox4 activation promotes a pro-inflammatory phenotype by the increase of monocyte chemotactic factor genes expression in 3T3-L1 cells (Han *et al*, 2012). This is similar to the effects found in aldosterone/MR signalling, which may occur via ROS production. The levels of Nox2 are increased in AT in obesity due to the elevated macrophage infiltration and participates in promoting ROS as well as cytokine synthesis. Nox1 expression is also increased in obesity. Nox1 is sometimes referred to as the

pathological Nox, due to its increased expression being associated with a variety of diseases including diabetes and hypertension (Matsuno *et al*, 2005; Gray *et al*, 2013)

1.3.2 Role of MR signalling

Emerging evidence supports a shift in our understanding of the crucial role of the elevated circulating levels of aldosterone in promoting insulin resistance and resistant hypertension. Aldosterone enhances tissue generation of ROS and systemic inflammation. This increase contributes to impaired insulin signalling, reduced endothelial-mediated vasorelaxation, and associated cardiovascular and renal structural and functional abnormalities (Whaley-Connell *et al*, 2010). As mentioned previously, AT releases a soluble active compound that promotes the release of aldosterone form the adrenal gland. The release of this unknown compound is increased in obesity thus increasing the release of aldosterone. Adipose tissue itself also shows an increase in aldosterone synthesis in obesity. Interestingly MR mRNA expression was increased in obese *db*/*db* mice leading to over-activation of MR in obesity (Guo *et al*, 2008; Hirata *et al*, 2009). MR activation promotes proliferation, inflammation and fibrosis in many organs such as the heart, kidneys, and the vasculature via ROS production (Park *et al*, 2008; Callera *et al*, 2005; Hirata *et al*, 2009). Chronic low grade inflammation associated with overproduction of ROS in fat tissue contributes to the development of metabolic syndrome. Aldosterone may increase ROS production in AT by increasing the expression of Nox subunits as shown in studies on other tissues (Park *et al*, 2008).

It is well established that aldosterone promotes inflammation in a variety of tissues and that chronic low grade inflammation is key in obesity alongside imbalanced production of bioactive factors by adipocytes. A study on obese mice showed increased expression of inflammatory adipokines TNF-α and MCP-1 and reduced expression of adiponectin and PPARy compared to the lean control were reversed by MR inhibition (Guo *et al*, 2008). Another study on two genetic models of obesity, *ob*/*ob* and *db*/*db*, by Hirata *et al* (2009) demonstrated that MR inhibition by eplerenone improved insulin sensitivity and reduced ROS production, restoring the balance in adipokine synthesis. In 3T3-L1 cells,

stimulation with aldosterone and H_2O_2 increased pro-inflammatory adipo(cyto)kines and TBARS which was blocked by eplerenone. Interestingly, H_2O_2 in 3T3-L1 cells increased ROS production while decreasing antioxidants catalase, Cu and Zn-SOD gene expression (Hirata *et al*, 2009). This indicates that ROS can modulate Nox and antioxidant expression promoting oxidative stress in disease state. This study also indicates that MR activation promotes ROS production.

In obesity macrophages in a polarized state play a key role in inflammation and adipocyte dysfunction. Interestingly macrophages also express MR on their surface. Macrophages are classically activated (M1) when exposed to aldosterone and alternatively activated (M2) with the MR inhibitor eplerenone but a GR antagonist had no effect (Usher *et al*, 2010). Consequently, MR activation has the capacity to increase AT inflammation as well as oxidative stress (Marzolla *et al*, 2014). A paradoxical study by Kuhn *et al* (2014) showed protective effects against obesity in transgenic mice that overexpress MR specifically in macrophages upon high fat diet (decrease in fat mass, smaller adipocytes, improved glucose tolerance). Furthermore, co-cultured macrophages and adipocytes from these transgenic mice showed a decrease in adipocyte differentiation. These results suggest that MR is a pro-adipogenic and pro-inflammatory effector mediating effects on adipocyte development and functions.

1.4 Rationale for the studies.

Despite the growing evidence that oxidative stress, hyperaldosteronism and inflammation are associated with obesity and metabolic and cardiovascular disease, molecular mechanisms linking these processes still remain unclear. In the present studies, we examined the relationship between ROS, aldosterone and adipocytes in the context of obesity. In particular we questioned whether ROS influence aldosterone production by adipocytes and whether these processes influence adipocyte function related to cell maturation, inflammation and fibrosis. We also explored the enzymatic source of ROS in adipocytes, focusing specifically on Nox isoforms, Nox1 and Nox4.

1.5 Aims and hypothesis of the study

Overall hypothesis

In obesity associated with metabolic syndrome (obesity-MS), adipocytes generate aldosterone through Nox1/4-dependent processes, contributing to a pro-inflammatory and pro-fibrotic adipose phenotype (Figure 1.7).

Specific aims.

To address our hypothesis, 4 specific aims were proposed:

1. To evaluate whether obesity/metabolic syndrome is associated with hyperaldosteronism and increased adipocyte-derived aldosterone through processes that involve Nox1/4;
2. To determine whether increased Nox1/4-derived ROS influences aldosterone biosynthesis by stimulating aldosterone synthase in adipocytes;
3. To assess whether aldosterone and ROS promote adipokine production and adipogenesis and if this is increased in obesity;
4. To elucidate whether redox-sensitive processes and aldosterone promote a pro-inflammatory and pro-fibrotic adipose phenotype.

Experimental approach

To address these aims, we performed studies in adipose tissue and mature adipocytes from obese mice that also have features of metabolic syndrome (*db*/*db* mice). To translate our findings to humans, we also studied differentiated human adipocytes. A dual Nox1/4 inhibitor, GKT137831, and other Nox/ROS modulators were used to elucidate the role of Nox isoforms.

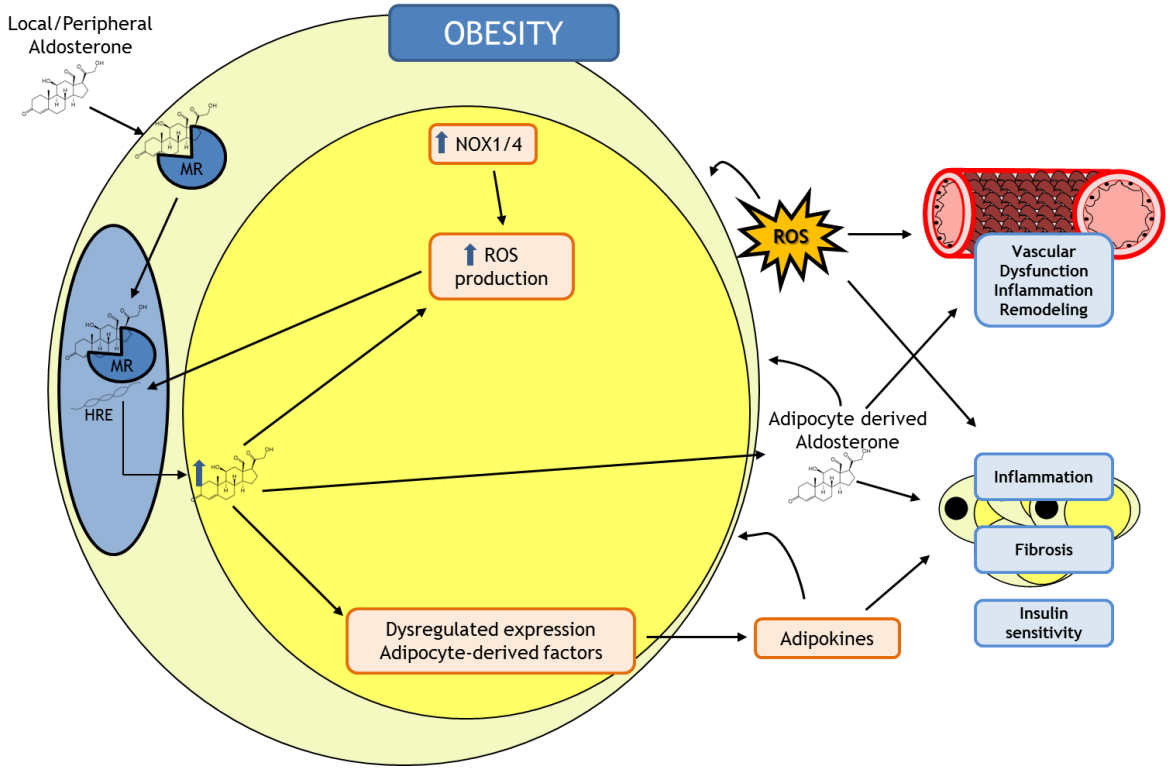


Figure 1.7. Hypothetical representation of the link between obesity, oxidative stress and aldosterone. Nox1/4-derived ROS play a central role in obesity by regulating aldosterone production by adipocytes and are an early instigator of metabolic syndrome. Redox state in adipose tissue is a potentially useful therapeutic target for obesity-associated metabolic syndrome. HRE: Hormone responsive element; MR: Mineralocorticoid receptor; NOX: NADPH oxidase; ROS: reactive oxygen species

2 Materials and Methods

We tested our hypothesis using an *in vivo* and *in vitro* approach.

2.1 *In Vivo*

2.1.1 Mice

In vivo studies were performed in obese *db*/*db* mice treated with GKT137831 a Nox1/4 inhibitor provided by Genkyotex S.A, Geneva, Switzerland (Page *et al,2008*). The animal studies were conducted by Drs Nguyen Dinh Cat and Montezano in the Touyz lab in Ottawa (Canada), before moving to Glasgow. The study was approved by the Animal Ethics Committee of the University of Ottawa. Tissue from the animal studies were processed and studied in Glasgow. 8 weeks old male B6.BKS(D)-*Lepr*^{db}/J (Stock Number 000697) (or obese *db*/*db* mice) and control were C57BL/6J heterozygotes from the colony (or lean *db*/+ control) mice were purchased from Jackson Laboratories (Maine, USA) and housed individually at ambient temperature under 12h light/dark cycles. Animals had free access to drinking water and A04 normal diet. Obese and lean animals were separated into three groups: none treated, low dose treatment with Nox1/4 inhibitor and high dose treatment with Nox1/4 inhibitor each comprising ten mice.

2.1.2 Treatment

The *db*/+ (lean) and *db*/*db* (obese) mice, at 10 weeks, were fed with a low (20 mg/kg/day) or high dose (60 mg/kg/day) of dual Nox1/Nox4 inhibitor (GKT137831), for 16 weeks. The animals were sacrificed by decapitation. After treatment, adiposity was determined and plasma samples obtained and stored at -80^{o}C for determination of aldosterone and corticosterone concentrations Tissues were harvested for further analysis.

2.2 Biochemical assays

Blood samples were collected at the end of the study by cardiac puncture, under anaesthesia. Glucose, total triglycerides, cholesterol, LDL and HDL were

determined in plasma samples using an automated analyser (Synchron CX5 PRO; Beckman and Fullerton).

2.3 Histology

Epididymal visceral adipose tissue (EVAT) from *db*/+ and *db*/*db* mice was removed and drop-fixed in 10% formalin solution, left for 48 hours at room temperature. For embedding, the tissues were dehydrated through a series of graded ethanol baths to displace water. They were then infiltrated with wax and finally embedded in wax blocks. EVAT tissue was sectioned to 5µm slices using a microtome. Fibrosis was assessed using Picrosirius red staining. Sections were stained momentarily with Weigert's haemotoxylin to stain the nucleus (Sigma-Aldrich), washed then stained in 0.1% picrosirius red solution under dark conditions. Fibrosis/collagen content was determined from five to eight fields of view on each section using ImageJ. Fibrosis was further assessed by use of polarised microscopy on the Picrosirius red stained slides in order to differentiate between older and recently made collagen. The results were analysed from five to eight fields of view on each section using ImageJ and Cell-D.

2.4 Cell Culture

2.4.1 Cultured mouse mature adipocytes

Mouse abdominal EVAT was obtained from male obese *db*/*db* and their littermates lean control (*db*/+) mice. Adipose tissue was cleaned of blood vessels and fibrous tissue and thoroughly minced with scissors in DMEM/F12 medium. The tissue was digested with collagenase type I (Worthington Biochemical Corporation, Lakewood, NJ) (0.5 mg/mL/g tissue) in Krebs-Ringer HEPES buffer pH7.4 (in mmol/L: 125 NaCl, 4.8 KCl, 0.5 NaH2PO4, 1.2 MgSO4, 2.6 CaCl2, 25 HEPES, 2 NaHCO3, 5.5 Glucose) containing 1% BSA for 20-30 min in a rotating shaker at 37^{o}C. After digestion, the same volume of DMEM/F12 was added to the sample mixture which was filtered through a 200µm mesh and centrifuged at 50 g for 5 min. The pellet was discarded. The floating mature adipocyte fraction was transferred into a new tube containing DMEM/F12 and centrifuged (50 g, 5 min). The floating mature adipocytes fraction was washed in DMEM/F12 twice.

After the last centrifugation, the mature adipocytes were placed in 500μL of DMEM containing 15 mmol/L HEPES pH7.4, 17 nM insulin and 1% FBS and incubated in 5 % CO2, at 37°C for 24 hr. After 24 hrs, culture media were collected and stored at -80°C until aldosterone and corticosterone concentrations determinations. Cells were washed with 1X PBS and collected with 250 mL of Trizol for RNA extraction.

2.4.2 Cell lines

SW872 cells (pre-adipocytes from a human liposarcoma cell line) were purchased from the American Type Culture Collection (ATCC, Manassas, VA). SW872 cells were cultured on DMEM low glucose supplemented with 10% fetal bovine serum (FBS) and 1% Pen/Strep in 5% CO_2, at 37° C.

2.4.3 Cell Culture and Passage

Cell culture was performed under sterile conditions. Cells were cultured in normal growth serum at 37°C in 5% CO_2 and 95% air. Cells were cultured until 70% confluence for differentiation protocol and 90% confluence for passage or cryopreservation. The medium was removed and the cells washed with 1% phosphate buffer saline (PBS) which was also discarded. The cells were detached by adding 0.025% trypsin (Gibco) to cell flasks and incubated at 37°C for 5mins. Cells were resuspended in normal growth medium, to inactivate the trypsin, and then centrifuged for 3min at 200 g. The supernatant was discarded and the cell pellet resuspended in normal growth medium and placed in appropriate containers.

2.4.4 Cryopreservation

Cells were stored using a freezing medium containing the normal growth medium supplemented with 5% of dimethyl sulphoxide (DMSO) to prevent crystal formation in the freezing process. Cells were stored at -80°C or liquid nitrogen. For resuspension, cells were rapidly thawed in a water bath at 37°C and added to a sterile tube with growth media and centrifuged for 3min at 200 g in order to remove DMSO. The supernatant was removed and the cells resuspended into a certain volume of normal growth medium and transferred to a T25 flask.

2.4.5 Differentiation protocol for SW872 preadipocytes

At 80% confluence, SW872 were subjected to a differentiation process by culturing them in DMEM supplemented with 10% FBS, 0.5 mmol/L 3-isobutyl-1-methylxanthine (IBMX, Sigma Chemical Co), 0.25 µmol/L dexamethasone, and 1 µmol/L insulin for 2 days. Medium was replaced by DMEM supplemented with 10% FBS, and 1 µmol/L insulin for an additional 2 days. Subsequently, the medium was replaced by DMEM containing only 10% FBS for 4 days. At the end of the differentiation period, the cells exhibited adipocyte morphology and differentiated human SW872 adipocytes were maintained in DMEM supplemented with 1% FBS and antibiotics overnight before stimulation.

2.4.6 Cell stimulation protocols

SW872 adipocytes were stimulated with 10 nmol/L Ang II for 24hrs in the presence and in absence of various ROS inhibitors: Nox1/4 inhibitor (1 µmol/L GKT137831), Nox1/Nox2 inhibitor (1 µmol/L ML171), and SOD mimetic (10 mmol/L Tempol). After stimulation, culture media were collected and kept at -80°C for aldosterone and cortisol determinations. Cells were washed with 1X PBS further processed for protein extraction.

2.5 General Molecular biology

2.5.1 RNA extraction

Total RNA was extracted from EVAT and perivascular mesenteric (PVAT) adipose tissues from obese and lean mice treated or not with Nox1/4 inhibitor and from SW872 cells with 750 µL and 250 µL Trizol, respectively (Invitrogen), following manufacturer's instructions. Mouse adipose tissues were homogenised using lysis tubes, containing micro-beads and a lysing homogenizer (Precellys 24, Bertin technologies, UK) programmed at for 2x20sec at 5000 rpm. To retrieve the RNA from homogenised samples, 200µL chloroform was added after homogenisation. After 2min at RT, samples were centrifuged at 12000g for 15min at 4°C. Aqueous phase was collected and transferred to new tubes to which 500µL isopropanol was added to precipitate nucleic acids. Samples were mixed, and after 15min at

RT were centrifuged for 10min at 1200g at 4°C. Supernatants were removed, and the pellets washed with 500 µL of 75% ethanol and centrifuged at 7500g for 5min at 4°C. Supernatants were removed and the pellets dried at 37°C. Samples were resuspended in 20-30 µL of RNAse free water depending on pellet size. RNA concentrations were measured using Nanodrop ND-1000 or spectrophotometer at 260nm absorbance. Averages of duplicate or triplicate readings were taken for each sample. Samples were stored at -80°C until next step.

2.5.2 RNA purification and reverse-transcriptase reaction

To ensure no DNA was present in samples, total RNA samples (10 µg) were treated with DNase I with DNA-free kit as described by the manufacturer (Ambion). DNase treated RNA (1 µg) was reverse-transcribed with a high capacity cDNA reverse transcription kit (Applied Biosystems) using 10X Reverse Transcription Buffer, 25XdNTP Mix, 10X Random Primers, RNase inhibitor, MultiScribe Reverse Transcriptase and nuclease free water. Reverse transcription was performed in a thermocycler (DNA Engine Tetrad Peltier thermocycler (MJ Research, PTC-225) using the following parameters: 10 mins at 25°C, 120 mins at 37°C, and 5 mins at 85°C. Samples were stored at -20°C.

2.5.3 Quantitative real time Polymerase Chain Reaction (PCR)

Real time PCR was carried out on a HT Fast Real-Time PCR machine with application of the 384-Well Block Module (Applied Bio systems, Life Technologies) using gene specific primers to quantify the relative abundance of each gene with FAST SYBR Green Master Mix (Applied Biosystems) as the fluorescent molecule. Primers used were designed using the software Primer 3. PCR was performed in duplicate for each sample using a PCR Master Mix containing nuclease free water, 300 nmol/L primers, and 3 µL template cDNA in 25 µL total volume. PCR conditions consisted of an activation step of the Hot Gold Start *Taq* DNA polymerase (95°C) for 10 min, followed by 40 cycles of 10 secs at 95°C (denaturation step) and 1 min at 60°C (primer annealing, extension, and fluorescence acquisition). Serial dilution of pooled cDNA was used in each experiment to assess PCR efficiency. F-box and leucine-rich repeat protein-10/Importin-8 (*Fblx10*/*Ipo8*), Ubiquitin-conjugating enzyme E2 (*Ubc*) or hypoxanthine guanine-phosphoribosyl-transferase (*Hprt*) housekeeping genes

were used as the reference gene for normalisation. The relative copies number of the target genes were calculated with the $2^{(-\Delta\Delta Ct)}$) method, after assessment that PCR efficiency was 100%.

2.6 Enzyme immunoassays: Aldosterone, corticosterone and cortisol measurements

Aldosterone (#10004377), corticosterone (#500651) and cortisol (#500360) concentrations were determined in plasma and the study was approved by the Animal Ethics Committee of the University of Ottawa. Mature adipocytes from *db*/*db* and *db*/+ mice as well as CCM of stimulated SW872 cells by enzyme immunoassay (EIA) (Cayman Chemical) according to the manufacturer´s instructions. CCM from adipocytes was concentrated in a speed vacuum system (Vacufuge Eppendorf). These immunoassays are based on a competition between the steroid hormone of interest (aldosterone will be used as an example here) and Ache (aldosterone-acetylcholinesterase conjugate, a tracer). Sample aldosterone was first extracted to prevent interference by other steroid hormones. The plate used was pre-coated with a goat polyclonal antibody to which was added AChE and either standard or sample. The sample and tracer compete to bind to the aldosterone monoclonal antibody which binds to the goat polyclonal antibody attached to the bottom of the wells. The plate was then washed and the Ellman's reagent was added producing a yellow colour read with a spectrophotometer (Dynex, Magellan Bioscience) at 412 nm absorbance. The intensity of the colour was proportional to the amount of bound AChE and therefore inversely proportional to the amount of sample aldosterone present in the wells. A standard curve was derived and non-conditioned medium as well as blank measurement of non-conditioned medium was subtracted from all samples

2.7 Protein concentration

SW872 adipocytes were lysed with appropriate buffer depending on technique used. Protein concentrations were determined using a detergent compatible (DC) Protein Assay kit according to the manufacturer's instructions (Bio Rad). Serial dilutions of albumin protein ranging from 0.125mg/ml to 6mg/ml were prepared (in lysis buffer) to make a concentration standard curve for each assay. DC mixture was prepared by mixing reagents A and B (provided) at ratio of 1:8

(25µl and 200µl respectively). For every ml of reagent A used 20µL of reagent S was added (used when detergent is present in lysis buffer). To a 96-well plate, in duplicate, were added 5µl of the albumin standards / protein samples / lysis buffer (blank) to which were added the DC reagent mix. Plates were subsequently incubated at 37°C for 15min. The DC assay is based on the Lowry assay where copper reduction by proteins and Folin reduction by the copper-protein complex producing a blue colour. The optical density (OD) of the colour is directly proportional to the quantity of protein in a sample. Absorbance at 750nm was determined using a Wallac Victor2 plate reader (Wallac, Turku, Finland). The average value of the two replicates was calculated. Concentration of protein in each sample was assessed from the linear equation of the standard curve and the degree of dilution.

2.8 ROS measurements

2.8.1 Superoxide ions levels measurements

Superoxide measurement was evaluated using lucigenin-dependent chemiluminescence (LgCL) and the experiments were performed in SW872 cells protein lysates. The SW872 cells were stimulated according to protocol and scraped on ice using 70µl of ROS lysis buffer (Drugs and solutions section). Basal O_2^- levels were measured with 50 µl sample with 100µl ROS Buffer in a white 96 well plate. 75µl of lucigenin mix (lucigenin 5µM) were added away from light. Basal O_2^- signal was measured using the AutoLumat LB 953 microplate luminometer (Berthold Technologies, Bad Wildbad, Germany) and the Simplicity 4.2 software. O_2^- production was measured by adding 100 µM NADPH to each sample in microplate luminometer machine. The basal O_2^- reading was subtracted from the active O_2^- production and normalised to protein concentration.

2.8.2 Measurement of hydrogen peroxide levels

Hydrogen Peroxide levels were evaluated in PVAT, EVAT, PVF and SW872 cells using the Amplex Red Hydrogen Peroxide/Peroxidase assay kit (Invitrogen #A22188) the experiments were performed in adipose tissue and SW872 cell protein lysates, according to the manufacturer's instructions. Amplex red

reagent in combination with horse radish peroxidase HRP reacted to the presence of H_2O_2 producing the red fluorescent oxidation product, resofurin (Figure 2.1).

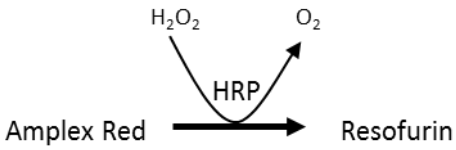


Figure 2.1. Measurement of hydrogen peroxide levels

In a clear 96 well plate 50µl of sample protein lysate was added with 50µl of amplex red and HRP working solution as advised in the kit manual. The plate was incubated in the dark for 30mins at RT and read at 560nm absorbance using a plate reader (Dynex, Magellan Bioscience). The results were analysed with the H_2O_2 standard curve as described in the kit manual and normalised to protein concentration.

2.8.3 Determination of TBARS levels

Malondialdehyde (MDA) is a natural result of lipid peroxidation which can be used as an indirect measurement of systemic oxidative stress. Lipid peroxidation was evaluated using a Thiobarbituric Acid Reactive Substances (TBARS) assay kit (Cayman #10009055) and the experiments were performed in adipose tissue protein lysates and plasma according to the manufacturer's instructions. The reaction between thiobarbituric acid (TBA) mix and malondialdehyde from samples formed the MDA-TBA adduct under high temperature and acidic conditions (Figure 2.2).

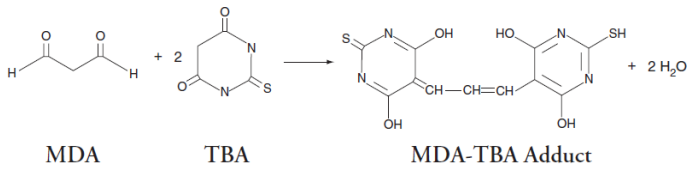


Figure 2.2. Determination of TBARS levels

The reaction produced a pink colour which was measured colorimetrically at 530-540nm. The density of the colour is directly proportional to the amount of MDA in the sample. The levels of MDA in the samples were estimated using the linear equation obtained from the MDA standard curve provided by the manufacturer.

2.9 Statistical analysis

Tissue samples were each analysed in duplicates with 10 mice in each group. Cell experiments for lucigenin and amplex red had an n=8 while aldosterone and cortisol measurements had an n=7. Statistical comparison of multiple groups with one factor variance was analysed using one-way-ANOVA followed by the Newman-Keuls post-test. Statistical comparison of multiple groups with a two factor variance was analysed using two-way-ANOVA followed by Bonferonni's multiple correction test. All statistical analysis was carried out with Micosoft Excel and Graphpad prism 6 commercially available software. Significance threshold for all analysis was considered at p value < 0.05.

2.10 Drugs and solutions

2.10.1 Agonists and inhibitors

Angiotensin II was purchased from Bachem (Torrance, CA, USA). IBMX, insulin, dexamethasone, Tempol and ML171 were purchased from Sigma-Aldrich Chemical, Co. GKT137831 was supplied by Genkyotex (Switzerland, Geneva). Dexamethasone was dissolved in 100% ethanol. GKT137831 and ML171 were dissolved in dimethylsulfoxide (DMSO). Ang II and tempol were dissolved in distilled water. Insulin was dissolved in 0.01N HCl.

2.10.2 Lucigenin solutions

ROS Phosphate buffer, pH7.4 in 100 ml dH_2O

1.36g 50mM KH2PO4 with 0.076g 1mM EGTA and 10.26g 150mM sucrose.

ROS lysis buffer

10ml of ROS Phosphate buffer, pH7.4, 10µl of 1µg/ml Aprotinin/Pepstatin/ Leupeptin respectively and 100µl PMSF 1mM.

Basal reading mix per well

50µl sample, 100µl ROS Phosphate buffer, pH7.4 and 75µl lucigenin mix

Lucigenin mix per well

73.75µl ROS Phosphate buffer, pH7.4 with 1.25µl lucigenin (10^{-3}M diluted in dH_2O)

2.10.3 Software

Simplicity 4.2 reference

ImageJ

Cell^D from Olympus

Graphpad Prism 6

Microsoft Word Excel

3 Results

3.1 Metabolic, biochemical parameters and blood pressure in obese *db/db* mice.

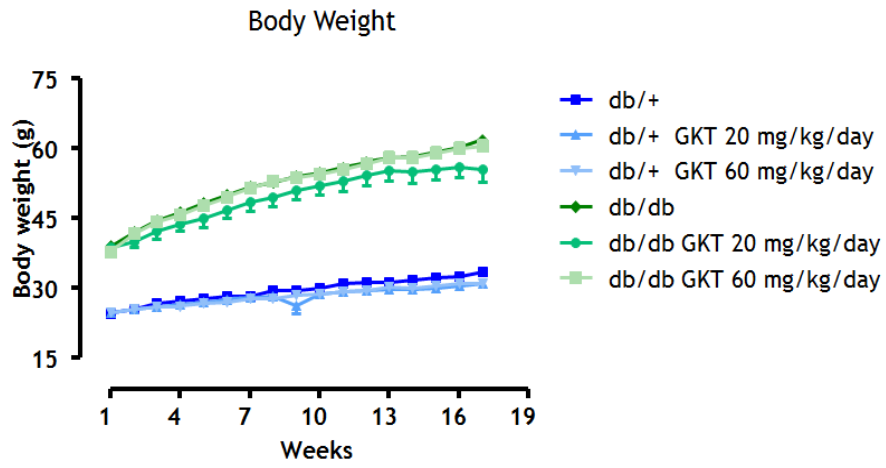
Obese *db*/*db* mice displayed higher body weight compared to lean *db*/+ controls (**Figure 3.1A**). This was associated with increased epididymal visceral adipose tissue (EVAT) mass (**Figure 3.1B**) and adipocyte hypertrophy as evidenced by histological analysis of EVAT sections (Picro-sirius red staining discussed below). Treatment with Nox1/4 inhibitor did not reduce body weight, whereas visceral adiposity, as measured by EVAT mass, showed a strong tendency (p value = 0.054) to be reduced in *db*/*db* mice treated with the high dose of Nox1/4 inhibitor (**Figure 3.1B**).

Obese *db*/*db* mice displayed features of metabolic syndrome where glucose, total cholesterol, HDL, LDL and triglycerides plasma levels were increased compared to lean *db*/+ control mice (**Figures 3.2A-E**). These were not attenuated by the Nox1/4 inhibitor. Plasma glucose levels of *db*/db mice were elevated versus *db*/+ controls, however still below 15mmol/L plasma glucose and thus could be labelled as mildly diabetic. The C57/bl6 background of this mouse model is known to cause a certain resistance to the development of diabetes.

Plasma levels of sodium, potassium and other electrolytes were determined in obese and lean mice. Plasma chloride levels in *db*/*db* mice were decreased compared to *db*/+ mice. Serum levels of potassium, calcium, magnesium and phosphate were increased in obese *db*/*db* mice versus lean *db*/+ control mice. Nox1/4 inhibition decreased plasma potassium in obese *db*/*db* mice versus non treated *db*/*db* mice, and interestingly increased plasma sodium levels and decreased plasma potassium levels in lean *db*/+ mice at high dose treatment. These results may have been caused by slight renal impairment.

Obese *db*/*db* mice displayed microalbuminuria (**Figure 3.4A**) compared with lean *db*/+ mice, indicating significant renal dysfunction Nox1/4 inhibition did not reduce microalbuminuria. Blood pressure was similar in *db*/*db* and *db*/+ mice, without effect of GKT (**Figure 3.4B**).

A



B

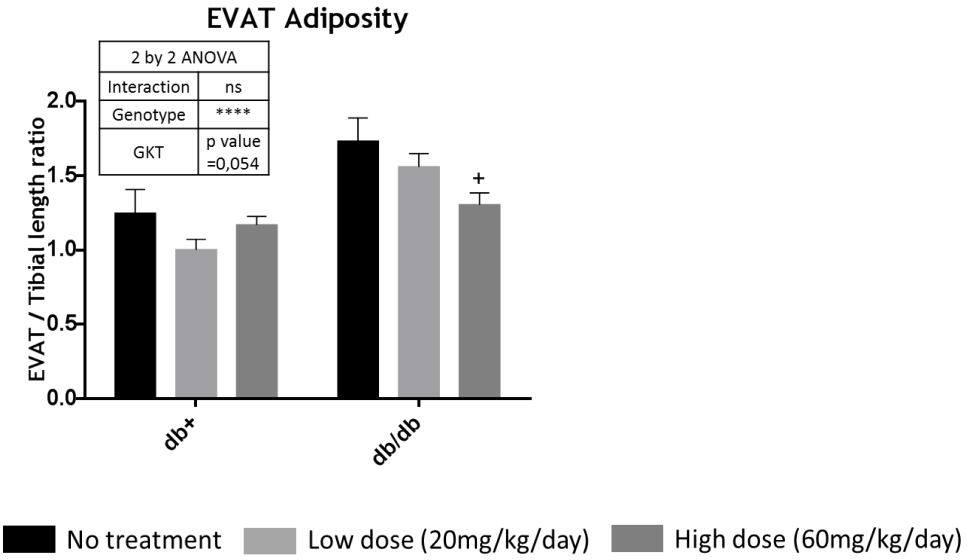


Figure 3.1. Obese *db/db* mice displayed reduced visceral adiposity by Nox1/4 inhibitor (GKT137831). Body weight (A) and EVAT adiposity (B) in *db/db* obese and *db/+* lean mice treated or not with Nox1/4 inhibitor (GKT137831). Values are means±sem, n=10 mice per group; **** $p<0.0001$, two-way ANOVA followed by Bonferonni's multiple test.; + $p<0.05$ vs *db/db* NT. (NT: No treatment)

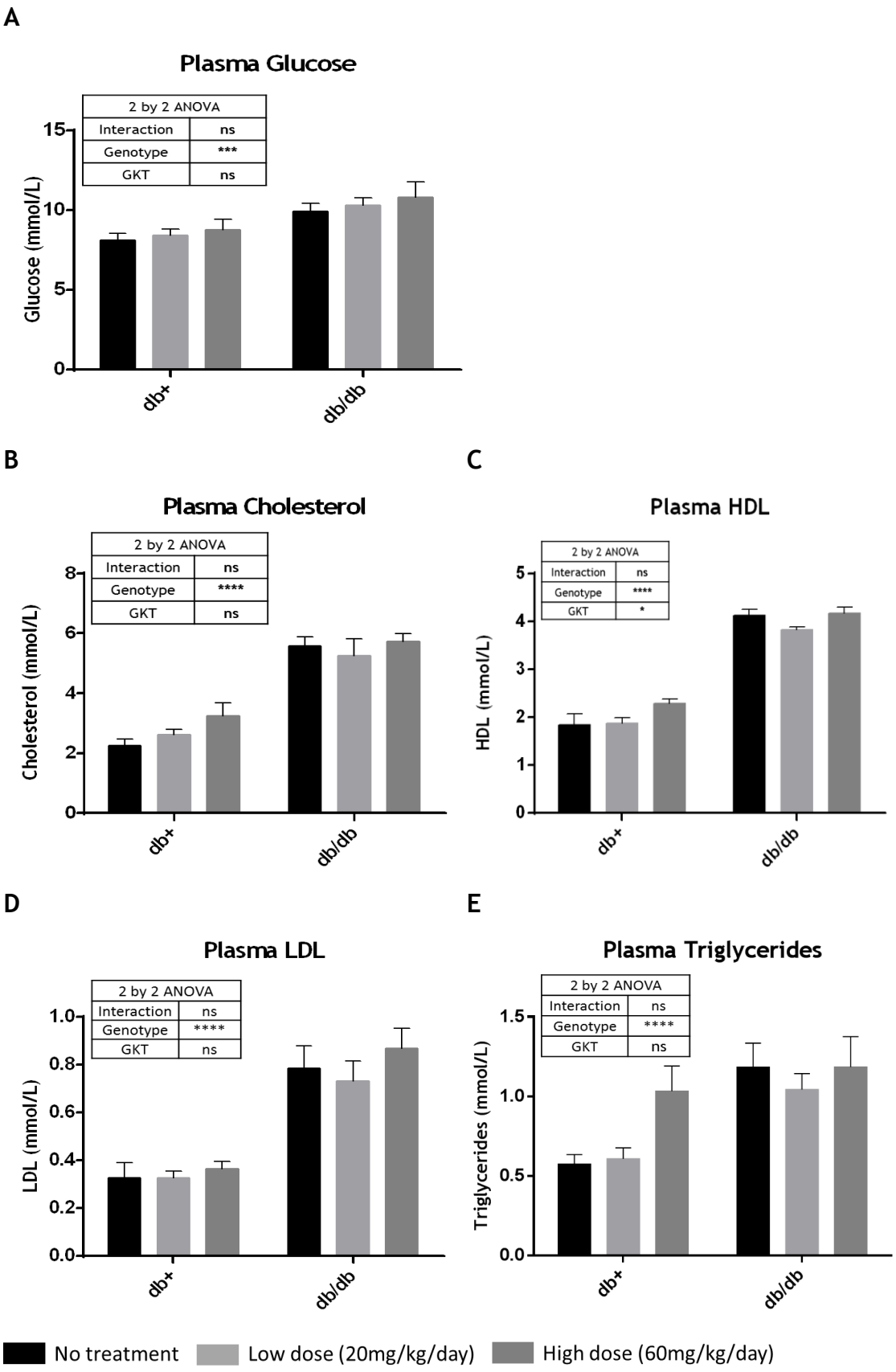


Figure 3.2. Obese *db/db* mice were not diabetic but displayed features of metabolic syndrome. Plasma levels of glucose, cholesterol, HDL, LDL and triglycerides in in *db/db* obese and *db/+* lean control mice treated or not with Nox1/4 inhibitor (GKT137831). Values are means±sem, n=10 mice per group. *** $p<0.001$, **** $p<0.0001$, two-way ANOVA followed by Bonferonni's multiple comparison test. (NT: No Treatment)

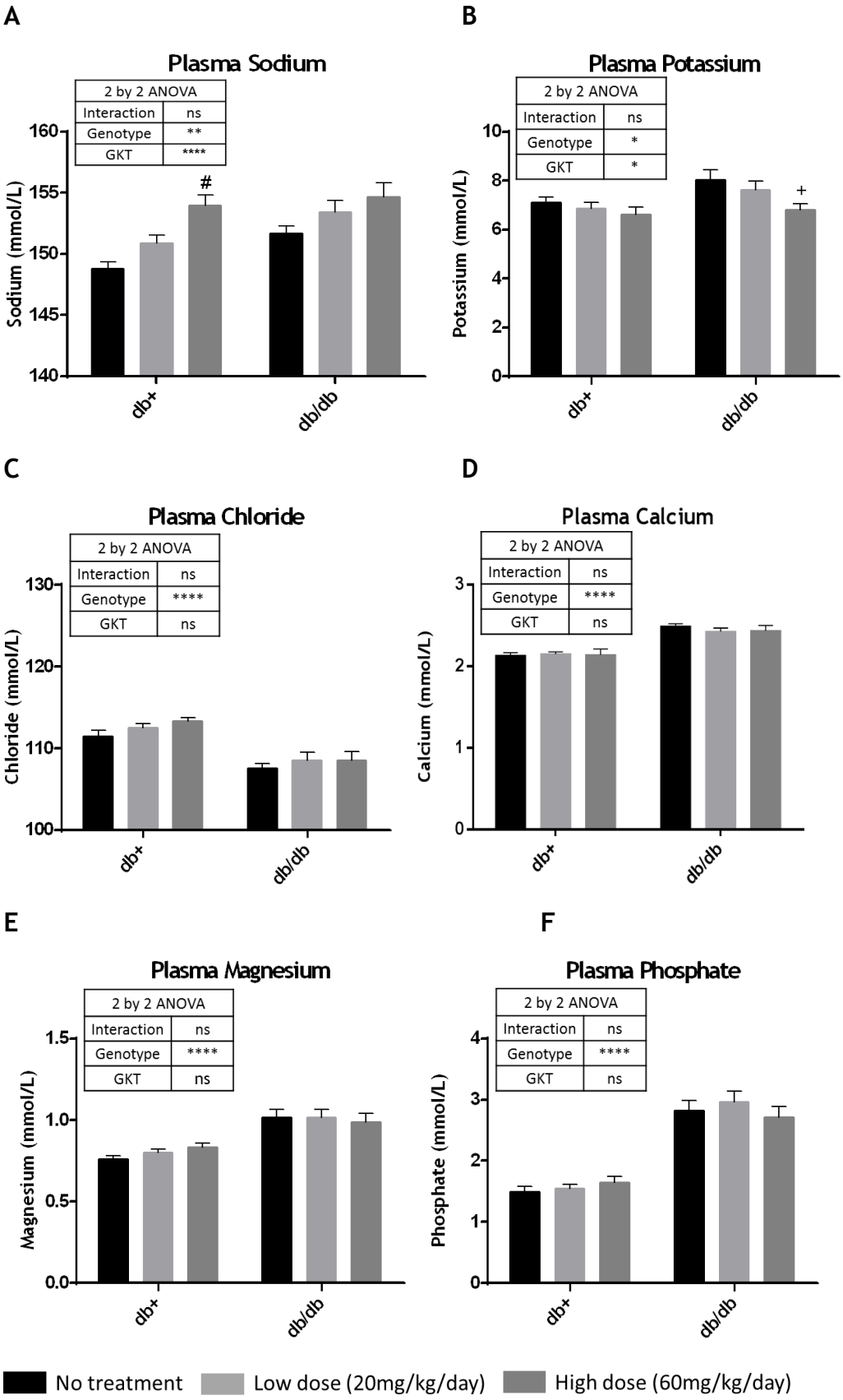
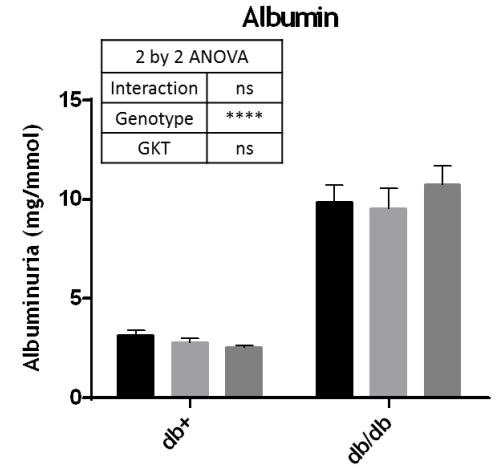


Figure 3.3. Plasma levels of electrolytes in *db/db* obese and *db/+* lean control mice treated or not with Nox4/1 inhibitor (GKT137831). Levels of sodium (A), potassium (B), chloride (C), calcium (D), magnesium (E) and phosphates (F) in plasma serum from obese *db/db* and lean *db/+* mice treated or not with Nox1/4 inhibitor (GKT137831). Values are means±sem, n=10 mice per group. * $p<0.05$, ** $p<0.01$, **** $p<0.0001$ two-way ANOVA followed by Bonferonni's multiple comparison test; + $p<0.05$ vs *db/db* NT, # $p<0.05$ vs *db/+* NT. (NT: No Treatment)

A



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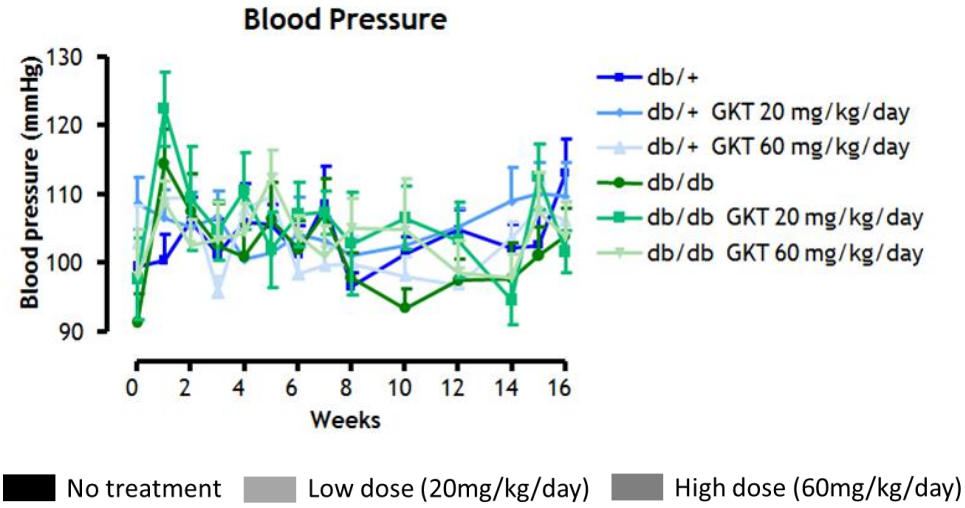


Figure 3.4. Microalbuminuria and Blood Pressure. Microalbuminuria (A) and blood pressure (B) in obese *db/db* mice and lean *db/+* mice treated or not with Nox1/4 inhibitor (GKT137831). Values are means±sem, n=10 mice per group. **** $p<0.0001$, two-way ANOVA followed by Bonferonni's multiple comparison test. (NT: No Treatment)

3.2 Aldosterone and corticosterone concentrations in plasma and adipocyte conditioned media were increased in obese *db/db* mice.

Circulating levels of aldosterone are increased in obese individuals (Goodfriend *et al*, 1999; Krug *et al*, 2008; Stiefel *et al*, 2011) and in different animal models of obesity (Guo *et al*, 2008). In addition, high levels of aldosterone have been positively correlated with high body mass index (Rossi *et al*, 2008). The Touyz laboratory previously demonstrated that adipocytes express all the components of the renin-angiotensin-aldosterone system (RAAS) including the aldosterone synthase gene *Cyp11b2*, and secrete aldosterone, an effect enhanced in obesity (Briones *et al*, 2012). We sought to study the possible implication of Nox-derived ROS in aldosterone production by AT as it was recently shown *in vitro* in adrenocortical cells (Rajamohan *et al*, 2012). Thus, aldosterone levels were measured by ELISA Kit in plasma and mature adipocytes culture media from *db*/*db* obese and *db*/+ lean mice treated with or without Nox1/4 inhibitor. Obese mice displayed a two-fold increase in plasma aldosterone levels compared to lean controls (**Figure 3.5A**). Low and high dose of Nox1/4 inhibitor decreased the production to the same capacity. To assess the local production of aldosterone, mouse mature adipocytes were isolated from EVAT and cultured for 24 hours. Levels of aldosterone were determined in culture media concentrated five times. Aldosterone levels were increased five-fold in mature adipocytes medium (MAM) from *db*/*db* obese mice compared to *db*/+ lean controls. It was completely prevented by Nox1/4 blockade in a dose dependent manner (**Figure 3.5B**). Whether Nox-derived ROS regulates aldosterone-induced effects in adipocytes is the next question we wished to address.

Determining corticosterone levels was of interest as it precedes aldosterone in the steroid hormones synthesis pathway as well as shares an equal selectivity for the mineralocorticoid receptor (MR). In MAM from obese *db*/*db* mice, corticosterone levels were five-fold higher than in MAM from *db*/+ lean controls. Interestingly, Nox1/4 inhibition had no effect on corticosterone levels (**Figure 3.5C**). Therefore, Nox1/4-derived ROS does not seem to impact on corticosterone secretion by adipocytes.

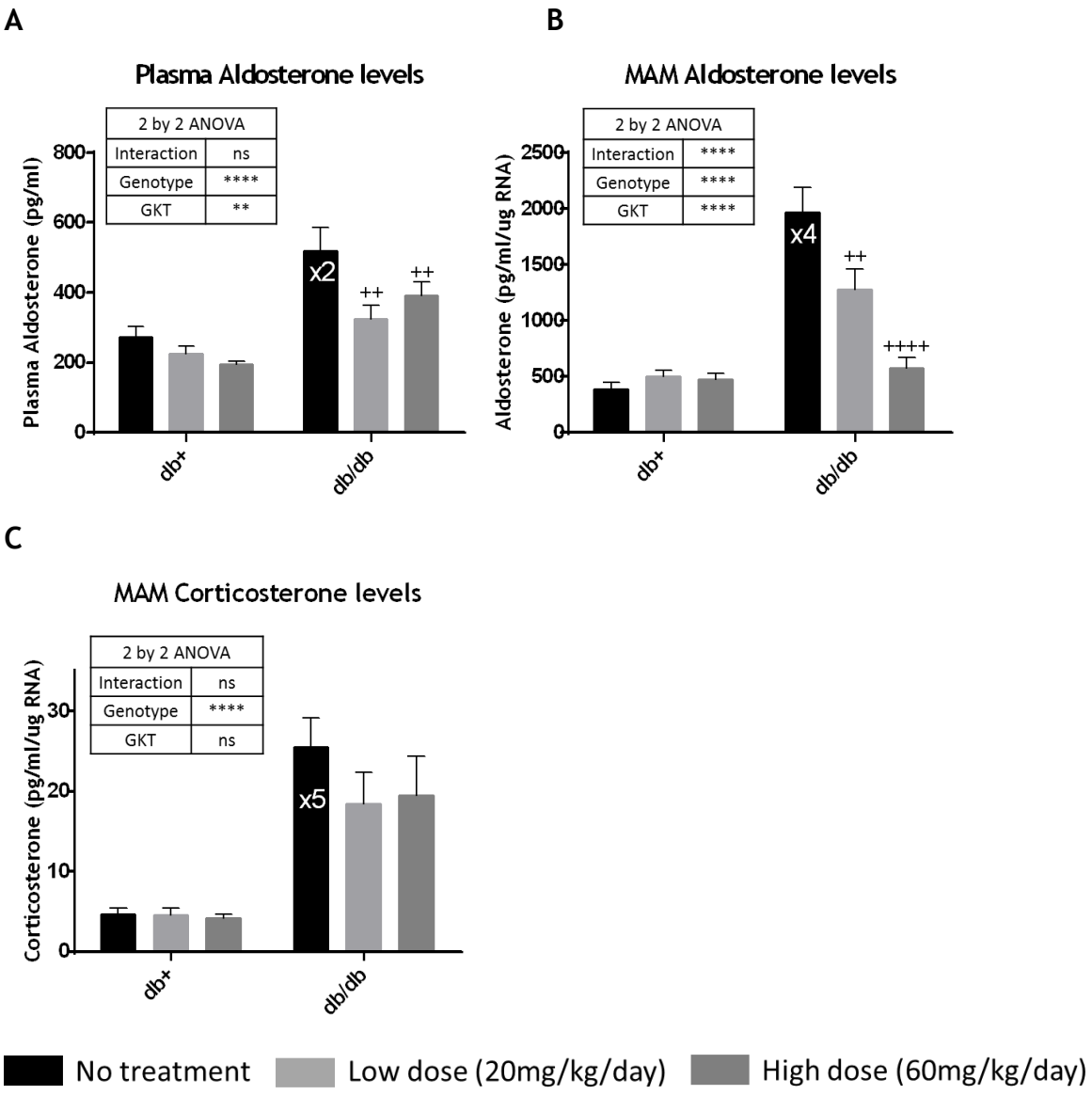
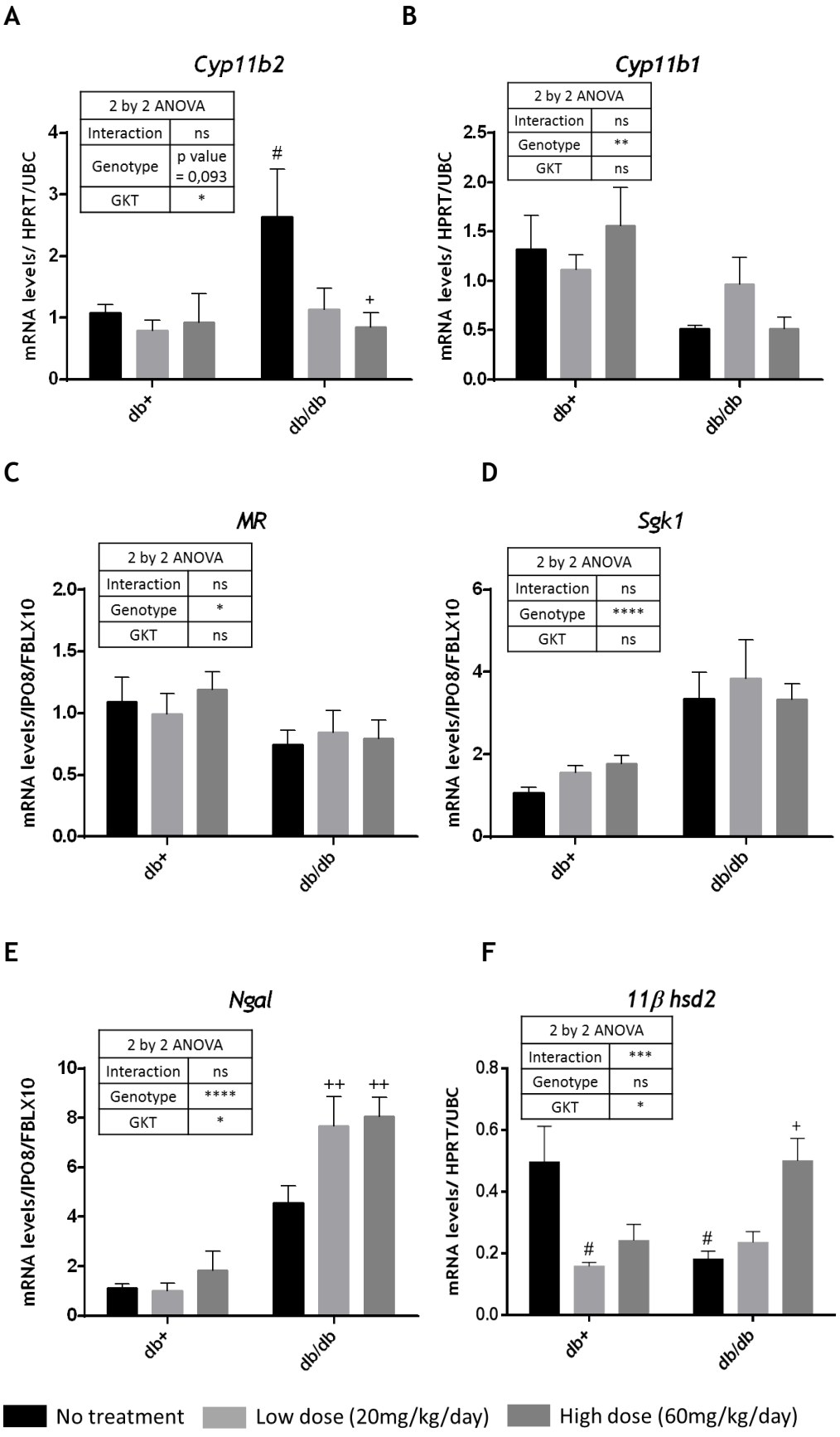


Figure 3.5. Aldosterone and Corticosterone levels in plasma and mature adipocytes media (MAM). Aldosterone (A, B) and corticosterone (C) concentrations were determined by ELISA in obese *db/db* and lean *db/+* mice. Levels in MAM were normalised by the amount of RNA. Values are means±sem, n=10 mice per group. ** $p<0.01$, **** $p<0.0001$; two-way ANOVA followed by Bonferonni's multiple comparison test; ++ $p<0.01$, ++++ $p<0.0001$ vs *db/db* NT. (NT: No Treatment)

3.4 Nox1/4-derived ROS regulated mRNA levels of aldosterone synthase, markers of MR activation and selective enzymes for aldosterone/MR in perivascular adipose tissue from obese *db/db* mice.

Aldosterone synthase C*yp11b2* mRNA levels showed a tendency to increase (p value = 0.0934) in PVAT from obese mice compared to lean controls. The levels were normalised in obese mice treated with Nox1/4 inhibitor (**Figure 3.6A**). Moreover, mRNA levels of C*yp11b1*, the enzyme responsible for corticosterone synthesis, were decreased in obese *db*/*db* mice and unchanged by Nox1/4 inhibition (**Figure 3.6B**). These results support the concept that aldosterone concentration is regulated by Nox1/4-derived ROS through regulation of the aldosterone synthase gene, but not corticosterone levels. *MR* gene expression was decreased in obese animals (**Figure 3.6C**). However, mRNA levels of different markers of MR activation, *Sgk1* and *Ngal* (or *lipocalin-2*), were elevated in obese *db*/*db* mice compared to *db*/+ lean mice. Nox1/4 inhibition further increased *Ngal* mRNA levels in *db*/*db* mice; *Sgk1* gene expression was increased in *db*/*db* but not in *db*/+ mice (**Figures 3.6D and 3.6E**). In addition, mRNA levels of the glucocorticoid inactivating enzyme *11βhsd2* were decreased in obese *db*/*db* compared to lean control mice. Interestingly, the high dose of Nox1/4 inhibitor normalised it (**Figure 3.6F**). Thus, Nox1/4 inhibition seems to promote the inactivate state of corticosterone and potentially promote the action of aldosterone through MR rather than corticosterone in obese mice. On the other hand mRNA levels of *11βhsd1,* the glucocorticoid activating enzyme, were not different between *db*/*db* and *db*/+ non treated mice (**Figure 3.6G**). Interestingly, Nox1/4 inhibition decreased both *11βhsd1* and *11βhsd2* mRNA levels in lean *db*/+ mice.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Cyp11b2
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Interaction ns
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GKT *
mRNA levels/ HPRT/UBC
#
+
db+
db/db
B
Cyp11b1
2 by 2 ANOVA
Interaction ns
Genotype **
GKT ns
mRNA levels/ HPRT/UBC
db+
db/db
C
MR
2 by 2 ANOVA
Interaction ns
Genotype *
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mRNA levels/IPO8/FBLX10
db+
db/db
D
Sgk1
2 by 2 ANOVA
Interaction ns
Genotype ****
GKT ns
mRNA levels/IPO8/FBLX10
db+
db/db
E
Ngal
2 by 2 ANOVA
Interaction ns
Genotype ****
GKT *
mRNA levels/IPO8/FBLX10
++
++
db+
db/db
F
11β hsd2
2 by 2 ANOVA
Interaction ***
Genotype ns
GKT *
mRNA levels/ HPRT/UBC
#
#
+
db+
db/db
No treatment
Low dose (20mg/kg/day)
High dose (60mg/kg/day)

G

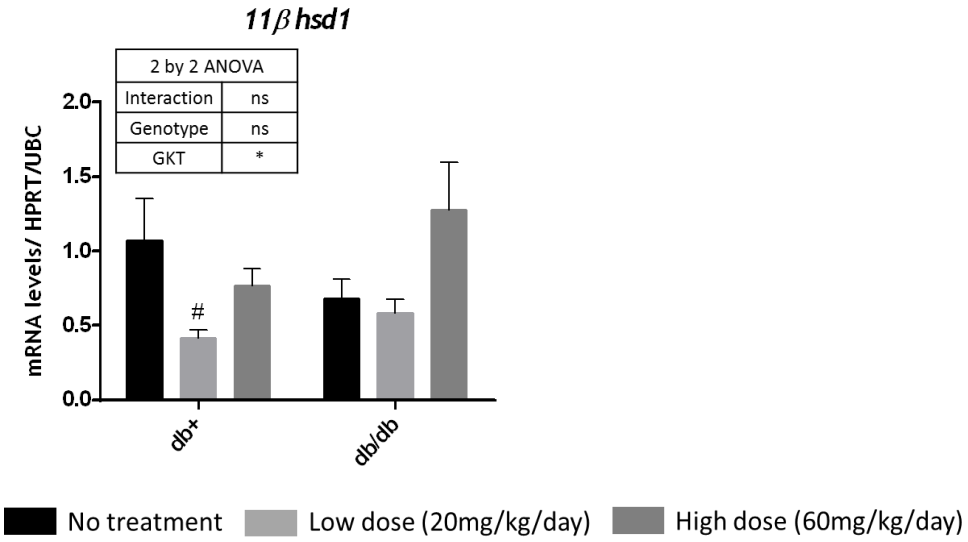


Figure 3.6. mRNA levels analysis by real time PCR. *Cyp11b2* (A), C*yp11b1* (B) enzymes, mineralocorticoid receptor (C) and markers of *MR* activation (*Sgk1* and *Ngal*) (D, E), glucocorticoid inactivating *11Bhsd2* (F) and activating *11Bhsd1* (G) enzymes were determined. Values are means±sem, n=10 mice per group. * p<0.05, ** p<0.01,*** p<0.001,**** p< 0.0001, two-way ANOVA followed by Bonferonni's multiple comparison test; # p<0.05 vs *db*/+ NT, + p<0.05, ++ p<0.01 vs *db*/*db* NT. (NT: No Treatment)

3.3 Increased adipose oxidative stress in obese *db/db* mice.

Clinical and experimental studies reported that obesity is associated with elevated ROS levels and oxidative damage (Furukawa *et al*, 2004; Hirao *et al*, 2010; Fernandez-Sanchez *et al*, 2011). We evaluated ROS generation in non-treated and treated animals. In EVAT from obese *db*/*db* mice TBARS levels were elevated, a marker of lipid peroxidation, indicating systemic increase of oxidative stress. This was not reduced by Nox1/4 inhibition (**Figure 3.7A**). TBARS and H_2O_2 levels in PVAT from obese *db*/*db* mice were not different from lean *db*/+ controls (**Figure 3.7B**) Interestingly, H_2O_2 levels were enhanced in PVAT from *db*/+ mice when treated with high dose of Nox1/4 inhibitor while treated *db*/*db* mice levels were unaffected (**Figure 3.7C**).

3.4 Gene expression of Nox 1, 2 and 4 is differentially regulated in perivascular adipose tissue (PVAT) from *db/db* obese mice.

Nox4 and *Nox2* mRNA levels were increased, whereas *Nox1* mRNA levels were decreased in *db*/*db* obese mice compared to *db*/+ lean mice (**Figures 3.8A-C**). GKT treatment in *db*/*db* mice showed a tendency to increase PVAT *Nox4* mRNA levels at high dose (p value = 0.058) with no effect on Nox1 gene expression. Indicating a feed forward system where Nox1/4 inhibition promotes *Nox4* and *Nox2* expression.

3.5 Role of Nox1/4-derived ROS on adipocyte differentiation and adipokine expression.

mRNA levels of adiponectin (*adipoq*), an adipocyte-specific anti-inflammatory mediator, (**Figure 3.9A**) were decreased in PVAT of obese *db*/*db* mice compared to lean *db*/+ mice while levels of *Ppar-γ* (important in adipocyte maturation) remained unchanged (**Figure 3.9B**). Nox1/4 inhibition had no effect on *adiponectin* and *ppar-γ* mRNA levels. Adipocyte protein *aP2*, another adipogenesis marker was increased in obese *db*/*db* mice compared to lean *db*/+ mice. Treatment had no effect (**Figure 3.9C**).

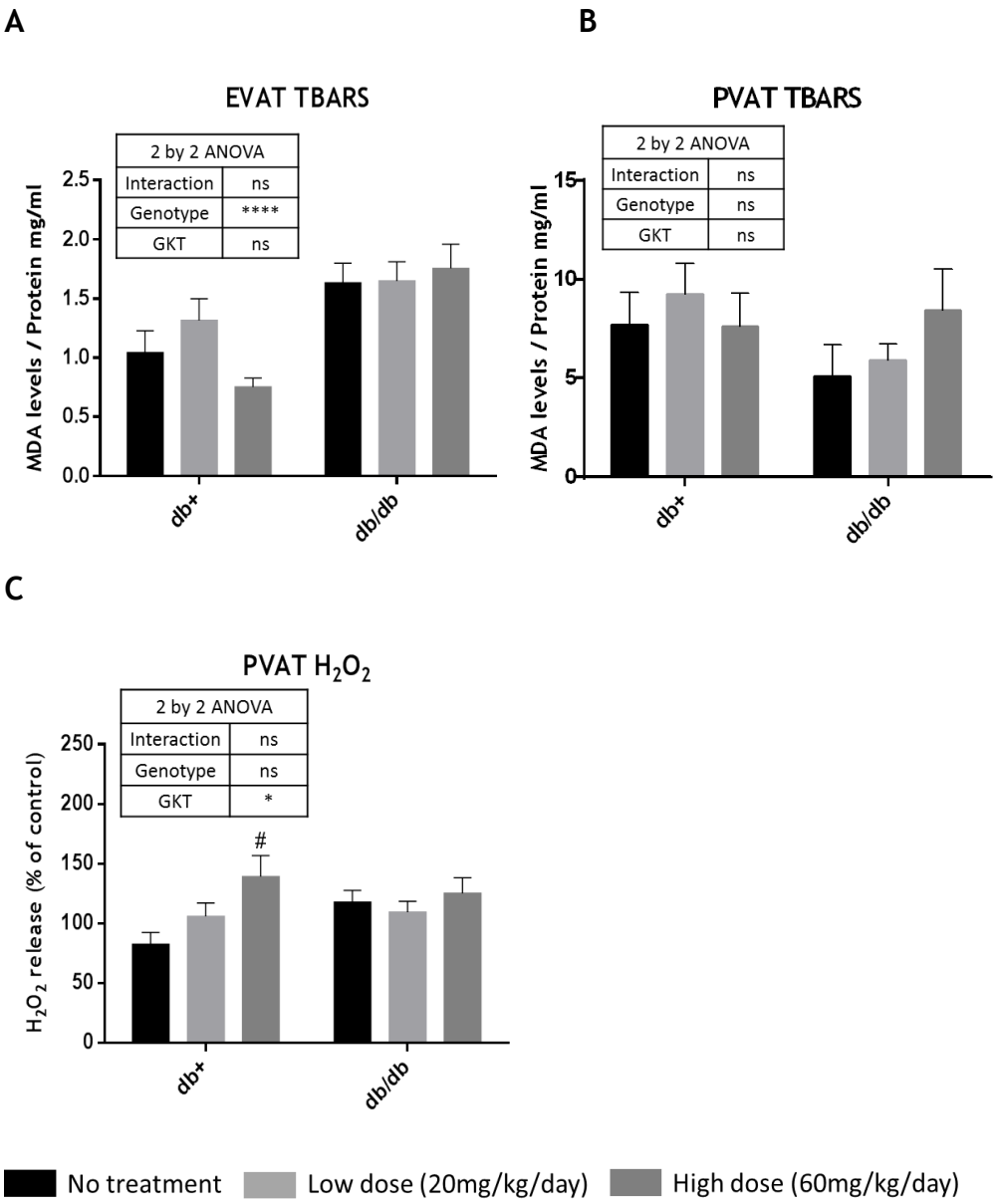


Figure 3.7. Obese *db/db* mice displayed systemic and adipose oxidative stress. Systemic oxidative stress was evaluated by TBARS in EVAT (A) and in PVAT (B) from obese *db/db* and lean *db/+* mice. Hydrogen peroxide (H_2O_2) levels were determined in PVAT by Amplex Red. Values are means±sem, n=10 mice per group. **** p< 0.0001, two-way ANOVA followed by Bonferonni's multiple comparison test, # p<0.05 vs *db/+* NT. (NT: NoTreatment)

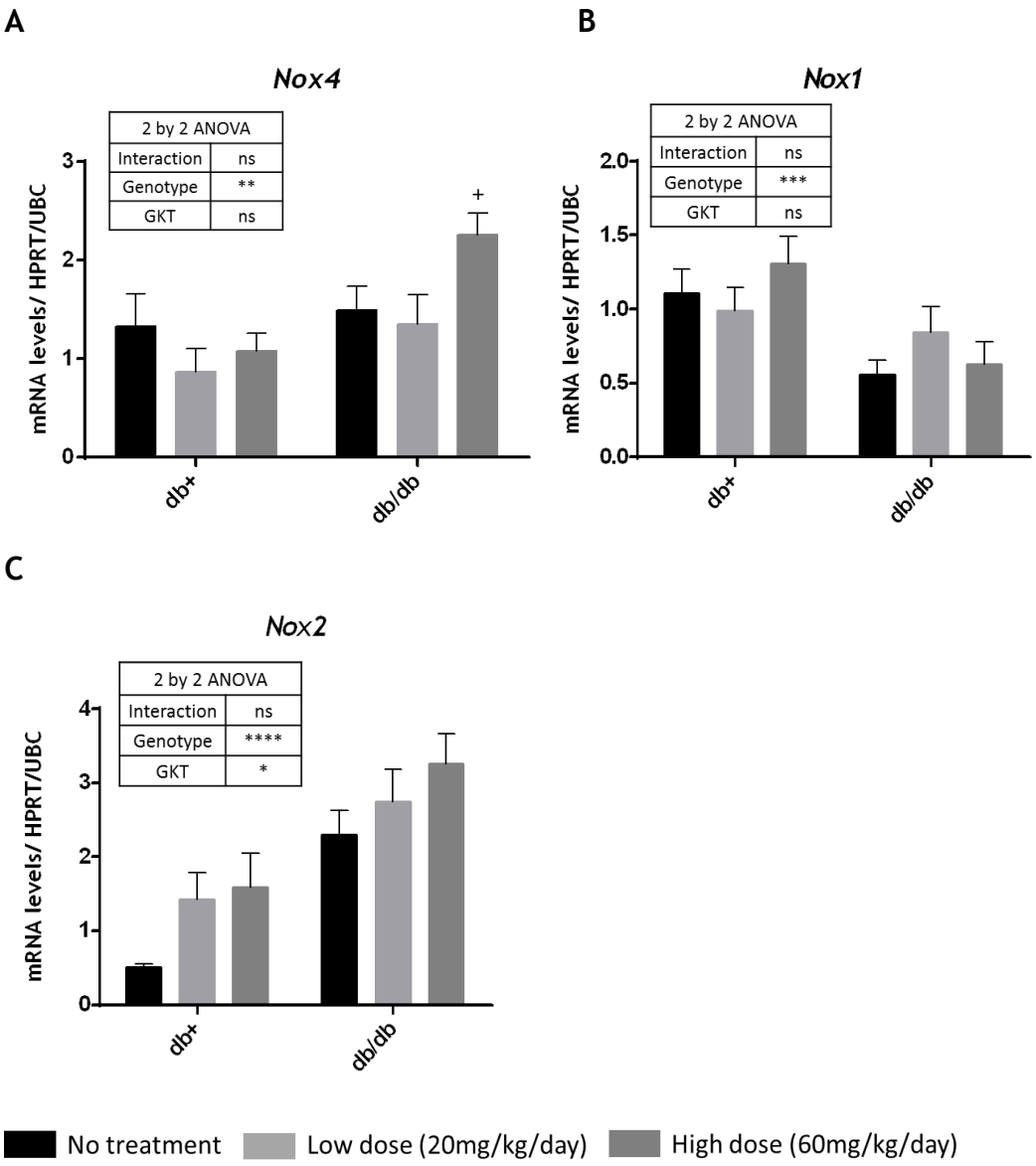


Figure 3.8. NADPH oxidase isoforms mRNA levels by real time PCR. *Nox4* (A), *Nox1* (B) and *Nox2* (C) gene expression were determined. Values are means±sem, n=10 mice per group * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$, two-way ANOVA followed by Bonferonni's multiple comparison test, + $p<0.05$ vs *db/db* NT. (NT: No Treatment)

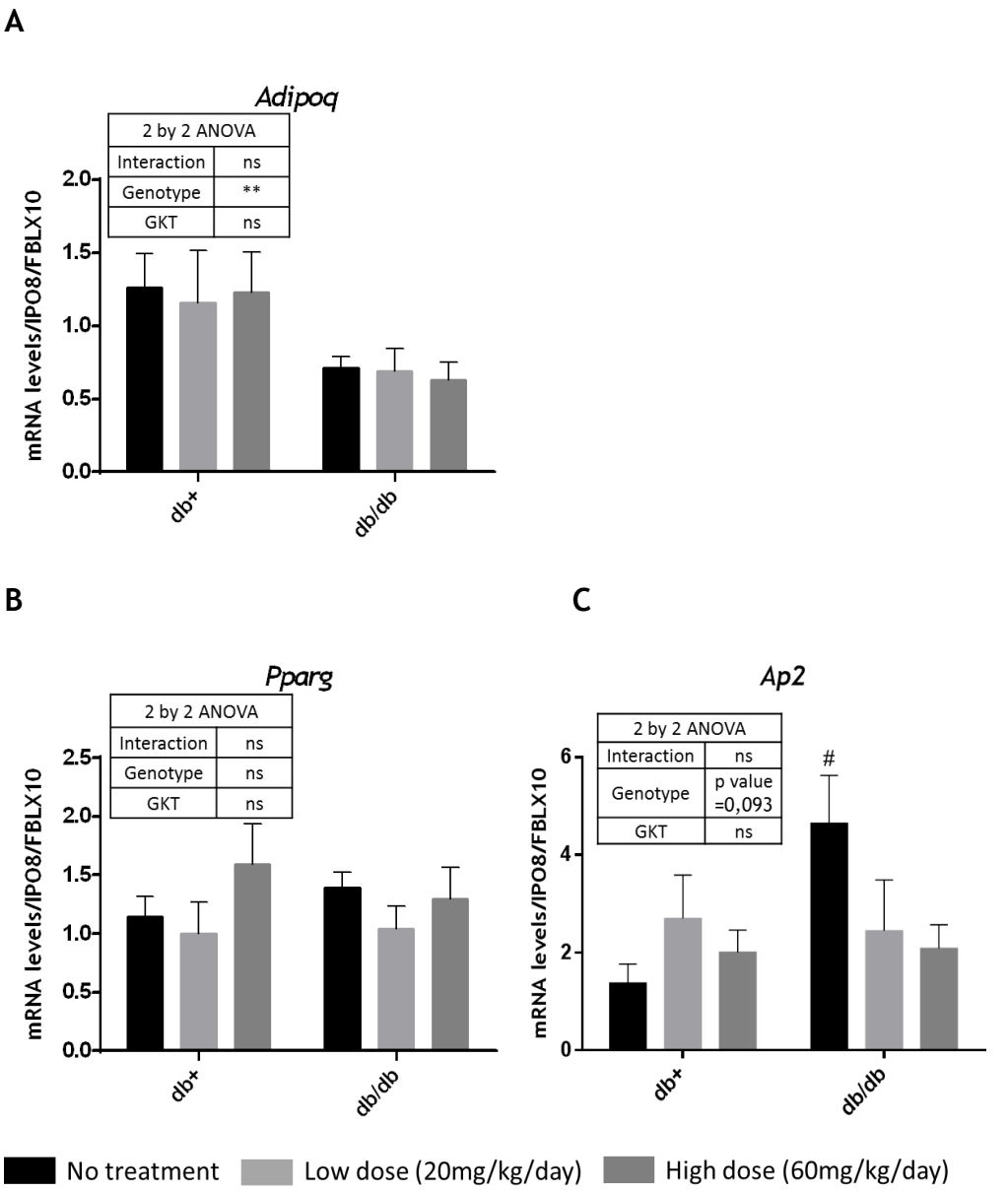
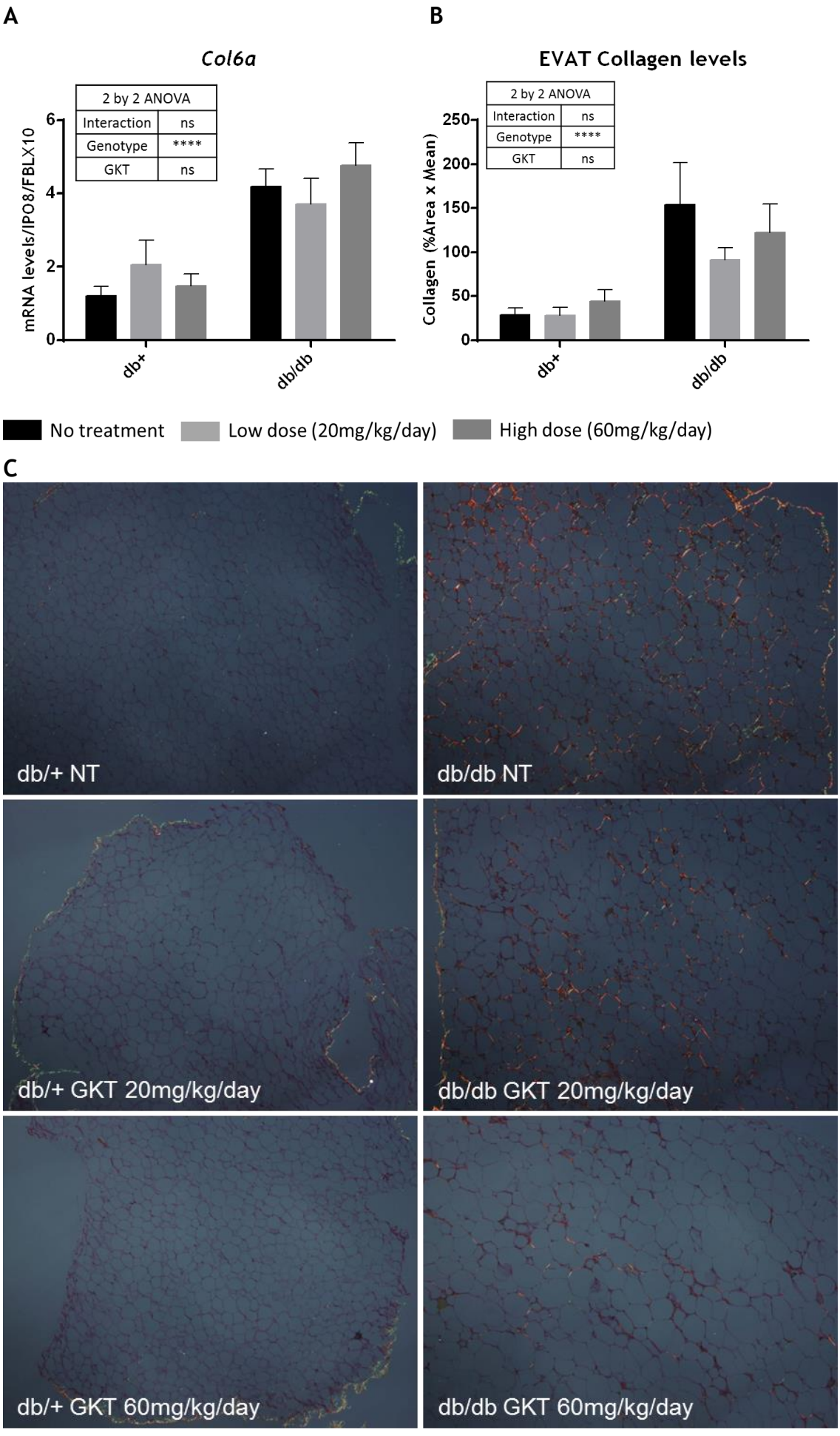


Figure 3.9. Adipokines and adipocyte differentiation markers mRNA levels in *db/db* mice. *Adipoq* (adiponectin) (A) *Ppar-γ* (B) and *Ap2* (C) mRNA levels in obese *db/db* and lean *db/+* mice were determined by Real Time PCR. Results were normalised to IPO8/FBLX10 used as housekeeping genes. Values are means±sem, n=10 mice per group. ** $p<0.01$, two-way ANOVA followed by Bonferonni's multiple comparison test # $p<0.05$ vs *db/db* NT. (NT: No Treatment)

3.6 Fibrosis in visceral adipose tissue from *db/db* obese mice is not attenuated by Nox1/4 inhibition.

During adipose tissue expansion, the extracellular matrix requires remodelling to accommodate adipocyte growth. Here, we assessed general upregulation of several extracellular matrix components in adipose tissue in the "obese" state, therefore implicating "adipose tissue fibrosis" as a hallmark of metabolic dysfunction. Collagen VI is a highly enriched extracellular matrix component of adipose tissue. Moreover, it is known that aldosterone has pro-fibrotic effects in several target tissues (kidney, heart, vessels) in the context of metabolic syndrome (Krug *et al*, 2008; Essick *et al*, 2011). Thus we questioned if Nox1/4 inhibition is able to impact on aldosterone-induced deleterious effects in adipose tissue.

Obese *db/db* mice presented higher mRNA levels of *collagen 6a* in PVAT than lean *db/+* mice (**Figure 3.11A**), which is not affected by Nox1/4 inhibition. Collagen content in EVAT was measured by polarized light microscopy and quantified by image-analysis software and showed an increase in obese *db/db* versus lean *db/+* mice (**Figures 3.11B and C**). From the picro Sirius red slides the collagen levels in EVAT from *db/db* mice seem to reduce with Nox1/4 inhibitor but the changes where not statistically significant. However, the difference between *db/+* NT mice and *db/db* low dose treated mice was lost (p value= 0.820) and thus indicates a mild reduction in collagen levels (**Figure 3.11C)**.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Col6a
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0
2
4
6
db+
db/db
B
EVAT Collagen levels
2 by 2 ANOVA
Interaction ns
Genotype ****
GKT ns
Collagen (%Area x Mean)
0
50
100
150
200
250
db+
db/db
No treatment
Low dose (20mg/kg/day)
High dose (60mg/kg/day)
C
db/+ NT
db/db NT
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db/+ GKT 60mg/kg/day
db/db GKT 60mg/kg/day

D

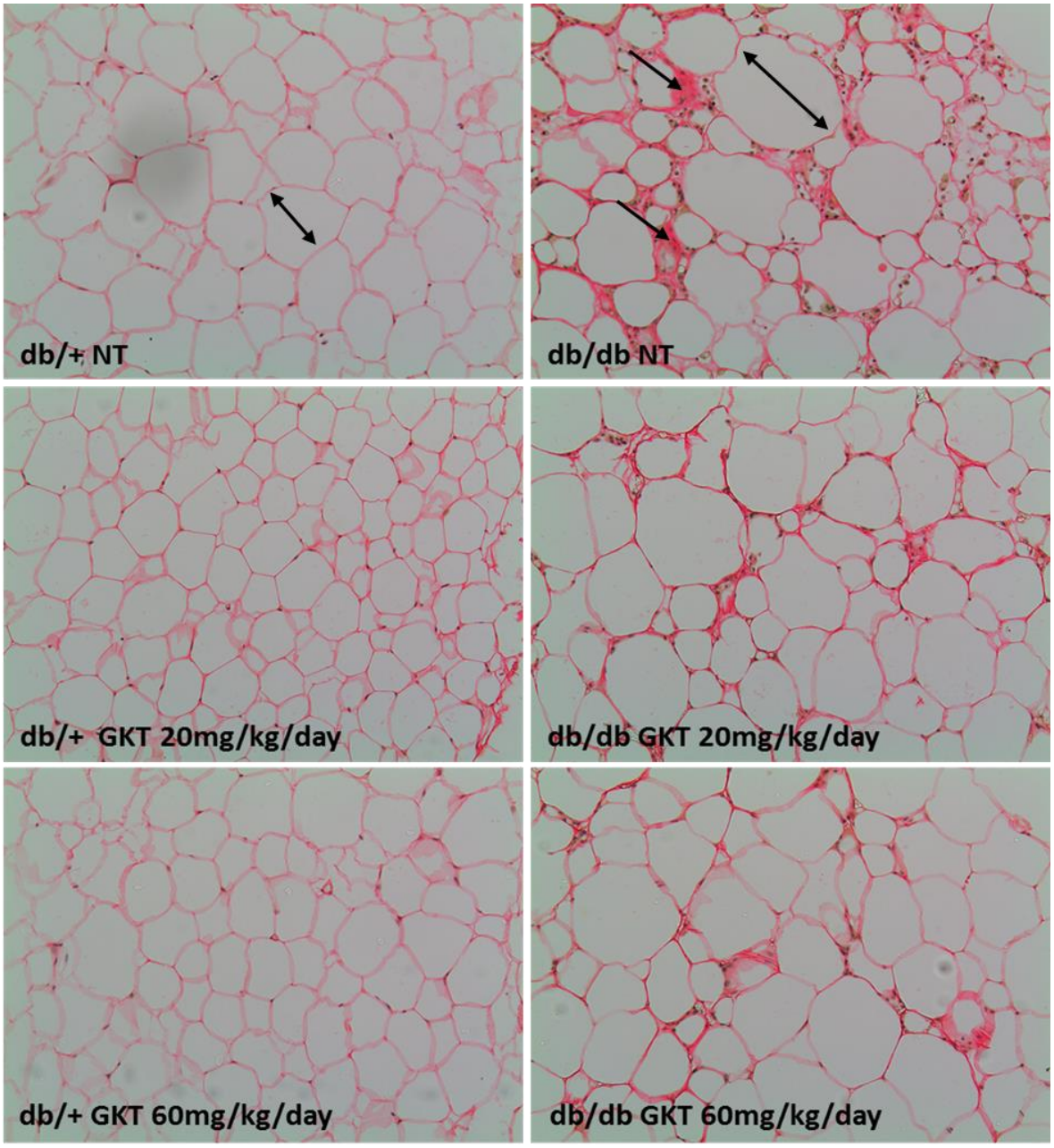


Figure 3.10. Obese *db/db* mice displayed adipose tissue fibrosis. C*ol6a (*collagen 6a*)* (A) mRNA levels were assessed by real time PCR in PVAT from obese *db/db* and lean *db/+* mice. Results were normalised to IPO8/FBLX10 housekeeping genes. EVAT collagen content (B-C) were visualised by polarized light microscopy and quantified using image analysis software (D) Picro-sirius red stained EVAT slides. The single head arrows indicate collagen, an index of adipose tissue fibrosis in *db/db* mice. Double head arrow indicates diameter of adipocytes, showing larger size (hypertrophy) in *db/db* mice versus controls. Values are means±sem, n=10 mice per group. **** p<0.0001, two-way ANOVA followed by Bonferonni's multiple comparison test. (NT: No Treatment)

3.7 Effects of Nox1/4 inhibitor on visceral fat inflammation and macrophage polarisation in obese mice.

Since obesity is associated with chronic low-grade inflammation of adipose tissue, where both adipocytes and macrophages contribute to local inflammation as well as promoting further inflammatory activity from each other, we sought to investigate changes in expression of specific markers of macrophages in PVAT from obese and lean mice.

F4/80 mRNA levels were increased in PVAT from obese *db*/*db* mice compared to lean *db*/+ controls, an effect that was not changed by Nox1/4 inhibition (**Figure 3.12A**). These results indicate that macrophages are more abundant within adipose tissue from obese mice than from lean mice. However, this does not differentiate between macrophages type 1 (M1, pro-inflammatory) and type 2 (M2, anti-inflammatory). Thus, we analysed mRNA levels of markers for M1 and M2 macrophages by real time PCR.

3.7.1 Classically activated (M1) macrophages markers

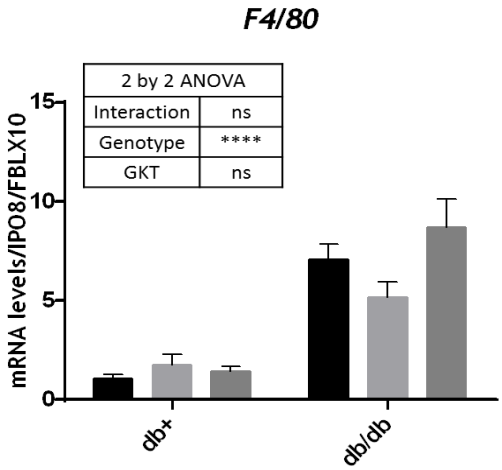
iNOS mRNA levels demonstrated a tendency to increase in PVAT from obese *db*/*db* mice (p value = 0.068), an effect normalised with high dose of Nox1/4 inhibitor (**Figure 3.12B**). *TNF-a* mRNA levels were increased in *db*/*db* obese mice and treatment had no effect (**Figure 3.12C**). Nox1/4-derived ROS may participate in adipose tissue inflammation in obesity, through iNOS-dependent signalling pathways and potentially M1 polarisation in PVAT.

3.7.2 Alternatively activated (M2) macrophages markers

M2 markers A*rg1* and *CD206* mRNA levels were elevated in PVAT from *db*/*db* obese mice (**Figures 3.12D, 3.12E**). The expression of *Arg1* in obese mice was further enhanced with high dose of Nox1/4 inhibitor. As iNOS and arginase-1 compete for L-arginine the decrease found in *iNOS* mRNA levels is further supported by the increase in *Arg1*. Arginase-1 blocks iNOS activation, thus preventing nitric oxide production. *Fizz1* mRNA levels were decreased in PVAT from obese mice, while *Il-10* mRNA levels were not significantly different across the groups (**Figures 3.12F** and **3.12G)**. Nox1/4 inhibition had no effect on

CD206, *Fizz1* and *Il-10* gene expression. Hence, these data suggest that inhibition of Nox1/4-derived ROS generation promotes an anti-inflammatory phenotype through an increase of arginase1 and a decrease of iNOS.

A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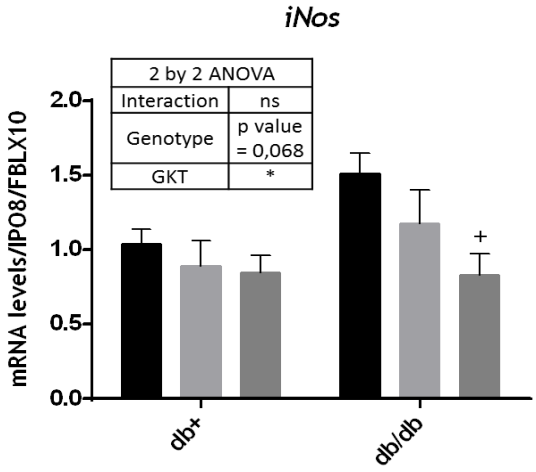
db+
db/db

B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2 by 2 ANOVA
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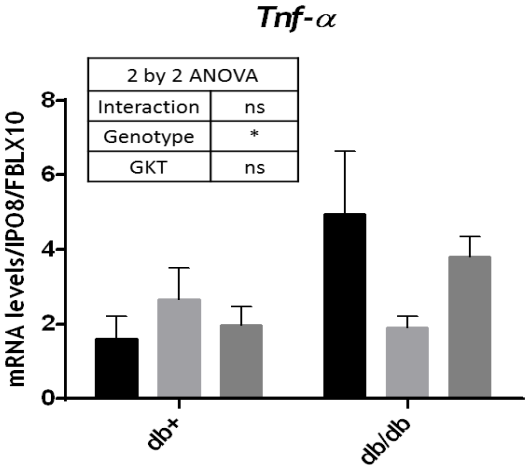
+
db+
db/db

C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2 by 2 ANOVA
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Genotype *
GKT ns
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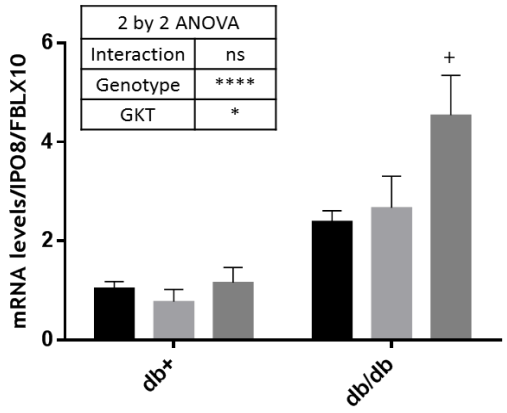
4
2
0
db+
db/db

D


Arg1
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GKT *
mRNA levels/IPO8/FBLX10
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2
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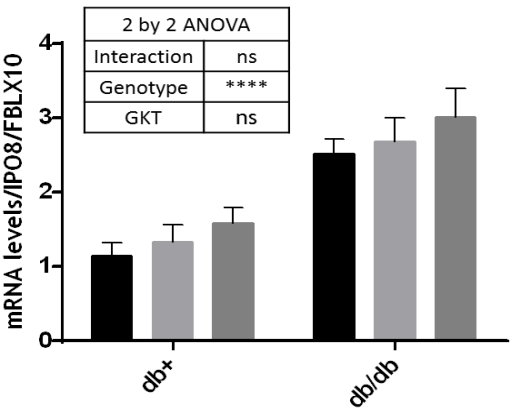
+
db+
db/db

E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2
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db+
db/db

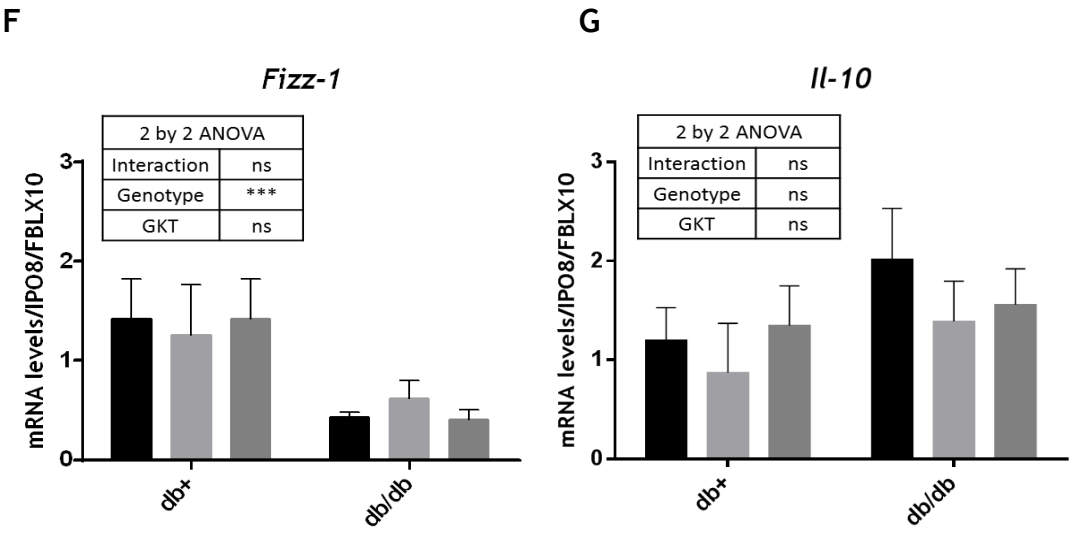

Figure 3.11. Nox1/4 inhibitor (GKT137831) promoted an anti-inflammatory phenotype in PVAT from obese *db/db* mice. mRNA levels of *F4/80* (A), M1 markers (B-C) and M2 markers (D-G) were determined by real time PCR. Values are means±sem, n=10 mice per group. * $p<0.05$, *** $p<0.001$, **** $p<0.0001$ two-way ANOVA followed by Bonferonni's multiple comparison test, + $p< 0.05$ *db/db* NT. (NT: No Treatment)

3.8 Studies in human SW872 adipocytes: Angiotensin II induces aldosterone and cortisol production and ROS generation, through NADPH oxidases-dependent mechanisms.

To explore some of the findings in our mouse models in human cells, we studied SW872 cells, an adipocyte line resembling white adipocytes. These cells were used to assess some mechanisms involved in aldosterone and cortisol production. Adipocytes were stimulated with angiotensin II (Ang II, 10^{-8}M) for 24 hours with or without Nox1/4 inhibitor (GKT137831, 10^{-6}M), Nox1 inhibitor (ML171, 10^{-6}M) and SOD mimetic (Tempol, 10^{-6}M). Levels of aldosterone and cortisol were determined by ELISA in culture media.

Figure 3.13A showed that Ang II-induced aldosterone production by adipocytes was blocked by tempol, GKT and ML171, while Ang II-induced cortisol production was blocked by tempol and GKT, but not ML171 (**Figure 3.13B**). Therefore, in human differentiated adipocytes, Ang II promoted aldosterone in a Nox1/4-dependent manner and cortisol in was Nox4-dependent. Figures 3.13 C and D show that stimulation for 24h with angiotensin II increased H_2O_2 and O_2^- production in human mature adipocytes. H_2O_2 levels were decreased with GKT (Nox1/4 inhibitor) but not by ML171 (Nox1 inhibitor) while O_2^- production was normalised with both GKT and ML171. Thus, Ang II in differentiated human adipocytes induced hydrogen peroxide (H_2O_2) through Nox4-dependent mechanisms and superoxide ions (O_2^-) production via Nox1-dependent mechanisms. These results support findings in the literature stating that Nox4 seems to produce H_2O_2 rather than O_2^- unlike other Noxes (Serrander *et al*, 2007).

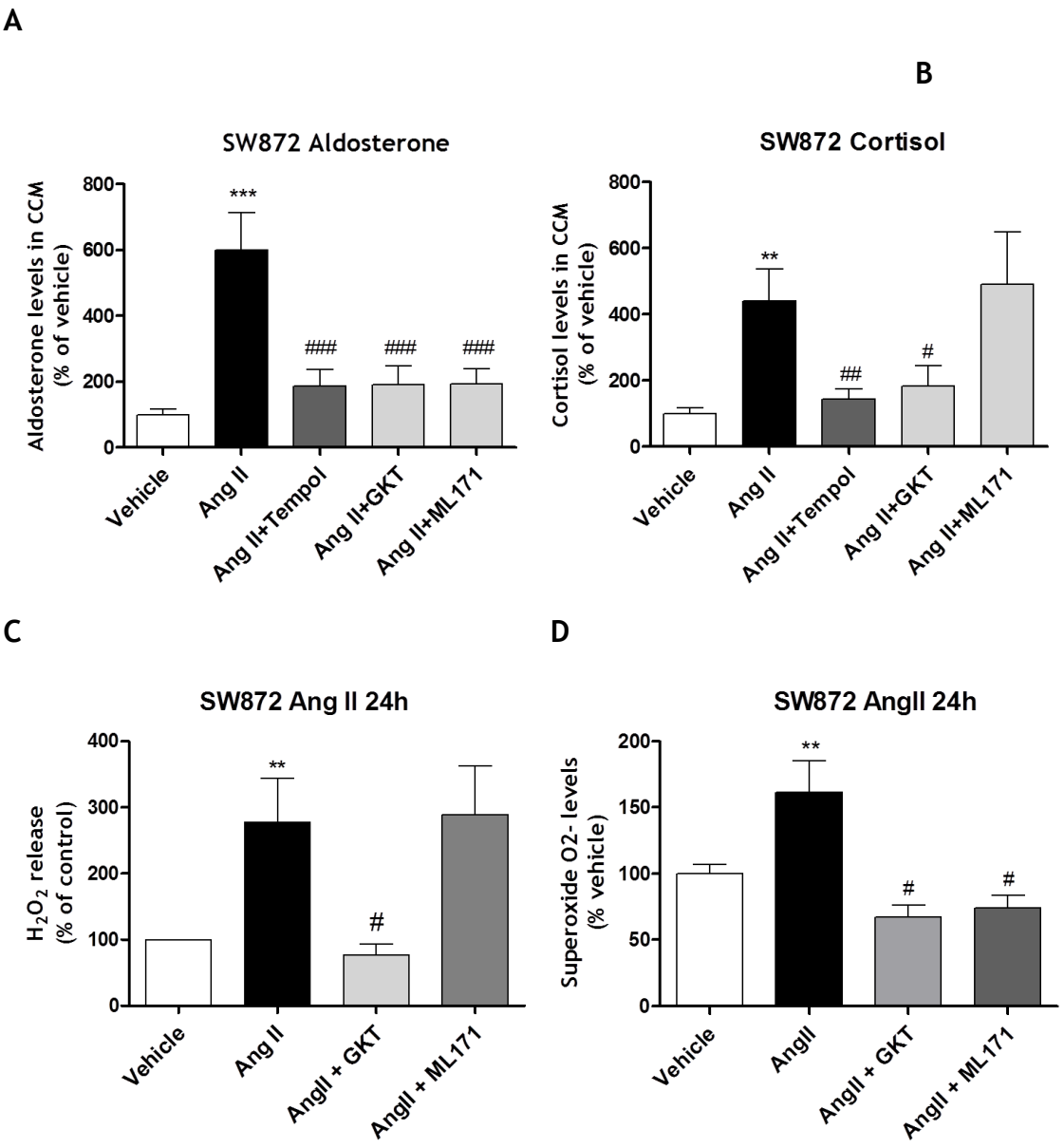

Figure 3.12. Angiotensin II enhanced aldosterone and cortisol levels and ROS generation by human differentiated adipocytes cell line SW872. Aldosterone (A) and cortisol (B) concentrations were determined by ELISA in culture media (CCM) from human differentiated adipocytes (SW872) stimulated with Ang II for 24h. Levels were normalised to protein levels. H_2O_2 (C) and O_2^- production (D) were determined by Amplex red and lucigenin assay respectively. GKT137831 1 µmol/L. Values are means±sem, n=7-8 individual sets of experiments. * $p<0.05$, *** $p<0.001$ vs vehicle, # $p<0.05$, ## $p<0.01$ vs AngII, one-way ANOVA followed by Newman-Keuls multiple comparison test. (NT: No Treatment)

4 Summary of Results

We hypothesized that in obesity associated with metabolic syndrome, adipocytes generate aldosterone through Nox1/4-dependent processes, contributing to a pro-inflammatory and pro-fibrotic adipose phenotype.

Aim 1: To evaluate whether obesity/metabolic syndrome is associated with hyperaldosteronism and increased adipocyte-derived aldosterone through processes that involve Nox1/4.

- Nox1/4 participates in adipocyte aldosterone production, an effect exacerbated in obesity.
- In Ang II-stimulated human adipocytes, Nox1/4-derived ROS contributes to aldosterone production, confirming studies in mouse adipocytes.
- In Ang II-stimulated SW872 cells, increased ROS generation was normalised by Nox1/4 inhibition.
- In Ang II-stimulated human adipocytes, Nox1/4-derived ROS contributes to aldosterone production, confirming studies in mouse adipocytes.

Aim 2: To determine whether increased Nox1/4-derived ROS influences aldosterone biosynthesis by stimulating aldosterone synthase in adipocytes.

- Aldosterone production is regulated by Nox1/4 at the gene level via aldosterone synthase.
- Corticosterone synthesis by adipocytes was not Nox1/4 dependent however, the gene expression of the glucocorticoid regulating enzymes were.
- MR signalling markers and ROS generation were increased in obese animals.

Aim 3: To assess whether aldosterone and ROS promote adipokine production and adipogenesis and if this is increased in obesity, and

Aim 4: To elucidate whether redox-sensitive processes and aldosterone promote a pro-inflammatory and pro-fibrotic adipose phenotype.

- In obese animals the anti-inflammatory adipokine, adiponectin was decreased while the adipogenic adipokine aP2 was increased. Nox1/4 inhibitor had no effects on adiponectin levels but normalised aP2 levels.

- Nox1/4 inhibitor promoted the polarisation of macrophages towards an anti-inflammatory phenotype in obese *db*/*db* mice.

5 Discussion

Metabolic syndrome – model of obesity used

Leptin is secreted from fat cells and acts on the hypothalamus to suppress the appetite and thus regulates food intake and energy expenditure. The leptin receptor deficiency (homozygous mutation of the leptin receptor (*Lepr*^{db})) in *db*/*db* mice leads to morbid obesity by preventing the homeostatic control of adipose tissue mass by leptin. Mice model, B6.BKS(D)-*Lepr*^{db}/J, used had a premature termination of the long cellular domain of the leptin receptor Ob-Rb splice form leading to the loss of its signal transducing function. In mice, the leptin receptor is alternatively spliced into five isoforms: Ob–Ra, Ob–Rb, Ob–Rc, Ob–Rd, and Ob–Re (Lee *et al*, 1996). Ob–Rb is the longest isoform expressed at high level in the hypothalamus and at lower level in other tissues including: testes, adipose tissues, pancreatic b cells, heart, lung, and lymph nodes (Fei *et al*, 1997; Lollmann *et al*, 1997). The inbred C57BL/6 genetic background arbitrated the severity of the disease by suppressing the otherwise spontaneous diabetic phenotype. Hence, the mice were morbidly obese and pre-diabetic with elevated glucose levels but would have required to be challenged in order to become diabetic (Hummel *et al*, 1972; Clément *et al*, 1998). The C57BL/6 background leads to compensatory hyperplasia of pancreatic islet beta with elevated plasma insulin as well as glucose levels starting from 10-14 days and 16 weeks respectively. Affected mice are polyphagic, polyuric and polydipsic (The Jackson laboratory, 2014; Clément *et al*, 1998). They also show hypercortisolaemia, dyslipidaemia and infertility due to hypogonadotropic hypogonadism (Coleman, 1978). The phenotype displayed by the *db*/*db* model is found in humans as a rather rare mutation in leptin or the leptin receptor genes(Farooqi *et al,* 2002). This disease model is reflected in humans only in rare genetic cases causing a genetic leptin deficiency while obesity is a form of leptin resistance and not an absolute lack off (Friedman, 2009).

The *db*/*db* mice model used exhibited features of metabolic syndrome as evidenced by elevated plasma levels of glucose, triglycerides and cholesterol (Figures 3.2 and 3.3). As expected, body weight of obese mice was greater than lean controls. The Nox1/4 inhibitor GKT137831 had no effect on body weight, but significantly reduced EVAT adiposity of *db*/*db* mice, suggesting an effect on white adipose tissue mass (Figure 3.1). Visceral fat depots have lower

hyperplasic capacity than other fat pads (DiGirolamo *et al*, 1998) resulting in greater stress on visceral than subcutaneous adipocytes in obesity. Adipocytes become hypertrophic and more rigid, as seen in our study where adipocytes in *db*/*db* mice were increased in size with increased fibrosis, thus reducing their capacity to expand with increased lipid uptake. Elevated plasma potassium in obese *db*/*db* mice was associated with high levels of plasma and adipocyte-derived aldosterone. Interestingly, the release of aldosterone is known to be stimulated by high potassium concentrations, while infusion of aldosterone lowers plasma potassium. Upon treatment with GKT, potassium and aldosterone levels were decreased. This may be due to the renoprotective effects of GKT in the kidney in *db*/*db* mice (Jha *et al*, 2014; Thallas-Bonke *et al*, 2015, Briones *et al*, 2012).

Role of Nox1/4 in adipocyte-aldosterone synthesis in obesity

Adipocytes possess Nox1, Nox2 and Nox4. The function of these Nox isoforms in adipose tissue is unclear. Our data demonstrate that Nox1/4 inhibition (GKT137831) reduces adipocyte-derived aldosterone production and inflammation in obesity. This suggests a key role for Nox1/4 in aldosterone biosynthesis and function in adipocytes. Our findings in adipose tissue were further supported by results obtained related to aldosterone levels in adipocyte conditioned media and in plasma from obese mice. To further explore the relationship between aldosterone, ROS and Noxs in adipocytes, we performed *in vitro* studies in human adipocytes (SW872), stimulated with angiotensin II in the absence and presence of the Nox inhibitor. Findings from these studies confirmed a role for Nox1/4 in ROS-mediated aldosterone production in Ang II-stimulated adipocytes.

From the systemic and local decrease in aldosterone concentrations caused by the Nox1/4 inhibitor, GKT137831, in mice, adipose tissue and cultured adipocytes, we could argue that aldosterone production by adipocytes is a Nox1/4-dependent process. Similar findings were observed in adrenal cells. Rajamohan *et al* (2012) demonstrated that Nox-derived hydrogen peroxide participates in Ang II-induced aldosterone production in adrenocortical cells. Hence adipocytes may share common redox-sensitive aldosterone-producing pathways as adrenal cells. Adipocyte-derived aldosterone production was

decreased in a dose dependent manner while plasma aldosterone was not further decreased by high dose of the Nox1/4 inhibitor. The adrenal gland (AG) is known to be the main source of aldosterone. However, due to its interaction with AT and the production of aldosterone by the AT one may wonder if, in obesity, the AT participates in systemic aldosterone levels. The increase in adipocyte number (hyperplasia) and size (hypertrophy) could further promote aldosterone production in obesity. Furthermore, AT stimulates release of AG-derived aldosterone via mineralocorticoid-releasing factors (Ehrhart-Bornstein *et al*, 2003). Thus, in obesity AT may participate in peripheral aldosterone actions.
All components of the RAAS are expressed in adipose tissue along with the AT_1R, AT_2R, renin, mineralocorticoid and glucocorticoid receptors. In obesity, adipose RAAS is over-activated and thus may participate in metabolic syndrome along with aldosterone/MR activation (Kulapahana & Mustaid-Mussa, 2012; Rahmouni *et al*, 2004).

To assess the significance of adipocyte-derived aldosterone and its specific physiopathological role in obesity, the study of a transgenic mouse model with adipocyte-specific aldosterone synthase deletion on a high fat diet would be relevant as well as obese *db*/*db* mice submitted to adrenalectomy. These studies would help elucidate what component of aldosterone is adrenal-independent.
In obesity, Nox1/4-derived ROS promote adipocyte-aldosterone synthesis and could induce AT damage such as fibrosis and inflammation, and this may be through aldosterone-dependent signalling pathways. The effects of Nox1/4-derived ROS on aldosterone production may trigger a vicious circle as aldosterone has been shown to promote oxidative stress as well. ROS feeds into aldosterone production and *vice versa* leading to a vicious feedforward circle. This may contribute to the overall increase in AT dysfunction and inflammation in obesity.

Role of Nox1/4 in Mineralocorticoid signalling in obesity

The mineralocorticoid receptor (*MR*) gene expression in adipose tissue was decreased in obese *db*/*db* mice. However, the gene expression of MR activation markers (*Sgk1* and *Ngal*) was increased, indicating augmented MR signalling in obese *db*/*db* mice. Nox1/4 inhibition increased *Ngal* and had no effect on *Sgk1* gene expression. Ngal also known as lipocalin-2 is associated with a variety of

diseases as well as promoting antioxidant enzymes superoxide dismutase (SOD) and heme oxygenase (HO-1) gene expression (Bahmani P *et al*, 2010). GKT may promote antioxidant effects of Ngal, thus reducing oxidative stress occurring in obesity. Moreover, high levels of corticosterone in obese mice were unaltered by GKT, suggesting a targeted action of GKT treatment on aldosterone production. We cannot exclude the possibility that MR may also be activated by glucocorticoids (GCs). GCs exert pleiotropic effects on adipocyte metabolic, endocrine and immune functions and may explain why GKT did not completely decrease inflammation and had little effects on fibrosis in adipose tissue from *db*/*db* mice, despite reduced aldosterone production. Future studies will evaluate the GC/GC receptor in adipocytes in obesity and the role of Nox-ROS.

Nox1/4 and ROS production in adipocytes in obesity

NADPH oxidase (Nox)-derived reactive oxygen species (ROS) have been implicated in adipocyte biology including adipogenesis and adipocyte differentiation (Mouche *et al*, 2007; Schröder *et al*, 2009; Liu *et al*, 2012). Noxes tend to be upregulated in AT from obese mice (Furukawa *et al*, 2004).

In our study only the phagocytic *Nox2* mRNA levels were increased in adipose tissue from obese *db*/*db* mice. This may be due to macrophage infiltration in adipose tissue in obesity, which contributes to adipose inflammation (Figure 3.8c). As Nox4 is a constitutively active enzyme, its gene expression is expected to reflect changes in protein levels (Serrander *et al*, 2007). It would be of interest to determine Poldip2 levels of expression and co-localisation with Nox4. Lyle *et al* (2009) demonstrated that Poldip2 co-localizes with Nox4 and Nox1 at p22phox at sites in SMCs and enhances Nox4 –ROS production by three-fold. Nox1 mRNA levels in EVAT were not different across the groups and were decreased in PVAT from *db*/*db* NT mice compared to controls.

In the present study there were some disparities in oxidative stress between fat depots, EVAT and PVAT. Obese *db*/*db* mice displayed elevated TBARs in EVAT while PVAT TBARS and hydrogen peroxide levels were not significantly different from the lean control mice. These data indicate that differences in redox status exist between adipose tissue depots and their contribution to metabolic complications may be different. It is also possible that different types of adipocytes (white, brown or beige) may exhibit different redox state and inflammatory phenotype. The lack of decrease in ROS production seen in PVAT

from treated *db*/*db* mice may also be due to the lower levels of *Nox1* gene expression in obese mice compared to lean mice. The lack of effect might be because there was no increase in ROS/TBARS in PVAT and that blocking Nox1/4 would have been countered in order to maintain ROS levels. It could have been caused by a decrease in antioxidants or increase in other sources of ROS. Thus looking into antioxidants as well as the protein levels of the Noxes would be of interest. The selectivity of GKT137831 for Nox1/4 may also be questioned even if GKT137831 has been used in a few studies as a Nox1/4 inhibitor (Gorin *et al*, 2015; Jiang *et al,* 2012). The effects of GKT can be assessed by the indirect means of ROS production as seen in the cell experiments. Mature human adipocytes stimulated with angiotensin II for 24h demonstrated that superoxide ions O_2^- production was decreased with Nox1 and Nox1/4 inhibitors while H_2O_2 levels were decreased only with Nox1/4 inhibitor. These results follow the trend of thought that Nox4 produces hydrogen peroxide and Nox1 superoxide thus showing be indirect measurements that the inhibitors used in the study inhibit the expected targets. For further clarity labelling the GKT would permit to see if it does specifically bind only to Nox1 and Nox4 in mice which could be of interest.

Adipocyte inflammation, fibrosis, aldosterone and ROS in obesity

Adipose tissue and inflammation

Several studies have demonstrated in obesity, dysregulation in production of pro-inflammatory adipokines such as TNF-α, IL-6, MCP-1 and angiotensinogen, and in protective adipokines such as adiponectin and leptin. The underlying mechanisms from excess fat storage to AT dysfunction have not yet been fully elucidated. Systemic oxidative stress has been shown to be correlated with fat accumulation and thus may participate to the development and progression of metabolic syndrome (Keaney JF *et al* 2003; Furukawa *et al* 2004). Our results confirm others by showing a decrease in protective adipokine adiponectin levels and increased macrophage infiltration in obese mice. A study by Yamauchi *et al* (2007) showed adiponectin receptor 1 and 2 deficient mice had enhanced oxidative stress. While, Furukawa *et al* (2004) demonstrated that the decreased adiponectin levels can be reversed by apocynin, a ROS scavenger. However, in our study, inhibition of Nox1/4 did not normalise adiponectin levels in obese *db*/*db* mice. It is possible that other sources of ROS such as the mitochondria,

xanthine oxidase enzymes, endoplasmic reticulum and other Noxes for example could affect adiponectin levels. The loss of protective effects by adiponectin could have promoted pro-inflammatory cytokine production and possibly insulin resistance thus leading to AT damage in *db*/*db* mice.

The increase in macrophage marker F4/80 in AT from obese *db*/*db* mice indicate that obese mice have more macrophages in the AT compared to lean controls. On the other hand it does not necessarily indicate the type of macrophage polarisation (Figure 3.11A). Macrophages are commonly found in obese as well as lean WAT (Feuerer *et al*, 2009), although the prevalence increases with adipocyte hypertrophy (Weisberg *et al*, 2003). M1 macrophages play a central role in innate immunity while M2 macrophages are associated with anti-inflammatory processes, tissue remodelling and fibrosis (Natoli & Monticelli, 2014). The M1/M2 ratio would provide indications with regards to the inflammatory status of the AT. In obese *db*/*db* mice the M1 markers *iNOS* and *TNF-a* gene expression were elevated and iNOS was normalised by GKT137831. M2 markers *Arginase-1* and *CD206* mRNA levels were elevated and *Arginase-1* was further increased with treatment hinting at a push towards M2 type polarisation of macrophage with GKT treatment. IHC complementary studies would enable us to determine if there are crown like structures indicating increased adipocyte cell deaths and further AT dysfunction (Cinti *et al*, 2005). Whether the changes in macrophage polarisation are due to Nox1/4-derived ROS or aldosterone, or both, cannot be determined from these experiments. *In vitro* experiments using culture media from differentiated adipocytes stimulated with aldosterone along with GKT to stimulate monocytes would be further studies to determine the effects specific to aldosterone on differentiation of these stimulated monocytes into macrophages. Further more FACS experiments and protein levels would be of interest and give stronger information on the status of macrophage polarisation than mRNA levels.

Adipose tissue and fibrosis

Fibrosis is an important feature in dysfunctional adipose tissue in obesity. Previous studies demonstrated that Nox1/4 plays an important role in fibrosis in various organs, e.g. liver, kidney, vessels, effects that are blocked with GKT137831 (Aoyama *et al*, 2012; Babalola *et al*, 2014; Jiang *et al*, 2012).

Whereby, the semi-quantitative analysis of Picro Sirius red stained collagen with thick fibres (collagen I) was seen as an orange/red colour compared to collagen with thinner fibres (collagen III) had green/yellow colours. Divoux *et al* (2010) demonstrated a build-up of thick fibres (Red coloured collagen) in AT of obese subjects leading to the focus on red coloured collagen seen via polarised light microscopy. From the Picro-Sirius Red staining and the polarised microscopy data, we showed pronounced remodelling in AT from obese mice indicated by the increase in fibrillary collagen content in obese mice compared to lean controls (Figure 3.10). Obese treated mice did not show a significant decrease in thick fibered collagen contents. We also assessed collagen 6 levels as studies on *ob*/*ob* mice lacking collagen 6 have demonstrated significantly improved glucose metabolism and lacking fibrotic depots (Khan *et al*, 2009). Collagen 6 gene expression was enhanced in obese *db*/*db* animals as seen in other studies. However the treatment had no effect. Rather than affecting collagen 6, Nox1/4 role may impact on other collagens or matrix metalloproteinases which have the capacity to degrade the extracellular matrix.

Translating findings to human adipocytes

From the *in vitro* studies we showed that adipocyte-derived aldosterone production is Nox1/4 dependent while adipocyte-cortisol production is Nox4-dependent in human adipocytes. These data are in line with the mouse studies and highlight a potentially important role for Nox1/4 in adipose biology, especially in conditions associated with obesity and hyperaldosteronism. Nox1/4 may be an interesting therapeutic target in obese patients with associated hyperaldosteronism.

General conclusion

Obesity is a global problem that is reaching pandemic proportions. It is a major contributor and risk factor for metabolic and cardiovascular diseases. Central to the development of obesity is adipose tissue – a dynamic and metabolically active organ. Thus, it is necessary to understand the mechanisms leading to adipose tissue dysfunction in obesity. Compelling experimental evidence indicates that ROS and hyperaldosteronism are elevated in adipose tissue in obesity and may participate in obesity associated inflammation leading to metabolic and cardiovascular diseases. In this study we explored molecular mechanisms whereby adipocytes become pro-inflammatory and pro-fibrotic in obesity, focusing on the role of Nox-derived ROS and aldosterone.

Our findings demonstrate that in a mouse model of obesity:

- Nox1 and Nox4 participate in adipocyte aldosterone production.
- Nox1/4 inhibition decreased epididymal visceral fat adiposity.
- Adipose tissue ROS levels differ between fat depots.
- Nox1/4 inhibition had a tendency to decrease fibrosis and promote macrophage polarization towards an anti-inflammatory phenotype.

In conclusion our novel findings highlight an important role for Nox1 and Nox4 in adipocyte-derived aldosterone production and inflammation in obesity. Thus, Nox1/4 inhibition may be a putative therapeutic target in obesity associated hyperaldosteronism and associated cardiovascular disease.

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