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THE LIPID BILAYER IN GENETIC HYPERTENSION

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AIMS OF STUDY

Membranes of cells isolated from genetically hypertensive rats or patients with essential hypertension when compared with those from normotensive controls, have been shown consistently to have greater microviscosity (lower fluidity), as monitored by the fluorescence polarisation or electron spin resonance spectra (Tsuda *et al;* 1987). The lipid bilayer of cells is, however, complex in nature and its study often confined by this complexity. Many cellular biochemical and biophysical events occur in the membrane where strict structural and dynamic features provide the control mechanisms and there is a reported correlation between changes in membrane fluidity and normal membrane functions (Dominiczak *et al;* 1991). Two methods used to measure membrane fluidity in this study are; fluorescence anisotropy and fluorescence recovery after photobleaching (FRAP). The cells of study are cultured vascular smooth muscle cells (VSMC), VSMC membranes and erythrocyte membranes.

Objectives of the study are to examine fluidity in intact membranes and membrane preparations of cultured vascular smooth muscle cells (VSMC) from stroke-prone spontaneously hypertensive rats (SHRSP) and Wistar-Kyoto (WKY) rats. Membranes from a segregating (F_2) hybrid produced by crossing SHRSP and WKY rats are used to determine whether elevated microvisosity is associated with elevated arterial pressure. These studies may contribute to an understanding of the genetic and possible causal relationship between membrane fluidity, hypertension and the possible effects on cellular homeostasis. Extensive *in-vitro* studies described that

significant modification of membrane physico-chemical structure may be achieved by small changes in fatty acid composition within the membranes (Remmers *et al;* 1990, Stubbs & Smith, 1984). Analysis of fatty acid composition in plasma membranes of VSMC from SHRSP and WKY was carried out in parallel with FRAP to see if fatty acid composition could be related to abnormalities on membrane fluidity. Also the possible effects of cholesterol on membrane fluidity will be investigated by enriching cultured VSMC from SHRSP and WKY rat strains.

The hypothesis is that the generalised dysfunction of diverse protein transport systems of the membrane in hypertension is caused by a fault in the common matrix in which they all function, the lipid bilayer.

INTRODUCTORY REVIEW

Hypertension

Coronary heart disease is the leading cause of death in many Western industrialised countries, including Scotland. High blood pressure or hypertension is associated with increased risk of coronary heart disease. Hypertension is characterised by normal cardiac output and an elevated total peripheral resistance. The "normal" arterial pressure in humans is in a region whereby enough blood can reach the critical organs and maintain their function and also be low enough to avoid excessive "wear and tear" on the vasculature. Higher pressures are associated with higher mortality and among the reasons why some individuals have higher blood pressure are: an individual's genetic make-up, lifestyle and/or disease.

Hypertension frequently accompanies renal disease and a number of complex biological pathways have been implicated. One of the factors associated with hypertension is that of vascular hypertrophy (increase in smooth muscle mass of a blood vessel) which is a major contributor to increase in blood pressure (Naftilan, 1992). It is unclear whether these vascular changes are a cause or consequence of elevated blood pressure (Bruner *et al;* 1986). The vasculature itself is under control of extrinsic factors (neuronal and hormonal influences) or intrinsic factors to the blood vessel (that is structural or functional alterations) (Bruner *et al;* 1986). It is apparent that there is a complex balance of vasodilator and vasoconstrictor

substances in the body. The renin-angiotensin (RAS) has been linked to cell growth. Renin, acts upon angiotensinogen (produced mainly by the liver) and catalyses the release of the decapeptide angiotensin I (ANG I). The physiologically active octapeptide, angiotensin II (ANG II) is released from ANG I by an angiotensin converting enzyme (ACE), which is located mainly on the surface of endothelial cells (Caldwell *et al;* 1976). Ang II affects blood pressure in a number of ways such as increasing total vascular resistance by direct effects on the resistance vessels and by facilitation of the sympathetic nervous system (Skøtt & Jensen, 1993). The molecular mechanism by which Ang II increases smooth muscle cell growth in not well defined (Naftilan, 1992). Excretion of salt and water is influenced by stimulation of the adrenal production of aldosterone and renal medullary blood flow. The RAS system has received attention as a local growth factor that may be involved in hypertrophy of heart and blood vessels during chronic heart failure and hypertension.

Hypertension has a multifactorial aetiology. Changes in blood pressure are produced when resistance of vessels is affected. These changes control the smooth muscle of the vasculature and involve three regulatory systems. These are:

1.nervous regulation (vascular agonists involved here include catecholamines and serotonin),

2. humoral regulation (blood volume is regulated by this system and adaption of the resistance vessels to change in order to maintain arterial pressure. Agonists here include angiotensin and vasopressin present within the bloodstream),

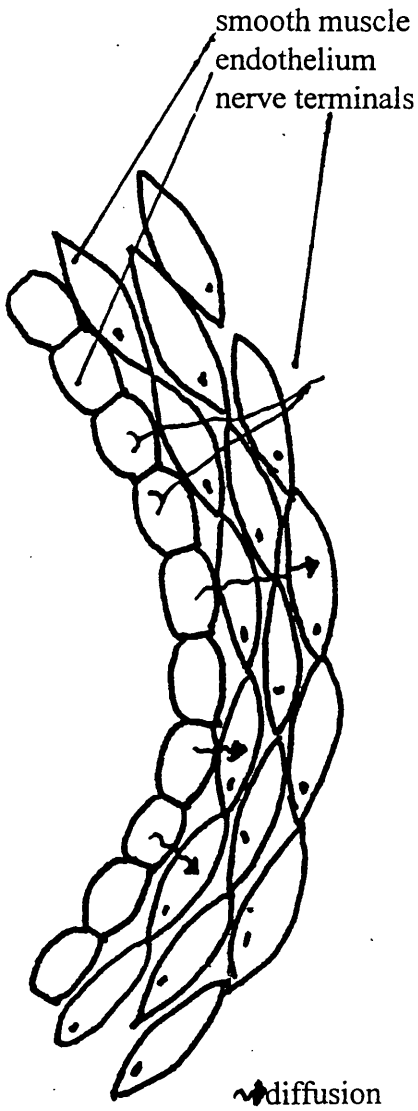
3. there is a local regulatory system that maintains the flow of blood through the tissues. These hormones and peptides reach the smooth muscle by simple diffusion (such as prostacyclin and kallikreins).

Recent years have revealed the major biological roles of endothelium derived relaxing factor, now identified as nitric oxide (NO) by Moncada and colleagues (see Snyder & Bredt, 1992). This chemical is an important messenger molecule and it has been linked as a neurotransmitter which dilates blood vessels (Snyder & Bredt, 1992). Production responds to changes in blood flow. Acetylcholine is a convenient laboratory tool for stimulating NO production but is probably not an important physiological agonist. Acetylcholine binds to endothelial cells on the blood vessel walls and these release NO which travels to adjacent muscle cells and causes them to relax. A normal artery responds to these agonists with a sensitivity which depends on aspects of physiological status such as sodium balance, posture and/or genetic background. This sensitivity is enhanced in hypertension. Figure 1 is a diagrammatic representation of the complexity of the regulatory systems involved in the homeostasis.

Hypertension and the regulation of smooth muscle cells

The elevated vascular resistance of hypertension is at least partly the result of increases in vessel wall thickness and in vascular smooth muscle reactivity (Dominiczak *et al;* 1991). These structural and functional changes occur in resistance vessels and have been attributed to increases in intracellular calcium concentration (which can stimulate cell growth and also activate smooth muscle contraction).

At the cellular level, hypertension has also been associated with abnormalities in many membrane transport systems (Dominiczak & Bohr, 1990), such as pumps, channels and exchangers.



Lumen

Systemic factors

+angiotensin II
+vasopressin
neurotransmitters:
(serotonin)
-acetylcholine
noradrenaline etc)
corticosteroids

Local factors

+angiotensin II
prostacyclin
-nitric oxide
(neurotransmitters)

+ vasoconstrictor
- vasodilator

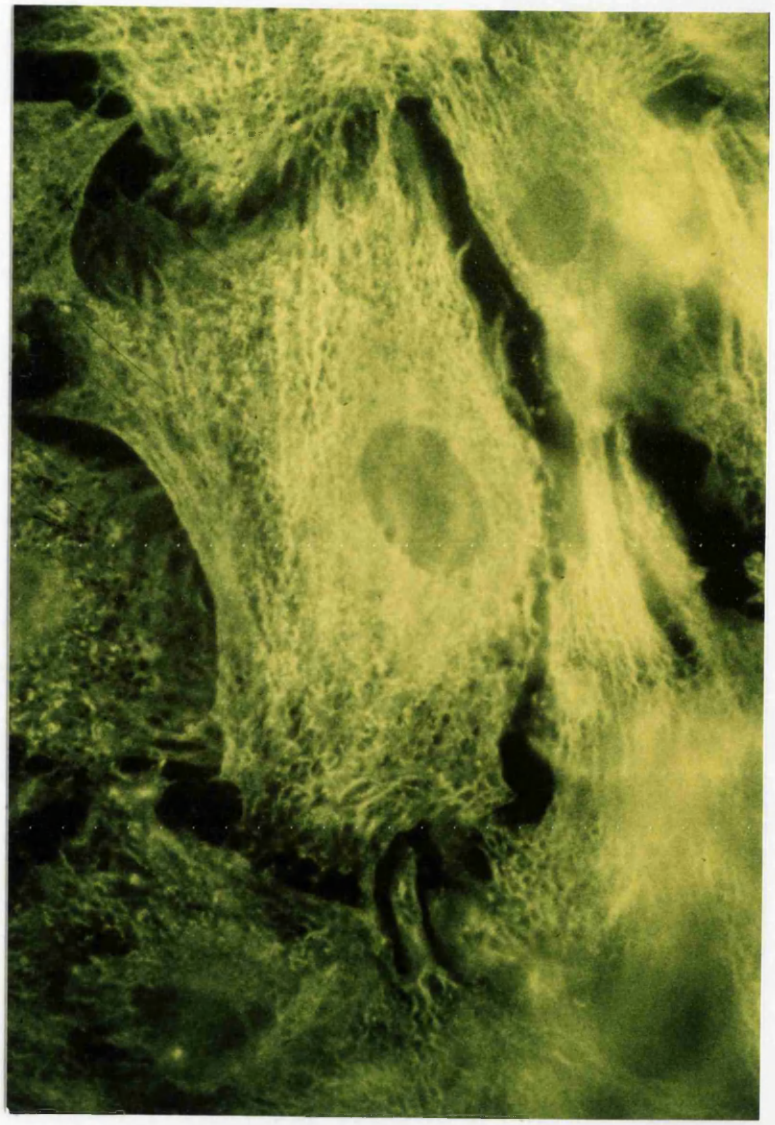
Fig 1 Major regulatory systems controlling vascular tone

These abnormalities may cause major alterations of vascular structure and function.

Vasoconstrictor agonists can attach to specific receptors and through second messengers release calcium ions from the sarcoplasmic reticulum or other calcium stores. Muscle cells of the body's vasculature have a specialised organelle, the sarcoplasmic reticulum, which sequesters calcium ions from the cytosol. The Ca-ATPase that pumps calcium ions into the organelle from the cytosol is the major membrane protein in the sarcoplasmic reticulum. Calcium release from the sarcoplasmic reticulum plays some role in tension development, but most agonists depend on an increase in calcium uptake for maintained tension development. The release of calcium from this organelle and its subsequent re-uptake from the cytosol mediates the rapid contraction and relaxation of contractile proteins during a muscle contraction. Force generation results from cyclic interactions of myosin and actin. The energy for the reaction is the hydrolysis of adenosine 5'-triphosphate (ATP) which is regulated by phosphorylation of the myosin light chain. The enzyme responsible for the phosphorylation (myosin light chain kinase) is activated by calcium. There is also a Ca^{2+} activated phospholipid-dependent protein kinase C which may regulate smooth muscle contraction. Figure 2 shows mesenteric VSMCs in culture using an anti-α smooth muscle actin antibody followed by an anti-mouse immunoglobulin G fluorescein isothiocyanate conjugate.

The increase in calcium ions in the cytosol is transient because calcium ions are rapidly pumped back by the abundant Ca-ATPase in the sarcoplasmic reticulum (Alberts *et al;* 1989). Calmodulin binds up to four molecules of calcium ions and mediates calcium ion signalled responses in all cells (Alberts *et al;* 1989). It is responsible for maintaining calcium homeostasis during the life of the cell and for resting levels of intracellular calcium. Sodium-calcium (Na-Ca) exchange has also been proposed as an additional calcium

Fig.2 Photomicrograph of mesenteric smooth muscle cells that reacted with anti-α smooth muscle actin antibody followed by an anti-mouse immunoglobulin G fluorescein isothiocyanate conjugate (Mag x1800) Courtesy of Dr. A. Dominiczak.



extrusion mechanism (Vemuri & Philipson, 1990). Changes in sodium permeability of cells and in activity of sodium-potassium pumps have been considered as possible cellular processes responsible for the changes in vascular reactivity associated with hypertension (Dominiczak *et al;* 1991). Dominiczak and Bohr, 1991, observed that the protein channels for sodium, potassium and calcium are more active than normal in hypertension, causing the membrane to be more permeable to these ions, thereby giving rise to an increase in vascular reactivity (Dominiczak & Bohr, 1991).

Bialecki *et al;* 1991, showed that enrichment of VSMC with free cholesterol resulted in significant increase in accumulation of intracellular calcium. Vascular reactivity was also noted to be increased, resembling the hypertensive state.

It is therefore possible that an alteration in the physical property of a hypertensive lipid bilayer influences the transport functions of the integral proteins leading to an increase in $[Ca^{2+}]_i$ in vascular smooth muscle cells. This in turn contributes to increased vascular tone and reactivity (Dominiczak & Bohr, 1990).

Lipid bilayer of the cell membrane

The observation that the channels (eg. Ca channels), exchangers (eg. Na/Ca exchangers) and pumps are situated in the lipid bilayer and that their abnormal function will have profound effects on the cell has highlighted the composition of this bilayer as a generalised membrane abnormality (Naftilan *et al;* 1986).

Cell membranes contain a lipid bilayer which has an amphiphilic structure. It was first described, in 1972 by Singer and Nicholson

(Singer & Nicholson, 1972) as proteins in a 'sea' of lipid. Although nowadays their description is seen as an oversimplification, it provided a model from which emerged more detailed models of the structure of the cell membrane and thus how it performed its plethora of functions.

The membrane is a highly selective filter and an active transport device and essentially controls the cell in accordance with its environment. Dynamically, the lipid bilayer is highly anisotropic- much of the interior of the bilayer is well ordered and only a small region in the middle is liquid (Yeagle, 1989). Cell membranes usually work best when the lipid bilayer is in the liquid crystalline state (Yeagle, 1989). Lipids and proteins can diffuse in the plane of the membrane over fairly long distances or they may be anchored to the cytoskeleton showing little capability of lateral diffusion (Alberts *et al;* 1989). The embedded integral proteins serve as receptors, channel, exchangers and active pumps.

The lipid bilayer characteristics make it an ideal structure for cell membrane in that it is fluid, which is crucial for any of its functions. Lipid composition of the two halves of the lipid bilayer have been shown to be different in a number of membranes studied. Phospholipids, sphingomyelin (SP) and phosphatidylcholine (PC) tend to predominate in the outer membrane (exofacial leaflet) of the lipid bilayer. The fatty acid tails of SP and PC are more saturated (that is no double and/or triple bonds) than those of the phospholipids- phosphatidylethanolamine (PE) and phosphatidylserine (PS) and this creates an asymmetry in distribution of hydrocarbon tails. This asymmetry in distribution of hydrocarbon tails may make the endofacial monolayer somewhat more fluid than the exofacial monolayer. In addition, because negatively charged PS is located in

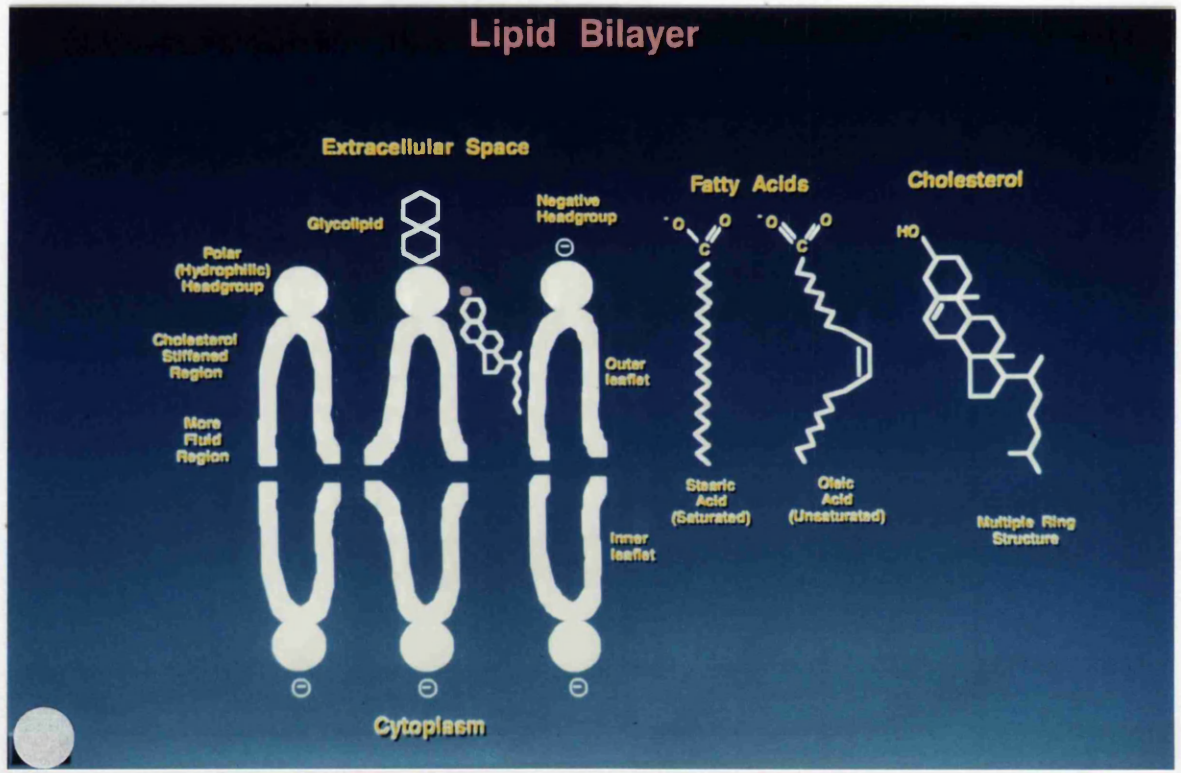
the monolayer, there is a significantly different charge between the two monolayers (Yeagle, 1989)

Cholesterol and glycolipids are two other major types of lipids in the plasma membrane. Cholesterol is a steroid with a planar ring structure which is an important addition into the membrane of many cell types. It inserts in the exofacial leaflet of the lipid membrane only and has a ridgifying effect. Cholesterol leads to an increase in anisotropic motional ordering of the lipid bilayer of the membrane due to the effects of the rigid sterol ring structure on the lipid components of the membranes (Yeagle, 1989). Figure 3 shows a diagrammatic model of the lipid membrane with cholesterol inserted into the exofacial leaflet

Many cells have receptors for the uptake of cholesterol. In human blood the major cholesterol-carrying particle is low-density lipoprotein (LDL). LDL plays a vital role in transporting cholesterol in the body and cholesterol is an essential component of all cell membranes (Lawn, 1992). Cholesterol level and high levels of LDL have been established as a definite risk for coronary heart diseases. Incorporation of free cholesterol into VSMC membrane has been shown to lower membrane fluidity and this may affect the function of membrane proteins situated in the lipid structure (Bialecki *et al;* 1991)

Glycolipids are oligosaccharide-containing lipid molecules found only in the exofacial leaflet of the bilayer (see Figure 3). Their sugar groups are exposed at the cell surface thus suggesting some role in interaction of the cell and its surroundings. There is remarkable variety between cells and in animal cells they are always produced from ceramide. Glycolipids are distinguished from one another by their polar head group, which consists of one or more sugar residue

Fig.3 Diagram of the lipid bilayer. It shows the features of the bilayer- outer and inner leaflet, asymmetric charge and cholesterol inserted in the outer leaflet. Fatty acids can be either saturated (no double bonds) or unsaturated (containing double and/or triple bonds).


Lipid Bilayer
Extracellular Space
Glycolipid
Negative Headgroup
Polar (Hydrophilic) Headgroup
Cholesterol Stiffened Region
More Fluid Region
Outer leaflet
Inner leaflet
Cytoplasm
Fatty Acids
Stearic Acid (Saturated)
Oleic Acid (Unsaturated)
Cholesterol
HO
Multiple Ring Structure

(Alberts *et al,* 1989). The most complex of the glycolipids, the gangliosides, contain one or more sialic acid residue which gives them a negative charge. Glycolipids may serve as receptors for normal signaling between cells.

Fatty acid composition of the lipid bilayer

Fatty acids are important sources of [illegible] energy in the cell and they are also necessary as building bricks for cell structure. There are a great variety of fatty acids which vary in the number of carbon atoms that make up the molecule and in the number of double bonds present. Oleic (C18:1), a monounsaturated fatty acid and stearic (C18:0), a saturated fatty acid, for example, have 18 carbon atoms in their acyl chain whereas palmitic fatty acid has only 16 (C16:0) carbon atoms. This number of carbon molecules in a fatty acid chain affects its length and the presence/absence of double/triple bonds affects the fluidity of the lipid bilayer. If one single genetic defect is responsible for a generalised abnormality, Naftilan *et al;* 1986, speculates that a defective fatty acid desaturase or elongase may be critical determinant of the hypertensive state. Failure to incorporate fatty acyl chains of the appropriate length and degree of saturation would lead to changes in membrane fluidity and consequently changes in membrane protein conformation and function (Naftilan *et al;* 1986).

The two important lipid determinants of fluidity in cell membranes are cholesterol and *cis* unsaturated fatty acyl group ratios. The presence of even a single double bond is sufficient to exert a profound influence on physical properties (Stubbs & Smith,

1984) on the membrane. The position of the double bonds appear to be more important. Remmers *et al;* 1990, showed *cis* fatty acids to be more potent than *trans* isomers in decreasing microviscosity of the membrane. This is because of steric hindrance of *cis* isomers disturbing the lipid packing density and thus strongly affecting membrane microviscosity (Stubbs & Smith, 1984).

The term "order" as applied to fatty acyl chains, implies a restriction of mobility and forms the basis for the interpretation of certain aspects of physical data on membranes. The fluid mosaic model, as previously mentioned, (Singer & Nicholson, 1972), led to the idea that membrane proteins possessed almost complete freedom of lateral motion. Cytoskeletal elements have now also been incorporated into the story as imposing severe restrictions on lateral protein mobility (Stubbs & Smith, 1984). A quantative analysis of membrane fluidity is more useful and is given by a description of lipid motion in two terms- one describing the rate of motion and another which relates to an orientation parameter. An increase in rate of motion of acyl chains and decrease in acyl chain order is related to the presence of double bonds. Further analysis of fatty acid composition within the membranes of the SHRSP and WKY can be quantatively analysed by high performance liquid chromatography (HPLC). This form of analysis is sensitive and was used in the study to investigate the possibility that one or more fatty acid is prevelant in either the SHRSP and WKY rat strains.

The majority of studies of lipid bilayer composition and its functional properties in hypertension have been formed in circulating blood cells such as red blood cells and platelets. Red cells (erythrocytes) are frequently used as more is known about them than any other eukaryotic cell and also because they have no

nucleus- the plasma membrane is the only membrane. One factor contributing to or potentially causing hypertension is an inherent membrane structure abnormality that alters the physical state of the membrane and thereby modifies membrane related cellular functions (Dominiczak *et al;* 1991). Dominiczak *et al,* 1991, studied red blood cells (and suggests as Naftilan *et al,* 1986 does for platelets) that the abnormality may be generalised, affecting the membrane of all cell types in the body, including smooth muscle cells.

The vascular smooth muscle cell is the most relevant cell to study as VSMC are directly involved in increased vascular reactivity and medial hypertrophy (both of which are associated with hypertension) (Dominiczak *et al;* 1991). The study of this cell can be facilitated by the use of animal models and the culturing of cells *in vitro.*

Animal models in genetic hypertension

Many human diseases show a strong familial pattern of transmission and must therefore be considered, at least in part, to be genetically determined heritable diseases (Lindpaintner, 1992) and identification of the offending genes will allow a more targeted approach and ultimately provide the basis for prevention and cure (Lindpaintner, 1992). Hypertension can be induced artifically in rats by successive segregation and recombination of genes. Selective breeding of several species, principally the Wistar rat has resulted in the development of a genetically hypertensive strain, spontaneously hypertensive rat (SHR) and its stroke prone substrain (SHRSP).

The SHR and SHRSP rat have been widely studied animal models of essential hypertension and its cerebrovascular complications (Nabika, *et al;* 1991). Blood pressure and the incidence of stroke are strongly influenced by genetic factors in SHR and SHRSP (Okamoto *et al;* 1974) and thus extensive effort has been taken to identify the genes responsible for hypertension.

Inbred strains

Experimentally, inbred strains, (that is genetically homogenous) are crossbred to yield a uniformly heterozygous F_1 population which is then brother- sister mated. This results in an F_2 generation which is tested for cosegregation of the phenotype and genotype (the marker) (Lindpaintner, 1992). If the marker is in direct association with the phenotype then the candidate gene is implicated as the pathogenic basis of a given condition and further analysis should reveal a mutation which affects structure and function or regulation of the gene product (a peptide or protein). The value of inbred animal strains, provided environmental factors are stringently controlled, is the lack of interindividual variation regarding both the property under investigation and the genetic background (Lindpaintner *et al;* 1992). The test to show if a polymorphic marker is relevant to the hypertensive phenotype is that the marker must show cosegregation with blood pressure in a freely segregating F_2 cohort bred from the two parental strains (Lindpaintner *et al;* 1992).

Bruner *et al;* 1986, reported observations consistent with the hypothesis that a single genetic locus determines the norephinephrine-induced oscillatory activity difference between the

WKY and SHRSP strains. Montenay-Garestier *et al;* 1981, reported elevated microviscosity in erythrocytes membranes in SHR. In a fluorescent probe technique study, Orlov *et al,* 1982, showed membrane fluidity to be increased with DPH (the 'core' probe) in erythrocyte membranes isolated from spontaneously hypertensive rats (SHR) compared with those membranes in the commonly used normotensive strain, the Wistar-Kyoto (WKY) rat. The use of ESR with a fatty acid spin label revealed that erythrocyte membranes of SHR and human subjects with essential hypertension had different spin motions than from those of normotensive controls and that fluidity was decreased in hypertension (Tsuda *et al,* 1987). Calcium loading of erythrocytes caused decreased membrane fluidity which was pronounced in erythrocytes from SHR and humans with essential hypertension with respect to normal controls. The authors suggest an abnormality of calcium handling at cellular level might affect the physical properties of biomembranes in subjects with hypertension. However, hypertension is considered to be polygenic in origin, where multiple genes contribute to the individuals phenotype.

Use of molecular probes to measure membrane microviscosity

Fluorescent probes have been used to evaluate the fluidity of lipid bilayers in biological membranes. Membranes are highly anisotropic 3-dimensional systems. Fluorescence anisotropy requires an extrinsic fluorescent probe to be embedded in the phospholipid bilayer and the fluorescence depolarisation is observed under continuous illumination by vertically polarised light. The technique is widely

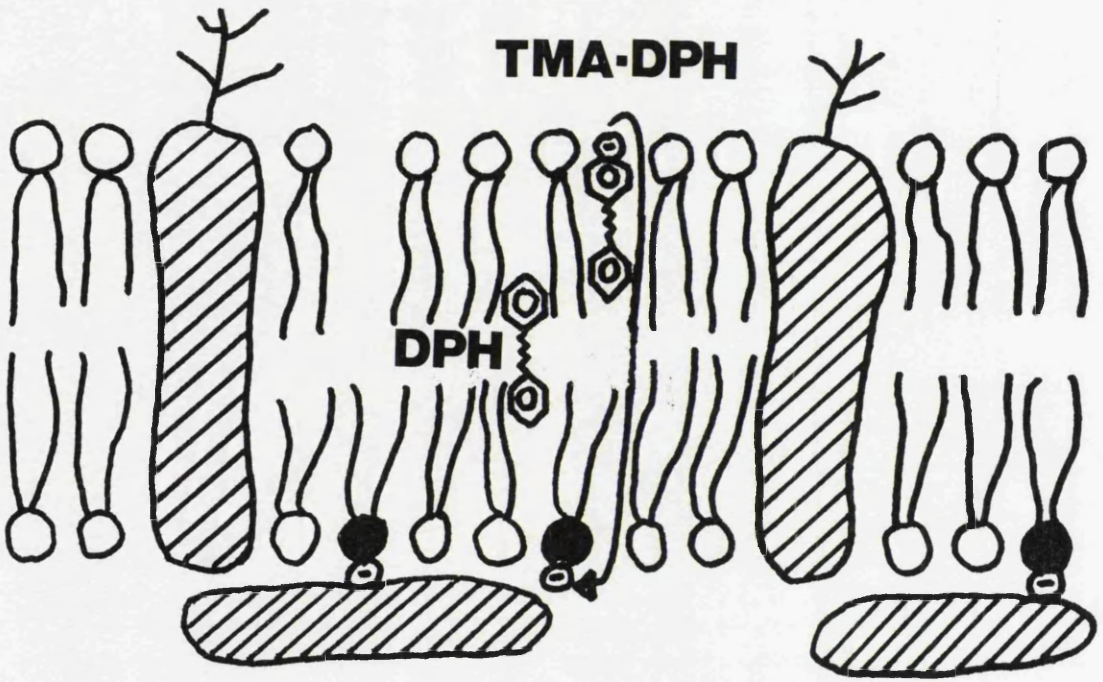
employed because of its sensitivity and flexibility, although it fails to resolve the dynamic from the static state (Kuhry *et al;* 1985).

The fluidity of the bilayer is basically a property of the motional state of the lipid acyl chains, but the lipid headgroups also have a significant effect on acyl chain motion (Stubbs & Smith; 1984). The value of a physical parameter obtained is highly dependent on the type of probe and its location in the membrane.

Molecules of known characteristics can be used to probe an unknown environment. DPH (1, 6-diphenyl-1, 3, 5-hexatriene) is the classic "fluidity" probe which localises within the hydrophobic core of the membrane and does not provide specific information (Kubina *et al;* 1987). DPH has derivatives which are incorporated into membranes with a well defined orientation and exhibit negligible background fluorescence in aqueous solution. However when these probes are incorporated into the lipid bilayer they have sensitive fluorescence (anisotropy) responses to phospholipid orientational order. Prendergast *et al;* 1981, synthesized a fluorescence probe (1,4-trimethyl ammoniumphenyl-6-phenyl-1,3,5, hexatriene, p-toluenesulfonate) (TMA-DPH), that would be attracted to the lipid-water interface and yet intercalate into the lipid matrix (Prendergast *et al;* 1981). The cationic DPH analog, TMA-DPH, has spectral characteristics similar to the neutral parent probe DPH. The introduction of the charged substituent in TMA-DPH provides a surface anchor to improve the localisation of the probe in the membrane, relative to DPH (see figure 4 for possible membrane localisation of DPH and TMA-DPH). As with DPH, its long hydrocarbon tail structure and its high fluorescence ability in biological membranes have lead to its extensive use for membrane fluidity measurements.

Fig.4 Possible localisation of DPH and its analog, TMA-DPH. Cationic TMA-DPH binds first to the outer layer of the plasma membrane and then gradually penetrates into the cytoplasmic side by a flip process, binding to the negatively charged cytoplasmic surface of the membrane. Neutral DPH is localised within the hydrophobic core of the membrane. (Based on Kitagawa *et al,* 1991).

Localisation of **DPH** and its analog



fig·4

TMA-DPH is reported to incorporate instantaneously in the outer monolayer of the plasma membrane (Kubina *et al;* 1987, Kuhry *et al;* 1983, Trotter & Storch; 1989). Higher polarisation values of TMA-DPH as compared to DPH are thought to reflect its localisation near the surface of the membrane (Prendergast *et al;* 1981). Transbilayer movement of the cationic trimethylammonium derivative of DPH (TMA-DPH) was first shown by Bevers *et al;* 1990 (Bevers *et al;* 1990). It first binds to the exofacial leaflet of the plasma membrane and then gradually penetrates to the cytoplasmic side by a flip-flop process, binding to the cytoplasmic surface of the membrane which has a negative surface potential (Kitagawa *et al;* 1991, Kitagawa & Takegaki; 1992) (see figure 4). Further studies revealed that ionomycin (calcium ionophore) stimulated rapid flip-flop of TMA-DPH in human platelets which induced more than a 3x increase in the total amount of its membrane binding (Bevers *et al;* 1990). Kitagawa *et al;* 1991, observed ionomycin inducing a 2x increase in fluorescence intensity of the probe in bovine platelets. These results support the anionic attraction of TMA-DPH for the cytoplasmic surface of the bilayer. The TMA-DPH probe provides information in a relatively short time interval on the physico-chemical properties of biological membranes and has well defined characteristics; thus it was the molecular probe of choice for the study.

Fluorescence recovery after photobleaching (FRAP)

The fluid mosaic model, as previously described is useful for description of the membrane but oversimplified. Some proteins can diffuse freely, others are restricted and some immobolised by

underlying cytoskeletal elements. Diffusion of integral membrane proteins may also be impeded by a carbohydrate rich layer (glycocalyx) as found in many cell types. The different classes of lipids in a membrane may not be homogeneously distributed, for example, one class of lipid may exist in domains which have different physical properties to the rest of the membrane lipid (Klausner *et al;* 1980). Physical properties may be very much dependent on the composition of the membrane and the temperature of the membrane and thus it is not strictly accurate to talk of a single bulk fluidity.

Lateral diffusion is the process that enables molecules to move from one part of the system to another by means of random motions (Brownian movement) (Foley *et al;* 1986). Frye and Edidin, 1970, provided the first evidence that membrane proteins could diffuse laterally. The mouse cell-human cell experiment using different coloured fluorescent antibodies against mouse cell membrane proteins and human cell membrane proteins demonstated lateral diffusion beautifully. The two cell types were induced to fuse to form a heterocaryon and complete mixing of the two fluorescent antibodies was observed within a couple of hours. It demonstrated lateral diffusion in the fluid "sea" of lipid (Ditta, 1987).

The movement of membrane molecules can be measured by fluorescence recovery after photobleaching (FRAP). The technique of FRAP allows the study of lateral mobility within intact membranes. It can also be applied to model membrane bilayer and monolayer systems and by focussing the laser in different places, lateral diffusion of components in different regions of membrane can be monitored (Koppel *et al;* 1976).

The experimental basis of FRAP was originally developed to measure the lateral diffusion of rhodopsin, a protein with an intrinsic

and bleachable chromophore, in the retinal rod outer segment disc membrane (Poo & Cone, 1974). They found the D_L value for rhodopsin to be 2.6 x 10^{-9} cm^2 s^{-1} at 20^0C. The theoretical basis and a method for analysing the results were derived by Axelrod *et al;* 1976. The technique requires that the molecules of study on the cell surface must fluoresce when excited by visible light. Very few molecules have properties like rhodopsin and so a fluorescent labelled probe is employed. In the simplist of terms, the membrane of study is labelled with a fluorescent chemical attached to its proteins or lipids and it is photobleached, that is, the fluorescent area of the membrane is made nonfluorescent with a short and intense flash of light in a small area (Ditta, 1987). Recovery of fluorescence is observed by lateral diffusion of unbleached molecules from neighbouring areas into this region. The time course of this recovery is used to calculate lateral diffusion of the labelled component.

During this study, small areas of membrane from cultured SHRSP and WKY VSMC, about 1μm in radius were illuminated by a low power argon laser beam and the fluorescent (amino-fluoroscein labelled) molecules in the spot are excited to fluoresce. The fluorescence intensity of the membrane is monitored before the laser power is increased briefly by a 10^4 fold for about 100ms and then immediately switched back to the low monitoring beam. The brief pulse of light serves to irreversibly bleach a proportion of the molecules in the spot. Any subsequent recovery must be due to diffusion of unbleached molecules into the spot. The data from an experiment are displayed as a fluorescence intensity versus time graph (termed a recovery curve). Figure 5 shows an idealised curve from a FRAP experiment and the movement of molecules into the bleached area. Typical D_L values are in the range of 10^{-10} - 10^{-12}

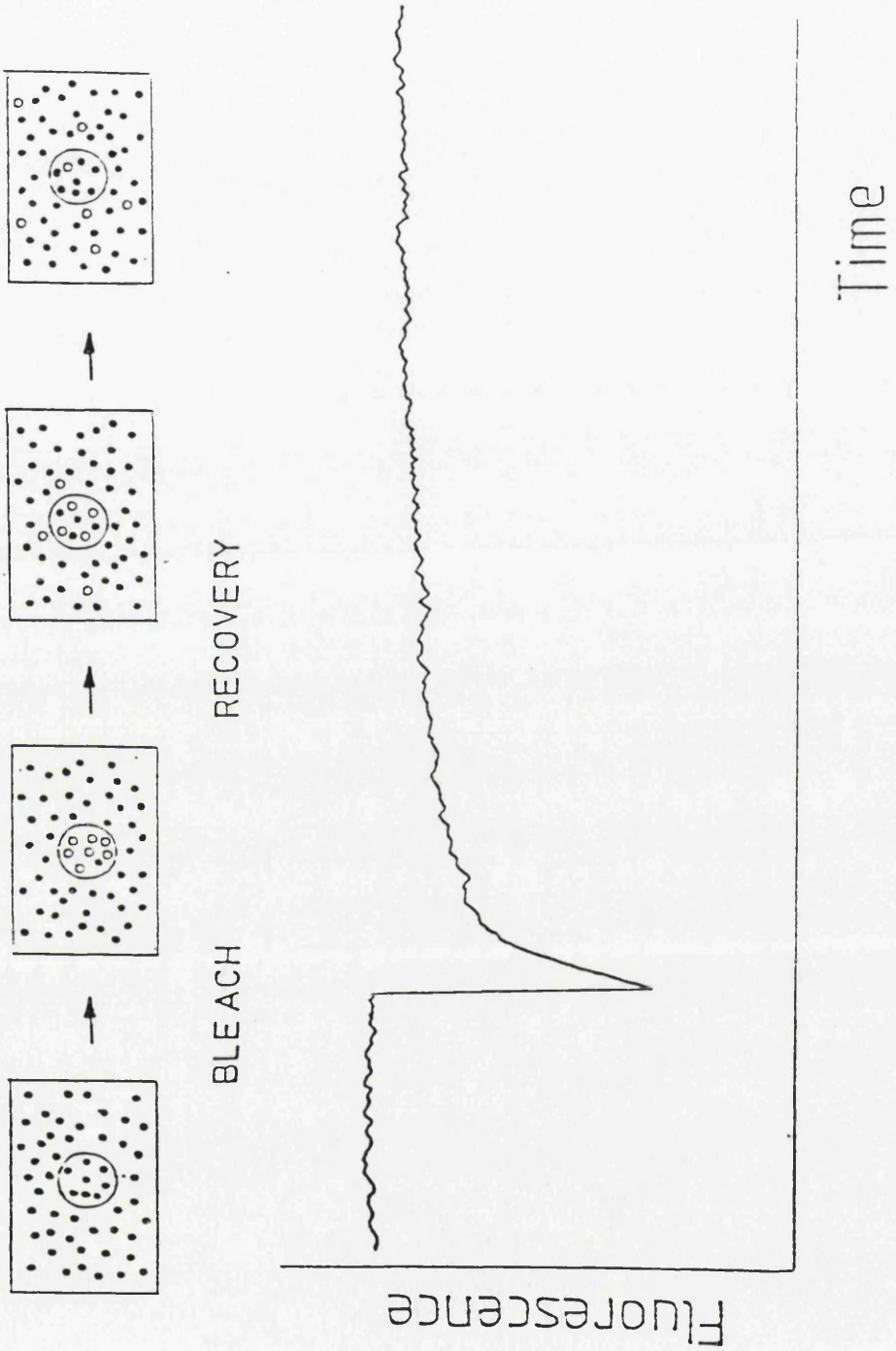


Figure 5 A diagram illustrating the principles of a FRAP experiment, the closed circles are unbleached chromophores and the open circles are bleached molecules, the large circle represents the laser spot. These events are represented in a recovery curve shown.(Foley *et al;* 1986)

cm^2/s^{-1}. Analysis of the kinetics of such a FRAP curve enables detemination of the lateral diffusion coefficient i.e. a measure of the rate of diffusion of the molecules and also determination of the immobile fraction i.e. the proportion of molecules that are immobile in the time scale of the experiment (Foley *et al;* 1986). It has been emphasised (Wier & Edidin, 1986) that the lateral motions of most membrane proteins are highly constrained and diffusion coefficients for membrane proteins are generally 10-100 times slower than those measured for lipid probes in the same cells.

Doubt about the damage due to photobleaching of cells have caused concern. Jacobson, *et al,* 1978, reported evidence that damage in fact does not occur during a typical FRAP experiment. Also Schlessinger *et al;* 1976, showed that recovery kinetics are not altered by repetitive photobleaching of the same spot or by a ten-fold increase in bleaching intensity. Local heating effects on the surface could also conceivably occur during a FRAP experiment but Axelrod *et al;* 1976, indicated that these were negligible.

The FRAP system was therefore used in the study as a novel method to measure membrane fluidity (in terms of lateral diffusion) in intact cultured vascular smooth muscle cells to compare membrane fluidity from SHRSP and WKY rats.

METHODS AND MATERIALS

PART 1

MEMBRANE MICROVISCOSITY: A COSEGREGATION STUDY

Experimental animals

The SHRSP and WKY rats used in all experiments were obtained from the colony at the University of Heidelberg (original Aoki-Okamoto stock (Okamoto & Aoki, 1963) maintained under strict inbreeding conditions for more than 25 generations).

The rats were housed three to four in a cage with free access to food and water throughout the experimental period at the German Institute for High Blood Pressure Research and Dept. of Pharmacology, University of Heidelberg, Heidelberg. Rats were separated according to sex, except for breeding experiments. Regular 12-hour diurnal light-dark cycles were maintained using an automatic light-switching device. During experiments requiring the maintenance of an indwelling arterial catheter, rats were housed one to a cage.

Crossbreeding experiments

The method used has been described previously (Jacob *et al;* 1991). Briefly, one male WKY rat was mated to two female SHRSP (cross 1) and one male SHRSP was mated to two female WKY rats (cross 2). The F_1 progeny (n=38) were matched brother-to-sister,

again one male with two females. The resultant F_2 offspring (n=139) fell into four groups: 33 males and 42 females having an SHRSP grandfather, and 34 males and 30 females having a WKY rat grandfather. Starting with the founders, all rats were ear-tagged with consecutive numbers.

Direct blood pressure measurements

The method used has been described in detail by Jacob *et al;* 1991. After reaching the age of 4 months all F_2 rats underwent catheterization of the right femoral artery under light ether anaethesia. The femoral artery was dissected and a piece of PE-10 tubing was inserted. A connecting length of PE-50 tubing was tunnelled subcutaneously and exteriorized at the neck. The rats were allowed to recover for 24-hours. On two consecutive days, three sets of blood pressure and heart rate measurements were made, twice between midnight and 4a.m. and once between 9a.m. and 1p.m. The rats then had 1% NaCl added to their drinking water for 12 days. After cannulation of the left femoral artery as in the first procedure, the haemodynamic measurements were repeated.

Recordings of blood pressure were obtained using a model P23PD Statham pressure transducer (Gould-Statham, Hato Rey, Puerto Rico) connected to a Hellige polygraph (Hellige GmbH, Freiberg, Germany). All measurements were taken by Dr. Reinhold Kreutz, at the University of Heidelberg, who was unaware of the genotype of the rat.

Blood Sampling and Erythrocyte Membrane Preparation

Six weeks after the last set of haemodynamic measurements, rats were anaethetised with ether and exsanguinated via the aorta into ethlenediaminetetra-acetic acid (EDTA)-coated tubes. Approximately 5-8ml was collected from each rat and the samples were kept on ice. Samples were coded and the blood pressure status of donors was unknown except to Dr R. Kreutz. The blood samples were sent on ice via courier from Heidelberg to Glasgow and the blood samples analysed 24-48 hours after withdrawal from the animal. Validation of the assays was observed from expected differences found between erythrocytes from parental strains. Blood samples were centrifuged at 1000g for 10 minutes at 4^0C . Plasma and the buffy coat were removed and the packed erythrocytes were used for membrane preparation following the method of Saito *et al;* 1986. The packed erythrocytes (1 volume) were suspended in 5 volumes 150mmol/l NaCl and 5mmol/l phosphate buffer (pH8), and were centrifuged for 10 minutes at 1000g at 4^0C. After two additional washes by this procedure, the erythrocytes were lysed in 40 volumes 5mmol/l phosphate buffer (pH8) and centrifuged at 15000g for 20 minutes at 4^0C. The pellet was then washed twice in 40 volumes of the same buffer and centrifuged at 15000g for 20 minutes. All erythrocytes membranes were homogenised in a Polytron-Kinematic system (Polytron-Kinematic GmbH, Kreins, Switzerland) for 30 seconds on a medium setting, and then protein was estimated by the method of Lowry *et al;* 1951. Membrane samples were stored at -70^0C until microviscosity measurements.

Measurement of Membrane Microviscosity

The fluorescence anisotropy of the fluorophore trimethyl ammonium diphenylhexatriene (TMA-DPH, Molecular Probes Inc., Eugene, Oregon, USA) was used to measure microviscosity. TMA-DPH (solution in N, N-dimethylformamide (Sigma Chemical Co, Poole, Dorset) at a final concentration of 5nmol/l was incubated at either 25^{0}C or 37^{0}C for 30 minutes with the erythrocyte membrane suspended in 50mmol/l Tris-HCl (pH 7.4) at a protein concentration of 1 mg/ml.

The molecular ratio of probe to membrane phospholipid was estimated to be approximately 1:200, this ratio having been determined (Dominiczak *et al;* 1991) so as not to perturb membrane microviscosity. Estimation of relative membrane fluidity for the experiments were measured for each membrane preparation in quadruplicate and calculated after fluorescence polarisation measurements using a Perkin-Elmer LS-50 luminescence spectrometer (Perkin-Elmer LS-50 Beaconsfield, Buckinghamshire, UK) fitted with a thermoregulated sample chamber and computer controlled excitation and emission polarisers. Specially formulated software was written by Steven Upstone, Perkin-Elmer for the acquisition of data.

The fluorescence intensity (I) was measured at the optimum wavelengths for the TMA-DPH fluorophore, that is, excitation at 340 nm and emission wavelength of 450 nm (slits 20nm). Anisotropy (A) was calculated according to the equations:

$$A = (I_{vv} - GI_{vh}) / (I_{vv} + 2GI_{vh})$$

$$G = I_{hv}/I_{hh}$$

where the first element of the subscript pair is the excitation orientation and the second element is the emission orientation (v, vertical; h, horizontal). G is the correction factor for the optical system and was calculated before each experiment. The autofluorescence of erythrocyte membranes was checked before each fluorescence measurement and the dedicated computer program was used to subtract the autofluorescence of each membrane preparation before anisotropy calculation. The coefficient of variation was 4.8% for microviscosity measurements performed at 25^{0}C and 6% for the measurements performed at 37^{0}C .

Statistical analysis

Values were expressed as means +/- SEM or as medians (range), as appropriate. Statistical analysis was performed using unpaired Student's t-test for comparison of parental strains. The Mann-Whitney U-test and Spearman's rank correlation were used for analysis of F_2 data. Confidence intervals for Spearman's rank correlation coefficients were calculated according to Altman and Gardner, 1988. $P < 0.05$ was considered statistically significant.

PART 2

LATERAL DIFFUSION AND FATTY ACID COMPOSITION IN VASCULAR SMOOTH MUSCLE FROM WISTAR-KYOTO NORMOTENSIVE AND STROKE PRONE SPONTANEOUSLY HYPERTENSIVE RATS

Experimental animals

SHRSP and WKY rats were obtained from colonies in the Department of Anatomy and Cell Biology at the University of Michigan. Six breeding animals of each strain were transported to Glasgow in December 1991, the inbred colonies have been established from these founder animals. Rats were decapitated and the mesenteric arteries were removed under aseptic conditions and were placed in ice-cold Hank's balanced salt solution (HBSS).

Isolation and culture of rat mesenteric artery smooth muscle cells.

VSMC were harvested from enzymatically dissociated rat mesenteric arteries as described previously by Dominiczak *et al;* 1991. This involved the superior mesenteric artery with its major branches was excised en bloc, from the origin to the aorta to the mesenteric border of the intestine and was placed in a petri dish containing ice-cold HBSS with 0.2mM added Ca^{2+}. Fat, adventitia and veins were removed by blunt dissection, the branches were cut off close to the main trunk of the superior mesenteric artery, and the artery was cut open lengthwise to expose the endothelial layer. The cleaned mesenteric arteries (five per preparation) were transferred into a petri dish containing 5ml enzyme dissociation mixture: HBSS

with 0.2mM Ca^{2+}, 1.25mg/ml collagenase, 0.25mg/ml elastase, 0.5 mg/ml soybean trypsin inhibitor, and 2 mg/ml bovine serum albumin. The arteries were incubated at 37^{0}C in a humidified 5% CO_2 -95% air atmosphere for approximately 30 minutes. Arteries were transferred to fresh HBSS and titrated 10 times through a plastic Pasteur pipette. They were cut into small pieces, transferred to fresh enzyme dissociation mixture, and incubated at 37^{0}C with periodic trituration until a single cell suspension was obtained (usually 45-60 minutes). The tissue suspension was sieved through 106µm stainless steel mesh to separate dispersed cells from undigested vessel wall fragments and debris. The filtered suspension was diluted in 20ml Dulbecco's modified Eagles medium (DMEM) (Gibco, Paisley) supplemented with 10% (vol/vol) foetal calf serum, 10% (vol/vol) equine serum, 2mM L-glutamine, 100 units/ml penicillin, and 100µg/ml streptomycin and then centrifuged (400g, 5 minutes). The cell pellet was resuspended in 5ml DMEM supplemented as described above.

The dispersed cells were plated in a 25cm^2 culture flask and were maintained in a humidified atmosphere of 5% CO_2-95% air at 37^0 C. After the first 24 hours, the medium was replaced to remove nonadherent cells. Plating efficiency was consistently above 50%. Medium was changed at 48-72 hour intervals. VSMC had the usual growth characteristics, took 7 days to reach confluency after plating and at confluence they had the "hill and valley" pattern. Cells up to the 9th passage were harvested once a week with trypsin-EDTA and passaged at 1:3 ratio in 62cm^2 flasks. Before experiments, cells were deprived of serum by a 24 hour incubation in fresh DMEM supplemented with 0.4% foetal calf serum.

Fluorescence microscopy and fluorescence recovery after photobleaching

Fluorescence photomicrographs were taken with a Leitz Ortholux II fluorescence microscope, a standard camera attachment and Kodacolor film.

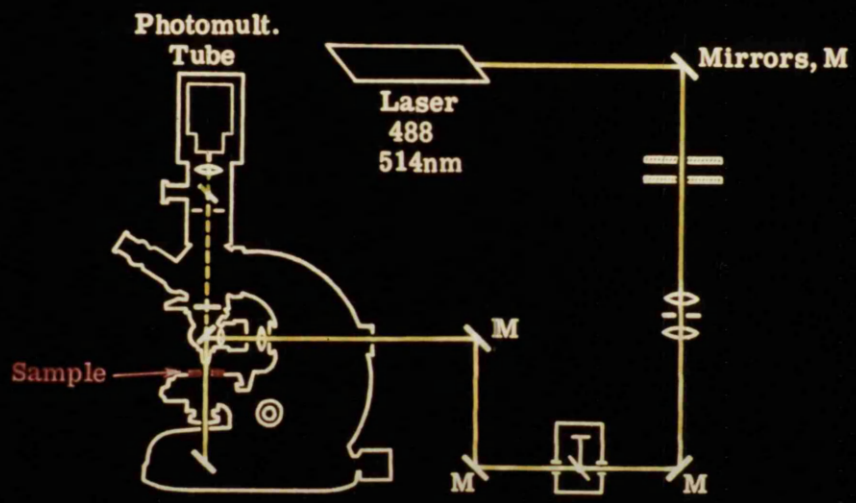
VSMC were grown to 50% confluence on a 2cm diameter petri dish. VSMC were labelled with 5µl of the fluorescent lipid probe 5N-(octadecanoyl) aminofluorescein (Sigma Chemical Co. Poole, Dorset) at a concentration of 5µg/ml probe in ethanol to 1ml of DMEM for 15 minutes at 37^0 C. The cells were washed four times in serum free DMEM and observed under x40 water immersion objective lens of the Leitz Ortholux microscope adapted for photobleaching. The $1/e^2$ radius of the focussed laser spot of the sample was 1-1.2µm as calculated from measurements using the FRAP apparatus by two-dimensional diffusion of fluoresceinated bovine serum albumin in thin films of glycerol of known viscosity (Thompson *et al;* 1980). Measurements of the two dimensional lateral diffusion of fluorescent lipid probe incorporated into membranes of the VSMC in culture were made based on the principles of Koppel *et al;* 1976. Figure 6 is a schematic representation of the apparatus required and was set up in the Dept. of Biochemistry, University of Glasgow by Dr. John Kusel and colleagues.

The FRAP technique involves an area of the membrane, (as measured at the start of each experiment was ~ 1µm in radius) which is illuminated by an argon laser beam and the fluorescent molecules in a spot are excited to fluoresce. After several seconds of monitoring fluorescence intensity, the laser power is increased 10^4 fold for

Fig.6 Schematic diagram of apparatus required for FRAP which was set up by Dr. John Kusel in the Dept. of Biochemistry, University of Glasgow. Based on Koppel *et al,* 1976.

Fluorescence Recovery after Photo Bleaching

APPARATUS



100ms and then immediately switched back to the low power monitoring beam. The pulse of light serves to irreversibly bleach a proportion of the molecules in the spot, thus fluorescence intensity decreases sharply. Any subsequent recovery of fluorescence is a measure of lateral mobility of fluorescence molecules. This lateral mobility (D_L) is calculated according to the equation derived by Axelrod *et al;* 1976:

$$D_L = (w^2/ 4\, t_{1/2}) y$$

where w is the e^{-2} radius of the laser beam, $t_{1/2}$ is the half time of full fluorescence recovery, and y is a factor that represents the beam profile and degree of bleaching. Factors which influence the % bleach are laser intensity, duration of the bleaching pulse, quantum efficiency of bleaching and fluorescence extinction coefficient.

Fluorescence quenching

The depth of insertion of the lipid probe in the membrane was determined by measuring the ability of a non-permeant molecule trypan blue (Sigma Chemical Co. Poole, Dorset) (0.25% wt/vol) to quench probe fluorescence by Forester resonance energy transfer as detailed (Foley *et al;* 1986; Schroeder *et al;* 1979). Fluorescence of cells labelled with 5-N (octadecanoyl) aminofluorescein was measured using the FRAP apparatus in the presence and absence of trypan blue by photon counting from an area of membrane, 1μm in diameter. These photon counts were corrected for the background

and all fluorescence values were measured within 30 minutes. The equation:

$$\% \text{ quenching} = \frac{F(-) - F(+)}{F(-)} \times 100$$

where F(-) and F(+) are fluorescent intensities in the presence and absence of trypan blue respectively, was employed to measure quenching.

Preparation of membrane fractions

Membrane fractions were prepared as previously described (Gleason *et al;* 1991; Harrison *et al;* 1983). Cells were removed with a rubber policeman and pelleted at 700g for 10 minutes. The cells were suspended in cold (4^{0}C), hypo-osmolar sucrose (0.1M) containing 10 mM Tris (pH 7.4) for 6 minutes, followed by disruption with 20 strokes of a type B pestle in a small Dounce homogeniser. An equal volume of 0.4 M sucrose solution containing 10mM Tris, pH 7.4, was then added to disrupted cells, and the resultant crude homogenate was centrifuged at 700g for 10 minutes. The resulting nuclear pellet was resuspended in 0.25 M sucrose, 10mM Tris, pH 7.4 (as were subsequent pellets), and the supernatant was centrifuged at 9750g for 10 minutes. The resulting mitochondrial/ lysosomal pellet was resuspended, and the supernatant was centrifuged at 110,000g for 30 minutes. The resulting microsomal pellet was resuspended after removal of the post microsomal supernatant (soluble fraction). All fractions were frozen for chemical and enzymatic analysis at a

later time, with the exception of cytochrome c oxidase activity, which was measured on the day (Cooperstein & Lazarow, 1951). 5' -nucleotidase was used as a marker of the plasma membrane (Bodansky & Schwartz, 1968) using a Sigma nucleotidase kit (Sigma Chemical Co. Poole, Dorset), and DNA content was used as a marker for the nuclear fraction as measured by acridine orange incorporation (Molecular Probes, Cambridge).

Fatty acid composition of membrane fractions

All fatty acid analysis were performed at the Williams S. Memorial Veterans Hospital, Madison, WI. This was possible due to collaboration with Theodore Goodfriend and his colleagues. Analysis of the fatty acid composition of the fractions was accomplished by alkaline methanolysis of total membrane lipids followed by conversion of the fatty acid methyl esters to nitrophenyl esters for chromatographic separation and detection. Methanolysis was carried out according to the method of Kates, 1964. Samples stored at -80^{0}C were thawed in wet ice and concentrated to a volume of 0.5 - 1.0ml using a vaccum centrifuge. Following concentration, 100 nanamoles of nonadecanoic acid (C19:0) was added as an internal standard to control for recovery. Each sample then received 2.0ml absolute methanol and 100µl of 33% (w/v) KOH / water. Samples were sealed under argon and refluxed 2 hours at 100^{0}C to hydrolyse complex lipids and form methyl esters of saponified fatty acids.

After reflux, samples were cooled, diluted with 2ml H_20, and the nonsaponifiable lipids extracted into 3 successive aliquots of 4ml n-heptane. To the remaining aqueous portion, one drop of phenol red

solution was added and 6 N HCl was added until the indicator changed colour. Two additional drops of acid were added and the mixture allowed to stand for 10 minutes. The acidified mixture was treated with three successive aliquots of 4ml n-heptane to extract fatty acid methyl esters. The three heptane fractions were pooled and dried in a vaccum centrifuge.

Fatty acid methyl esters were converted to the chromophore esters by reconstituting in methylene chloride, 300µl, and adding 50µl of a solution of 0-p-nitrobenzyl-N,N'-diisopropylisourea 0.036M in methylene chloride (Knapp & Krueger, 1975). The vials were capped with a teflon-lined screw cap under a blanket of argon gas and refluxed 2 hours at 80^0C. Solvent was removed under vaccum. To each dried sample was added an aliquot of the p-nitrobenzyl ester of undecanoic acid (C11:0) as an external standard and chromatographic identification. The derivatised fatty acids were chromatographed in a Waters HPLC through a Novapak C-18 radial compression cartridge (Waters/8NVC184); Waters Chromatography Div. Millipore Corp, Milford, Mass, USA. The elution scheme was as follows: injection time is 0 minutes, flow rate is constant at 2ml/min; 0-9 minutes in solvent A: methanol(absolute): acetonitrile: water (71:10:19) and trifluoroacetate acid 100ppm; 9-39 minutes in solvent B: methanol: tetrahydrafuran: acetonitrile: water (80:2:2:16) and trifluoroacetic acid 100ppm; 39-57 minutes- linear gradient from solvent B to 100% solvent C: methanol: acetonitrile: water (91:1:8) and trifluoracetic acid 50 ppm; 57-70 minutes solvent C. The chromatograph ended at 70 minutes and the column was re-equilibrated in solvent A for 15 minutes between chromatographic runs. Peaks were identified by running derivatised samples of standards purchased from Sigma Chemical Co., St Louis, USA. Fatty

acid masses were assessed by peak height compared to a standard mixture run the same day and corrected for recovery of undecanoic and nonadeconoic.

PART 3

Cholesterol Enrichment of cultured VSMC from SHRSP and WKY rats

Cultured VSMC between passages 5-8 were used throughout the experiment. Cholesterol measurements were based on the method of Heider & Boyett; 1978. Cells were seeded in 3.5cm diameter plastic culture dishes at a density of 100,000 cells/dish and grown in DMEM medium (Gibco, Paisley) containing antibiotics and 20% serum (foetal calf and horse serum) (Gibco, Paisley). in a humidified atmosphere under 5% CO_2 /95% air (37^0 C). On this day and for the following two days 20µg/ml of LDL and Ox-LDL and appropriate amounts of control PBS were added to the dishes. Cells reached confluence in 24 hours. Medium was replaced with serum free DMEM and antibiotics for a further 48 hours before the cholesterol assay commenced to remove the influence of growth factors contained in FCS. LDL and 0x-LDL was freshly prepared by ultracentrifugation of whole human blood samples by Mr. Jim McCulloch Dept. Medicine and Therapeutics, Western Infirmary, Glasgow. Concentrations were determined by fluorescence colorimetry.

Confluent cultured cells were scraped from culture dishes on day 4 with a rubber policeman using 250µl of buffer (K_2HPO_4 0.1M, pH7.4) after 2 washes with this buffer. A further 250µl of the above buffer was added to each sample and then 500µl of

chloroform/methanol (2:1) was added to each tube, spun at ~10000g before a brief vortex. The samples were then left overnight (~18-24 hours) to allow extraction. Tubes were centrifuged at~1500g for 15 minutes allowing separation of the layers where the bottom layer contained the chloroform and the lipids, including the cholesterol. 275µl of the bottom layer was removed to a clean tube. (The residue was subsequently dissolved in 0.1N sodium hydroxide and an aliquot taken for protein determination by the method of Lowry *et al;* 1951). Tubes containing the chloroform and lipids were vaccum dried and then 400µl of ethanol added to each with vortexing to mix. The suspension was sonicated in a water bath containing ice for 30 minutes to ensure cell disruption.

Free and total standard values were calculated from a standard solution of cholesterol (Sigma Chemical Co., Poole, Dorset) at a concentration of 0.4 mg/ml in 95% ethanol which was used to make a standard curve. These standard values were read at 325nm and 415nm for excitation and emission respectively. (Esterified cholesterol can be determined from total value minus free value).

Fluorometric Assay

Free and total cholesterol measurements were carried out in parallel and the required reagents shown below:

FREE (µl)	TOTAL (µl)	REAGENT & CONC.
500	400	K_2HPO_4 (0.1M, pH7.4)
100	100	oxidase (1U/ml)
100	100	peroxidase (10U/ml)
-	100	esterase (0.1U/ml)
50	50	triton (X100) 0.5% in H_2O

50	50	Na cholate (20mM in H_2O)
150	150	p-hydrophenyl acetic acid (4mg/ml in K_2HPO_4)

(All reagents from Sigma Chemical Co. Poole, Dorset)
Oxidase, peroxidase and esterase prepared as stocks in K_2HPO_4 (0.1M, pH7.4).

1ml of the above solutions were added to 100µl of the prepared sample for free and total cholesterol assays. This mixture was vortexed and free values were read after 15 minutes at room temperture whereas, total was read after 30 minutes at 37^{0}C (with gentle agitation) in a water bath. Fluorescence excitation and emission wavelength was set as for standard curve and samples measured using the LS-50 Perkin-Elmer fluorescence spectrometer (slits 5nm). These fluorescence values were calculated as µg cholesterol/µg protein by use of the equation from the standard curve and the Lowry protein values obtained for each individual sample. All assays of additions of PBS, LDL and 0x-LDL to SHRSP and WKY cultured cells were carried out in quadruplicate (N=4).

Analysis of data

Statistical significance was determined by Mann-Whitney for unpaired comparisons. P values< 0.05 were considered significant. Data was presented as means +/- sem.

RESULTS

Macroscopic appearance of SHRSP and WKY aorta and mesenteries.

Upon dissection and removal of aorta and mesenteries for culturing it appeared that SHRSP had thicker aorta/mesenteric branches as compared to the normotensive control, WKY. This was apparent because it was easier to incise the SHRSP vessels for disaggregation. Also during enzymatic breakdown of the vessels, WKY tended to disaggregate into single cells quicker.

Membrane microviscosity in erythrocyte cells from SHRSP and WKY rats

Measurements with the cationic fluorescent probe TMA-DPH revealed a significant difference in microviscosity between membranes obtained from SHRSP erythrocytes and those obtained from WKY rat erythrocytes (Fig. 7a). Corresponding systolic blood pressures (SBP) values (tail-cuff method) are shown in Fig. 7b.

Membrane microviscosity in F_2 rats.

Microviscosity was measured at 25^{0}C in erythrocyte membranes from 86 F_2 rats and at 37^{0}C in erythrocyte membranes from 77 of the same F_2 rats. At 37^{0}C the membrane microviscosity was lower for each membrane preparation studied: at 25^{0}C, median 0.286

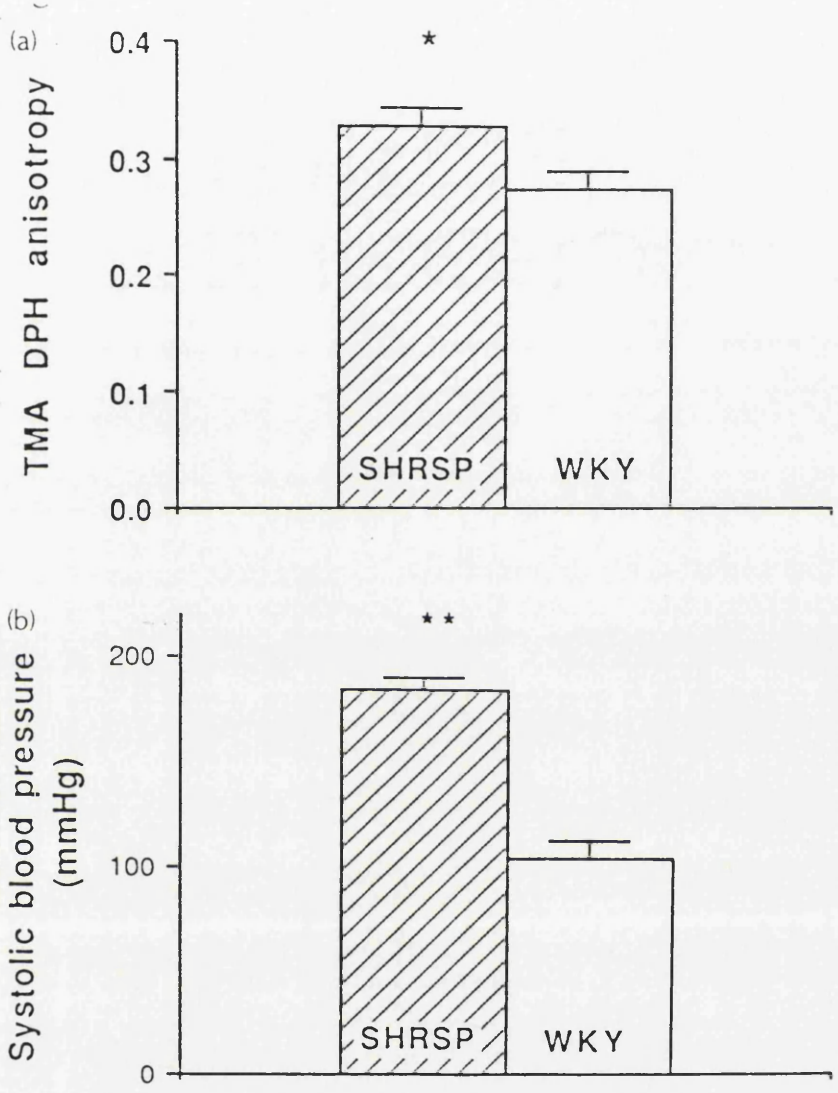


Fig. 7. (a) Membrane microviscosity measured as an anisotropy of timethylammonium diphenylhexatriene (TMA DPH) in stroke-prone spontaneously hypertensive rats (SHRSP, n = 6) and Wistar–Kyoto rats (WKY, n = 5); $^{*}P = 0.01$, versus WKY. (b) Systolic blood pressure measured by tail-cuff method under light ether anaesthesia in SHRSP and WKY; $^{**}P < 0.001$, versus WKY.

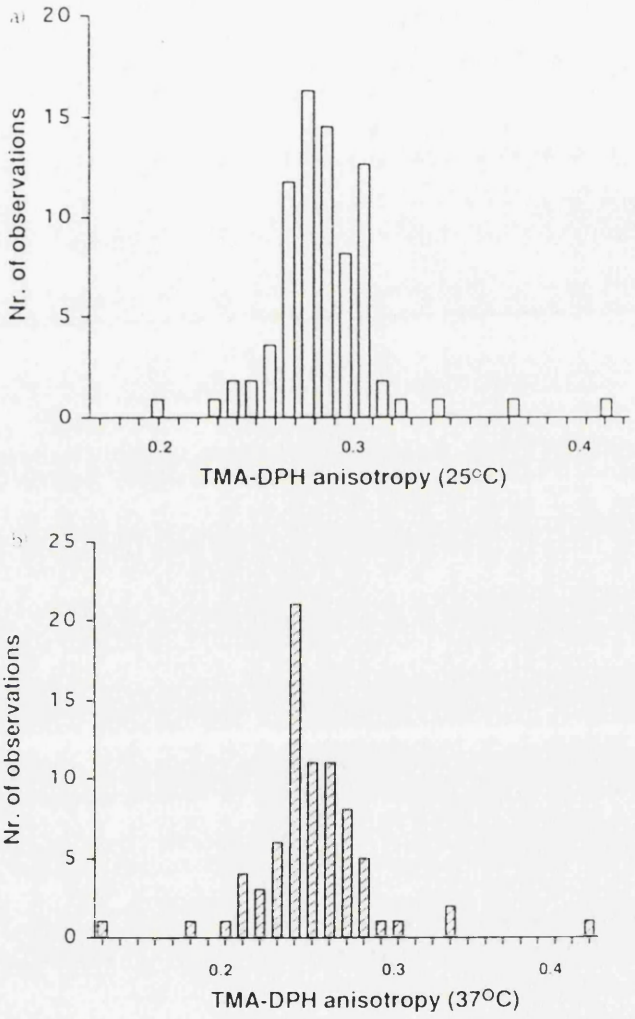


Fig. 8 Histograms of trimethylammonium diphenylhexatriene (TMA-DPH) anisotropy of erythrocyte cell membranes performed (a) at 25°C in 86 rats from the F_2 generation and (b) at 37°C in 77 rats from the F_2 generation.

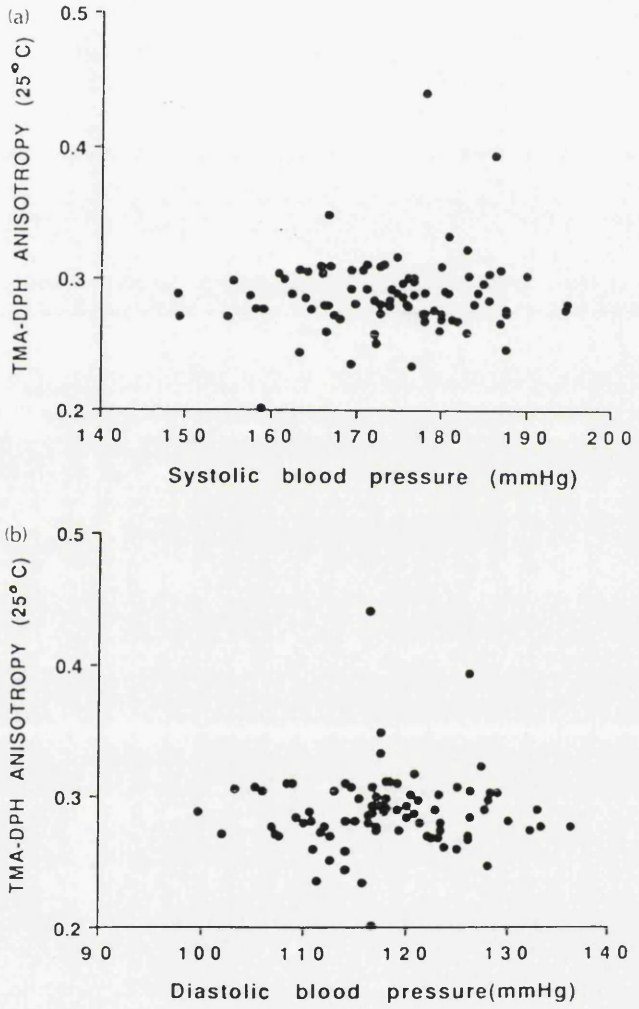


Fig. 9. The relationship between trimethylammonium diphenylhexatriene (TMA-DPH) anisotropy measured at 25°C and (a) systolic blood pressure ($r = 0.02$, 95% confidence interval -0.20 to 0.23), and (b) diastolic blood pressure ($r = 0.05$, 95% confidence interval -0.17 to 0.26), both on normal-salt diet.

(range 0.201-0.441); at 37°C , median 0.263 (range 0.13-0.347); $p<0.001$; 95% confidence interval (CI) 0.017 to 0.031.

The frequency distributions of microviscosity measurements in membranes at 25°C and 37°C are shown in Fig. 8a & 8b. For a group as a whole there was no significant correlations between membrane microviscosity at 25°C and SBP (r=0.02, 95% CI -0.20-0.23) or diastolic blood pressure (DBP; r=0.05, 95% CI -0.17 to 0.26; Fig. 9a & 9b). Similarly, no significant correlation between membrane microviscosity at 25°C and salt-loaded SBP and salt-loaded DBP, or between membrane microviscosity at 37°C and SBP, DBP, salt-loaded SBP and salt-loaded DBP were found (data not shown).

In a subgroup analysis males (group 1) and females (group 2) were studied separately, as were male WKY rat progenitor and males (group 3) and females with a male SHRSP progenitor (group 4). There was no significant correlations between microviscosity measured at 25°C and 37°C and SBP, DBP salt-loaded SBP and salt-loaded DBP in any of the four subgroups studied.

Lateral diffusion and fatty acid composition in vascular smooth muscle membrane from SHRSP and WKY rats.

Lateral diffusion

Membrane fluidity of intact VSMC was measured as a lateral diffusion (D_L) of 5N (octadecanoyl) aminofluorescein in cells obtained from 18 SHRSP and 18 WKY rats. Figure 10 shows VSMC in culture labelled with the lipid probe. Lateral diffusion was significantly lower in VSMC membranes from SHRSP (Fig. 11). Lateral diffusion

Fig.10 5-N (octadecanoyl)-aminofluorescein (C_{18}-Fl) stained VSMC. The VSMC were labelled with the fluorescent lipid by incubating them for 10 minutes at 37°C in 1ml of DMEM to which 5µl of ethanol containing 2mg/ml probe was added.

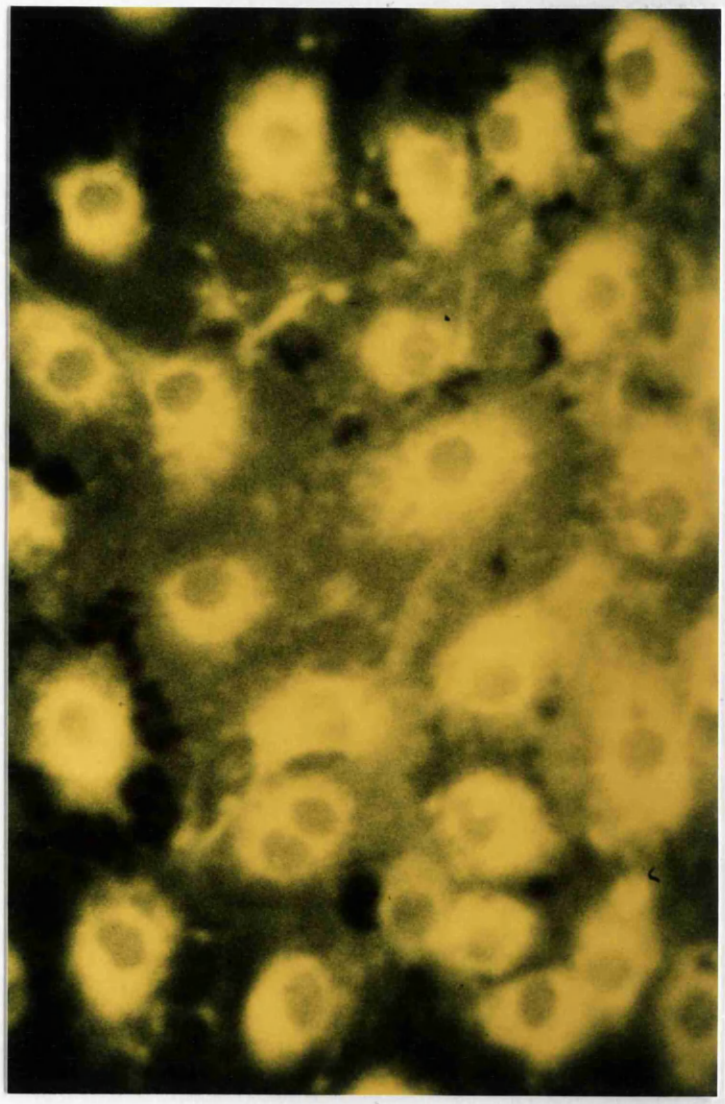


Fig.11 Lateral diffusion and % recovery in VSMC membranes. Lateral diffusion coefficient which is a measure of membrane fluidity was 2.83 +/- 0.22 in VSMC from SHRSP and 3.2 +/- 0.19 x 10^{-9} cm^2 /sec in those cells isolated from WKY (N=18; $p<0.01$). % recovery is ~ 70% for both SHRSP and WKY (not statistically significant).

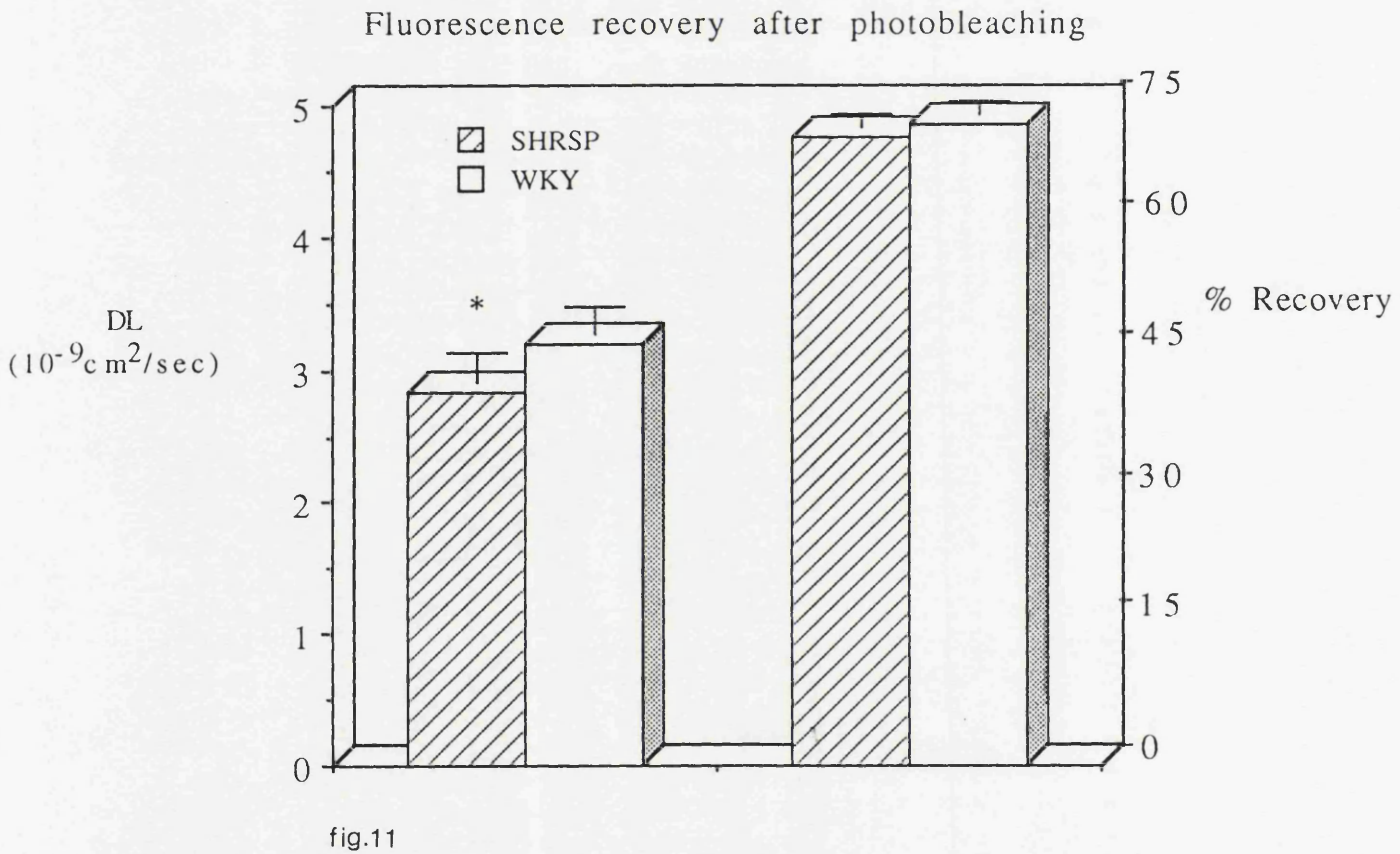

Fluorescence recovery after photobleaching
DL
(10-9c m2/sec)
5
4
3
2
1
0
SHRSP
WKY
*
75
60
45
30
15
0
% Recovery

fig.11

coefficient which is a measure of membrane microviscosity was 2.83 +/- 0.22 in VSMC from SHRSP and 3.2 +/- 0.19 x 10 $^{-9}$ cm ^{2}/sec in those cells isolated from WKY (n=18; p<0.01). % recovery was 69.7% +/-0.9 for SHRSP and 70.2% +/-1.2 for WKY (no statistical significance difference, Fig. 11)

Addition of trypan blue, a non-permeant energy receptor, resulted in significant quenching of the fluorescence in cells labelled with 5-N (octadecanoyl) aminofluorescein. In three separate experiments, 45 VSM cells isolated from SHRSP and WKY were examined, the percentage quenching was 97% for cells isolated from SHRSP and 98% for cells isolated from WKY.

Fatty acid composition

Eight samples from four different cell harvests were the basis of the data obtained. Data are expressed as ratios to emphasize qualitative aspects of lipid composition, and to reduce between sample-variation. The only statistically significant difference in plasma membrane fatty acid composition between SHRSP and WKY cells was the proportion of arachidonic acid to total fatty acid content. This was evident whether arachidonic acid was expressed as a molar proportion of total fatty acids (Fig. 12a) where the ratio was 1.6 : 1, or as a ratio of arachidonic to oleic (C16:0) or palmitic (C18:1) acids (Fig. 12b). In VSMC plasma membrane fractions isolated from SHRSP the ratio of arachidonic : total fatty acids was significantly greater than this ratio in cells from WKY (n=9, 0.06 +/- 0.007 vs 0.04 +/- 0.005; p=0.02 (see Fig. 12a). Most other fatty acids were found in the same proportion in SHRSP and WKY membranes.

Fig.12A & B. Fatty acid composition of membranes from SHRSP and WKY. Fatty acid content was measured using HPLC.

12A. In plasma membrane fractions isolated from SHRSP the ratio of arachidonic:total fatty acids was significantly greater than this ratio in cells from WKY (n=9, 0.06 +/- 0.007 vs 0.04 +/- 0.005; p=0.02).

12B. This significance was also found when arachidonic acid was expressed as a ratio with palmitic (C18:1) acid or oleic (C16:0) acid ($p<0.05$).

FATTY ACID COMPOSITION OF PLASMA MEMBRANE LIPIDS

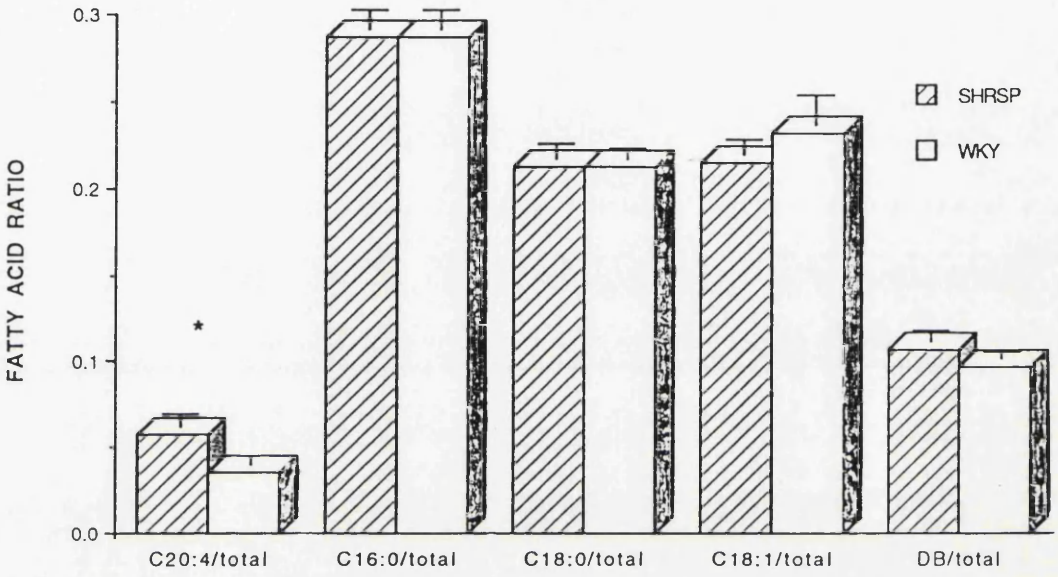


fig.12a

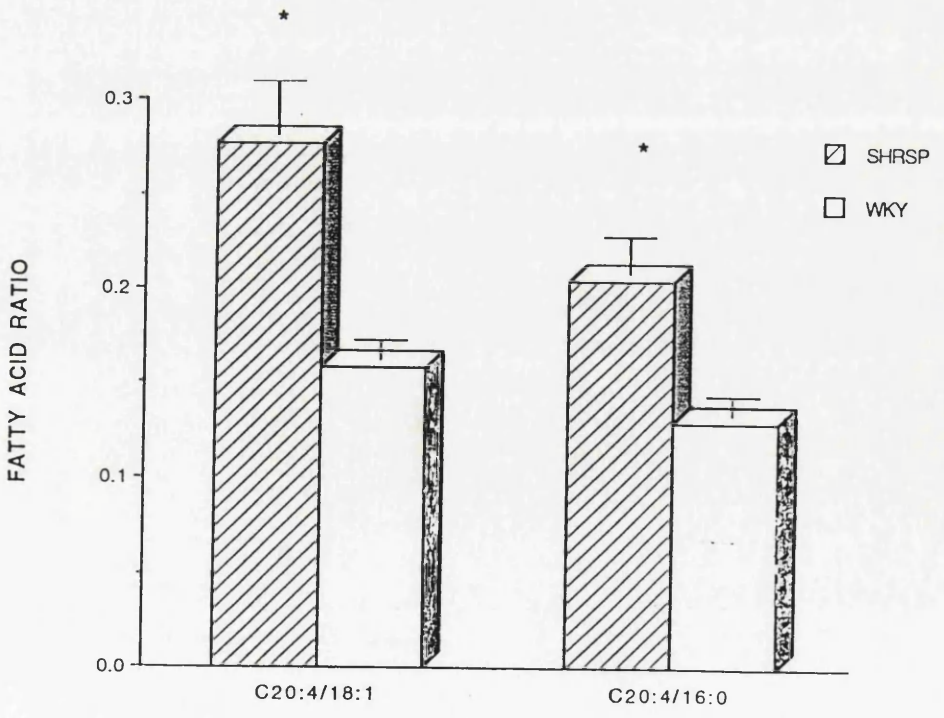


fig.12b

In mitochondrial/lysosomal fractions there was more palmitic and oleic acid in VSMC from SHRSP as compared to VSMC from WKY (n=9, 0.28+/- 0.003 vs 0.26 +/- 0.007; p=0.04 and 0.21 +/- 0.01 vs 0.24 +/- 0.005; p=0.02).

Calculations of the molar ratio of unsaturated to saturated fatty acids, or the molar ratio of monounsaturated to saturated fatty acids, or the molar sum of double bonds per total moles of fatty acid showed no difference between SHRSP and WKY membranes.

Determination of free and total cholesterol in cells in culture from SHRSP and WKY rats.

Figures 13a and 13b show results from the analysis of cholesterol (total and free) incorporation of four different experiments (N=4) where PBS, LDL and OX-LDL was incubated with cultured smooth muscle cells from the mesenteries of SHRSP and WKY rats at a concentration of 20μg/ml. Data are expressed as μg cholesterol (free and total) to μg protein. WKY has greater incorporation of cholesterol from Ox-LDL particles for free cholesterol measurements as compared to SHRSP (WKY 32% and SHRSP 20% increase). The percentage incorporation of free cholesterol from LDL particles is 26% for WKY and 30% for SHRSP.

There is a statistically significant difference between SHRSP and WKY free cholesterol uptake from Ox-LDL particles ($P<0.005$) and total cholesterol uptake of Ox-LDL ($p<0.05$). There is also statistical significance between SHRSP and WKY for PBS group free cholesterol measurements ($p<0.05$) from cultured VSMC.

Fig.13A & 13B Determination of total and free cholesterol in cells in culture from SHRSP and WKY VSMC. There is a statistically significant difference between total and free measurements from SHRSP as compared to WKY for incorporation of cholesterol from Ox-LDL particles ($p<0.05$ and $p<0.005$ respectively)

TOTAL CHOLESTEROL MEASUREMENTS-CULTURED SHRSP & WKY VSMC

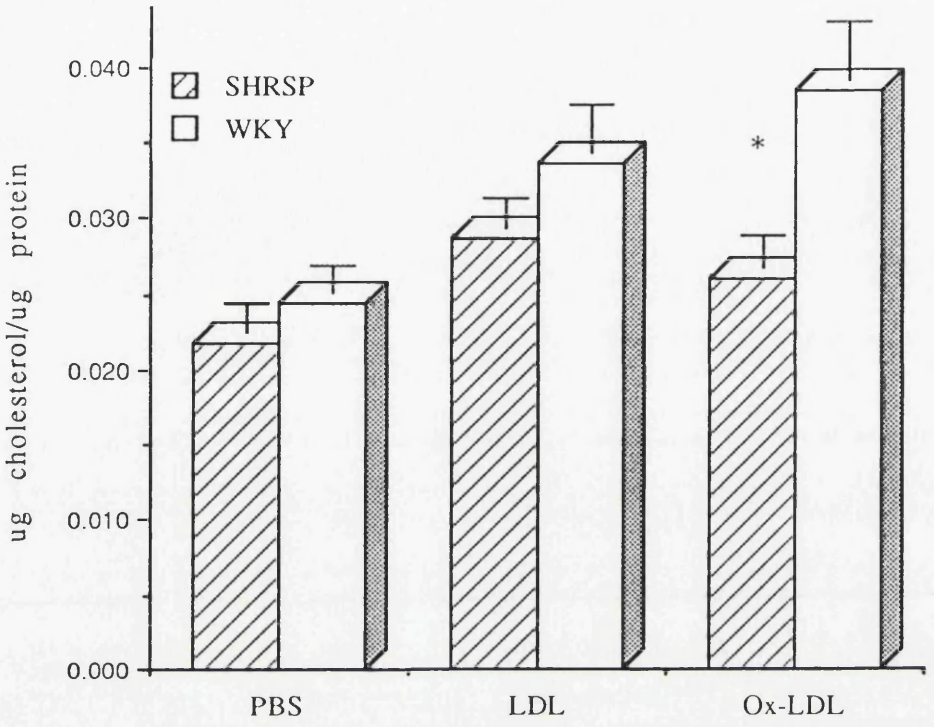


fig.13a

FREE CHOLESTEROL MEASUREMENTS-CULTURED SHRSP & WKY VSMC

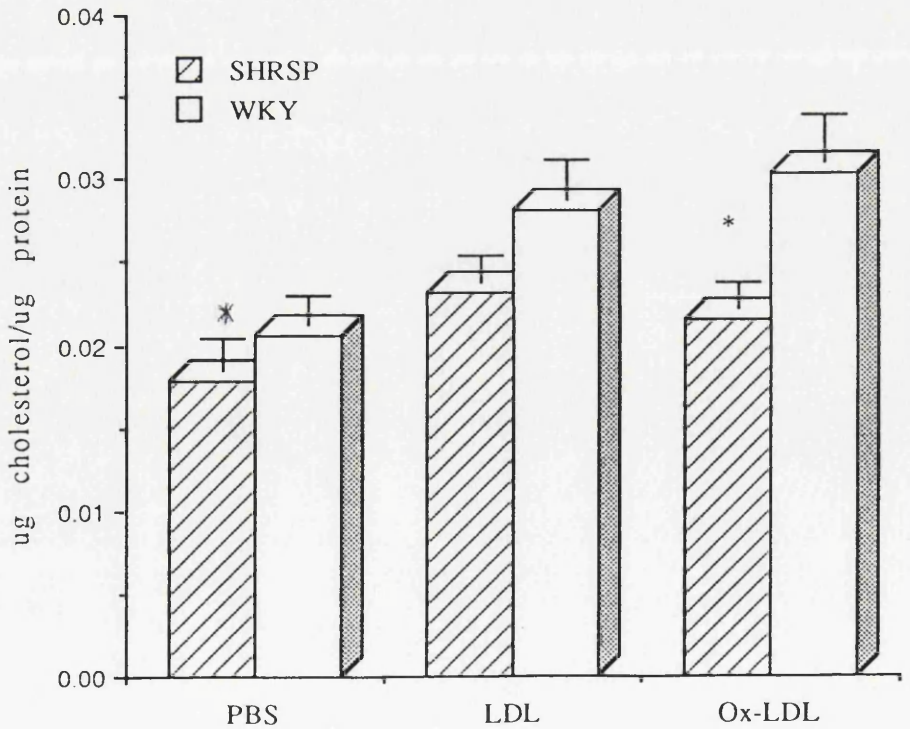


fig.13b

DISCUSSION

Many studies have shown that the biochemical and biophysical abnormalities of cell membranes are important factors in the etiology of hypertension in animals and humans (Devynck *et al;* 1981 Tsuda *et al;* 1987). The major features of cell membrane structure that are known to be important are that cell membranes contain a lipid bilayer and that it is hydrophobic in nature. The bilayer imparts the essential permeability control to the cell (Yeagle,1989). Any loss of integrity of the lipid bilayer would make the cell unstable and could affect the protein and lipid associations within the complex assemble.

Membrane microviscosity: a cosegregation study

The study confirmed previous findings that membrane microviscosity as measured with the surface probe TMA-DPH was significantly higher in membranes obtained from SHRSP than in those obtained from WKY rats, the control strain (Dominiczak & Bohr, 1991; Dominiczak *et al;* 1991). The result (McLaren *et al;* 1993) is the first fluorescence polarisation study, with TMA-DPH as a probe, showing increased microviscosity of erythrocyte membranes from genetically hypertensive rats. These results are similar to previous data on vascular smooth muscle cell membranes (Dominiczak *et al;* 1991) and platelet membranes (Dominiczak & Bohr,1991) in SHRSP. In addition to measurements performed at 25^{0}C, membrane microviscosity was also measured at physiological temperature. In agreement with previous observations by Shinitzsky, 1989,

membrane microviscosity was lower (fluidity higher) at 37^{0}C than at 25^{0}C for each sample studied. Thus, the physiological properties of erythrocyte membranes are similar to those of membranes from other cells and species (Shinitzsky, 1989).

To test the hypothesis that the membrane physicochemical structure trait is under control of the same genetic mechanism that regulates blood pressure, the study (McLaren *et al;* 1993) measured membrane microviscosity in erythrocytes isolated from 86 F_2 rats derived from crossing SHRSP and WKY rats. The F_2 progeny is called segregating progeny, since it is in these rats that the genes controlling blood pressure and membrane properties have a chance to recombine (Rapp, 1991). A variety of phenotypes related to electrolyte transport and vascular reactivity in genetic hypertension have been subjected to a cosegregation study. Bruner *et al;* 1986, examined oscillatory contractile activity to noradrenaline of tail arteries in the F_2 generation of the same cross. The oscillatory activity cosegregated with blood pressure in the F_2 (Bruner *et al;* 1986). Other phenotypes that cosegregated with blood pressure in SHRSP x WKY were oscillatory behaviour induced by noradrenaline in mesenteric resistance vessels (Mulvany, 1988). Certain traits have also been measured and found not to correlate with blood pressure. Lymphocyte sodium influx in SHRSP x WKY was found not to correlate (Furspan *et al;* 1987), and also whole body exchangable sodium and sodium intake (Harrap, 1986).

This is the first study in which the relationship between blood pressure and membrane microviscosity has been subjected to a cosegregation analysis. We (McLaren *et al;* 1993), found no correlation between membrane microviscosity and SBP or DBP in the F_2 generation. In the segregating F_2 population, gene(s) controlling

the expression of membrane properties and blood pressure segregated independently. This was true for the F_2 cohort as a whole and when the F_2 generation was divided into four groups dependent on two factors: the sex of the rat and whether the rat had a WKY rat or SHRSP male progenitor. In these four subgroups we (McLaren *et al;* 1993) found no correlation between membrane microviscosity measured at 25^{0}C or 37^{0}C and SDP, DBP, salt-loaded SBP and salt-loaded DBP. It seems that the genetic loci controlling blood pressure and membrane physicochemical structure (microviscosity) are separable and they therefore distribute randomly with respect to one another (Rapp, 1991). These results eliminate the membrane microviscosity trait from being causally related to blood pressure differences.

Lack of reproducibility and precision in blood pressure measurements generally represents a larger problem in cosegregation studies than in studies in parental strains, because of the importance of measuring blood pressure accurately in individuals (Lindpaintner, 1992). Therefore blood pressures in this study were obtained by indwelling femoral catheters. Three sets of blood pressure measurements were taken by Dr. R. Kreutz on two consecutive days in order to minimise measurement errors in individual rats. The salt loading phase of the study was designed to test the effect of an important environmental variable. The blood pressure response to salt-loading may itself be genetically determined.

In conclusion, erythrocyte membrane microviscosity is elevated in SHRSP compared with membranes from WKY rats. In the segregating F_2 generation the membrane microviscosity trait does not correlate with blood pressure. Based on these data, the hypothesis that the

microviscosity trait is related to cause of genetic differences in blood pressure between WKY rats and SHRSP must be rejected.

Lateral diffusion and fatty acid composition in VSMC

The observation of decreased lateral diffusion coefficient (D_L) in VSMC membranes from SHRSP as compared to VSMC membrane isolated from WKY is consistent with previous data, and is complementary to the fluorescence polarisation technique (Dominiczak *et al;* 1991; McLaren *et al;* 1993).

The fluorescence polarisation measurements are performed on crude membrane preparations with a mixture of subcellular membranes. In contrast, FRAP allowed us to measure lateral diffusion specifically in cell membranes. There is approximately the same amount of molecules moving into the bleached area of membrane for SHRSP and WKY rat strains (~70% recovery). This means that nearly 30% of molecules may be immobile in both SHRSP and WKY. Lipid domains which retain a greater degree of immobility may be one possible way in which some membrane enzymes operate more efficiently as opposed to one domain in the bulk membrane lipid (Carruthers & Melchoir, 1986).

In order to examine the location of aminofluorescein in the membrane the non-permeant molecule trypan blue was used as a quencher of the fluorophore group of the lipid analogue. Trypan blue is able to quench fluorescence via an energy transfer mechanism (Schroeder *et al,* 1979). The absorption peak of trypan blue overlaps with the fluorescence emission of the fluorescent lipid (aminofluorescein)(Foley*et al,* 1986). Energy transfer fluorescence

quenching is proportional to r^{-6} where r represents the intermolecular distance (Bradley, 1976) and only those molecules very close to the surface (50 A or less) will be effectively quenched. From the data obtained, it can be seen that aminofluorescein is severely quenched in both SHRSP and WKY membranes (97% and 98% respectively). This indicates that the fluorescent lipid is located mainly in the exofacial leaflet of the bilayer, at least during the 30 minutes period over which time data was obtained. With greater time it is expected that the fluorescent probe would be internalised into inner membranes. Probes such as DPH have been found to be quenched by as little as 20% (Schroeder *et al,* 1979). This would tend to suggest the probe is located internally in the membrane which supports the idea that it gives information on the 'interior' core region of the bilayer.

The results of the two methodological approaches represent different types of molecular movement in the membrane but both confirm the hypothesis that membrane microviscosity is increased (fluidity is decreased or more accurately, motional ordering is decreased) in cell membranes isolated from SHRSP as compared to membranes from WKY. The motion as sensed by different probes, relates to different points along the hydrocarbon chain and this is reported to be markedly dependent on the number and position of double bonds (Stubbs & Smith, 1984). The reduction in membrane fluidity may thus affect the proposed hypothesis that these observed differences have profound influences on membrane protein function (that is receptors, channels and pumps), via increases in $\{Ca^{2+}\}_i$, which contribute to increased total peripheral resistance.

The use of different probes, (in this study, TMA-DPH for fluorescence anisotropy and the aminofluorescein for FRAP) is the

way forward as the characteristics of the individual probes are selective for specific cellular environments in the membrane. Caution is however required in interpreting results from different probes because of the different time scales involved in measurements (Stubbs & Smith, 1984).

It has been suggested previously that membrane microviscosity may be affected by small changes in fatty acid composition (Remmers *et al;* 1990; Stubbs & Smith, 1984). Naftilan *et al;* 1986, showed significantly less linoleic acid (18:2) in platelets from hypertensive than normotensive subjects and less oleic acid (18:1) in hypertensive subjects. A study showed a decrease in fluorescence polarisation of the hydrophobic 'core' probe DPH, in platelet membranes exposed to *cis* unsaturated fatty acid which was also associated with an inhibition of platelet function (McIntyre *et al;* 1984).

It has also been shown that arachidonic acid content is slighty increased in membranes isolated from hypertensive patients (Naftilan *et al;* 1986) but the sample size was small and significance was not achieved. Another study by Ollerenshaw *et al;* 1987, showed an increase in arachidonic acid content in erythrocyte membranes of patients with essential hypertension. These data are at least partially in agreement with the findings of the study that there is an increase in arachidonic acid content in VSMC membranes from SHRSP as compared to control WKY. Arachidonic acid acts as a precursor of prostaglandins and other eicosanoids which have a plethora of cellular functions and pathways. Ordway *et al,* 1989, reported that arachidonic acid and certain other fatty acids at concentrations similar to those required for metabolically mediated and direct effects of fatty acids, directly activate specific K^+ channels in smooth muscle cells. It is suggested that certain fatty acids liberated by

receptor regulated lipases and by other processes may be the second messenger molecules for the regulation of ion channels (Ordway *et al,* 1989). It is difficult to conclude that the increased arachidonic acid content in VSMC membranes is a sole cause of differences in membrane fluidity between SHRSP and WKY. It is however, possible that cells from SHRSP remain metabolically more active even in culture and thus retain a greater amount of arachidonic acid within the cell membrane. Our experiment was performed on cultured mesenteric VSMC alone and the cells were fractionated to isolate plasma membranes. Fresh arteries and veins may not show similar results and this is an area for further work.

Vemuri & Philipson, 1990, investigated the influence of membrane fatty acyl composition on the activities of sarcolemmal and sarcoplasmic reticular trasnsporters (ie Na^+-Ca^{2+} exchange and Na^+-K^+ ATPase). They found exchange activity to be rather sensitive to membrane fatty acyl composition in a systematic manner and that there was a requirement for cholesterol. They suggest a common lipid environment, as defined by the phospholipid fatty acyl chains is required for optimal activity for each of the cation transporters and that the environment allows the appropriate conformational changes for transport to occur (Vemuri & Philipson,1990). This would further support the hypothesis of a basic fundamental change in lipid bilayer composition in hypertension.

McIntyre *et al;* 1984, suggest that effects of saturated and *cis* and *trans* unsaturated fatty acids on platelet membranes are produced at the level of the membrane, where they may be related to perturbations of specific lipid domains. They further suggest that it is likely that perturbation of the platelet membrane in specific lipid domains by *cis-* unsaturated fatty acids suppresses the transduction

processes that mediate receptor induced platelet activation. This again would be in line with the proposed hypothesis that the abnormality in membranes is generalised and that novel techniques such as FRAP provide evidence for the importance of specific lipid domains. Different physical properties of fatty acids may account for different biological behaviour of different classes of free fatty acids (FFA) and are compatible with sequestration of different fatty acid classes into separate domains in the platelet membrane (Klausner *et al,* 1980).

Membrane fluidity appears to change during development (as in aging) or with drug treatment of cultured cells. Toplak *et al;* 1990, measured membrane fluidity as a fluorescence anisotropy of attached living cells using the surface probe TMA-DPH. They showed membranes of growing cells to be more fluid than those of stationary cells. Cell density had no effect except at very low numbers (Toplak *et al;* 1990) and calcium concentration increased in medium proportionally to a decrease in membrane fluidity (Toplak *et al;* 1990). Although the above observations are for the TMA-DPH probe, and fluorescence polarisation technique, it is possible that the same principle applies to cultured cells and the FRAP technique. When the VSMC grow on their culture dishes there is a variety of cell shapes. It seems that a possible source for variability in FRAP results may arise from the different stages of growth and also intracellular variation.

A further study to show that fatty acids seem to play a pivotal role in membrane regulation and function is that of Grunfeld *et al;* 1981. In 3T3H cells exposed to saturated fatty acids in culture, cells exhibited up to an 80% decrease in insulin binding and insulin 2 deoxyglucose uptake, whereas cells exposed to monosaturated fatty

acid of the same length showed less change but results suggest that modification of cellular lipids can produce profound effects of both insulin binding and insulin action (Grunfeld *et al;* 1981). These results suggest that the cell membrane is affected by changes in lipid composition and is therefore a valid area for further study on relationships between membrane structure and function.

Cholesterol enrichment of SHRSP and WKY VSMC

The levels of free cholesterol incorporation from LDL particles in SHRSP and WKY rat strains is in agreement with reported values for cholesterol incorporation in VSMC (Gleason *et al;* 1991). They showed a 29% increase in cholesterol content with addition of LDL to culture medium. Many investigators have demonstrated that cholesterol incorporation into cell membranes alters the physical state of the phospholipid bilayer, an alteration that can affect function of integral membrane proteins as well as other cellular functions. Although it is not possible to determine if SHRSP or WKY cells have been affected functionally in this study, it is of interest that there is a variation in uptake of cholesterol, which is statistically significant when cells are exposed to oxidised LDL.

Free (unesterified) cholesterol is a major lipid class of mammalian plasma membrane and in arterial smooth muscle it is thought to participate in the regulation of the physical state or "fluidity" of the phospholipid bilayer. Cholesterol leads to an increase in anisotropic motional ordering (Yeagle, 1989). Indeed cholesterol has been shown to regulate ion pumps which in some cases show an absolute

dependence on cholesterol for activities and this motional order may be the control mechanism.

The uptake of cholesterol following exposure to LDL and Ox-LDL by WKY and SHRSP VSMC is a normal function. It is noteworthy that there is a greater incorporation in cultured VSMC isolated from WKY rats. It may be that VSMC from WKY incorporate more free cholesterol when it is added to the medium because the cholesterol leads to the ability to open ion channels, such as voltage-operated calcium channels. VSMC isolated from the SHRSP are perhaps less able to incorporate free cholesterol as they might not have such a flexible membrane system due to some generalised dysfunction. This data is from a cultured population and *in vivo* may be different. The increase in SHRSP microviscosity (decreased fluidity) may affect their ability to transport cholesterol across the membrane. Gleason *et al;* 1989, found in cultured smooth muscle cells from rabbit aorta that enrichment of free cholesterol with a combination of cholesterol-rich liposomes and human LDL-cholesterol caused a selective enrichment of the plasma membrane. It is likely that there is an inter-relationship between cholesterol content, phospholipid headgroup compositon, asymmetric distrubution across the membrane and the level of unsaturation with respect to membrane functions and physical properties (Stubbs & Smith, 1984).

Shattil and Cooper, 1976, showed that in platelet membranes exposed to cholesterol rich liposomes there was an increase in fluorescence polarisation of DPH and increased sensitivity to agonists. An increasing body of evidence has been accumulating suggesting a role for Ox-LDL in mediating some of the vasomotor properties known to accompany the atherosclerotic state (Resink *et al;* 1992, Witztum & Steinberg. 1991). The atherosclerotic coronary artery

appears to be characterised by a reduced responsiveness to endothelial-dependent relaxing factor (EDRF)-mediated vasodilation (Witztum & Steinberg. 1991). The mechanism remains to be worked out. There is evidence for direct effects of Ox-LDL on vascular smooth muscle. Ox-LDL is reported to stimulate phosphoinositide turnover in cultured human VSMC (Resink *et al;* 1992). Resink *et al;* 1992, noted that this effect of Ox-LDL could be blocked by compounds such as Ca^{2+} antagonists, inhibitors of endocytosis, and some second messengers or their analogues. These insights may provide possible pharmalogical regulation of Ox-LDL in the vessel wall.

Although this study did not measure membrane microviscosity following exposure to different forms of cholesterol, it is a topic for further investigation. In a study of enrichment of SMC membranes with cholesterol, Gleason *et al;* 1991, found that fluorescence anisotropy was increased and membrane fluidity decreased at all temperatures studied. This decreased membrane fluidity was associated with an increase in FC:PL ratio, as was shown by Shattil and Cooper, 1976. Gleason *et al;* 1991, hypothesise that arterial SMC calcium permeability is sensitive to changes in the lipid dynamics of the plasma membrane bilayer.

Conclusions

The findings of the study are:

Membrane microviscosity is a marker of hypertension and vascular damage in animal models of genetic hypertension but it is not causally related to genetic differences in blood pressure.

There is association between increased microviscosity and increased arachidonic acid content in plasma membranes of VSMC from SHRSP. These differences may contribute to abnormal membrane transport functions and intracellular signalling in genetic hypertension.

Cholesterol plays an important role in the fluidity and functioning of the lipid bilayer.

Publications arising from the study

McLaren, Y; Kreutz, R; Lindpaintner, K; Bohr, D; Ganten, D; Reid, J.L; Dominiczak, A.F. (1993) Membrane microviscosity does not correlate with blood pressure: a cosegregation study. *J. Hypertens. 11*, 25-30.

Abstracts

Dominiczak, A.F; McLaren, Y; Kreutz, R; Lindpaintner, K; Reid, J.L. (1993) Membrane microviscosity does not correlate with blood pressure: a cosegregation study.*J. Endocrin. 137, suppl.*

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McLaren, Y; Kreutz, R; Lindpaintner, K; Bohr, D; Ganten, D; Reid, J.L; Dominiczak, A.F. (1993) Membrane microviscosity does not correlate with blood pressure: a cosegregation study. *J. Hypertens. 11*, 25-30.

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RAPID PUBLICATION

Membrane microviscosity does not correlate with blood pressure: a cosegregation study

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Objective: To determine whether elevated microviscosity is associated with elevated arterial pressure in segregating (F_2) hybrids produced by crossing stroke-prone spontaneously hypertensive rats (SHRSP) and Wistar–Kyoto (WKY) rats.

Methods: SHRSP and WKY rats were obtained from the colony at the University of Heidelberg. F_2 progeny were obtained by brother–sister mating of the F_1 progeny of the cross between SHRSP and WKY rats. Membrane microviscosity (the inverse of fluidity) was measured as a fluorescence anisotropy of trimethylammonium diphenylhexatriene incubated with the erthrocyte membranes. The measurements were made using a luminescence spectrometer with computer-controlled excitation and emission polarizers.

Results: Membrane microviscosity was significantly greater (fluidity was lower) in erythrocyte membranes obtained from SHRSP than in those obtained from WKY rats. In the F_2 cohort there were no significant correlations between membrane microviscosity and systolic blood pressure, diastolic blood pressure, salt-loaded systolic blood pressure or salt-loaded diastolic blood pressure. A similar lack of relationship between these parameters was shown in a subgroup analysis, in which males or females with a male WKY rat progenitor and males or females with a male SHRSP progenitor were analysed separately.

Conclusions: Erythrocyte membrane microviscosity is elevated in SHRSP compared with WKY rats. In segregating F_2 hybrid rats the membrane microviscosity trait does not correlate with blood pressure. These results eliminate the microviscosity trait as being directly related to the cause of genetic differences in blood pressure between WKY rats and SHRSP.

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Keywords: Membrane fluidity, genetic hypertension, trimethylammonium diphenylhexatriene.

Introduction

Hypertension has been associated with multiple abnormalities in cell membrane function [1–3]. These abnormalities include increased vascular reactivity to various agonists [4,5], increased intracellular calcium [6,7], changes in Na^+,K^+ cotransport, Na^+–Li^+ countertransport and Na^+–H^+ exchanger [8–10]. It has been suggested that these multiple membrane abnormalities may be due to generalized physicochemical alteration of the lipid bilayer, the matrix in which all the membrane proteins function [2,3,11,12].

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Changes in the lipid composition of erythrocyte membrane have been shown to alter the physical properties of the membrane, resulting in modifications of the lipid–protein interaction and enzymatic activity of membrane-bound proteins [13,14].

Membrane fluidity (the inverse of microviscosity) is a property of the membrane that describes quantitatively the mobility and rate of rotational movement of lipid molecules within the membrane [15]. Membrane fluidity has been consistently found to be increased in erythrocyte, platelet and vascular smooth muscle cell membranes isolated from spontaneously hypertensive rats (SHR) or stroke-prone SHR (SHRSP) compared with those membranes in the commonly used normotensive reference strain, the Wistar–Kyoto (WKY) rat [11,12.16–21]. In addition, membrane microviscosity was found to be elevated in 3-week-old SHR, at the time when blood pressure is barely increased [21], whereas this membrane abnormality was not observed in secondary forms of hypertension such as deoxycorticosterone acetate hypertension or renal hypertension [17,20]. These studies are in accord with the notion that abnormalities of the membrane physicochemical structure may be related to the pathogenesis of genetic hypertension [11,12].

Essential hypertension in man is also characterized by increased membrane microviscosity of erythrocyte and platelet membranes [20,22,23]. However, one recent study on intact human platelets has shown lower microviscosity at the lipid–water interface of the cell membrane in untreated hypertensive patients than in normotensive control subjects [24].

It is not clear whether the abnormalities of membrane physicochemical properties described are causally related to genetic hypertension. The aim of the present study was to determine whether elevated microviscosity is associated with elevated arterial pressure in the segregating (F_2) generation of a cross between SHRSP and WKY rats.

Methods

Experimental animals

The SHRSP and WKY rats used in all experiments were obtained from the colony at the University of Heidelberg (original Aoki–Okamoto stock [25] maintained under strict inbreeding conditions for more than 25 generations).

The rats were housed three to a cage with free access to food and water throughout the experimental period. Rats were separated according to sex, except for breeding experiments. Regular 12-h diurnal light–dark cycles were maintained using an automatic light-switching device. During experiments requiring the maintenance of an indwelling arterial catheter, rats were housed one to a cage.

Crossbreeding experiments

The methods used have been described in detail previously [26,27]. Briefly, one male WKY rat was mated to two female SHRSP (cross 1) and one male SHRSP was mated to two female WKY rats (cross 2). The F_1 progeny (n = 38) were matched brother-to-sister, again one male with two females. The resultant F_2 offspring (n = 139) fell into four groups: 33 males and 42 females having an SHRSP grandfather, and 34 males and 30 females having a WKY rat grandfather. Starting with the founders, all rats were ear-tagged with consecutive numbers.

Direct blood pressure measurements

The method used has been described in detail elsewhere [26,27]. Briefly, after reaching the age of 4 months, all F_2 rats underwent catheterization of the right femoral artery under light ether anaesthesia. The femoral artery was dissected and a piece of PE-10 tubing was inserted. A connecting length of PE-50 tubing was tunnelled subcutaneously and exteriorized at the neck. The rats were allowed to recover for 24 h. On two consecutive days, three sets of blood pressure and heart rate measurements were made, twice between midnight and 4 a.m. and once between 9 a.m. and 1 p.m. The rats then had 1% NaCl added to their drinking water for 12 days. After cannulation of the left femoral artery as in the first procedure, the haemodynamic measurements were repeated.

Recordings of blood pressure were obtained using a model P23PD Statham pressure transducer (Gould–Statham, Hato Rey, Puerto Rico) connected to a Hellige polygraph (Hellige GmbH, Freiburg, Germany). All measurements were taken by a single observer (R.K.) who was unaware of the genotype of the rat.

Blood sampling and erythrocyte membrane preparation

Six weeks after the last set of haemodynamic measurements, rats were anaesthetized with ether and exsanguinated via the aorta into ethylenediaminetetraacetic acid-coated tubes. Approximately 5–8 ml was collected from each rat and the samples were kept on ice. Samples were coded, and the blood pressure status of donors was unknown to the laboratory performing microviscosity measurements. The blood samples were sent on ice and via courier from Heidelberg to Glasgow and were analysed 24–48 h after withdrawal of the blood. These assays have been validated by the observation that the expected differences were found between erythrocytes from the parental strains.

The blood samples were centrifuged at 1000 **g** for 10 min at 4°C. Plasma and the buffy coat were removed and the packed erythrocytes were used for membrane preparation following the method of Saito *et al.* [28]. The packed erythrocytes (1 vol) were suspended in 5 vol 150 mmol/l NaCl and 5 mmol/l phosphate buffer (pH 8), and were centrifuged for 10 min

1000 **g** at 4°C. After two additional washings by is procedure, the erythrocytes were lysed in 40 vol mmol/l phosphate buffer (pH 8) and centrifuged at 000 **g** for 20 min at 4°C. The pellet was then washed ice in 40 vol of the same buffer and centrifuged at 000 **g** for 20 min. All erythrocyte membrane sam- es were homogenized in a Polytron-Kinematic sys- m (Polytron-Kinematic GmbH, Kreins, Switzerland) r 30 s on a medium setting, and then protein was timated by the method of Lowry *et al.* [29]. Mem- ane samples were stored at −70°C until microvis- sity measurement.

easurement of membrane microviscosity

embrane microviscosity was measured as a flu- escence anisotropy of the fluorophore trimethyl- mmonium diphenylhexatriene (TMA-DPH; Molecu- r Probes Inc., Eugene, Oregon, USA) [11]. TMA- PH (solution in N,N-dimethylformamide) at a final ncentration of 5 nmol/l was incubated at either or 37°C for 30 min with the erythrocyte mem- anes suspended in 50 mmol/l TRIS–HCl (pH 7.4) a protein concentration of 1 mg/ml. Under these nditions the molar ratio of probe : membrane phos- holipid was approximately 1 : 200. It was determined at this ratio of probe : membrane phospholipid did ot perturb membrane microviscosity [11]. Fluo- escence anisotropy was measured in quadruplicate ith a Perkin–Elmer LS-50 luminescence spectrome- r (Perkin–Elmer Ltd, Beaconsfield, Buckinghamshire, K) with computer-controlled excitation and emission olarizers and dedicated software for data acquisition.

he fluorescence intensity (I) was measured at exci- tion and emission wavelengths of 340 and 450 nm, espectively (slits 20 nm). Anisotropy (A) was calcu- ted according to the equations:

$$A = (I_{vv} - GI_{vh})/(I_{vv} + 2GI_{vh})$$

$$G = I_{hv}/I_{hh}$$

here the first element of the subscript pair is the xcitation orientation and the second element is the mission orientation (v, vertical; h, horizontal). G is he correction factor for the optical system and was valuated before each experiment. The autofluores- ence of erythrocyte membranes was checked before ach fluorescence intensity measurement and a ded- cated computer program was used to subtract the utofluorescence of each membrane preparation be- ore anisotropy calculation. The coefficient of variation vas 4.8% for microviscosity measurements performed t 25°C and 6% for the measurements performed at 7°C.

tatistical analysis

Values are expressed as means ± SEM or as medians range), as appropriate. Statistical analysis was per- ormed using unpaired Student's t-test for compar- son of parental strains. The Mann–Whitney U-test nd Spearman's rank correlations were used for analy- is of the F_2 data. Confidence intervals for Spearman's rank correlation coefficients were calculated according to Altman and Gardner [30]. $P < 0.05$ was considered statistically significant.

Results

Membrane microviscosity in erythrocyte cells from SHRSP and WKY rats

Measurements with the cationic fluorescent probe TMA-DPH revealed a significant difference in micro- viscosity between membranes obtained from SHRSP erythrocytes and those obtained from WKY rat erythrocytes (Fig. 1a). Corresponding systolic blood pressure (SBP) values (tail-cuff method) are shown in Fig. 1b.

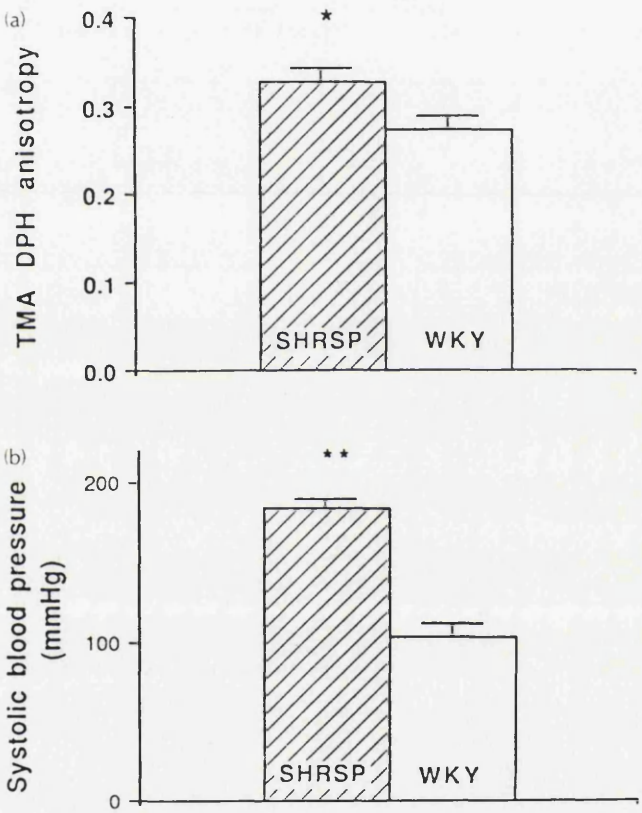

Fig. 1. (a) Membrane microviscosity measured as an anisotropy of timethylammonium diphenylhexatriene (TMA DPH) in stroke-prone spontaneously hypertensive rats (SHRSP, n = 6) and Wistar–Kyoto rats (WKY, n = 5); $^{*}P = 0.01$, versus WKY. (b) Systolic blood pressure measured by tail-cuff method under light ether anaesthesia in SHRSP and WKY; $^{**}P < 0.001$, versus WKY.

Membrane microviscosity in F_2 rats

Microviscosity was measured at 25°C in erythrocyte membranes from 86 F_2 rats and at 37°C in erythro- cyte membranes from 77 of the same F_2 rats. At 37°C the membrane microviscosity was lower for each membrane preparation studied: at 25°C, median 0.286 (range 0.201–0.441); at 37°C, median 0.263 (range 0.14–0.347); $P < 0.001$; 95% confidence interval (CI) 0.017 to 0.031.

The frequency distributions of microviscosity measurements in membranes at 25 and 37°C are shown in Fig. 2. For a group as a whole there were no significant correlations between membrane microviscosity at 25°C and SBP (r = 0.02, 95% CI −0.20 to 0.23) or diastolic blood pressure (DBP; r = 0.05, 95% CI −0.17 to 0.26; Fig. 3). Similarly, no significant correlations between membrane microviscosity at 25°C and salt-loaded SBP and salt-loaded DBP, or between membrane microviscosity at 37°C and SBP, DBP, salt-loaded SBP and salt-loaded DBP were found (data not shown).

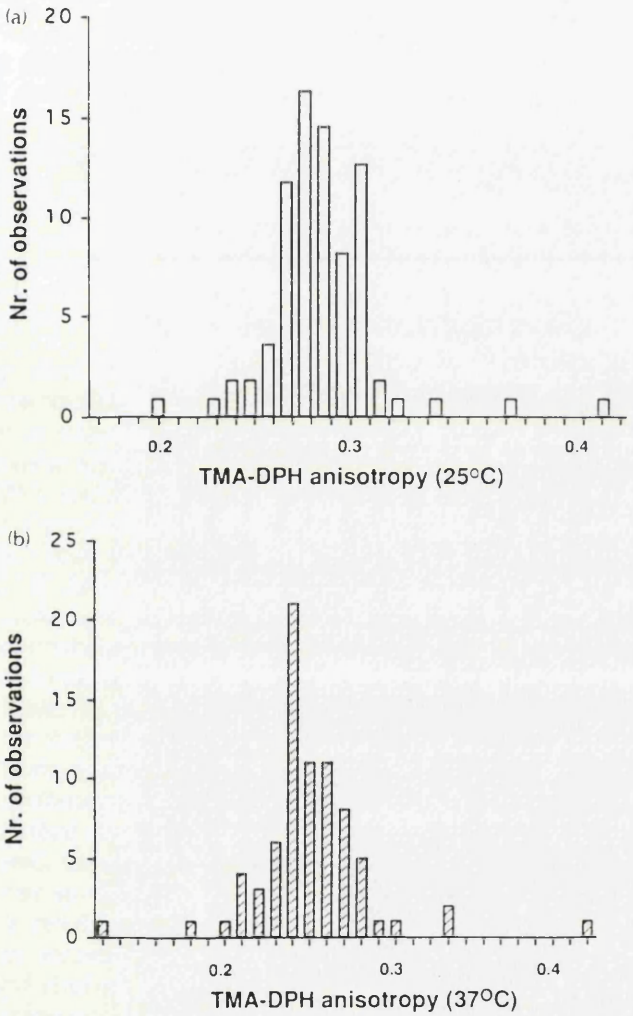

Fig. 2. Histograms of trimethylammonium diphenylhexatriene (TMA-DPH) anisotropy of erythrocyte cell membranes performed (a) at 25°C in 86 rats from the F_2 generation and (b) at 37°C in 77 rats from the F_2 generation.

In a subgroup analysis we studied separately males (group 1) and females (group 2) with a male WKY rat progenitor and males (group 3) and females (group 4) with a male SHRSP progenitor. There were no significant correlations between microviscosity measured at 25 and 37°C and SBP, DBP salt-loaded SBP and salt-loaded DBP in any of the four subgroups studied (Table 1).

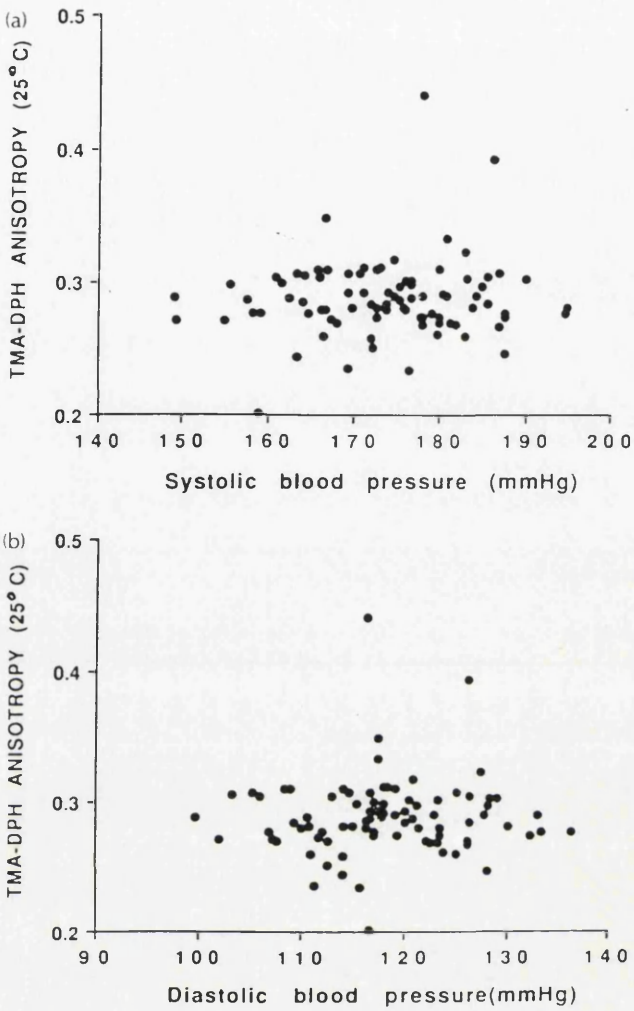

Fig. 3. The relationship between trimethylammonium diphenylhexatriene (TMA-DPH) anisotropy measured at 25°C and (a) systolic blood pressure (r = 0.02, 95% confidence interval −0.20 to 0.23), and (b) diastolic blood pressure (r = 0.05, 95% confidence interval −0.17 to 0.26), both on normal-salt diet.

Discussion

This study confirms our previous findings that membrane microviscosity, as measured with the surface probe TMA-DPH, was significantly higher in membranes obtained from SHRSP than in those obtained from WKY rats, the commonly used non-hypertensive reference strain [11,12]. The present study is the first fluorescence polarization study, with TMA-DPH as a probe, showing increased microviscosity of erythrocyte membranes from genetically hypertensive rats. These results are similar to previous data on vascular smooth muscle cell membranes [11] and platelet membranes [12] in SHRSP. The current results are also in agreement with earlier studies with DPH (the 'core' probe on erythrocyte membranes of SHR [16,17,19] or of patients with essential hypertension [20,22,23]) demonstrating increased microviscosities of the same order of magnitude. In addition to measurements

Table 1. Spearman rank correlations between membrane microviscosity and blood pressure in four subgroups of F_2 rats.

Fluorescence anisotropy	Blood pressure			
	Normal diet		High-salt diet	
	SBP	DBP	SBP	DBP
Group 1 (n = 23)				
At 25°C	0.06	0.13	0.05	−0.22
At 37°C	0.21	0.01	0.13	−0.26
Group 2 (n = 20)				
At 25°C	0.11	0.08	0.21	0.34
At 37°C	0.16	0.09	0.09	0.03
Group 3 (n = 23)				
At 25°C	−0.11	−0.03	−0.06	−0.06
At 37°C	−0.36	−0.37	−0.03	−0.01
Group 4 (n = 20)				
At 25°C	−0.19	−0.19	0.03	−0.33
At 37°C	−0.17	−0.05	0.32	−0.36

SBP, systolic blood pressure; DBP, diastolic blood pressure; group 1, males with a male Wistar–Kyoto (WKY) rat progenitor; group 2, females with a male WKY rat progenitor; group 3, males with a male stroke-prone spontaneously hypertensive rat (SHRSP) progenitor; group 4, females with a male SHRSP progenitor. To achieve significance at $P < 0.05$ the r-value should be: for groups 1 and 3, $r \geq 0.42$ or $r \leq -0.42$; for groups 2 and 4, $r \geq 0.45$ or $r \leq -0.45$.

performed at 25°C [11,12], we measured membrane microviscosity at physiological temperature. In accord with previous observations [15], membrane microviscosity was lower (fluidity was higher) at 37°C than at 25°C for each sample studied. Thus, the physicochemical properties of erythrocyte membranes are similar to those of membranes from other cells and species [15]. Devynck *et al.* [21] reported increments in microviscosity of the plasma membrane of SHR not only in erythrocytes, but also in hepatocytes, synaptosomes and cardiomyocytes. It appeared that the genetic substrate for this membrane abnormality had its effect on the membrane structure and function of all tissues. Further studies showed increased microviscosity in erythrocyte membranes isolated from 3-week-old SHR before hypertension developed [21]. In agreement with these findings, membrane microviscosity is normal in deoxycorticosterone acetate hypertension or in renal hypertension [17,20]. These observations are in accord with the possibility that the physicochemical abnormality of the membrane may alter electrolyte transport systems, thereby contributing to the pathogenesis of genetic hypertension.

To test the hypothesis that the membrane physicochemical structure trait is under the control of the same genetic mechanism that regulates blood pressure, we measured membrane microviscosity in erythrocytes isolated from 86 F_2 rats derived from crossing SHRSP and WKY rats. The F_2 progeny is called segregating progeny, since it is in these rats that the genes controlling blood pressure and membrane properties have a chance to recombine [31,32]. A variety of phenotypes related to electrolyte transport and vascular reactivity in genetic hypertension have been subjected to cosegregation analysis [5,33–36]. Furspan *et al.* [33] showed a significant correlation between lymphocyte potassium efflux and blood pressure in the SHRSP × WKY rat F_2 generation. Bruner *et al.* [5] examined oscillatory contractile activity to noradrenaline of tail arteries in the F_2 generation of the same cross. The oscillatory activity cosegregated with blood pressure in the F_2 generation [5]. Other phenotypes that cosegregated with blood pressure were cobalt-induced aortic smooth muscle contraction [34], ouabain sensitivity of aortic strips in SHRSP × WKY rats [35] and oscillatory behaviour induced by noradrenaline in mesenteric resistance vessels [36]. There are other traits that have been tested in the F_2 population and did not correlate with blood pressure. These traits include lymphocyte sodium influx in SHRSP × WKY rat F_2 [33], and whole-body exchangeable sodium and sodium intake [37] and various measures of salt appetite in SHR × WKY rat F_2 [38]. It is noteworthy that some of the previous studies were compromised by low numbers of F_2 rats.

This is the first study in which the relationship between blood pressure and membrane microviscosity has been subjected to a cosegregation analysis. We found no correlation between membrane microviscosity and SBP or DBP in the F_2 generation. In the segregating F_2 population, gene(s) controlling the expression of membrane properties and blood pressure segregated independently. This was true for the F_2 cohort as a whole and when the F_2 generation was divided into four groups dependent on two factors: the sex of the rat and whether the rat had a WKY rat or an SHRSP male progenitor. In these four subgroups we found no correlation between membrane microviscosity measured at 25 or 37°C and SBP, DBP, salt-loaded SBP and salt-loaded DBP. It seems that the genetic loci controlling blood pressure and membrane physicochemical structure (microviscosity) are separable and that they therefore distribute randomly with respect to one another [32]. These results eliminate the membrane microviscosity trait from being causally related to blood pressure differences.

Lack of reproducibility and precision in blood pressure measurements generally represents a larger problem in cosegregation studies than in studies in parental strains, because of the importance of measuring blood pressure accurately in individuals [39,40]. Therefore, blood pressures in the F_2 cohort were obtained by indwelling femoral catheters. Three sets of blood pressure measurements were taken on two consecutive days in order to minimize measurement errors in individual rats. The salt-loading phase of the study was designed to test the effect of an important environmental variable. The blood pressure response to salt-loading may itself be genetically determined.

In conclusion, erythrocyte membrane microviscosity is elevated in SHRSP compared with membranes

from WKY rats. In the segregating F_2 generation the membrane microviscosity trait does not correlate with blood pressure. Based on these data, the hypothesis that the microviscosity trait is related to the cause of genetic differences in blood pressure between WKY rats and SHRSP must be rejected.

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P161 MEMBRANE MICROVISCOSITY DOES NOT CORRELATE WITH BLOOD PRESSURE: A COSEGREGATION STUDY.

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Hypertension has been associated with multiple abnormalities in cell membrane function which may be due to a generalised defect in the lipid bilayer. Membrane microviscosity has been consistently found to be increased in stroke prone spontaneously hypertensive rats (SHRSP) as compared to their normotensive reference strain (WKY). To determine whether the microviscosity phenotype is causally related to genetic differences in blood pressure we measured membrane microviscosity in segregating (F_2) hybrids produced by crossing SHRSP and WKY rats.
Microviscosity of red cell membranes was measured as a fluorescence anisotropy of the trimethylammonium diphenylhexatriene with a Perkin-Elmer LS-50 spectrofluorometer. Direct blood pressure measurements were performed with indwelling femoral catheters in unanesthetized animals.
Membrane microviscosity was significantly greater in red cell membranes from the SHRSP as compared to those from WKY (0.33 ± 0.01 vs 0.27 ± 0.01 anisotropy units; $p = 0.01$). In the F_2 hybrids ($n = 86$) there were no significant correlations between membrane microviscosity and systolic [$r = 0.02$, 95% CI (-0.2 to 0.23)] or diastolic blood pressure [$r = 0.05$, 95% CI (-0.17 to 0.26)].
Membrane microviscosity is a marker of hypertension and vascular damage in animal models of genetic hypertension but is not casually related to genetic differences in blood pressure.

P160 FATTY ACIDS AND LATERAL DIFFUSION IN MEMBRANE IN HYPERTENSION.

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Membrane microviscosity is increased in vascular smooth muscle cells (VSMC) isolated from stroke prone spontaneously hypertensive rats (SHRSP) as compared to membranes isolated from normotensive reference strain (WKY). We hypothesised that this abnormality of membrane physicochemical structure is related to a difference in fatty acid composition of the plasma membrane in VSMC from SHRSP. Membrane microviscosity was measured as a lateral mobility of 5N (octadecanoyl) aminofluorescein using argon laser and fluorescence microscopy. Fatty acid content in membrane fractions was measured with high performance liquid chromatography. Lateral diffusion coefficient which is a measure of membrane microviscosity was 2.83 ± 0.22 in VSMC from SHRSP and 3.2 ± 0.19 x 10^{-9} cm^2/sec in those cells isolated from WKY (n = 18; p <0.01). In VSMC plasma membrane fractions isolated from SHRSP the ratio of arachidonic:total fatty acids was significantly greater than this ratio in cells from WKY (n=9, 0.06 ± 0.007 vs 0.04 ± 0.005; p = 0.02). In mitochondrial/lysosomal fraction there was more palmitic and oleic acid in VSMC from SHRSP as compared to VSMC from WKY (n = 9, 0.28 ± 0.003 vs 0.26 ± 0.007; p = 0.04 and 0.21 ± 0.01 vs 0.24 ±0.005; p = 0.02).
There is an association between increased microviscosity and increased arachidonic acid content in plasma membrane of VSM from SHRSP.These abnormalities may contribute to abnormal membrane transport functions and intracellular signalling in genetic hypertension.

P44 SODIUM/HYDROGEN EXCHANGE ACTIVITY, MEMBRANE MICROVISCOSITY AND LIPOPROTEINS IN ESSENTIAL HYPERTENSION AND TYPE 2 DIABETES.

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Sodium/hydrogen exchange activity has been shown to be elevated in essential hypertension and in type 1 diabetes. It has been suggested that the activity of this transporter may be related to changes in membrane microviscosity and plasma lipoproteins.

Platelet Na/H exchange activity (Vmax and Km) using ^{22}Na method, membrane microviscosity (anisotropy of trimethylammonium diphenylhexatriene), serum cholesterol and plasma lipoproteins were measured in 15 patients with essential hypertension, 17 patients with type 2 diabetes and 17 control subjects with the approval from the Western Infirmary Ethical Committee. Membrane microviscosity and Km for Na/H exchange correlated with HDL_3 ($r = 0.77$; $p<0.01$ and $r = 0.61$; $p <0.05$, respectively). In patients with type 2 diabetes, membrane microviscosity was inversely related to serum cholesterol and apoB ($r =-0.51$; $p<0.05$ and $r = -0.69$; $p =0.02$) and positively correlated with HDL_2 ($r = 0.84$; $p <0.01$)

There is an interrelationship between circulating lipoproteins, membrane microviscosity and Na/H exchange activity. These relationships are altered in Type 2 diabetes.

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