The characterization of altered cerebrovascular homeostasis associated with background pathology and its influence upon haemodynamic responsiveness in a rodent model of haemorrhagic stroke

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ABSTRACT OF THESIS

The presence of host disease, such as insulin dependent diabetes mellitus (IDDM) and chronic hypertension alters the incidence of stroke. Whether these factors also influence outcome of a cerebrovascular event is less clear. This possibility is even more obscure in relation to haemorrhagic stroke (ICH), because the presence of intraparenchymal blood is likely to present an additional factor that could potentially alter the pathophysiological response to stroke. The processes underlying the evolution of delayed perihaemorrhagic brain ischaemia, oedema and infarction are still speculative. The modification of these processes by the presence of cerebrovascular dysfunction, associated possibly with IDDM or hypertension, could be a contributing factor for an adverse influence upon outcome. It is also equally intriguing to hypothesize that host cerebrovascular dysfunction may alter the mechanisms of stroke evolution, offering an explanation for the failure to translate the spectacular successes of neuroprotective strategies from animal models of stroke into the clinical environment.

The aims of this thesis were (i) to investigate whether cerebrovascular dysfunction linked to a perturbation of nitric oxide (NO)-related control mechanisms, is associated with IDDM and chronic hypertension; (ii) to assess the extent to which any abnormal cerebrovascular physiology associated with IDDM and chronic hypertension contributes to the pathophysiological response to haemorrhagic stroke; and finally (iii) to determine if endothelin (ET) or neuronally derived NO, which may be implicated in the development of delayed perilesional ischaemia following ICH in normal animals, contribute to a potentiated ischaemic burden in ICH associated with IDDM or chronic hypertension respectively.

In a series of physiological studies, local cerebral blood flow (LCBF) was measured using the fully quantitative [^{14}C]-iodoantipyrine (IAP) autoradiographic technique, in areas within the vascular territories of the anterior, middle and posterior cerebral arteries in spontaneously diabetic (BB) and hypertensive (SHR) rats and appropriate

controls, under basal conditions or following manipulation of NO systems with the NOS inhibitor N^G-nitro-L-arginine methyl ester (L-NAME), the selective neuronal NOS inhibitor 7-nitroindazole (7-NI) and the NO donor 3-morpholinosydnonimine (SIN-1). In parallel intracerebral haematoma studies, volumes of significant striatal oligaemia were measured by computer-based planimetry in the same groups of animals injected with blood, or silicon oil as a control, and treated with saline, sesame oil, the non-peptide ET_A/ET_B receptor antagonist SB209670 or the selective neuronal NOS inhibitor 7-NI. In the same studies, sections adjacent to those used for autoradiography were taken for histological analysis, stained with either haematoxylin and eosin or cresyl violet with luxol fast blue. Differences in blood-brain barrier (BBB) permeability to α-aminoisobutyric acid (AIB) were measured in the same groups of animals subjected to experimental ICH, to determine if differences in permeability contributed to differences in pathology.

The physiological studies revealed that both IDDM and chronic hypertension are associated with NO-dependent cerebrovascular dysfunction. IDDM is associated with a reduced basal LCBF, linked to specific perturbation of endothelial NO, but with an intact NO-related vasodilatory capacity. In contrast, chronic hypertension is associated with an attenuated NO-associated vasodilatory reserve, in conjunction with an upregulated neuronal NO system. The haematoma studies revealed that the presence of intraparenchymal blood, rather than the mass effect, is responsible for the evolution of delayed perilesional oligaemia, which in turn is exacerbated by the presence of diabetes mellitus. Finally, although the endothelin antagonist and the neuronally derived NOS inhibitor resulted in haemodynamic improvement in the non-diseased animals, both the diabetic and hypertensive rats exhibited resistance to their beneficial effect.

In conclusion, there is abnormal cerebrovascular homeostasis associated with IDDM and chronic hypertension resulting in worse outcome following experimental haemorrhagic stroke. This cerebrovascular pathology results in resistance to neuroprotection with endothelin antagonists or neuronal NOS inhibitors which are

effective in otherwise healthy animals. These results are of particular importance since stroke commonly occurs in patients with assorted "risk" factors that can cause underlying alterations in cerebrovascular regulation. Extrapolation of the neuroprotective strategies that have evolved from tests in young healthy animals may therefore be inappropriate.

DEDICATION

I dedicate this thesis to my uncle Archbishop Methodios *PhD DD*, whose bravery and virtue is a constant source of inspiration and pride for me.

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Finally, I would like to pay tribute to the late Professor JD Miller, whose tragic death deprived the medical scientific community of a truly great leader.

DECLARATION

I, Ioannis Panayiotou Fouyas, hereby declare, that unless otherwise stated the work embodied in this thesis is the result of my own independent investigation. This is in accordance with the rule 3. 4. 7. of the University of Edinburgh Postgraduate Study Programme.

PUBLICATIONS AND PRESENTATIONS ARISING FROM THIS THESIS

FULL PAPERS

Fouyas IP, Kelly PAT, Ritchie IM, Whittle IR: Cerebrovascular responsiveness to N^G-nitro-L-arginine methyl ester in spontaneously diabetic rats. *British Journal of Pharmacology,* 1996; 118: 243-248.

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REVIEWS

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Fouyas IP, Kelly PAT, Ritchie IM, Whittle IR: Reduced basal cerebral blood flow in diabetes mellitus: Evidence for a potential involvement of the nitric oxide pathway. *Journal of Cerebral Blood Flow and Metabolism*, 1995; 15[Suppl 1]: S474

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PRESENTATIONS TO LEARNED SOCIETIES

British Neurosurgical Research Group, Chollerford, April 1995.

Brain '95 (XVII International Symposium on Cerebral Blood Flow and Metabolism), Cologne. July 1995.

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International Stroke Trial UK Collaborators Meeting, London, November 1995.

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ABBREVIATIONS

ADP	5' (Pyro)-diphosphate of adenosine
AGE	Advanced glycosylation end-products
AIB	[^{14}C]-α-aminoisobutyric acid
AMP	5'-phosphate of adenosine
AMPA	Alpha-amino-3-hydroxy-3-methyl-4-isoxasol
Ang II	Angiotensin II
AT	Angiotensin
ATP	5' (pyro)-triphosphate of adenosine
AVM	Arteriovenous malformation
BB	BioBred
BBB	Blood-brain barrier
BP	Blood pressure
$[Ca^{2+}]_i$	Intracellular calcium
CBF	Cerebral blood flow
CPP	Cerebral perfusion pressure
CVR	Cerebrovascular resistance
cAMP	3', 5'-cyclic adenosine monophosphate
cGMP	3', 5'-cyclic guanosine monophosphate
CNS	Central nervous system
CSII	Continuous subcutaneous insulin infusion
CT	Computerized tomography
DAG	Diacylglycerol
DCCT	Diabetes complications control trial
DOCA	Deoxycorticosterone acetate
DP	Diabetes prone
DR	Diabetes resistant
ECE	Endothelin converting enzyme
EDNO	Endothelium-derived NO
EDRF	Endothelium-derived relaxing factor

EM	Electron microscopy
ET	Endothelin
GSH	Glutathione
Hb	Haemoglobin
HbA_1	Glycosylated haemoglobin
H+E	Haematoxylin and eosin
HPLC	High performance liquid chromatography
IAP	[^{14}C]-iodoantipyrine
ICAM-1	Intercellular adhesion molecule-1
ICH	Intracerebral haemorrhage
ICP	Intracranial pressure
IDDM	Insulin-dependent diabetes mellitus
k_i	Blood-to-brain unidirectional transfer rate constant
LCBF	Local cerebral blood flow
L-NA	nitro-L-arginine
L-NAME	N^G-nitro-L-arginine methyl ester
L-NMMA	N^G-monomethyl-L-arginine
L-NNA	N^G-nitro-L-arginine
MABP	Mean arterial blood pressure
MCAO	Middle cerebral artery occlusion
NADPH	Reduced form of nicotinamide-adenosine dinucleotide phosphate
NMDA	*N*-methyl-D-aspartate
NO	Nitric oxide
NOS	Nitric oxide synthase
7-NI	7-nitroindazole
PAS	Periodic acid schiff
PET	Positron emission tomography
PKC	Protein kinase C
SAH	Subarachnoid haemorrhage
SD	Sprague-Dawley
SE	Standard error

SHR	Spontaneously hypertensive rat
SHRSP	Stroke-prone SHR
SIN-1	3-morpholinosydnonimine
SPECT	Single photon emission computed tomography
SRII	Sustained release insulin implant
STZ	Streptozotocin
VSMC	Vascular smooth muscle cell
WKY	Wistar Kyoto rat

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CHAPTER 1

INTRODUCTION

1.1 General Introduction

Spontaneous intracerebral haemorrhage (ICH) is a common condition and uncertainty exists regarding optimal therapeutic management (Mendelow, 1991). The initial bleed may cause limited brain dysfunction, but over the subsequent 24 to 48 hours there may be progressive deterioration in coma score and neurological deficit (Mayer *et al.*, 1994). These epiphenomena have been attributed to the development of perilesional brain ischaemia and oedema (Mendelow, 1993; Yang *et al.*, 1994). The aetiology of delayed perihaemorrhagic brain ischaemia, oedema and infarction is complex but relates to the effects of the "mass" lesion on local tissue perfusion pressure and the release of secondary "toxic" mediators from the haematoma (Nath *et al.*, 1986; Vanhoutte & Houston, 1985). The occurrence of ICH is commonly associated with the presence of host risk factors (such as chronic hypertension and diabetes mellitus; Juvela, 1996), but whether their presence alters the pathophysiological sequelae of ICH or the efficacy of therapeutic intervention is not known. This hypothesis was tested in a rodent model of ICH (Nath *et al.*, 1987), with the use of spontaneously diabetic (BB) and hypertensive rats (SHR). Pathophysiological events such as perilesional ischaemia and blood-brain barrier (BBB) disruption were measured 24 hours following experimental ICH using fully quantitative autoradiographic techniques (Blasberg *et al.*, 1983; Sakurada *et al.*, 1978). A 24h delay was used to allow any putative mediators of vasospasm and BBB disruption released from the blood clot to act upon the perilesional intraparenchymal cerebrovasculature.

The presence of endothelial dysfunction, affecting mainly extracranial tissues in a heterogeneous manner, is undoubtedly a complicating factor for both hypertension and diabetes mellitus (Cohen, 1993; Lüscher, 1992). However, the presence of cerebrovascular dysfunction, if it was established in diabetes mellitus and hypertension, could be an attractive mechanism for an altered pathophysiological response following an intracranial haemorrhagic insult. Nitric oxide (NO) plays a pivotal role in the basal control of vascular tone (Moncada & Higgs, 1993). In the brain, its role is more complex because NO of non-endothelial origin can affect blood flow (Faraci & Brian Jr, 1994) and possesses potentially neurotoxic properties

(Huang *et al.*, 1994). Perturbations of the NO systems have been found in extracranial tissues of humans and animals with diabetes mellitus and hypertension (Bucala *et al.*, 1991; Calver *et al.*, 1992). This thesis sets out to test the hypothesis that cerebrovascular dysfunction complicates diabetes mellitus and hypertension, with measurement of local cerebral blood flow (LCBF) following manipulation of the various NO pathways using selective NOS inhibitors and exogenous NO donors, and employing the same fully quantitative autoradiographic techniques in the rat strains mentioned above.

Various neuroprotective substances have been used in animal models of occlusive stroke with spectacular success, but their subsequent use in clinical trials has been rather disappointing (Hsu, 1993). Some potential neuroprotective agents have also been evaluated in experimental models of haemorrhagic stroke (Mendelow, 1993; Sinar *et al.*, 1988). The presence of host "risk" factors has not been taken into consideration in many of the animal studies, despite the fact that they are present in most human stroke victims (Juvela, 1996). It is conceivable that these risk factors can not only affect the outcome, but can also modify the pathophysiological cascades which are triggered following the pathological insult resulting ultimately in ischaemic cell death. Therefore, specific therapeutic strategies which take these individual host factors into account may be more appropriate (Muizelaar, 1996). Along these lines, antagonists of two putative mediators of ischaemic brain damage (endothelin-1, the bioavailability of which is promoted by factors released from the blood, Ohlstein *et al.*, 1991; and neuronal NO, Huang *et al.*, 1994) were tested using the same haematoma model and quantitative autoradiography, in order to identify any fundamental differences in the mechanisms that govern the haemodynamic responses of non-diseased, diabetic and hypertensive animals.

1. 2 Spontaneous Intracerebral Haemorrhage (ICH)

1. 2. 1. Incidence

Spontaneous intracerebral haemorrhage (ICH) is common in the United Kingdom (20 new cases per 100000 per year; Mendelow, 1991) and accounts for 10% of all strokes (Jørgensen *et al.*, 1995). Patients with ICH are younger, with a median age at presentation of 56 years, than patients with occlusive strokes (Wityk & Caplan, 1992) who have a median age of 65 years. The incidence of ICH is declining due to better control of risk factors such as hypertension and more appropriate use of oral anticoagulation (Furlan *et al.*, 1979). A transient increase in incidence occurred between 1961 and 1968 due to widespread use of anticoagulants. In the mid '70s a second increase was noted following the introduction of CT and subsequent realization that the incidence of ICH had previously been underestimated since many small haemorrhages had been considered to be thrombotic infarcts (Drury *et al.*, 1984). African-Americans have a higher risk for ICH, possibly due to a higher prevalence of hypertension (Broderick *et al.*, 1992). A higher incidence of ICH also seems to occur in Japan (Tanaka *et al.*, 1982), raising the possibility of a genetic predisposition to this condition.

1. 2. 2. Mortality and morbidity

Mortality from spontaneous ICH remains very high (over 50%) and is higher than that of a similar volume occlusive event (Findley & Weir 1990). This higher mortality is related to events occurring during the first 48 hours, with 27% of patients dying during the first day (Fogelholm *et al.*, 1992). In patients surviving this early post-ictal period however, the mortality becomes similar to that following cerebral infarction (Franke *et al.*, 1992). Morbidity is also very high. Of the patients surviving an ICH, 49% will be dependent on outside help (Fogelholm *et al.*, 1992). The volume of the haematoma is one of the crucial factors influencing subacute neurological deterioration and final outcome (Mayer *et al.*, 1994). Lobar haematomata with volumes of 40 ml or more are likely to be associated with a delayed deterioration, most commonly occurring within the first 24 hours of the ictus, unrelated to haematoma enlargement which invariably occurs within 6 hours after the original

bleed (Fujii *et al.*, 1994), and carry a worse prognosis. Other factors related to poor prognosis include location of haematoma, elevated plasma glucose and low Glasgow Coma Score on admission (Franke *et al.*, 1992; Tuhrim *et al.*, 1995), initial stroke severity (Jørgensen *et al.*, 1995), old age, intraventricular extension and hydrocephalus (Fogelholm *et al.*, 1992). The presence of ventricular blood probably reflects the size of the bleed and may not be independently important. Patients on oral anticoagulants often have large haematomata, with substantially higher mortality rates (Hart *et al.*, 1995; Rådberg *et al.*, 1991). Similarly, haemorrhages associated with the use of thrombolytic agents for the treatment of acute myocardial infarction have a grave prognosis (Kase *et al.*, 1992; Sloan *et al.*, 1995). Haemorrhages occurring from a pre-existing AVM are frequently non-fatal (Kase, 1995). Markedly elevated blood pressure on admission adversely affects prognosis (Dandapani *et al.*, 1995).

1. 2. 3. Pathophysiology

Studies addressing the cerebral pathophysiological sequelae following ICH have been conducted using various animal models. The main bulk of current information arises from experimental work in rodents, where the temporal evolution of the acute and subacute pathophysiological events that accompany an ICH have been investigated.

Following experimentally induced ICH, an area of early ischaemia develops in the perilesional brain parenchyma, possibly due to compression of the microcirculation immediately surrounding the "mass" lesion, even in the absence of generalized increase of intracranial pressure (ICP) (Bullock *et al.*, 1984; Kingman *et al.*, 1987; Nath *et al.*, 1986). No evidence of an acutely raised ICP has been found following injection of a mass lesion upto 100μl, which volume for volume is equivalent to a 100ml haematoma in the human brain (Kingman *et al.*, 1988). Gradually, this compression of the microcirculation around the haematoma settles. This is probably due to clot retraction (Wagner *et al.*, 1996) and lysis (Masuda *et al.*, 1988) associated with spatial rearrangements in the brain, so that by 4 hours after the insult the initial ischaemic zone around the clot has almost disappeared (Nath *et al.*, 1987). There is also strong speculation that "toxic" metabolites are released from the clot (Vanhoutte

& Houston, 1985) inducing vasospasm in the intraparenchymal perilesional arterioles and disruption of the BBB with development of vasogenic oedema (MacKenzie, 1996; Mendelow *et al.*, 1986). Experimental evidence supporting this hypothesis arises from studies in which an intrastriatal injection of silicon oil (with viscosity similar to that of blood), induces less acute perilesional oligaemia compared to an equivalent volume of unclotted arterial blood (Jenkins *et al.*, 1990).

There are reports from clinical studies, where these concepts of perilesional ischaemia and oedema can be applied to human sufferers of ICH. In these studies it has been reported that patients with ICH can suffer delayed neurological deterioration in the absence of rebleeding (Mayer *et al.*, 1994), independent of global ICP elevations (Papo *et al.*, 1979) and where the presence of perilesional ischaemia has been indicated with the use of transcranial doppler (Mayer *et al.*, 1996).

1. 2. 4. Ischaemia associated with ICH

Ischaemic brain injury is a rather complex event and intensive research is being pursued in an attempt to understand the nature of this process (see Siesjö *et al.*, 1995). Following cessation of blood flow (CBF) cell death due to energy failure occurs in the core of an ischaemic lesion (Siesjö, 1992). However, in the zone surrounding the core, which is perfused by significantly compromised CBF, cells can remain potentially viable for a variable but limited time, and eventually die (Fisher & Garcia, 1996). This area of constrained blood supply, where the metabolic demands are met for a limited period, is termed the "penumbra" (Hossmann, 1994). The availability of glucose in the presence of ischaemia can result in further compromise of the brain tissue, because the cells shift to anaerobic glycolysis with subsequent creation of tissue acidosis. This presents one potential mechanism explaining the detrimental effects of hyperglycaemia in relation to cerebral ischaemia. The role of hyperglycaemia in relation to cerebral ischaemia is also covered in the ***Insulin-dependent diabetes mellitus*** section of this thesis.

Experimental findings support the hypothesis that following the initial "stroke" ictus (occlusion of an intracranial artery or formation of an intraparenchymal haematoma) secondary processes evolve, upon an already vulnerable tissue, resulting in the liberation of excitotoxic neurotransmitters, presumably as a result of neuronal depolarization or spreading depression originating in the core of ischaemia. Upon activation of neuronal receptors (mainly the NMDA) abnormally large amounts of Ca^{++} enter the cells, with subsequent activation of proteolytic enzymes (Bartus *et al.*, 1994) and further cell damage.

There is also evidence that an occlusive cerebrovascular event is commonly followed by subsequent spontaneous reperfusion. Early reperfusion through a previously occluded artery is obviously essential for the salvage of ischaemically compromised tissue, but delayed reperfusion has various potentially deleterious effects due to generation of oxygen-derived free radicals (Kim *et al.*, 1996; Matsuo *et al.*, 1995) and inflammatory sequelae (Connolly Jr *et al.*, 1996), including activation of microglia (Gehrmann *et al.*, 1995) and cytokines (Yamasaki *et al.*, 1995). Finally, the as yet ill-defined role of apoptosis in relation to cerebral ischaemia is currently under intense investigation (Du *et al.*, 1996).

Ischaemic events associated with ICH may follow a somewhat different pathway because of the presence of a "mass" lesion compressing the surrounding microvasculature, with creation of local tissue pressure gradients, and also the presence of blood with potentially vasospastic properties (Mendelow, 1993). Interestingly, *in vitro* studies have also shown that endothelin (ET) production is induced by blood constituents in a time-dependent manner, and ET itself is a very potent vasoconstrictor in the cerebrovasculature (Sharkey *et al.*, 1993). There is additional evidence that haemoglobin quenches nitric oxide (NO) (Martin *et al.*, 1985), a very potent vasodilator, possibly providing further gravity to the concept of delayed perilesional ischaemia following ICH. The roles of both NO and ET in relation to cerebral ischaemia are covered more extensively in the ***Nitric Oxide*** section of this thesis.

It should also be considered that an analogy to the reperfusion injury of occlusive stroke might also exist in the perilesional brain following ICH, once the action of the putative spasmogens resolves. In addition, iron from haeme can catalyze lipid peroxidation of cellular membranes contributing to the severity of the ischaemic cell damage (Halliwell & Gutteridge, 1990). Although the brain has all the prerequisite systems (including astrocytes and microglia) to convert haemoglobin iron to ferritin and haemosiderin and thus inactivate it (Koeppen *et al.*, 1995), these enzyme systems may be adversely affected by ischaemia. It is also noteworthy, in relation to the role of inflammatory mediators upon the evolution of ischaemia following haemorrhagic stroke, that whole body irradiation (resulting in inactivation of white cells) attenuated ischaemic damage in the perilesional striatum following intrastriatal microballoon inflation in the rat (Kane *et al.*, 1990).

Studies evaluating the presence of delayed perilesional ischaemia following experimental ICH are lacking. A microballoon has been used in the rat to simulate the "mass" effect of the haematoma, and experiments have been undertaken with the use of this model addressing the role of surgical intervention in ICH. Perilesional ischaemia was evident at 24 hours, but animals in which the balloon was deflated 4 hours after the inflation had a much better outcome in terms of haemodynamic compromise (Nehls *et al.*, 1990). However, the microballoon is devoid of the putative spasmogens released from a blood clot. In another study, pretreatment with the calcium antagonist nimodipine improved striatal CBF 4 hours after the production of an experimental haematoma (Sinar *et al.*, 1988). In collagenase-induced striatal ICH in the rat (a model of haemorrhagic transformation in an existing infarct), ischaemic levels of LCBF were observed in the caudate at 24h (Rosenberg *et al.*, 1992).

1. 2. 5. Oedema following ICH

Brain oedema is generally divided into cytotoxic and vasogenic types depending upon whether the BBB is intact or disrupted (Kimelberg, 1995; Klatzo, 1967). In the context of ICH, an additional form of oedema should be considered generated by osmotic stress from the clot products (Yang *et al.*, 1994). This latter form of oedema

develops probably quite early (~1h) after the onset of the haemorrhage presumably as a result of clot retraction and release of soluble protein into the parenchymal tissue (Wagner *et al.*, 1996).

Oedema formation around the haematoma generally resembles, with certain exceptions, that occurring during focal ischaemia; it presents at 6 to 12 hours following the ictus and this initial phase results mainly from disruption of BBB presumably following release from the blood of putative mediators of vasogenic oedema (Wahl *et al.*, 1988) such as thrombin (Lee *et al.*, 1995; Lee *et al.*, 1996); it reaches a peak at 24 hours, and remains constant for around 5 days before it starts subsiding. At 24 hours following the bleed the oedema seems to be a result of direct cytotoxicity and BBB disruption (Yang *et al.*, 1994).

This temporal evolution of oedema is in contrast to what occurs in occlusive focal cerebral ischaemia, where the cytotoxic phase of oedema occurs early (Menzies *et al.*, 1993). A possible explanation for this discrepancy is that factors released in a *delayed* fashion from the clot cause vasospasm in the surrounding vessels, contributing to ischaemia. Studies to test this possibility have not been performed, and in those reports where the temporal evolution of oedema following experimental ICH was addressed (Yang *et al.*, 1994), LCBF in the perilesional area was not measured. Total ipsilateral striatal CBF was however measured, and although a reduction in CBF was noted at 24 hours compared to earlier time points, it did not reach ischaemic levels (Yang *et al.*, 1994). Not only did this paradigm fail to measure small, localized areas of perilesional ischaemia, it also failed to account for any possible reactive hyperaemia associated with the insult.

1. 2. 6. Potential modifications of pathophysiology of ICH by "host" risk factors

Hypertension, insulin-dependent diabetes mellitus (IDDM) and aging (Davis *et al.*, 1995, Sutherland *et al.*, 1996) are associated with increased incidence and worse outcome following cerebral ischaemia. Although chronic hypertension is very commonly associated with ICH and experimental studies have shown that occlusive

stroke in hypertensive animals is associated with worse outcome (Coyle, 1984), its association with increased morbidity following ICH is far from clear. In an experimental study using spontaneously hypertensive rats (SHR), there was a significantly higher acute mortality in the hypertensive group following striatal injection of a blood volume of 400μl (Gonzàlez-Darder & Duràn-Cabral, 1990). In contrast, studies in humans report that hypertensive patients have worse outcome following ICH probably because they have larger bleeds (Jørgensen *et al.*, 1995) and not because the pathophysiological response to the haemorrhage is different from that of non-hypertensives. Interestingly, studies have also emerged stressing the fact that inherited alterations of vascular function (manifest in an impairment of endothelium-dependent vasorelaxation) may, independently of blood pressure, contribute to the pathogenesis of stroke in hypertensive animals (Volpe *et al.*, 1996). Likewise, IDDM is associated with worse outcome following occlusive stroke in humans and animal models, but whether this is true following ICH has not been investigated.

Attempts to translate the spectacular successes of neuroprotective strategies from animal models of stroke into the clinical environment have, to date, been largely disappointing. Since most human stroke victims suffer from on-going vascular disease processes, which might be both actively responsible for the stroke and at least contribute to the pathology once the stroke has occurred, it is attractive to hypothesize that their presence may alter the pathophysiological sequelae of ischaemia. In relation to ICH this association is even more relevant, because if blood products trigger additional delayed processes (ET production, NO quenching, BBB disruption by thrombin and other mediators), the presence of "host" cerebrovascular dysfunction may alter the response. There is evidence of vascular dysfunction complicating both chronic hypertension and IDDM in extracranial vascular tissues, so, it is worth investigating whether this may also be the case in the cerebrovasculature. Additionally, these pathological processes seem to evolve at a time point that the possibility for neuroprotection becomes more realistic.

Experimental studies in support of the hypothesis that the presence of background vascular pathology can modify the pathophysiology of cerebrovascular events, arise from studies that show opposite patterns of response to reperfusion following experimental middle cerebral artery occlusion (MCAO) in SHR when compared to normal rats (Kurokawa & Tranmer, 1995; Selman *et al.*, 1994). In these studies SHR had a worse outcome following ischaemia/reperfusion compared to permanent ischaemia, whereas the non-hypertensive animals had a better outcome following ischaemia/reperfusion compared to permanent ischaemia. The SHR are also resistant to neuroprotection with excitotoxic neurotransmitter (glutamate) antagonists following focal ischaemia (Roussel *et al.*, 1992).

1. 2. 7. Experimental Models of ICH

Experimental models of ICH have been available since the 1960s and commonly involve the injection of autologous blood into the frontal lobe of anaesthetized dogs, cats or monkeys (Sussman *et al.*, 1974; Takasugi *et al.*, 1985; Whisnant *et al.*, 1963), and more recently smaller animals (rodents). Rodents have been found to provide a convenient and suitable model of ICH. They are inbred providing genetic homogeneity within strains, there is close resemblance of the cerebrovascular anatomy and physiology to that of higher species, and certain strains are associated with the presence of background pathology akin to human disease (i.e. the BioBred-BB rat and the SHR strain).

Several approaches have been developed for the production of ICH in rodents. Most groups inject autologous arterial blood in the striatum of the anaesthetized animal, with varying speed to avoid reflux. During the original attempts in Glasgow, blood was injected under arterial pressure, the whole injection lasting less than 1min (Nath *et al.*, 1987). In a subsequent modification of this model the blood was introduced in the form of an *infusion* rather than an injection with a speed of infusion ~10$\mu l.min^{-1}$ (Masuda *et al.*, 1988). In an attempt to overcome the problem of reflux, the group from Glasgow used a microballoon (Nehls *et al.*, 1988), which could be deflated, to simulate haematoma removal. However, despite the interesting results obtained with

the microballoon model, the physical characteristics of the balloon were substantially different from that of a blood clot.

Problems associated with the use of rats for experimental ICH include the fact that the brains of these animals have very limited amounts of white matter tissue, making it difficult to simulate the commonly occurring lobar haematomata in humans. In addition, the small size of the rat brain makes reflux of blood along the needle track problematic. In an attempt to reduce the backflux of injected blood along the needle track, the "double injection" model has been recently described. A small amount of blood is initially injected, followed by an interval of 7min before the final injection is performed, thus allowing the first injection to clot and block the potential pathways of backflux (Deinsberger *et al.*, 1996).

In an effort to overcome the problems of introducing exogenous blood, Rosenberg injected collagenase, a proteolytic enzyme that destroys the vascular wall, into the striatum of the rat (Chesney *et al.*, 1995; Elger *et al.*, 1994; Rosenberg *et al.*, 1990a,b; Rosenberg & Navratil, 1994). This is more akin to a haemorrhagic transformation of an occlusive infarct in the man, rather than a primary ICH. Bleeding occurred 10min after the injection, coalescing into a mass lesion around 4 hours, and at 24 hours there was a narrow band of poorly staining parenchyma surrounding the clot. The final lesion is a confluent area of multiple foci of microscopic haemorrhages. The temporal evolution of oedema and the presence of delayed (24h) striatal ischaemia associated with this model, resembles the other rodent models of ICH. However it must be stressed that with this model the volume of the haematoma is very extensive, occupying virtually the entire caudate nucleus (Lyden *et al.*, 1997).

The development of a rat strain, the stroke-prone spontaneously hypertensive rat (SHRSP) was reported by Yamori and his colleagues in 1974, that had very high blood pressure and a high incidence of stroke. This was derived from the spontaneously hypertensive rat (SHR) and exhibited an 80% to 90% incidence of cerebral lesions during the first year of life. However, most of the lesions that develop

are occlusive rather than haemorrhagic in origin and their incidence also depends on nutritional factors (Yamori *et al.*, 1984). This, along with the lack of reproducibility in the spontaneous haemorrhagic lesions, makes this model far from ideal for the study of spontaneous ICH.

Experiments in larger animals (pigs) have been combined with injection of thrombolytic agents and clot aspiration at certain time points following the injection of the blood. The purpose of this was to simulate haematoma evacuation, in an attempt to ascertain any benefits from surgical intervention (Wagner *et al.*, 1995 & 1996). Another advantage associated with the use of these animal models is that they have a comparable volume of white matter to that of the human brain. The disadvantage of using larger animals is that, appropriate models of spontaneous host diseases, like chronic hypertension or diabetes mellitus (Muizelaar, 1996), cannot be used.

The use of transgenic animals, is and will undoubtedly continue providing new insights in relation to cerebrovascular physiology and pathology. It is an area of scientific research in constant evolution, but since it is currently confined mostly to mice (Johns *et al.*, 1996; Shuldiner, 1996), it is technically difficult to incorporate them into studies that address the pathophysiology of ICH.

1. 3. Insulin-Dependent Diabetes Mellitus (IDDM)

1. 3. 1. Pathogenesis and role of nitric oxide (NO)

Diabetes mellitus is a metabolic disorder associated with functional and structural abnormalities in a variety of organs and systems of the body. The aetiology of the disease is still obscure, but the bulk of scientific evidence suggests that immunological factors play a major role (Pankewycz *et al.*, 1995). The histological evidence of "insulitis", the presence of islet cell antibodies and of increased levels of "activated" T lymphocytes provide supportive evidence that the destruction of the pancreatic beta cells is mediated by autoimmune processes. Current evidence suggests that the release of highly toxic free radicals (either directly by infiltrating macrophages or secondary to secretion of cytokines, such as interleukin-1, from activated macrophages) may be a critical event in the inflammatory destruction of islet β-cells (Rabinovitch, 1993). Nitric oxide (NO) has been proposed by several scientific groups as the specific free-radical species which acts as the primary effector molecule in cytokine-mediated β-cell dysfunction (resulting in decreased insulin secretion) and destruction *in vitro* (Corbett & McDaniel, 1992; Kroncke *et al.*, 1991; Sjöholm, 1996) and *in vivo* (Lindsay *et al.*, 1995; Wu, 1995).

1. 3. 2. General Complications

Diabetes is associated with the development of complications in several tissue organs. Areas rendered particularly vulnerable include the retina, the kidney and the peripheral nerves. The presence of both macro- and microvascular changes in relation to IDDM result in hypertension, accelerated atherogenesis, thrombosis and ischaemia (Colwell, 1991; Tooke, 1995), which can all cause tissue dysfunction. The pathogenic mechanisms for the evolution of these processes are still rather obscure, with earlier studies concluding that their appearance is independent of the degree of diabetic control (Kannel & McGee, 1979; Kroc Collaborative Study Group, 1984), suggesting that it was the disease process itself which was responsible and not exposure to hyperglycaemia or insulin treatment. However, more recent work (DCCT Research Group, 1993; Reichard *et al.*, 1993) suggests a more complex causal relationship between the disease process and therapeutic intervention.

1. 3. 3. CNS Complications

Although the cerebral circulation has been found to be subject to pathological change similar to that found in the periphery (Aronson, 1973; Grunnet, 1963), the effects upon cerebrovascular physiology were previously thought to be rather subtle, and often went unrecognized. More recently however, it has become apparent that neither the brain nor its vasculature are spared from the effects of diabetic pathology (McCall, 1992; Mooradian, 1988), and altered blood/brain transport (Boyle *et al.*, 1995; Gjedde & Crone, 1981; McCall *et al.*, 1982), cerebral blood flow (Grill *et al.*, 1990; Macleod *et al.*, 1994; Tallroth *et al.*, 1993), and brain metabolism, as well as effects on neurons and glia, are all associated with the disease process.

Diabetic patients are at higher risk of developing stroke, both occlusive and haemorrhagic (Juvela, 1996; Lavy *et al.*, 1973; Stegmayr & Asplund, 1995). Epidemiological studies reveal that the presence of the disease process seems to also alter the outcome following stroke (Jørgensen *et al.*, 1994). The complexity of functional and structural derangements associated with IDDM make it difficult to study the pathophysiological mechanisms responsible for the increased vulnerability of the diabetic brain to ischaemia.

1. 3. 4. Role of hyperglycaemia in the CNS complications

Hyperglycaemia is commonly associated with IDDM and in extracranial tissues seems to be correlated with the extent of vascular complications (Dahl-Jørgensen *et al.*, 1994; DCCT Research Group, 1993) although a direct cause-and-effect relationship is not evident in all the tissues. Its presence is also associated with exacerbation of ischaemic cell damage in animal models of cerebral ischaemia (Duverger & MacKenzie, 1988; Nedergaard & Diemer, 1987), with very few contradictory results (Kraft *et al.*, 1990).

In severe ischaemia, acute hyperglycaemia potentiates brain lactic acid production and increases tissue acidosis (Smith *et al.*, 1986). The increased H^+ generation leads to a sustained rise in intracellular Ca^{++} with all the detrimental effects that this carries. This

scenario occurs while cerebral blood flow (CBF) is reduced to a point where glucose delivery becomes limiting for glucose phosphorylation and subsequent glycolysis (Siesjö *et al.*, 1993). However, under less severe ischaemia, glucose phosphorylation, rather than glucose delivery and blood-brain barrier transport, is the limiting factor. Hyperglycaemia under these conditions will not affect brain lactate generation, but can still be associated with worsened brain damage, unrelated to acidosis potentiation (Hoffman *et al.*, 1990; Warner *et al.*, 1995). In some animal studies of occlusive stroke (Wagner *et al.*, 1992), but not others (Nedergaard *et al.*, 1988), hyperglycaemia was also associated with more depressed CBF in the area supplied by the occluded middle cerebral artery, as well as delayed blood flow recovery following reperfusion (Kittaka *et al.*, 1996).

Recent studies have also revealed that hyperglycaemia is associated with an increase in endothelin (ET) concentration in cultured endothelial cells *in vitro* (Yamaguchi *et al.*, 1990), a decrease in adenosine production following ischaemia and reperfusion *in vivo* (Hsu *et al.*, 1991), and with increases in inflammatory cell adhesion molecules (ICAM-1) *in vitro* (Baumgartner-Parzer *et al.*, 1995a). These may offer potential explanations for the potentiated cerebrovascular sequelae of ischaemia. *In vivo* studies in extracranial tissues have also raised the possibility that ET may be responsible for some of the microvascular abnormalities associated with drug-induced diabetes (Cameron *et al.*, 1994). However, contradictory reports of down-regulation of ET-1 receptors in the kidney in association with activation of protein kinase C (PKC) (Awazu *et al.*, 1991) and resistance to ET-1's biochemical actions induced by elevated glucose (De La Rubia *et al.*, 1992) have also emerged. High plasma glucose levels are also associated with apoptosis in cultured endothelial cells (Baumgartner-Parzer *et al.*, 1995b).

1. 3. 5. Role of oedema in the CNS complications

One of the potential mechanisms for the increased susceptibility of diabetics to brain injury is a "leaky" BBB associated with the disease. Certainly, in extracranial tissues of humans, IDDM is associated with increased vascular permeability, even at early

stages of the disease (Parving, 1976; Viberti, 1983). *In vitro* studies showed that vascular permeability was modestly increased in the brains of streptozotocin (STZ)-induced diabetic rats (Wautier *et al.*, 1996) but studies in BioBred (BB) rats revealed a relative resistance of brain to the increased vascular permeability, which was nevertheless present in extracranial tissues (Williamson *et al.*, 1987). However, *in vivo* studies in STZ rats showed a very subtle increase in BBB permeability in the hypothalamus of the diabetic animals (Lorenzi *et al.*, 1986), as well as selective extravascular escape of albumin into the cerebral cortex (Öztas & Küçük, 1995; Stauber *et al.*, 1981). In contrast, another study employing the same model of drug induced diabetes failed to reveal any increase in BBB permeability associated with the disease (Bradbury *et al.*, 1991).

In the context of experimental (Dietrich *et al.*, 1993; Warner *et al.*, 1987) and human (Berger & Hakim, 1986) cerebral ischaemia, acute hyperglycemia worsens acute BBB injury, but interestingly chronic hyperglycaemia may generate adaptive processes to counter this effect (Sieber *et al.*, 1994).

1. 3. 6. Role of endothelial dysfunction in the CNS complications

Microvascular pathology is commonly associated with IDDM. Evidence of endothelial dysfunction, i.e. the presence of abnormal vascular responses to agonists exerting their action via endothelial intermediates in the absence of structural abnormality, is present in various extracranial tissues (Cohen, 1993; Oyama *et al.*, 1986; Poston & Taylor, 1995). This suggests altered bioactivity of either vasodilators (endothelium-derived NO, prostacyclin, endothelium-derived hyperpolarizing factor) or vasoconstrictors (prostaglandin, endothelin). Potential mechanisms for this dysfunction include alterations in the polyol pathway, increased production of oxygen-derived free radicals (Cameron & Cotter, 1995) and protein kinase C, alterations in Na^+, K^+-ATPase (Xia *et al.*, 1995) and production of advanced glycosylation end products (AGE) (Bucala *et al.*, 1991), all associated with the disease process.

The presence of abnormal endothelial function in the brain of diabetics is not well established, but indirect evidence suggests that this may indeed be the case. Pathophysiological effects of the disease upon the cerebral circulation are manifest in an impaired autoregulatory response to alterations in systemic blood pressure (Bentsen *et al.*, 1975; Kastrup *et al.*, 1986), altered CO_2 reactivity even in young patients who lack structural vascular complications (Griffith *et al.*, 1987), impaired cerebrovascular reactivity to lidocaine (Kastrup *et al.*, 1990) and acetazolamide (Rodriguez *et al.*, 1993), and regionally distributed cerebral blood flow decreases in animal models (Duckrow *et al.*, 1987; Harik & LaManna, 1988; Jakobsen *et al.*, 1990), all being processes in which the endothelium may play an important role.

In the STZ-induced animal model of diabetes there is clear evidence, from both *in vitro* and *in situ* (cranial window) studies, of impaired cerebrovascular responsiveness to a variety of vasoactive compounds including ADP (Mayhan, 1989), serotonin (Mayhan, 1989; Rosenblum & Levasseur, 1984), β-adrenoceptor agonists (Mayhan, 1994), and acetylcholine (Mayhan *et al.*, 1991). Studies performed *in vivo* showed a reduced effect of muscarinic agonists upon blood flow, which was regionally variable (Pelligrino *et al.*, 1992). Interestingly however, the STZ rat model does not appear to display the same reduced cerebrovascular CO_2 reactivity found in humans (Pelligrino & Albrecht, 1991), although even in that model the CBF response to acute hypoglycaemia was attenuated in the diabetic animals. Moreover, in the STZ model, and contrary to findings in human sufferers of the disease, autoregulation is preserved (Rubin & Bohlen, 1985). It should be pointed out that the endothelial dysfunction in STZ-treated rats is rather complex especially in peripheral vascular beds, where *in vivo* studies have reported that the pressor response to an inhibitor of NO synthase (L-NAME) was attenuated (Kiff *et al.*, 1991a) whilst vasodilator responses to acetylcholine (which establishes some of its vasoactive effects through stimulation of NO release) remained intact (Kiff *et al.*, 1991b).

The potential presence of cerebrovascular dysfunction in IDDM would be of particular importance not only in relation to the physiological control of cerebral

circulation, but also because some of these endothelial mediators (NO, ET and prostaglandins) have recently been implicated in the pathophysiology of stroke (Barone *et al.*, 1995; Huang *et al.*, 1994; Warso & Lands, 1983). Recent evidence suggests that there is selective depression of endothelium-dependent dilatation in the cerebrovasculature during experimental cerebral ischaemia (Rosenblum & Wormley, 1995), rendering the presence of endothelial dysfunction under physiological conditions an even more important factor that could complicate the responses of the host to ischaemia.

1. 3. 7. Role of structural changes, biochemical and metabolic abnormalities in CNS complications

The disease is also associated with structural changes in the cerebrovasculature of both humans (Johnson *et al.*, 1982) and animals with drug-induced diabetes (Moore *et al.*, 1985; Mukai *et al.*, 1980). Interestingly, the expression of collagen IV and fibronectin in endothelial cells increases and the activity of enzymes involved in collagen synthesis is enhanced under high-glucose conditions. This effect of hyperglycaemia persists for several weeks after restoration of normoglycaemia (Roy *et al.*, 1990). Increased atherosclerosis has also been reported in association with STZ-induced diabetes (Kunjathoor *et al.*, 1996).

Reduction in the density of cerebral cortical microvessels has also been reported in STZ rats (Jakobsen *et al.*, 1987). The presence of rheological abnormalities, such as increased viscosity, hyperlipidaemia, decreased red blood cell deformability and increased procoagulant activity is also common in IDDM. An increase in plasma viscosity (Barnes *et al.*, 1977) and increased adhesion of platelets to endothelial cells (MacMillan *et al.*, 1978; Wautier *et al.*, 1981) may contribute to cerebrovascular dysfunction in diabetes. Interestingly, the haemorrheological changes associated with experimental diabetes reveal regional variations (Sutera *et al.*, 1992). Further experimental work has also shown that tissue plasminogen activator is downregulated in diabetic but not acutely hyperglycaemic rats following middle cerebral artery occlusion (MCAO), possibly linked to delayed restoration of CBF during reperfusion

(Kittaka *et al.*, 1996). Delayed metabolic recovery (Sutherland *et al.*, 1992) as well as slower recovery of ion homeostasis (Tyson *et al.*, 1996) after reperfusion, has also been observed in spontaneously and drug-induced diabetic rats following forebrain ischaemia.

In the aetiology of diabetic neuropathy, metabolic alterations such as decreased myo-inositol levels (secondary to an increase in sorbitol formation), decreased Na^+, K^+-ATPase (Greene *et al.*, 1987; Ver *et al.*, 1995) and Ca^{2+}-ATPase (Janicki *et al.*, 1994) activity, mitochondrial dysfunction and altered calcium signaling (Levy *et al.*, 1994), have been reported. Whether similar abnormalities exist in the diabetic brain is not clear. It should also be mentioned that contradictory reports have been published regarding the potential role of decreased Na^+, K^+-ATPase activity and decreased myo-inositol in the diabetic microvessels (Mooradian *et al.*, 1994; Sussman *et al.*, 1988). With respect to the potential role of metabolic alterations in relation to the excessive production of free radicals in diabetes, which can in turn be responsible for vascular damage, *in vitro* and *in vivo* evidence exists of a link between diacylglycerol (DAG)-PKC activation and production of free radicals, induced by hyperglycemia in extracranial vascular smooth muscle cells (VSMC) (Kunisaki *et al.*, 1996).

1. 3. 8. Role of advanced glycosylation endproducts (AGE) in CNS complications

The development of AGE in diabetes (Brownlee *et al.*, 1988) has rather complex effects (Cameron & Cotter, 1996; Kihara *et al.*, 1991), both haemodynamic but also directly cytotoxic. The haemodynamic effects are manifested primarily by the quenching of endothelial NO by subendothelial AGE, resulting in reduction in its bioactivity (Bucala *et al.*, 1991). However, studies have also shown that AGE are directly cytotoxic in the cerebral tissue (Zimmerman *et al.*, 1995). AGE-proteins and peptides may enhance tissue damage by binding to AGE-specific receptors on macrophages, endothelial cells (Schmidt *et al.*, 1995) and other cell types including VSMCs (Schmidt *et al.*, 1996), to mediate capillary leakage, cytokine production, enhanced procoagulant activity on the endothelial surface and increased generation of reactive oxygen intermediates (Vlassara & Bucala, 1996). Their potential involvement

in the vulnerability of the diabetic brain to exacerbated cerebral ischaemia is very probable, but whether their precise role is haemodynamic or cytotoxic is far from clear.

1. 3. 9. Animal models of diabetes mellitus

The most commonly used model of the disease is the STZ-induced diabetic rat. The development of diabetes in this model involves an autoimmune component, with elevated endogenous NO participating in that action (Lukic *et al.*, 1991; Tanaka *et al.*, 1995). However, the existence of other mechanisms for the diabetogenic function of STZ, such as the direct release of NO from the nitroso moiety of STZ (Turk *et al.*, 1993) complicate the interpretation of the pathogenesis of diabetes in this model.

Animals with STZ-induced diabetes are not dependent on exogenous insulin for survival, despite the presence of severe hyperglycaemia and malnutrition, and are ketosis-resistant (Pelligrino *et al.*, 1989). In an attempt to create an animal model with plasma glucose levels more closely related to those of diabetic subjects, some investigators have administered nicotinamide prior to the injection of STZ, which results in a milder form of diabetes (Pugliese *et al.*, 1989). Additionally, in most studies with STZ, the duration of diabetes is rather short. This makes it impossible to address the effects of AGE, which need at least 6 to 8 weeks to develop, in the evolution of any observed complications. Interestingly, treatment with insulin reverses the abnormalities found in peripheral tissues (Kihara & Low, 1995) and the brain (Bômont & MacKenzie, 1995; Warner *et al.*, 1992), indicating that this could be best described as a model of hyperglycaemia rather than diabetes *per se*. Finally, and probably most importantly, studies have shown that STZ has no diabetogenic effect in human β-cells (Eizirik *et al.*, 1994). Similarly, alloxan damages pancreatic β-cells by an oxidative mechanism and it has been used in a context similar to that of STZ, but again the same problems regarding the lack of any diabetogenic effect in human β-cells occur in this model too (Eizirik *et al.*, 1994).

Diabetes has also been induced in larger animals (dogs) following pancreatectomy. In this model the vascular pathology associated with the disease does not develop until 2 to 3 years of poorly controlled diabetes have elapsed (Sieber *et al.*, 1993 & 1994). It is evident therefore, that it is used as a model of hyperglycaemia rather than diabetes mellitus by most groups.

The BioBred (BB) rat strain provides an attractive model for the study of juvenile-onset IDDM. The involvement of genetic and immune aetiological factors in the pathogenesis of the disease (Crisa *et al.*, 1992; Lee, 1994), and especially the dependence on exogenous insulin for prevention of ketoacidosis together with the development of diabetic complications in a variety of organs (Marliss *et al.*, 1982), represent a condition more akin to the human disease process than that afforded by models of drug-induced diabetes (Eizirik *et al.*, 1994). Pathological changes in the retina, kidneys and peripheral nerves have been observed as early as 3 weeks following the onset of diabetes (Baird, 1989). There is no detectable circulating immunoreactive insulin (Baird, 1989) and endothelial dysfunction has been described in extracranial tissues of the diabetic animals (Kappagoda *et al.*, 1989; Lindsay *et al.*, 1997; Meraji *et al.*, 1987).

The transgenic mouse studies have provided insights into fundamental questions of how immune tolerance is established and broken down, delineating the potential effector mechanisms involved in autoimmune destruction associated with the pathogenesis of IDDM. However, transgenic technology has not yet produced models which have been so comprehensively characterized as for example the BB rat strain (Lipes & Eisenbarth, 1990). Moreover, the size of mice is prohibitive for the study of the associated central nervous system complications.

1. 4. Essential Hypertension

1. 4. 1. Pathogenesis of Hypertension

Chronic hypertension is characterized by altered haemodynamic balance, in particular increased peripheral vascular resistance (Pickering, 1936). The precise mechanisms for the raised peripheral resistance are not clear, with neural (e.g. sympathetic nervous system and neuropeptide Y), circulating (e.g. angiotensin II, noradrenaline and vasopressin) and local factors (e.g. nitric oxide, endothelin-1 and prostaglandins) potentially contributing to the increased vascular tone (Shepherd, 1990). Within the vascular wall, structural and functional alterations may also occur (Folkow, 1990).

The endothelium has a strategic anatomical position that allows it to regulate the function of both VSMCs and circulating blood cells (platelets, monocytes). The release by the endothelium of various mediators that effect either contraction (endothelin and certain prostaglandins) or dilatation (NO, prostacyclin and endothelium-derived hyperpolarizing factor) of the underlying VSMC, makes an imbalance between those opposing influences an attractive pathogenetic mechanism of the disease, as well as a source for its associated complications (Lüscher, 1994).

1. 4. 2. Role of Nitric Oxide (NO) in the pathogenesis of hypertension

Although it has been proposed that an impaired release of endothelial vascular relaxing factors might underlie the pathogenesis of chronic hypertension (Lüscher & Vanhoutte, 1986; Snyder, 1995; Taddei *et al.*, 1996; Woodman, 1995), the involvement of NO in the aetiology of the disease process remains controversial. Studies in hypertensive human subjects have shown that there is abnormal NO activity associated with hypertension (Calver *et al.*, 1992; Panza *et al.*, 1990), but divergent results have also emerged (Angus & Lew, 1992; Cockroft *et al.*, 1994). In experimental animal models of chronic hypertension, there is increasing evidence that endothelium-dependent relaxation is heterogeneously affected (Lüscher, 1992), with normal function maintained in the renal and coronary arteries (Kelm *et al.*, 1995; Nava *et al.*, 1995; Tschudi *et al.*, 1991), but impaired function in aorta, mesenteric, carotid and cerebral circulation (Crespo *et al.*, 1996; Cuevas *et al.*, 1996; Dohi *et al.*,

1990; Lüscher & Vanhoutte, 1986). These observations could explain not only contradictory experimental observations (Dominiczak & Bohr, 1995), but also the selective vulnerability of certain tissues, such as the brain, to hypertensive complications. Interestingly, recent findings of a reduced release of endothelial NO from aged aorta but not from aged pulmonary artery in response to agonists, is consistent with the hypothesis that elevated BP may cause a reduction in the production of endothelially derived NO (Tschudi *et al.*, 1996a). Overall, there is no clear consensus as to whether NO synthesis is impaired in human hypertension and, if it is, whether the defect is primary or secondary (Benjamin & Vane, 1996).

1. 4. 3. Role of endothelin (ET) in the pathogenesis of hypertension

The exact role of endothelin (ET) in the pathogenesis of hypertension is rather obscure, with experimental findings supporting its role only in fulminant forms or at discrete stages of the disease (see Rubanyi & Polokoff, 1994). In experimental models of hypertension the information available is equally conflicting. In the spontaneously hypertensive rats (SHR) chronic blockade of ET-1 did not alter MABP (Li & Schiffrin, 1995). In contrast, ET has been shown to play an enhanced role in deoxycorticosterone acetate (DOCA)-salt hypertension (Schiffrin *et al.*, 1995). It is also probable that it may possess a modulating role since subthreshold concentrations of ET have a potentiating effect on vasoconstriction induced by other agonists in hypertensive rats (Dohi *et al.*, 1992).

1. 4. 4. Hypertensive complications

The vasculature contributes both as a regulator of peripheral vascular resistance and as a target of high blood pressure. Alterations induced by hypertension in certain vascular beds are crucial events in the development of complications such as myocardial infarction and stroke (Doyle, 1992).

1. 4. 5. Hypertensive complications affecting the CNS

The brain is particularly vulnerable to complications associated with chronic hypertension and both the incidence (Whisnant, 1996) and severity of cerebrovascular

ischaemia (Coyle, 1984) are increased in human sufferers with the disease. The same applies to haemorrhagic stroke, where hypertension is a factor that increases the prevalence of this pathology (Juvela *et al.*, 1995; Juvela, 1996). However, recent clinical studies have questioned the association between hypertension and worse outcome following intracerebral haemorrhage and have raised the possibility that hypertensive subjects simply have larger strokes (Jørgensen *et al.*, 1995). In experimental models of the disease, the outcome following occlusive (Grabowski *et al.*, 1988) and haemorrhagic stroke (Gonzàlez-Darder & Duràn-Cabral, 1990) together with the response to neuroprotective agents (Roussel *et al.*, 1992) are influenced by the presence of hypertension.

1. 4. 6. Role of vascular dysfunction in the CNS complications

The presence of cerebrovascular dysfunction has been examined as a potential explanation for the vulnerability of the brain to hypertensive complications. Early *in vitro* investigations identified a decrease in NO-mediated activity in cerebral blood vessels taken from SHR (Malinski *et al.*, 1993b; Miyata *et al*, 1990). Subsequent examination of the basilar artery *in situ* showed that L-NAME, a non-selective NOS inhibitor, induced greater constriction in hypertensive rats (Kitazono *et al.*, 1995), an observation which suggests rather surprisingly that basal release of NO might be somewhat enhanced in SHR over that in WKY controls. *In vivo* studies confirmed that the cerebrovascular response to NO inhibition with L-NMMA was greater in SHR (Izuta *et al.*, 1995), although no significant difference in LCBF was found in SHR and WKY treated with nitro-L-arginine (L-NA).

Cerebrovascular dysfunction associated with hypertension is most probably multifactorial. Studies using pial arteries from hypertensive rats have identified altered dilator responses which appear to involve vasoconstrictor prostanoids (Mayhan *et al.*, 1988; Mayhan, 1992b; Yang *et al.*, 1991a) and altered responses to bradykinin, possibly related to an abnormal production of hydrogen peroxide (Yang *et al.*, 1991b). There is also recent evidence that NO and prostanoid pathways are not

independent of each other because *in situ* studies have shown that prostaglandin-induced pial arterial vasodilatation is related to NO production (Armstead, 1995).

Enhanced responses of the basilar artery to activation of ET_B receptors, independent of NO or prostanoid pathways (Kitazono *et al.*, 1995), as well as impaired responses of the same vessel to activation of ATP-sensitive potassium channels (Kitazono *et al.*, 1993) have also been observed in hypertensive rats. The same group also found that the mechanisms responsible for the impaired responses of the basilar artery in SHRs (Mayhan, 1990) are not the same as those responsible for the attenuated responses of the pial vessels in the same sub-strain. This observation is not surprising, since it has been shown that the cerebrovascular control mechanisms differ fundamentally between large conduit (basilar artery) and smaller resistance (small pial and intraparenchymal) vessels (Faraci, 1991; Iadecola *et al.*, 1994a). Several research groups have also reported enhanced intravascular generation of superoxide radicals in extracranial vascular beds of hypertensive animals (Grunfeld *et al.*, 1995; Nakazono *et al.*, 1991), with all the potential detrimental functions they can convey, including inactivation of NO and peroxidative damage to cell membranes (Halliwell, 1994).

1. 4. 7. Roles of altered white cell and platelet function in hypertensive complications

There is evidence for enhanced adhesiveness of monocytes onto endothelial cells in the cerebrovasculature of SHR, associated with altered expression of adhesion molecules by the endothelium (McCarron *et al.*, 1994a&b). The exact mechanism is unclear and there is a possibility that increased shear stress, associated with the increased vascular resistance, could be a factor for the stimulation of the endothelial cells (Alexander, 1995). Another potential explanation is that NO, being a possible paracrine mediator regulating adhesion of leukocytes to the vascular endothelium (Kubes *et al.*, 1991), is inactivated by superoxide anion, which may be increased in hypertension (Grunfeld *et al.*, 1995; Tschudi *et al.*, 1996b).

Platelets from hypertensive patients and animals are more reactive to stimuli that induce platelet aggregation (Vlachakis & Aledort, 1979). In addition, they release

more serotonin (Biondi *et al.*, 1986), adhere more readily to vascular endothelium (Hazama *et al.*, 1979), have a decreased survival time (Okuma & Yamori, 1976), and have augmented turnover as compared to platelets from normotensive patients and animals (Yamanishi *et al.*, 1985). Thus, platelet dysfunction during chronic hypertension could be contributing to the vulnerability of the brain to ischaemia.

1. 4. 8. Role of vascular structural changes associated with hypertension in CNS complications

Morphological analysis of cerebral vessels has been reported in the SHR strain, which is the most commonly used animal model of essential hypertension. It seems that in those cerebral blood vessels which are largely responsible for the control of LCBF, no structural differences have been found between normotensive (WKY) rats and SHR (Lin *et al.*, 1990a). It is obviously possible that subtle changes might go undetected in these rather small vessels, but in the larger intracranial vessels, structural changes have been reported in the same model of hypertension (Folkow, 1990; Harper & Bohlen, 1984). It has also been reported that structural changes appear even before frank hypertension has been established (Folkow, 1990).

Even if structural alterations play a role in the vascular dysfunction described in chronic hypertension, these changes in the vessel wall could not alone provide sufficient explanation for the preferentially impaired responsiveness to certain vasoactive agents whereas responsiveness to others is not affected (Calver *et al.*, 1992). If structural cerebrovascular changes were the sole determinant of vascular dysfunction in hypertension, it might be expected that the responses to all vasoactive agents (constrictor and dilator) would be affected in a non-specific manner (Folkow 1990; Harper & Bohlen, 1984). This is certainly not the case, and the current literature appears to support the concept of hypertension-induced functional changes in combination with structural changes in the cerebrovasculature (Winquist & Bohr, 1983).

1. 4. 9. Role of oedema in CNS complications

Increased vascular permeability has been reported in extracranial (Tedgui *et al.*, 1995) and cerebral (Nag, 1993) vascular beds of spontaneously hypertensive rats under physiological conditions. It is speculated that the endothelial cytoskeleton involved in blood-brain barrier (BBB) integrity is altered in hypertension (Nag, 1992). Under pathological conditions and following experimental middle cerebral artery occlusion (MCAO), hypertensive animals have significantly more brain oedema, as compared to normotensives (Olsson *et al.*, 1989). It seems possible therefore, that increased BBB permeability resulting in enhanced oedema may account for a worse outcome of hypertensives following stroke.

1. 4. 10. Animal models of chronic hypertension

The main bulk of information regarding both the pathogenesis of hypertension and the mechanisms underlying the hypertension-associated complications has been derived from the spontaneously hypertensive rat (SHR), which is a variant of the Wistar strain. As also stated in the ***Spontaneous Intracerebral Haemorrhage*** section of this thesis, a stroke-prone SHR sub-strain (SHRSP) has also been developed and studied extensively.

It appears that the brain renin-angiotensin system plays a role in the development and maintenance of hypertension in the SHR model. Brain angiotensin (Ang II) levels are higher, and the pressor response to intracerebroventricular administration of Ang II is augmented as is norepinephrine turnover in the anteroventral third ventricle (Tsukashima *et al.*, 1996) of SHR. Conflicting data exist regarding the bioactivity of NO in this model, but the weight of evidence suggests that it may be overactive in SHR (Dominiczak & Bohr, 1995) but surprisingly enough, depressed in SHRSP (Dominiczak & Bohr, 1995; Fozard & Part, 1991). Angiotensin infusion has been used experimentally to analyze cerebrovascular responses to acute hypertension (Kelly *et al.*, 1994b), and direct infusion of Ang II via osmotic minipumps to rats has also been used as a model of chronic hypertension (Simon & Abraham, 1995).

In an attempt to reproduce a situation akin to the human renovascular hypertension, the "one kidney one clip" (1K1C) model has been developed, in which a unilateral nephrectomy is combined with a placement of an occluding vascular clip to the contralateral renal artery. There is indirect evidence for preserved or even increased basal influence of NO upon vascular tone in extracranial tissues in this model of hypertension, which depends upon the duration of the condition (Dubey *et al.*, 1996). NO bioactivity seems to be increased at 2 weeks after the onset of hypertension but returns to normal levels at 5 weeks. Basal NO activity may be reduced after that time-point, either as a secondary adaptation of the system to the hypertensive environment or endothelial cell damage caused by a prolonged increase in blood pressure.

The Sabra rat develops hypertension following deoxycorticosterone acetate (DOCA)-salt treatment (Ben-Ishay *et al.*, 1987). The susceptibility of this strain to hypertension results probably from a decrease in NO generation (Rees *et al.*, 1996). Interestingly, sodium loading in this model results in defective excretion by the kidneys, a function which is mediated in part at least by NO (Lahera *et al.*, 1991). In contrast, sodium loading in the SHR results in increased sodium excretion, providing indirect evidence that in the SHR NO production may be adequate to preserve renal function.

Selective breeding and genetic selection has produced the San Juan hypertensive rat, which is associated with presence of endothelial dysfunction (Crespo *et al.*, 1996), but with advancing molecular genetics transgenic animals have also been developed, expressing the murine *Ren-2*^{d} renin gene, with severe hypertension (200-260mmHg). In this model, the adrenal glands show remarkable overexpression of mRNA encoding renin (Mullins *et al.*, 1990). They exhibit high plasma aldosterone concentrations, reduced renal renin production and low plasma renin activity.

The existence of a large variety of models of chronic hypertension indicates the complexity of the condition and the lack of a single ideal one. More importantly, the pathogenetic mechanisms of the disease and the role of NO in this context in particular, seem to differ dramatically between different models. It is of interest that

human studies have reached equally conflicting results when the peripheral vascular responsiveness of chronically hypertensive subjects was measured (Calver *et al.*, 1992; Cockroft *et al.*, 1994). It would seem reasonable to suggest that essential hypertension is an heterogeneous condition and despite the large amount of experimental work, it is likely that diverse conclusions will continue to be reached.

1. 5. Nitric Oxide

1. 5. 1. General

Nitric oxide (NO) is a ubiquitous molecular mediator involved in a wide variety of biological processes in several organ systems (Moncada & Higgs, 1993). It is synthesized from the amino acid L-arginine by a family of enzymes, the nitric oxide synthases (NOS). L-citrulline is also produced, which can in turn be converted to L-arginine, a process that prevents depletion of intracellular substrate for NO production (Hecker *et al.*, 1990). NO is freely diffusible, with a short half-life and is a highly reactive chemical species that is difficult to measure *in vivo* (Iadecola *et al.*, 1994a). Therefore, studies on the role of NO have most often utilized agents that inhibit the activity of NO synthases or agents that generate NO. More recently, the biological functions of NO have been clarified considerably by the use of mice with various forms of NOS inactivated through gene knockouts created by homologous recombination (Huang *et al.*, 1993; Huang *et al.*, 1995; MacMicking *et al.*, 1995).

To date, three isoforms of NOS have been identified. Two of them, the endothelial (eNOS, Type III) and the neuronal (nNOS, Type I), are constitutively expressed. They are calcium-dependent and release NO phasically from various cells (including the endothelium, neurons, peripheral nerves, glial cells and others). The third isoform, inducible (iNOS, Type II), is calcium-independent, is induced by certain cytokines and leads to a potentiated production of NO. It is released by cells of the macrophage-monocyte lineage and other cells too (including those present in the vascular wall) and acts upon mitochondrial respiratory-chain enzymes as well as nuclear DNA-synthesizing enzymes in the target cells by binding to their iron-sulphur centers (Garthwaite & Boulton, 1995). NO also inhibits NOS providing a feedback system to protect the body from an excess of the product (Buga *et al.*, 1993).

1. 5. 2. Cardiovascular physiological role of NO

It is rather intriguing that the discovery of endothelium-derived relaxing factor (EDRF) (Furchgott & Zawadzki, 1980) was probably a result of scientific curiosity, since the biological significance of the direct effect of acetylcholine upon the vascular

endothelium is of questionable physiological importance. The quest to identify the EDRF led to the discovery of an enzyme, nitric oxide synthase, that generates nitric oxide from the amino acid L-arginine (Moncada & Higgs, 1993). NO is one of several EDRFs including PGI_2 and EDHF (Palmer *et al.*, 1987). It acts via activation of guanylate cyclase in VSMC and subsequent increase in cGMP.

In VSMC, cGMP causes relaxation through various putative mechanisms such as activation of cGMP-dependent protein kinase (cG-PK) and activation of large calcium-dependent potassium channels which leads to membrane hyperpolarization and closure of voltage-sensitive calcium channels. This pathway seems to operate also in the cerebrovasculature, where cAMP has a similar action (Paternò *et al.*, 1996). There is recent evidence that NO can directly activate potassium channels in the VSMC (Bolotina *et al.*, 1994), independently of cGMP or cAMP generation.

Basal release of NO by the vascular endothelium (from eNOS) is responsible for regulation of blood flow and blood pressure (BP). Interestingly, recent findings suggest that non-endothelial sources of NO are also involved in maintaining blood pressure (Huang *et al.*, 1995; Sander *et al.*, 1995), the role of which may differ between species (Okamura *et al.*, 1996). It is clear though that eNOS knockout mice are hypertensive providing strong evidence for a basal secretion of endothelium-derived NO (EDNO) (Huang *et al.*, 1995), under normal conditions. The NO-dependent vasodilator tone seems to be maintained through the activation of endothelial cells by stimuli such as pulsatile flow (Cooke *et al.*, 1990; Hutcheson & Griffith, 1991; Noris *et al.*, 1995) and shear stress (Lüscher & Vanhoutte, 1990). EDNO also participates in the general homeostatic control of the vasculature by inhibiting aggregation of platelets (Radomski *et al.*, 1987), interaction of leukocytes with vessel walls and proliferation of smooth-muscle cells (Garg & Hassid, 1989).

Interestingly, there is an interplay between the vasodilatory effects of NO and adenosine (Smits *et al.*, 1995), as well as NO and the cyclooxygenase pathway (Garthwaite & Boulton, 1995; Salvemini *et al.*, 1993), which is of obscure

physiological significance. There is also evidence that Ang II plays a role in the hypertension maintained by chronic, but not acute NOS inhibition (Melaragno & Fink, 1996). Recent studies have also raised the possibility that Ang II promotes NO production via stimulation of non-angiotensin$_1$ (AT_1) receptors and activation of local kinin production (Gohlke *et al.*, 1996). This interaction between various paracrine mediators shows that it is futile to attribute vascular homeostasis to a single molecule. Along these lines, during chronic but not acute NOS inhibition, other homeostatic mechanisms including possible upregulation of NOS appear to be activated to restore, at least partially, cerebral blood flow towards normal levels (Kelly *et al.*, 1995c).

As stated above, NO produced by Type I NOS can be released from nitrergic nerves in the adventitial layer of peripheral vessels and probably the cerebrovasculature (Okamura *et al.*, 1996). NO from this particular source may be involved in the regulation of BP, but its significance may vary across animal species (Okamura *et al.*, 1996). It is also possible that NO released from these nerve terminals may induce vasoconstriction because NOS inhibition in eNOS knockout mice causes hypotension (Huang *et al.*, 1995), the nNOS knockout mice are prone to hypotension when exposed to anaesthesia (Huang *et al.*, 1994) and superfusion of pial vessels with NOS inhibitors in eNOS mutant mice causes vasodilatation (Huang *et al.*, 1996).

1. 5. 3. Role of NO in cerebrovascular regulation

The finding that the rat cerebellar cells stimulated with *N*-methyl-D-aspartate (NMDA) released an EDRF-like material, established the existence of an NO pathway in the brain (Garthwaite *et al.*, 1988). Neuronal excitation leads to elevations in cGMP in cerebellum, cerebral cortex, striatum, hippocampus and other brain areas. The function of this cGMP is unclear. It is speculated that it activates specific channels that lead to an inward current of sodium and calcium with subsequent depolarization of the neurone. cGMP may also activate cG-PK in the same way that it does in VSMC, activate or inhibit phosphodiesterases, and activate ADP ribosyl cyclase with subsequent calcium release. NO might also regulate cyclooxygenase-2 (inducible) in the brain (Garthwaite & Boulton, 1995). Studies have also shown that

activation of AMPA and kainate receptors is associated with NO production in the cerebellum (Southam *et al.*, 1991). Although these findings provide evidence that NO functions as a signaling molecule in the brain, it must be stressed that NO is fundamentally different from the concept of the classical neurotransmitter, since it spreads out from its site of production to influence many tissue elements - neuronal, glial, and vascular - that are not necessarily in close anatomical juxtaposition, it is not stored in vesicles, there is no known re-uptake mechanism (Paakkari & Lindsberg, 1995) and the tissue volume in which NO can exert physiological effects is probably equal to a sphere of diameter 1000μm (Garthwaite & Boulton, 1995).

Although this information could explain a cerebrovascular role for NO secondary to its effect upon metabolic activity, and has also been suggested that NO plays a central role as a mediator responsible for flow-metabolism coupling in response to activation of NMDA receptors (Ayata *et al.*, 1996; Faraci & Breese, 1993),it is surprising that NOS inhibition reduces basal LCBF without affecting resting cerebral glucose use (Kelly *et al.*, 1994b). More surprising even was the finding that inhibition of neuronal NOS similarly reduced basal LCBF with minimal effects upon cerebral glucose use (Kelly *et al.*, 1995b). It seems therefore very likely that NO produced from both endothelial and non-endothelial sources has a pivotal role in the regulation of resting cerebral blood flow, independent of its potential role as a neuromodulator. Although it could be argued that non-endothelial NO might be of more importance in the regulation of LCBF, studies have shown that NO continues to provide dilator tone in cerebral penetrating arterioles after all possible neuronal sources of NO have been removed (Kimura *et al.*, 1994).

There is evidence that NO plays a role in the cerebrovascular responses to hypercapnia possibly of a "permissive" (perhaps by maintaining resting levels of cGMP in the VSMC) rather than a directly vasodilatory nature (Iadecola *et al.*, 1994b). Its role in hypoxia-induced vasodilatation is probably minor, and adenosine plays a more important role (Iadecola *et al.*, 1994a). Reports of a potential role in the increase in CBF following hypoglycaemia in piglets (Ichord *et al.*, 1994) have been

followed by contradictory studies in rats (Horinaka *et al.*, 1997). There is also evidence that NOS inhibition may determine the upper limit of pressure autoregulation (Kelly *et al.*, 1994b).

1. 5. 4. Role of NO in cerebral ischaemia

The formation of NO is increased in brain after induction of focal and global cerebral ischaemia (Malinski *et al.*, 1993a; Tominaga *et al.*, 1993). This could be viewed as an appropriate homeostatic response to ischaemia. However, the role of NO is rather complex, with initial studies of experimental ischaemia reaching rather contradictory results (Dawson *et al.*, 1992; Iadecola *et al.*, 1994a; Kuluz *et al.*, 1993; Nowicki *et al.*, 1991).

The haemodynamic, and platelet and leukocyte antiaggregatory effects of NO (Zhang *et al.*, 1994) presenting acutely after the onset of ischaemia, mediated mainly by eNOS (Lo *et al.*, 1996) and in part by nNOS, are beneficial in reducing infarct size. However, as stated earlier, NO can inhibit iron-containing enzymes and alter DNA synthesis (Garthwaite & Boulton, 1995). Following reperfusion, it may also react with superoxide anion to produce peroxynitrite (Halliwell, 1994). Peroxynitrite can cause lipid peroxidation of cellular membranes (Beckman, 1991). It can also lead to irreversible nitration of critical tyrosine residues, which could disrupt cellular communication and integrity (Beckman *et al.*, 1994). Finally it can cause aggregation of platelets and reverse the action of endogenous inhibitors of aggregation, including NO and prostacyclin. Its formation under certain conditions can explain how, and under what circumstances, the same molecule (NO) may seemingly produce both physiological and pathological effects (Radomski, 1995). Another potentially detrimental effect of NO is that it may form hydrogen peroxide at subphysiological levels of L-arginine or tetrahydrobiopterin (Cosentino & Katusic, 1995). The sources of this NO are not absolutely clear but several studies using selective nNOS inhibitors or nNOS-knockout mice (Huang *et al.*, 1994; Yoshida *et al.*, 1994; Zhang *et al.*, 1996b), point towards a pathogenetic role for nNOS. Recent studies, extending the experiments with the eNOS deficient mice have provided further evidence for a

beneficial role of EDNO and a detrimental role of neuronally derived NO following experimental focal cerebral ischaemia (Huang *et al.*, 1996). One important feature regarding the detrimental role of NO in cerebral pathophysiology is the large amount of NO that is produced, and the redox state of the tissue exposed to NO. When the redox state of the tissue favours the production of $NO^{\bullet}$, neurotoxicity occurs mediated by peroxynitrite, whereas when NO^{+} is produced instead, neuroprotection results linked to S-nitrosylation of NMDA receptor thiol groups and subsequent downregulation of the NMDA receptor (Lipton *et al.*, 1993).

Although it seems from the above that the detrimental effects of NO produced by nNOS are at least partially linked to glutamate excitotoxicity (Dawson *et al.*, 1991; Lin *et al.*, 1996), its precise role in the NMDA-mediated toxicity *in vivo* is rather complex, depending on the NMDA dose and on the participation of haemodynamic mechanisms secondary to NMDA exposure (Globus *et al.*, 1995). It is also of interest that hypoxia (probably in relation to generation of oxygen radicals) impairs the NMDA-induced dilatation of pial arterioles in the rat (Bari *et al.*, 1996). Interestingly, neurons that release NO seem themselves to be resistant to its cytotoxic actions (Koh *et al.*, 1986).

NO produced following induction of iNOS has a detrimental effect in experimental focal cerebral ischaemia (Iadecola *et al.*, 1997; Zhang *et al.*, 1996a). Induction of iNOS (which is present in astrocytes, microglial cells, endothelium and VSMC) presents following experimental occlusive stroke. However the temporal characteristics of this phenomenon differ between permanent and temporary occlusion. Earlier studies reported that iNOS was not induced for the first 24h after the onset of permanent ischaemia (Iadecola *et al.*, 1995), but recent studies found that in focal ischaemia associated with reperfusion, the induction of iNOS starts much earlier (during the first 24h) and the cell type (vascular) in which it is expressed is also different (Iadecola *et al.*, 1996) from that in permanent ischaemia (inflammatory). Therefore, a contributory role of iNOS in ischaemic brain damage is probable.

With particular relevance to the presence of intracranial blood, haemoglobin (Hb) inactivates NO. NO reacts with the ferrous iron in the haeme group, an effect that also depends on the degree of glycosylation of Hb (Angulo *et al.*, 1996). This quenching towards NO effect of Hb has been investigated as a potential contributing factor for the pathogenesis of vasospasm following SAH (Hongo *et al.*, 1988; Kim *et al.*, 1989), although contradictory results have also emerged (Yoshimoto *et al.*, 1995). However, it must be stressed that in SAH the vasospastic arteries are large calibre vessels and the mechanisms governing EDRF function might be different to those of the intraparenchymal vasculature (Faraci, 1991; Hino *et al.*, 1996). Another issue relating to the presence of intracranial blood is that haemin, a prominent breakdown product of haemoglobin, activates iNOS in VSMC *in vitro* in a time-dependent manner (Suzuki *et al.*, 1995). Despite the ever-increasing number of studies, the role of NO in cerebrovascular regulation and in the pathophysiology of cerebral ischaemia is far from clear and a considerable degree of controversy remains.

1. 5. 5. Role of NO in oedema formation

Experimental evidence reveals that NO of both constitutive and inducible origin plays a role in the formation of oedema in extracranial tissues (Hughes *et al.*, 1990; Ialenti *et al.*, 1992; Mayhan, 1993). NO may also be involved in the regulation of BBB under physiological conditions (Janigro *et al.*, 1994) and also in the disruption of BBB following ischaemia (Chi *et al.*, 1994; Zhang *et al.*, 1995). It also seems likely that the neuronal NO is responsible for this effect, since an association between glutamate and BBB disruption has also been found recently (Mayhan & Didion, 1996).

1. 5. 6. Endothelin (ET): Interaction with NO and its role in ischaemia

The discovery of the endothelin (ET) oligopeptide family by Yanagisawa and his colleagues in 1988 was of great interest, in view of their profound vasoconstrictor potency. Their potential contribution to the maintenance of basal vascular tone has received considerable attention (Haynes & Webb, 1994; Hirose *et al.*, 1995), but initial enthusiasm regarding a contributory role in the pathogenesis of hypertension (Webb & Haynes, 1993) and ischaemia (Clozel *et al.*, 1993) has somewhat subsided

(Patel & McCulloch, 1996), although some rather interesting findings have come to light with respect to the links between the NO and ET pathways (Boulanger & Lüscher, 1990; Schini *et al.*, 1991). However, in support of endothelin's essential role for life, mice lacking the ET-1 gene cannot survive due to respiratory and craniofacial anomalies (Kurihara *et al.*, 1994).

Endothelin-1 is generated from a precursor polypeptide, proendothelin-1, through the action of endothelin-converting enzyme (ECE), a membrane-bound neutral metalloprotease inhibited by phosphoramidon (Fukuroda *et al.*, 1990). Endothelin-1 binds to at least two receptors (Haynes & Webb, 1993): the ET_A receptor appears to be the major receptor causing vasoconstriction in arteries (by increasing intracellular calcium in VSMC) as well as mitogenesis, the ET_B receptor mediates release of endothelium dependent vasodilator substances (including EDNO, which can in turn block the conversion of preproendothelin to proendothelin, Kurihara *et al.*, 1994; Rubanyi & Polokoff, 1994; Vanhoutte, 1994). The ET_B receptor also mediates vasoconstriction in some vessels (Noguchi *et al.*, 1993). In support of an interplay between NO and ET in the cardiovascular regulation, acute hypertension after inhibition of NOS is attenuated by ET_A/ET_B receptor antagonism (Banting *et al.*, 1996), and mice heterozygous to ET-1 gene disruption have elevated blood pressure, compared to wild-type animals (Kurihara *et al.*, 1994).

ET is a potent vasoconstrictor of cerebral arteries both *in vivo* and *in vitro*, with long-lasting effects. Exogenously administered ET results in extensive vasoconstriction and causes tissue damage similar to that observed following ischaemia in rat, cat and dog brain (Kurosawa *et al.*, 1991; Robinson *et al.*, 1991; Sharkey *et al.*, 1993). ETs could also be directly cytotoxic to neurons and glia, independent of their haemodynamic effects. Both ET-1 and ET-3 increase $[Ca^{2+}]_i$ in cultured glia and neuroblastoma cells (Marsault *et al.*, 1990; Yue *et al.*, 1990). They also stimulate the release of excitatory amino acids (Lin *et al.*, 1990b). In models of focal cerebral ischaemia tissue levels of ET start to rise after 4h, and are significantly increased by 24h (Barone *et al.*, 1994). ET-receptor antagonists reduce infarct volume following middle cerebral artery

occlusion in rats (Barone *et al.*, 1995) and cats (Patel *et al.*, 1996), although contradictory results have also emerged from the same group (McAuley *et al.*, 1994).

The presence of intraparenchymal blood following haemorrhagic stroke has further implications, in relation to the role of endothelin in the potential development of delayed perilesional ischaemia. Factors released from the blood (most probably the platelets) promote the bioavailability of ET in a time-dependent manner *in vitro* (Kurihara *et al.*, 1989; Ohlstein *et al.*, 1991). In support of this hypothesis, and in studies addressing the role of ET in the development of vasospasm following SAH, haemoglobin has been found to be responsible (amongst other factors) for arterial spasm by stimulating the synthesis/release of ET-1 from cultured endothelial cells (Ohlstein & Storer, 1992).

1. 5. 7. Potential mechanisms of NO dysfunction in IDDM

Conflicting data have been reported from *in vitro* experiments on the effects of hyperglycaemia, in which the production of NO is increased via amplification of agonist-induced Ca^{++} response in endothelial cells (Graier *et al.*, 1993; Wascher *et al.*, 1994), and *in vivo* experimental studies, where reduced NO in peripheral tissues associated with diabetic complications has been reported (Elliott *et al.*, 1993; Taylor *et al.*, 1995; Way & Reid, 1995). In contrast to the *in vitro* findings, acute hyperglycaemia does not affect NO mechanisms in humans *in vivo* (Houben *et al.*, 1996).

Potential mechanisms contributing to altered NO bioavailability in IDDM include increased synthesis/release of sorbitol via the polyol pathway (Sredy *et al.*, 1991), or exacerbated generation of oxygen-derived free radicals (Hattori *et al.*, 1991; Keegan *et al.*, 1995) and reduced L-arginine availability (Mans *et al.*, 1987; Matsunaga *et al.*, 1996; Pieper & Peltier, 1995; Wascher *et al.*, 1996). Upregulation of the polyol pathway associated with diabetes or hyperglycaemia can influence the bioavailability of NO through at least two mechanisms (Otter & Chess-Williams, 1994). This pathway consumes NADPH which is essential, first for the action of NOS, and second

for the action of glutathione reductase (Loven *et al.*, 1986) which is responsible for the production of the free radical scavenger glutathione (GSH).

Contradictory results have also emerged from studies of the cerebrovasculature (Mayhan *et al.*, 1996), where responses of pial vessels to superfusion with NOS inhibitors was preserved in drug-induced diabetic rodents, using *in situ* methodology. Some groups also report that the production of NO is increased in the peripheral tissues of diabetic animals, especially in the early stages of the disease, and are responsible for the pathogenesis of diabetic complications (Corbett *et al.*, 1992; Komers *et al.*, 1994; Tilton *et al.*, 1993; Williamson *et al.*, 1993). In order to explain these contradictory reports, it should be noted that important regional (conduit *vs* resistance vessels) differences exist between the mechanisms that contribute to cerebrovascular dysfunction associated with diabetes (Mayhan, 1992a) together with differences between extra- and intracranial vascular beds (Mayhan *et al.*, 1996).

Alterations of protein kinase C (PKC) activity associated with diabetes (Inoguchi *et al.*, 1994) can explain a reduction in NO bioactivity, since PKC is known to phosphorylate and inhibit endothelial and brain NOS (Bredt *et al.*, 1992; Hirata *et al.*, 1995). However, the interplay between NO and PKC is rather complex and recent studies have reported that cGMP can also inhibit the production of PKC, which in turn can elicit directly vasoconstriction to the VSMC (Nishizawa *et al.*, 1996).

Nonenzymatic glycosylation of long-lived proteins occurs in diabetes mellitus and aging (Eble *et al.*, 1983; Monnier *et al.*, 1984). Bucala and his colleagues have proposed that the formation of advanced glycosylation products in the subendothelial collagen associated with diabetes, quenches and inactivates NO before it can act upon the VSMC (Brownlee *et al.*, 1988; Bucala & Cerami, 1992; Bucala *et al.*, 1991).

Although the presence of a perturbed NO pathway in the aetiology of experimental diabetic neuropathy is evident, resulting from synergistic interactions between polyol pathway/nitric oxide and essential fatty acid/cyclo-oxygenase systems (Cameron *et*

al., 1996), the characterization of NO dysfunction in the diabetic brain is not clear to date. There is more clear evidence of impaired cerebrovascular responsiveness associated with abnormalities of the cyclo-oxygenase pathway in drug-induced diabetes (Mayhan *et al.*, 1991). This might be relevant to NO, because the effects of prostaglandins in the cerebrovasculature are mediated by both cAMP and cGMP, and the cGMP production is NO dependent (Armstead, 1995). Another observation of relevance to NO is that Ang II has been implicated in the pathogenesis of diabetic complications in the periphery, because ACE inhibition improved the regulation of vascular tone in the mesenteric bed of diabetic rats (Olbrich *et al.*, 1996). NO in turn has a regulatory role in modulating some of the Ang II effects (Ho *et al*, 1995).

1. 5. 8. Insulin and NO

Insulin-induced vasodilatation in extracranial tissues, independent of its effect on plasma glucose, is probably mediated by NO production (Scherrer *et al.*, 1994; Trovati *et al.*, 1995). The possible mechanism is through activation of the Na^{+}-K^{+} pump at the endothelial surface (Tack *et al.*, 1996), which results in cell hyperpolarization and influx of Ca^{++} due to electrogenic driving force. Calcium channels in the endothelium are primarily voltage independent, but in contrast, hyperpolarization of VSMC would result in closure of voltage-dependent Ca^{++} channels. There is also recent evidence that NO may be a novel effector of the insulin signaling pathways involved in glucose metabolism (Zeng & Quon, 1996). This concept is supported by studies in healthy humans suggestive of a physiological link between endothelial nitric oxide synthesis and insulin sensitivity (Petrie *et al.*, 1996). Hyperinsulinaemia is associated with an increase in cerebral blood flow (CBF), independent of blood glucose level (Kerr *et al.*, 1993). Despite all of these reports, a recent study from Sokoloff's laboratory provided evidence against a direct role of insulin to raise CBF, or a role of NO in the increased CBF following hypoglycaemia in awake animals (Horinaka *et al.*, 1997).

1. 5. 9. NO and hypertension

There is an extremely large body of experimental and clinical work regarding the role of NO in both the pathogenesis of hypertension and its associated complications with conflicting results depending on the species/models, tissue and duration of the disease. This work has been reviewed in the ***Essential Hypertension*** section of this thesis.

1. 5. 10. Concluding remarks

NO is a most extensively investigated molecule, and it is impossible to present all the information available regarding its physiological and pathological functions. The purpose of this introduction was to include only those facts which are relevant to the work presented in this thesis.

1. 6. Aims and objectives

The aims of this thesis are (i) to investigate whether cerebrovascular dysfunction linked to a perturbation of NO-related control mechanisms is associated with IDDM and chronic hypertension (ii) to assess the extent to which any abnormal cerebrovascular physiology associated with IDDM and chronic hypertension contributes to the pathophysiological response to haemorrhagic stroke, and finally (iii) to determine if ET or neuronally derived NO, which may be implicated in the development of delayed perilesional ischaemia following experimental ICH in normal animals, potentiate the ischaemic burden following ICH in diabetic or hypertensive animals respectively.

CHAPTER 2

MATERIALS AND METHODS

General

The experiments presented in this thesis represent two discrete, but related studies: firstly, an investigation of the physiology of the cerebrovascular endothelium, and secondly an investigation of the pathophysiology of ICH.

In the physiological studies, local cerebral blood flow (LCBF) was measured using the fully quantitative [^{14}C]-iodoantipyrine (IAP) autoradiographic technique, in areas within the vascular territories of the anterior, middle and posterior cerebral arteries in spontaneously diabetic (BB) and hypertensive (SHR) rats and appropriate controls, under basal conditions or following manipulation of NO systems with the NOS inhibitor N^G-nitro-L-arginine methyl ester (L-NAME), the selective neuronal NOS inhibitor 7-nitroindazole (7-NI), the NO donor 3-morpholinosydnonimine (SIN-1) and the non-peptide ET_A/ET_B-receptor antagonist SB209670 [(+)-(1*S*,2*R*,3*S*)-3-(2-carboxymethoxy-4-methoxyphenyl)-1-(3,4-methylenedioxyphenyl)-5-(prop-1-yloxy) indane-2-carboxylic acid]. Electron and light microscopy using the periodic acid schiff (PAS) stain were used to identify any structural vascular abnormality in the diabetic vessels which might reflect cerebrovascular responses to the above pharmacological agents. Finally, whole blood viscosity and plasma L-arginine levels were obtained in the diabetic and non-diabetic controls to further elucidate the mechanisms responsible for cerebrovascular dysfunction associated with IDDM.

In the parallel intracerebral haematoma studies, volumes of striatal oligaemia were measured by computer-based planimetry in the BB and SHR groups of animals injected with blood, or silicon oil as a control. Rats carrying the burden of ICH were treated with saline, SB209670, sesame oil or 7-NI. In the same studies, sections adjacent to those used for autoradiography were examined histologically, stained with either haematoxylin and eosin or cresyl violet with luxol fast blue. Differences in blood-brain barrier (BBB) permeability to α-aminoisobutyric acid (AIB) were measured in the parallel groups of animals subjected to experimental ICH, to determine if the presence of IDDM or chronic hypertension were associated with alterations in permeability. In all groups of animals, volumetric analysis of the mass

lesion was performed *ex vivo* using tissue planimetry. Intracranial pressure (ICP) was also measured in pilot experiments, to ensure that the volume of blood injected into the striatum (50µl) did not induce global cerebral perfusion pressure (CPP) reductions.

All of the experimental methods used in this thesis, as well as the animal models, represent established procedures which have been previously validated and described in detail in the scientific literature.

2. 1. Animals

All experiments were performed using adult male rats, weighting between 230 and 495 g. Diabetic animals and their controls were purchased from the British Diabetic Association BB (Edinburgh) U.K. Resource Unit. Hypertensive animals and their controls were purchased from Charles River. All animals had free access to food and water until the day of the experiment and environmental control (room temperature, light/dark cycle) was maintained throughout.

2. 1. 1. Spontaneously diabetic BioBred rats

The BB/E colony consists of two lines derived by selectively breeding for and against diabetes. In the high-incidence diabetes-prone (DP) main line, the incidence of IDDM is 50-60%, and the age at onset of diabetes is 96 ± 18 days (mean ± s.d.). In the diabetes-resistant (DR) subline, the incidence of diabetes is < 1%. Animals from the DR subline were used as controls for diabetes, and were age-matched to the diabetics used in the experiments presented in this thesis.

Both DP and DR rats were maintained at 20^0C on a 12h light/dark cycle and fed rat and mouse Number 1 Expanded Feed (Special Diet Services, Witham, U.K.). Animals were weighed twice weekly from 40 days of age. Failure to gain weight, or loss of weight, was taken as an indication of the possible onset of diabetes, and such rats were tested for glycosuria. If glycosuria was detected, the blood glucose concentration was measured from a sample obtained by tail-tipping. A blood glucose concentration >324mg.dl^{-1} is invariably associated with ketonuria, weight loss and the absolute requirement for daily injection of insulin. These variables constituted the criteria for classifying an animal as having IDDM. The diabetic animals were maintained on a single subcutaneous injection of medium-acting insulin (U40 Bovine Ultralente insulin, NOVO), the injection given at 9.00 am each morning in the subcutaneous tissue of the neck. The exact dosage (2.4 to 11 units), was specifically titrated for each individual animal to prevent ketoacidosis and weight loss. Blood glucose concentration and body weight were measured three times per week in each diabetic animal and the insulin dose adjusted accordingly. This protocol is in

accordance with those employed by other groups using animals from BB colonies elsewhere (Hall *et al.*, 1996).

In those rats in which haematomata were induced, the daily insulin injection was given 4h prior to the intracerebral injection of blood or silicon oil. On the final day of experiments, all diabetic rats were injected with insulin 4h prior to the measurement of LCBF or BBB permeability. This time interval was chosen to allow the insulin time to act, and normalize the plasma glucose.

Some of the diabetic complications, such as the appearance of advanced glycosylation endproducts develop only after approximately 6 weeks of established diabetes (Bucala & Cerami, 1992). The diabetic animals used for all the experiments were between 18 and 36 weeks old and had a duration of diabetes between 8 and 20 weeks. The processes of aging are themselves associated with endothelial dysfunction, therefore older animals were not used. As mentioned earlier, the DR rats used as controls were age-matched to the diabetic animals.

In a further group of diabetic animals long lasting sustained release insulin implants (SRII, Mollegaard, Denmark) were used, instead of the daily dosing regimen described above. These implants deliver a basal dose of insulin continuously, thereby preventing periods of hyperglycaemia, eliminating the large diurnal fluctuations in plasma glucose concentrations, and achieving a better overall glycaemic control. The insulin implants are in the form of rods (7x2mm) consisting of a 17% powder admixture of Bovine insulin compressed into a pellet with 83% palmitic acid. The insulin release rate is approximately 2 units per day per implant for 6 weeks. The sites of implantation were subcutaneous in the neck region or in the sternal area. Because the diabetic animals used in this study had a duration of disease of over 8 weeks, a new rod was implanted at 6 weeks. This protocol of continuous subcutaneous insulin infusion (CSII) was employed in an attempt to create a model of diabetes with more effective metabolic control.

2. 1. 2. Hypertensive rats

The hypertensive animals used in the experiments outlined in this thesis were from the spontaneously hypertensive strain (SHR). Hypertension presents before the age of 6 weeks in this animal strain. The rats were purchased from Charles River at the age of 10 to 14 weeks, and used for experiments 4 weeks later, having become re-acclimatised to their new surroundings. The age of the SHR rats at the time of experimentation was between 14 and 18 weeks. There was no dietary supplement in these animals.

WKY animals were used as controls. These were purchased from the same supplier at the same age, were age-matched to the SHRs at the time of experimentation and housed in the same way as the SHRs.

2. 2. Production of experimental ICH

Experimental intracerebral haemorrhage was produced by injecting blood from a donor rat into the right caudate nucleus of recipient animals using stereotactic procedures.

2. 2. 1. Donor

The donor of the blood was a syngeneic animal from each of the groups examined. In a series of preliminary experiments, it was established that the response to experimental ICH in diabetic or DR rats was independent of the source of blood, i.e. whether the donor was a diabetic or DR and the recipient diabetic or DR. Subsequently, all donors were DR, thus eliminating the blood variable from the treatment effect. Similarly, it was found that the same occurred with the SHR and WKY groups, where the response to the experimental ICH was independent of the source of blood. Therefore all donors in these studies were from the WKY group.

Donor animals were anaesthetized with intraperitoneal pentobarbitone (45-60mg. kg^{-1}). A 15cm long PE50 cannula (Portex polythene tubing i.d. 0.58mm, e.d. 0.96mm) was inserted in the right femoral artery and kept patent by connecting it to a syringe

(1.0ml) containing heparinized saline (10IU.ml^{-1}), the whole procedure taking place near the stereotactic frame where the recipient of the haematoma was to be placed. 0.2ml of blood was withdrawn from the arterial cannula into the heparinized syringe. A further 50µl of unheparinized arterial blood was withdrawn, with a small bubble of air separating the blood from the saline, into a 30cm length cannula tubing graduated in 25µl steps and filled with saline. The other end of the 30cm long cannula was connected by way of a three-way tap to a 20ml syringe containing saline.

2. 2. 2. Recipient

The recipient animal was anaesthetized with pentobarbitone (30-45mg.kg^{-1}) injected intraperitoneally. Depth of anaesthesia was assessed by lack of movement of the animal upon exertion of stimuli in the lower limb paws. The scalp was shaved and cleaned with an antiseptic swab, containing 5% chlorhexidine. The animal was placed prone in a stereotactic frame, restrained by use of ear bars and an incisor bar set at 0mm, according to Paxinos and Watson (1986). A thermal blanket was placed under the torso of the animal and a rectal probe was also inserted, ensuring that, with appropriate adjustments of the heat source, the temperature of the animal remained at 37^{0}C. Oxygen, at 4 litres per minute, was administered via a face mask. The scalp was infiltrated with 1% lignocaine hydrochloride, incised longitudinally along the midline and retracted. The bregma was identified. Using a 1mm diameter burr cleaned with 5% chlorhexidine a burrhole was made 3.5mm to the right of the midline, on the coronal suture, while the skull was continuously irrigated with normal saline. The underlying dura was identified, and incised with the tip of a 25G needle (e.d. 0.5mm).

The needle (25G) which was connected to the cannula tubing containing the 50µl of blood was cleaned with alcohol, air dried and placed on the stereotactic frame. It was introduced into the burrhole and initially lowered to a depth of 6mm below the dura, but subsequently withdrawn to a depth of 4.5mm, creating a "pocket" of 1.5mm below the tip of the needle.

In preliminary experiments, 25μl of blood was injected in the right caudate nucleus of control animals for 24 and 48h. The animals were sacrificed by decapitation and the brains were removed intact and immersed in 10% formalin for 24h. Coronal sections were cut and processed routinely into paraffin wax, from which 5 micron (μ) sections were cut and stained with haematoxylin and eosin (H+E). No perilesional structural abnormality was evident in any of these groups, so in the subsequent procedures 50μl of blood or silicon oil was injected in control, diabetic and hypertensive rats and the animals sacrificed at 24h. Perilesional cell damage of variable extent between treatments and groups was evident, so this volume, which is also more realistic in being equivalent to a 40-50ml lesion in the human brain (Kingman *et al.*, 1988), was used for all the subsequent experiments.

In determining the mode of injection, two parameters were found in preliminary experiments to be important for reproducibility of the lesions: the rate of the injection and the time interval between the withdrawal of the blood from the donor and its injection into the striatum. Initially the method used was to inject the blood under 100mmHg pressure generated by a saline column connected by a three-way tap to the tube containing the blood (Nath *et al.*, 1986). The injection was normally complete within 20sec. However, the reproducibility was poor using this method because of variable backflux of the blood, especially when the injection was performed within a short time after the withdrawal of the blood from the donor. It was also found during these preliminary experiments that, if the blood was left for 2min in the collection tube before it was injected, the occurrence of backflux was greatly reduced. Therefore, the protocol for the experiments presented in this thesis was as follows; the donor blood was drawn into the injection apparatus, left in the tube for 2min, and then injected over 1min. The needle was left *in situ* for another 5min and then withdrawn, the burrhole sealed with sterile bonewax and the wound sutured with 4/0 silk. The animal was removed from the stereotactic frame and placed in an incubator, set at 37^0C until it regained consciousness. Thereafter, it was placed in an individual cage, and allowed free access to food and water for 24h, at which point the final experiment for the

measurement of LCBF, BBB permeability or histopathology was performed (see appropriate sections).

2. 2. 3. Controls for ICH - Silicon oil injection

In a group of animals, 50µl of inert silicon oil was injected in order to differentiate the effects of the blood products from the effects of the "mass" lesion. The silicon oil was of similar viscosity to blood (7.6 centipoise at 37°C) and was kindly provided by Professor Gordon Lowe from the University of Glasgow. The oil has the same flow rate as blood, and when injected around the pial vessels of a cat, no vasoconstrictive or dilatating effects are observed (Jenkins *et al.*, 1990). The oil was injected into the animals in exactly the same way as the blood. However, in preliminary experiments, it was noted that if the needle was withdrawn 5min after the completion of the injection, there was considerable backflux (presumably due to lack of clotting of the oil). Therefore, the needle was withdrawn much more slowly, at a rate of 1mm every 5min. Since the original depth at the start of the injection was 4.5mm, the procedure lasted for 25min. The animals were treated afterwards in the same way as those injected with blood, and were allowed to recover for 24h prior to further experimental manipulation.

2. 3. Surgical preparation for final experiment

Rats were initially placed in a perspex chamber into which an anaesthetic gas composed of 5% halothane in a nitrous oxide: oxygen mixture (70%: 30%) flowed for induction of anaesthesia. Once the animals became unconscious, they were removed from the box, placed supine on an operating bench, and anaesthesia maintained with 1.5-2% halothane in the same nitrous oxide: oxygen mixture delivered by a face mask. Both lower limbs were immobilized with adhesive tape and the skin over both groins shaved and cleaned with antiseptic solution. The femoral vessels were exposed bilaterally through small incisions in the skin overlying the pelvic muscles, proximal to the femur. Polyethylene cannulae (Portex, external diameter 0.96mm, internal diameter 0.58mm), 15cm in length and filled with heparinized saline ($10IU.ml^{-1}$), were inserted approximately 3cm into both femoral arteries and veins. The cannulae were

secured with fine suture material, the wounds infiltrated with local anaesthetic (lignocaine hydrochloride, 2%) and sutured (Ethicon, W586) closed. Externally, the wounds were covered with gauze pads soaked in lignocaine. The lower torso was enveloped in a surgical stocking (Tubigrip, size E), and a plaster cast (Gypsona) was applied loosely around the hindlimbs and lower abdomen. With the rat in prone position, the plaster cast and hindlimbs of the animal were taped to a weighted block for purposes of restraint and support, and a rectal thermometer was inserted. Core temperature of around 37°C was maintained with the use of a heating lamp. Anaesthesia was terminated and the animals were allowed to regain consciousness. A recovery period of at least 2h were allowed to elapse, prior to further manipulation or injection of isotopes or pharmacological agents. During this recovery time and subsequent experimental procedures, arterial blood pressure was monitored and/or recorded from one of the arterial femoral cannulae using Macintosh, MacLab 4e Chart. Body temperature was displayed continuously.

In the subgroup of animals to be treated with 7-nitroindazole (7-NI), an intraperitoneal cannula (PE 50) was inserted via a trocar into the abdominal space, secured with suture at the insertion site, and led caudally to lie with the femoral cannulae, to which it was also secured.

2. 4. Measurements of physiological variables

After the end of the recovery period from anaesthesia, 100-150µl of arterial blood was withdrawn for the measurement of pH, pO_2, pCO_2, base excess, bicarbonate, plasma glucose and haematocrit. Mean arterial blood pressure (MABP), heart rate and core temperature were also noted just prior to the initiation of subsequent LCBF or BBB permeability experiments. In the groups of animals in which an acute injection or infusion of a pharmacological agent was performed (i.e. L-NAME, 7-NI, SIN-1 or SB209670), these physiological parameters were measured prior to the drug injection (or infusion) and again just prior to the LCBF experiment.

2. 5. Measurement of local cerebral blood flow (LCBF)

Local cerebral blood flow (LCBF) was measured using the fully quantitative [^{14}C]-iodoantipyrine autoradiographic technique (Sakurada *et al.*, 1978).

Rats already supported on the weighted blocks were positioned under a guillotine. One femoral venous cannula was connected to a syringe containing 50μCi of the freely diffusible tracer [^{14}C]-iodoantipyrine (Tocris Cookson, specific activity 55mCi.mmol^{-1}) diluted in 0.5ml of normal saline. One arterial femoral cannula was secured to a metallic block to facilitate subsequent sampling.

The tracer was infused over a 45sec period on a "ramp" schedule, i.e., the rate of infusion was continuously accelerating so that the arterial tracer concentration would rise steadily and be highest at the end of experiment. Thus, the possibility of reaching a steady-state arterial tracer concentration, or of the arterial concentration falling at any stage of the experiment was avoided. During administration of the isotope, blood was allowed to flow freely from the arterial cannula that was secured on the metallic block. Timed samples (16 to 18) were collected intermittently onto preweighed filter discs (Whatman AA, 13mm, Merck) held flat in polystyrene auto-analyzer cups (automatic assay type, 14mm, 1.5ml, Merck) held around the perimeter of a perspex wheel (12cm diameter). The sequence of all drops, emanating from the arterial cannula, including the numbered ones collected on the discs, was called out and recorded on audiotape.

At the end of the infusion (around 45sec after the onset), the animal was decapitated by guillotine. To avoid equilibration of the isotope in the brain structures or reduction in arterial concentration, the animal was decapitated just before the tracer infusion ended. Because of the time lag inherent in the sampling cannula, care was taken to obtain a blood sample after the decapitation, in order to estimate as accurately as possible (without the need for extrapolation), the amount of isotope present in the brain at the instant of death. The brain was dissected intact, and frozen in pre-cooled isopentane (-45 °C) within 2 to 3 min of death to prevent diffusion of the isotope. The

frozen brain was transferred onto dry ice. A layer of approximately 3mm M-1 embedding matrix (Lipshaw) was allowed to freeze on the top of a 13mm object holder, the matrix being held in place by a collar of masking tape. Another few drops of matrix were put on the frozen surface and the brain, cerebellum down, placed into the liquid matrix. Solid CO_2 was crumbled over the brain and liquid matrix to ensure rapid freezing. The brain, now held in an upright position, was brushed free of excess CO_2, dipped quickly in a small reservoir of matrix and returned immediately to be covered in crumbled CO_2. The frozen brain was left for 5-10min before being either stored in a small self-sealing, labeled polythene bag at -70°C until required, or being transferred to a cryostat at -22°C for sectioning.

In the preliminary experiments performed in the WKY and SHR animals, there was a tendency for the blood pressure to fluctuate and sometimes to drop considerably during the haemorrhaging necessary for the collection of blood samples. In these groups the protocol was modified as follows; the infusion period was reduced from 45 to 30sec, and although the same amount of isotope was infused, by careful crimping of the sampling cannula, the flow rate of arterial blood was reduced (40-48 drops per minute instead of 60-64 drops per minute employed in the diabetic and non-diabetic BB groups). With this modification, MABP remained steady during each experiment. MABP and time of death were read from recorded computer trace (Macintosh, MacLab 4e). In the recording of physiological parameters, the MABP measures that were included were those obtained at the exact time of death.

The filter discs were returned quickly to the appropriate scintillation vials in which they had been preweighed, capped tightly to prevent evaporation, and then reweighed. Hydrogen peroxide (0.4ml, 30% w/w, Sigma) was added to bleach the blood, and reduce the possibility of colour quenching. The vials were left at room temperature for 2h to allow completion of bleaching, and then 10ml of liquid scintillant (Picofluor, Packard) was added. The vials were placed in a cold room at 4 °C for 24h to allow elution of isotope into the scintillation fluid and then placed in a liquid scintillation analyzer (Tri-Carb 1600TR, Packard). Each sample was counted for two minutes

over each of three cycles, and the mean d.p.m. (disintegrations per minute minus background) of each sample used for the subsequent calculations.

The timing of the numbered samples and the total number of drops, from the start of the isotope infusion, were noted from the audiotaped record of the experiment. From mean sample weight (corrected to volume by the specific gravity of blood, 1.05) and the number of drops issuing from the cannula, the cannula flow rate could be calculated and lag-time for blood to pass through the cannula determined. This was necessary because blood sampled from the catheter is delayed relative to that in the brain by the amount of time it takes to flow through the catheter (lag-time). A correction for this lag-time, which varies from animal to animal, can be determined from the flow rate (number of drops/ min x volume/ drop) and the volume of the catheter.

2. 6. Measurement of blood-brain barrier (BBB) Permeability

Blood-brain barrier (BBB) permeability was measured using the fully quantitative [^{14}C]-α-aminoisobutyric acid (AIB) autoradiographic technique (Blasberg *et al.*, 1983). The AIB method is excellent for measuring moderate to large increases in BBB permeability, since this amino acid (molecular weight 104 daltons) has a low rate of transport across normal brain capillaries (carrier-mediated, the same that mediates flux of neutral amino acids) and undergoes rapid concentrative uptake by brain cells (Blasberg *et al.*, 1983).

The production of the haematoma 24h prior to these experiments, the surgical preparation of the animal, and the measurement of the physiological variables have been described in sections 2. 2., 2. 3. and 2. 4. respectively. MABP was continuously monitored before and during the experiment until the animal was dead and core temperature measured by a rectal thermometer throughout. The isotope, 40μCi dissolved in 0.7ml of normal saline, was injected intravenously over a period of 30sec. Timed arterial blood samples (100-150μl over 1-2sec) were taken by allowing arterial blood to flow from the open cannula into a heparinized centrifuge tube (Beckman

EHF-26). The exact times of starting and stopping each collection were noted. The first sample coincided with the beginning of the injection (0sec), and then at 15sec intervals until 60sec. Subsequent samples were taken at progressively longer intervals (2, 3, 5, 7.5, 10, 15, 20, 25 and 30min), to make 14 samples in total. The animal was killed with an overdose of barbiturate immediately after the last sample. The exact time of the animal's death was noted. The brain was dissected and prepared for cryostat sectioning as described in section 2. 5.

The arterial blood samples were centrifuged for 1min (Beckman microfuge E) and 0.02ml aliquots of the plasma were withdrawn, placed in plastic vials (Pico-prias 6.5ml, Packard) containing 0.5ml of distilled water, and 5ml of liquid scintillant (Picofluor 40) were then added. Liquid scintillation analysis was performed as described in section 2.5.

2. 7. Preparation of tissue for autoradiography

Brains which had been stored at -70°C were transferred to a cryostat at -22°C and 30 to 45min were allowed to elapse before the brains were sectioned.

2. 7. 1. Protocols to generate LCBF autoradiograms from physiological studies

Semi-serial brain sections (20 microns thick) were cut starting at the olfactory bulbs and ending at the medulla oblongata. The first three consecutive sections in every 13 were mounted on glass cover slips and dried on a hotplate (90 °C). The other 10 sections were discarded, apart from the diabetic and non-diabetic (DR) groups treated with saline (for measurement of basal blood flow). In those groups, sections from several brain areas were mounted onto poly-L-lysine - coated glass slides and stained with periodic acid schiff (PAS, see 2. 10. 3.) in order to investigate whether any significant deposition of advanced glycosylation end-products was evident in the subendothelial layer of the diabetic animals. The cover slips were glued onto thin cardboard (20x25cm), together with a set of 8 [^{14}C]-containing (40-1069 nCi.g^{-1}), methylmethacrylate plastic standards (Amersham), precalibrated for 20 micron brain sections. Sections and standards were exposed to X-ray film (Kodak SB-5) in light-

tight cassettes stored at 4°C for seven days. At the end of the exposure period, cassettes were brought to room temperature. The films were developed, under red safety light (Kodak GBX-2 filter) conditions, for 5min in LX-24 developer (Kodak, 1:4 with tap water), washed quickly in water to remove excess developer, fixed for 2x5min in UNIFIX (Kodak, 1:4 with tap water) then washed in running tap water for 15min before being hung up to dry at room temperature.

2. 7. 2. Protocols to generate LCBF and BBB permeability autoradiograms from haematoma studies

In these studies, sectioning of the brain was performed in largely the same way as described before. However, when the beginning of the clot was noted at the anterior part of the caudate nucleus, the frequency with which sections were collected onto coverslips was increased to three brain sections in every 8. All intervening sections were mounted on glass slides (coated with poly-L-lysine to avoid dislodging of the section during the staining procedure) and stained with haematoxylin and eosin or luxol fast blue with cresyl violet, in order to correlate directly the blood flow or BBB permeability in autoradiograms with these neuropathological slides in each individual animal. Once the haematoma had ended, the frequency of collection was reduced and the protocol was again as described previously.

In the BBB permeability protocol, brain sectioning started at the anterior caudate and ended at the end of the globus pallidus. Initially the first three consecutive sections out of every 13 serial sections were mounted on glass coverslips and dried on a hotplate (90°C). However, when the haematoma was encountered, a protocol similar to that used for the LCBF studies was adopted.

The cover slips were processed in the same way as described in section 2. 7. 1., apart from the BBB permeability autoradiograms, which were exposed to X-ray film (Kodak SB-5) and kept in light-tight cassettes at 4°C for twenty days. In preliminary experiments the films were left in the cassettes for ten days, but the radioactivity was

found to be too low for provision of reasonable discrimination from the background of the film.

2. 8. Analysis of autoradiographic images

Quantitative densitometric analysis of all autoradiographic images was performed using a computer-based image analysis system (Quantimet 720, Cambridge Instruments).

2. 8. 1. Analysis of autoradiograms of LCBF from physiological studies

The images on the X-ray film were analyzed by quantitative densitometry (Quantimet 720, Cambridge Instruments), relative to the precalibrated standards. The optical densities of the plastic ^{14}C standards were measured before and after the measurement of the optical densities of the brain structures. The mean of these two values for each standard was entered in the computer. This procedure was carried out to avoid errors associated with measurements of optical density consistency. In all the experiments analyzed, the difference between the two values obtained for each standard was less than 10%. For each region of the brain which constituted an area of interest, twelve optical density readings were made bilaterally on six consecutive sections in which the structure could be anatomically defined by reference to a stereotactic atlas (Paxinos & Watson, 1986). The mean optical density of these 12 measurements was used to calculate the tissue ^{14}C concentrations. The size of the measuring frame varied depending upon the brain region to be analyzed. For the caudate nucleus, a frame of 50 square picture points (pixels, 500 pixels representing a distance of 15mm) was used; 20 square pixels were used for globus pallidus, 10 square pixels were used for nucleus accumbens, lateral geniculate and hypothalamus and 5 square pixels were used for parietal cortex, subcortical white matter and the hippocampal structures. The size of the measuring frame was maintained constant for the same region in different animals. Blood flow was calculated using the appropriate operational equations. Tracer concentrations in arterial blood samples were taken from the liquid scintillation analyzer as described previously (section 2.5.). These d.p.m. together with the blood drop weights and corresponding collection times were entered into a computer.

Knowing the specific gravity of whole blood (1.05) (Sakurada *et al.*, 1978), and the drop weight, the sample volume was calculated. The computer then calculated the best fit between the optical densities and the known ^{14}C concentrations. Using a specifically designed computer programme, in which the partition coefficient (the ratio of the solubility of the diffusible tracer in the brain tissue to that in the blood) was considered to be 0.8 (Sakurada *et al.*, 1978), the calculation of the CBF was determined for the corresponding tissue ^{14}C concentrations of the tracer (Sakurada *et al.*, 1978) from the equation

$Ci(T) = \lambda K \int_0^T C_A e^{-K(T-t)} dt$, where $Ci(T)$ equals the tissue concentration of the chemically inert diffusible tracer in a homogeneous tissue at a given time, T, after the introduction of the tracer into the circulation; λ equals the tissue:blood partition coefficient; C_A is the concentration of the tracer in the arterial blood; t equals the variable, time; and K equals a constant that incorporates within it the rate of blood flow in the tissue. The constant K is defined as follows $K = mF/W\lambda$ where F/W equals the rate of blood flow per unit mass of tissue and m equals a constant between 0 and 1 that represents the extent to which diffusion equilibrium between blood and tissue is achieved during passage from the arterial to the venous end of the capillary. In the absence of diffusion limitations or arteriovenous shunts, $m = 1$.

2. 8. 2. Calculation of striatal ischaemia from LCBF autoradiograms in haematoma studies

In this method of analysis, volumes of striatal tissue with blood flow below predetermined thresholds were derived for each animal. These thresholds (15, 25, and 35ml.100g^{-1}.min^{-1}), represent widely acceptable values for the ischaemic penumbra in the rat (Ginsberg & Pulsinelli, 1994; Hossmann, 1994). It is understood that in the original study by Astrup, Siesjö and Symon in 1981 the actual CBF values for the penumbra were lower. However it seems that in the rat, because of a higher metabolic rate (Nilsson & Siesjö, 1976; Siesjö, 1992; Sokoloff *et al.*, 1977), these CBF values are higher. Moreover, as was found in this work, these CBF thresholds corresponded

very closely to the area of neuropathological evidence of ischaemic injury obtained from adjacent sections to those used for autoradiography.

A preliminary analysis of the autoradiograms was conducted to measure the optical densities of the eight [^{14}C]-containing standards. It was also necessary to include a measure of the background optical density of each film, and this represented zero [^{14}C] concentration. Using the blood data for the individual animal to which the film related, and Sakurada's operational equation (1978), blood flow values were derived which corresponded to the optical densities of the standards. The operational equation was then reversed to derive by an interactive process, optical density values which corresponded to the chosen blood flow thresholds.

The image analyzer was calibrated to absolute units of length. In most experiments 500 picture points (i.e. the units which comprise a video image) were equivalent to 15mm, and optical density thresholds were set, which had previously been calculated to correspond with the predetermined blood flow thresholds. Using a light-pen linked to a computer mouse, the perimeter of the striatum containing the haematoma (or silicon oil) was delineated, and the area within the boundary was calculated by the computer. The computer then generated the area of striatum within the boundary which had an optical density below each of the three thresholds, expressed both as percentage of total striatal area and also in absolute units of area, i.e. mm^2. These measurements were conducted over three consecutive sections, and the mean values derived for this level of striatum. Volumes (in mm^3) of striatal tissue in which blood flow was below the same thresholds were derived by repeating the above procedures at all levels of the brain in which the haematoma was present. Volumes were calculated from the measured areas, using the known distance between each of the levels (100μm).

2. 8. 3. LCBF measurements in areas distant to the mass lesion

LCBF was also measured in areas distant to the haematoma/silicon oil injection (i.e., contralateral caudate nucleus). The procedure employed was similar to that described

in section 2. 8. 1., with the exception that only 6 instead of 12 optical readings were made on the same side (left, opposite the mass lesion) on six consecutive sections, for each brain structure studied.

2. 8. 4. Analysis of AIB (BBB permeability) autoradiograms

Analysis of the images on the X-ray film was performed using the same computer-based densitometer described above (section 2. 8. 1.). Tissue tracer concentrations were derived by densitometric analysis relative to the precalibrated ^{14}C-containing standards. Because the optical densities of "normal" tissues were so low, the optical density of the film background (tracer concentration = zero) was included. Three consecutive images containing the haematoma were analyzed separately at each brain level using a measurement area of 5 square pixels, and the mean optical density obtained from these three readings. The area of measurement was defined as that with the most obvious AIB concentration in the lesioned caudate. At each brain level the optical density of the contralateral non-lesioned striatum was also measured, in order to obtain an index of concentrations of AIB accumulated in normal tissue in each animal. Permeability to AIB was calculated using the appropriate operational equation;

$K_i = FV_f [1 - \exp(-PS/FV_f)]$, where K_i: blood-to-brain transfer rate constant; F: blood flow; V_f: fraction of the whole blood flowing through the capillaries that is involved in the blood-brain transfer process of AIB (1 - haematocrit); P: permeability; S: surface area, (see Blasberg *et al.*, 1983) using the timed arterial concentrations of the tracer taken from the liquid scintillation analysis as described previously (section 2. 6.).

2. 9. Calculation of striatal volume of haematoma or silicon oil

The coverslips containing the sections of the lesioned (with blood or silicon oil) animal brains were placed on the densitometer. The volume occupied by blood or silicon oil, excluding that in overlying cortex or adjacent white matter, was derived by planimetry. Areas of haematoma or silicon oil, and the caudate nucleus (in mm^2) were delineated at each brain level and volumes (in mm^3) calculated from these areas with

knowledge of the distance between brain levels (100μm). Striatal haematoma or silicon oil was expressed as percentage of the volume of the whole caudate nucleus.

2.10. Neuropathology

All the staining procedures were performed in the Department of Neuropathology at the Western General Hospital, using standard histological techniques (Bancroft & Cook, 1994; Cox, 1982; Stevens, 1982).

2. 10. 1. Haematoxylin and eosin (H+E)

The sections (20μ thick) were initially placed in water for 30sec (to wash off any excess poly-L-lysine) and then stained in haematoxylin solution for 1.5min. They were then washed in water, and "blued" in tap water to which 5-10ml saturated aqueous lithium carbonate was added. They were again washed in water and then stained in eosin solution for 2min, washed in water and eosin fixed in tap water with 5-10ml potassium alum added, for 10sec. Finally, the sections were washed, dehydrated, cleaned and mounted.

The haematoxylin solution was prepared as follows: 3g of haematoxylin powder was dissolved in 20ml of absolute alcohol, with the aid of gentle heat. This was added to 2000ml of distilled water and oxidized by adding 0.4g sodium iodate. 100g potassium alum was added as a mordant. The mixture was boiled for 2min, allowed to cool and finally 2ml acetic acid was added to 100ml of the solution before use.

The eosin solution was prepared from two solutions, the first containing 5% eosin L in distilled water and the second containing a saturated solution of eosin ethyl in 95% alcohol. Two volumes of the first solution were mixed with one volume of the second solution and the resultant solution was ready for use.

With this method nuclei appear blue and cytoplasm, connective tissue, red blood cells and muscle appear pink.

2. 10. 2. Luxol fast blue with cresyl violet (LFB)

The sections (20μ thick) were placed in water for 30sec and then stained in luxol fast blue (1g, dissolved in 5ml of 10% acetic acid and 1000ml of alcohol and filtered before use) for 30min. They were afterwards washed in water and differentiated in 0.1% lithium carbonate for a few seconds, followed by 70% alcohol for 20-30sec. This procedure was repeated until the required degree of differentiation was achieved. The sections were again washed in water and counterstained with 0.1% cresyl violet for 2min. Finally, they were washed, dehydrated, cleaned and mounted. With this method myelin is stained deep blue and neuronal cell bodies (Nissl substance) are purple.

2. 10. 3. Periodic Acid Schiff (PAS) Stain

One of the effects of long-standing hyperglycaemia is the non-enzymatic glycosylation of long-lived tissue proteins. This process also occurs with aging. With the PAS stain it is sometimes possible to visualize this deposition of glycogen and periodate reactive carbohydrates (appearing *magenta*) in the bodily tissues. Brain sections of the diabetic animals used for autoradiographic measurement of basal LCBF (section 2. 7. 1.) were examined, with non-diabetic (DR) brain sections of animals used for measurement of basal LCBF serving as controls.

The sections were initially washed with water for 30sec. They were then put to 1% periodic acid for 2-3min. They were afterwards washed well in running water for 5-10min. Schiffs reagent (0.5M in water at 20°C, Sigma) was added for 2-3min. They were again washed well in running water for 5-10min. Then, it was counterstained lightly in haematoxylin (prepared as described in section 2. 10. 1.). Following that they were differentiated in acid alcohol and "blued" in lithium carbonate. Finally, they were washed, dehydrated, cleared and mounted.

2. 10. 4. Electron microscopy (EM) of microvessels in diabetic and DR rats

For these studies, the animals were anaesthetized with pentobarbitone (30-45mg.kg^{-1}) and the right femoral artery and vein were cannulated in the same way described in

section 2. 2. 2. The arterial cannula was used to monitor the MABP continuously and for withdrawal of blood for physiological measurements.

Initially, 2ml of heparin (2000IU) was injected through the right femoral vein. Subsequent to that a thoracotomy was performed and the pericardium opened. The apex of the left ventricle was incised and a cannula was introduced into the ascending aorta. The cannula was clamped in the ascending aorta. The atria were subsequently opened. 50ml of sodium phosphate buffer (0.1M) at room temperature was rapidly perfused transcardially in order to clear the intravascular space from the blood. Care was taken to keep the MABP between 100 and 160mmHg, by adjusting the rate of perfusion. Afterwards, transcardiac perfusion of 300ml of a solution containing 0.2% gluteraldehyde and 4% parformaldehyde in 0.1M sodium phosphate buffer was performed.

After the end of the perfusion the brain was dissected intact and immersed in perfusate for 24h at 4°C to ensure complete fixation. Previous studies performed by others in this laboratory using the same perfusion fixation technique, have revealed that there were sometimes perfusion artifacts in the brain parenchyma (dark neurons) when the brain was left in the perfusate for more than 4h. However, no artifacts relating to the structure of the vessels have ever been noted.

The brain was finally cut in gross coronal blocks 2mm apart, and specimens of tissue ($1mm^3$) were taken from the cortex, the basal ganglia, the hippocampus and the white matter. The tissues were processed using standard approaches for EM by Dr James Ironside, Consultant Neuropathologist in Western General Hospital, Edinburgh.

2. 11. Analysis of neuropathological sections

As already described, most of the neuropathological sections that were analyzed were obtained from frozen tissue, so the criteria set by Brown & Brierley in 1968 regarding the microscopic appearance of ischaemic/hypoxic cell damage could not be used in these studies. As an alternative approach, the area of striatal tissue in which pallor and

cell loss was evident in the lesioned brains was calculated in each section with the use of the densitometer, in the same way that the volume of striatal blood or silicon oil were analyzed (section 2. 9.). The boundary of this area was sharply demarcated in the haematoxylin and eosin and luxol-fast blue sections from the adjacent normal brain tissue. This procedure was undertaken primarily to study to which extent the CBF thresholds set for the calculation of striatal ischaemia (section 2. 8. 2.) corresponded to presence of structural abnormality. This analysis was conducted "blind" to either animal strain or treatment group.

2. 12. Drugs (L-NAME, 7-NI, SIN-1, SB209670)

7-NI and SB209670 were used in experiments investigating both physiology and pathology. In contrast, L-NAME and SIN-1 were only used in relation to physiology.

2. 12. 1. N^G-nitro-L-arginine methyl ester (L-NAME)

The non-selective NOS inhibitor L-NAME (Sigma) was dissolved in saline at a concentration of 30mg.ml^{-1}. It was injected at a dose of 30mg.kg^{-1} i.v. over 30sec and LCBF was measured 20min later. MABP and core temperature were monitored constantly and the rest of the physiological parameters were measured before the injection of L-NAME and before the measurement of LCBF. The control animals were injected with an equal i.v. volume of saline.

L-NAME, at the dose used in the experiments presented in this thesis has been previously shown to reduce LCBF significantly by 15min post-injection and the effect is maintained stable for at least 3h (Kelly *et al.*, 1994b; Macrae *et. al.*, 1993).

2. 12. 2. 7-nitroindazole (7-NI)

The selective nNOS inhibitor 7-NI (Lancaster Synthesis) was dissolved in sesame oil on a hot-plate at 70°C at a concentration of 12.5mg.ml^{-1}. It was injected fast i.p. at a dose of 25mg.kg^{-1} and LCBF was measured 40min later. MABP and core temperature were monitored constantly and the other physiological parameters were measured just

before the injection and before the LCBF measurement. The control animals were injected i.p. with the same volume of sesame oil, at the same temperature.

In the experimental ICH studies, the same dose was injected i.p. 30min after the induction of the haematoma, while the animals were under the influence of the anaesthetic.

7-NI is selective for neuronal NOS *in vivo* (Moore *et al.*, 1993a,b) and at the dose used in the experimental procedures outlined in this thesis, produces significant reductions in LCBF (Kelly *et al.*, 1995b). Doses of 7-NI higher than that used here have been shown to produce further reductions in cerebral blood flow in normal rats (Kelly *et al.*, 1995b; Wang *et al.*, 1995), but they may also produce anomalous focal hyperaemia (Kelly *et al.*, 1994a; 1995b). In the haematoma studies, the dose of 7-NI and the timing of the injection relative to the induction of the haematoma were chosen on the basis of reports of a neuroprotective effect in a rat model of focal cerebral ischaemia (Kelly *et al.*, 1994a).

2. 12. 3. 3-morpholinosydnonimine (SIN-1)

The NO donor compound SIN-1 (donated by Cassella AG) was dissolved in saline at a concentration of 250μg.ml^{-1} or 62.5μg.ml^{-1}. It was infused i.v. over 20min using an infusion pump set at a rate of 1.8mg.kg^{-1}.h^{-1} in the diabetic and non-diabetic groups, and at a rate of 1.8mg.kg^{-1}.h^{-1} or 0.54mg.kg^{-1}.h^{-1} in two groups of WKY and SHR. MABP and core temperature were monitored constantly throughout this infusion period. Equal volumes of saline (~40 or 48μl.min^{-1}) were infused in the control animals.

In a series of preliminary studies, it was found that an i.v. infusion of SIN-1 at 1.8mg kg^{-1} h^{-1} for 20 to 30 min completely blocked the expected cardiovascular and cerebrovascular effects of subsequent i.v. injection of L-NAME (30mg.kg^{-1}), indicating saturation by exogenous NO. However, when this dose was used in the SHR, it produced a reduction in MABP very close to the lower limit of pressure

autoregulation reported for this sub-strain (Barry *et al*, 1982; Harper & Bohlen, 1984). Therefore, a second lower dose (0.54mg.kg^{-1}.h^{-1}) was also used for the SHR, which produced similar magnitude of reduction in MABP in WKY and SHR.

2. 12. 4. SB209670

The ET-antagonist SB209670 (donated by Dr Ohlstein, Smith-Klein Beecham Pharmaceuticals, King of Prussia) was dissolved in saline at a concentration of 10mg.ml^{-1}. It was injected i.v. in the physiological studies at a dose of 10mg.kg^{-1} over 30sec. LCBF was measured 20min later. MABP and core temperature were monitored throughout this period. The rest of the physiological parameters were measured before the injection and before the LCBF measurement. Because both the mode of administration and vehicle were similar to that of L-NAME, the same saline controls were used. SB209670 is a non-peptide endothelin antagonist, so delivery across BBB was not of concern (Ohlstein *et al.*, 1994).

For the haematoma studies, SB209670 was administered i.p. at the same dose (10mg.kg^{-1}), starting 30min prior to the haematoma induction and continuing every 6h for 24h. The last i.p. injection was administered 2h prior to the LCBF experiment. Control animals received i.p. injections of saline. Barone and his colleagues reported neuroprotection with the use of a very similar ET antagonist in 1995, and the protocol employed in the haematoma studies was based on that report.

2. 13. High performance liquid chromatography (HPLC) for L-arginine

The aminoacid analysis of plasma was kindly performed by Mr Douglas Thompson, at the Department of Clinical Neurosciences in the Western General Hospital. Briefly, the procedure was as follows:

Venous blood (200μl) was obtained by tail-tipping from awake diabetic and non-diabetic animals. The blood specimens were placed in individual heparinized tubes and centrifuged at 10.000 rpm for 10min. 100μl of the supernatant plasma was placed into polythene vial and stored at -20°C for 5 days. Each specimen was then brought to

room temperature and 30μl of that plasma together with 70μl of methanol (to precipitate the proteins) were vortex-mixed for 1min in a polythene vial. The mixture was then centrifuged at 10000 rpm for 10 min. 10μl of the supernatant was removed and mixed with 70μl of borate buffer (accq-tag kit) and then, 20μl of accq-tag reagent was added and vortex mixed for 10 sec. 30μl of that mixture was placed in an autosampler vial and loaded in an autosampler tray, and 10μl was injected into the accq-tag column for aminoacid analysis using the Waters HPLC system (Waters Corporation, Milford, Massachussets).

2. 14. Whole blood viscosity for the BB rats

IDDM is associated with increased viscosity in humans. Several studies have raised the possibility that raised viscosity may be associated with alterations in blood flow and predisposition to cerebrovascular occlusion (see appropriate section in ***Introduction***). Therefore, whole blood viscosity was measured in groups of diabetic and non-diabetic BB rats.

Venous blood (1.5ml, obtained by tail-tipping in awake animals, 30min after the daily injection of insulin in the diabetic rats) was introduced into a heparinized tube and taken to the Royal Infirmary of Glasgow, where Professor Gordon Lowe measured the blood viscosity in each animal, relative to standard fluids of known viscosity.

2. 15. Measurement of glycosylated haemoglobin

Glycosylated haemoglobin values were determined from some diabetic animals and non-diabetic BB controls in order to assess the degree of metabolic control achieved with the insulin treatment. These measurements were performed by Dr Mark Lindsay, at the Metabolic Unit of the Western General Hospital.

Briefly, blood samples were obtained by tail tipping in awake rats using heparin as an anticoagulant. The samples were centrifuged for 5min at 10,000 revs.min^{-1} and the plasma collected for glucose determination (Beckmann Glucose Analyser 2). The remaining red blood cells were washed three times in 0.9% sodium chloride and

incubated overnight at room temperature with an equivalent volume of saline. The cells were then centrifuged and the supernatants discarded. The resulting packed cells were haemolyzed by the addition of three volumes of a 1:10 dilution of haemolyzing reagent (0.1% saponin and 0.05% EDTA in distilled water, Ciba Corning Diagnostics Corp., Palo Alto, CA.). After vortex mixing for 15sec the haemolyzed cells were spun at 10,000 revs.min^{-1} for 5min and 1µl of the resulting haemolysate was applied to individual sample wells on an electrophoretic plate (Ciba Corning glycosylated haemoglobin citrate agar gel electrophoresis film). The rat haemoglobin components were separated by electroendosmosis using 0.1M citrate buffer (pH 6.3) under 60V for 40 min. The percentage of glycosylated haemoglobin was determined by automatic scanning densitometry on a Corning 720 fluometer/densitometer using a 420 nm filter.

2. 16. Assessment of physiological status during the induction of haematoma

All haematoma recipients were expected to survive for the following 24h and for ethical and practical reasons these rats could not be fully instrumented. As physiological status at the time of haematoma induction could seriously influence the final outcome (Bileviciute *et al.*, 1995), a series of experiments were performed using exactly the same protocols outlined in section 2. 2., but in which the animals were cannulated to allow physiological variables to be measured.

Animals were anaesthetized with Pentobarbitone (30-45mg.kg^{-1}). The right femoral artery was cannulated as described above. Following that, continuous MABP monitoring was initiated and arterial blood was taken for estimation of arterial blood gases and plasma glucose. The animals were placed on the stereotactic frame as described previously (on a thermal blanket, an oxygen mask delivering 4 litres of oxygen per minute and with a rectal thermometer *in situ*) and 50µl of blood was injected in the striatum using the protocol already described. Arterial blood gas measurements were repeated 30min after the injection of the blood, and then the animals were sacrificed with an overdose of barbiturate.

2. 17. Measurement of intracranial pressure (ICP)

ICP was measured at the time of the haematoma induction in two pilot experiments, since extensive relevant information is already available in the literature (see ***Introduction***). The results of these experiments are therefore not presented in the ***Results*** section, but are only briefly considered in the ***Discussion***.

The rats were initially prepared in the same way as for induction of the haematoma. A burr-hole was made in the left posterior corner of the bregma. A 24G sprott spinal needle with side port, the side with the opening placed medially, was secured on the stereotactic apparatus and lowered until the tip touched the brain. The output of the needle was connected through a fluid link to external strain gauge transducer which was zeroed at the level of the earbars to atmospheric pressure. The constant infusion method was employed, with the infusion pump delivering $5ml.h^{-1}$ "mock" CSF while the needle was lowered slowly into parenchyma in 1mm increments observing the intraparenchymal pressure on a chart recorder. The needle was lowered progressively to a maximum depth of 4mm from the dural surface, until a step drop in pressure was observed. This was taken to be the point at which the needle entered the ventricle. At that point the constant infusion was stopped and the ICP returned to physiological levels in approximately 10min. After that time point, the haematoma was induced as described in section 2. 2. The animal was finally sacrificed with an overdose of barbiturate 45min later. ICP was recorded constantly throught the experiment.

2. 18. Statistical analysis

Physiological and LCBF data (presented as mean ± standard error of mean) were analyzed by Student's *t* test with Bonferroni correction (Wallenstein *et al.*, 1980) applied to allow multiple pair-wise comparisons between appropriate groups (maximum number of comparisons = 3). Differences in the LCBF responses to L-NAME, 7-NI and SIN-1 treatment between the rat sub-strains were analyzed by Mann-Whitney U-test (Jennrich *et al.*, 1990). Acceptable levels of significance were set at $P < 0.05$ for all statistical tests. The number of animals in each treatment group ranged from 4 to 8 and is reported in the Appendix for each treatment group.

CHAPTER 3

RESULTS

General conduct of the experiments

Physiology of cerebrovascular endothelium

There were no unexpected physiological consequences of drug treatments in the diabetic and DR groups used in the quantitative autoradiographic studies of cerebrovascular dysfunction. The use of intensified insulin treatment in BB/E rats did however present problems. One third of the animals originally included in this protocol died during the intensive treatment regime, possibly due to hypoglycaemia. Nonetheless, satisfactory data were acquired from the remaining two thirds which survived.

Experimental ICH

The preliminary experimental ICH studies performed in diabetic and non-diabetic rats, with injections of 25μl of blood showed no detectable histopathological changes at 24h. Increasing survival time to 48h did not alter the outcome. In contrast, 50μl injections resulted in clearly discernible changes in tissues surrounding the haematoma with survival periods of 24h. In a single diabetic rat subjected to ICH during these preliminary studies, presence of fibrinoid necrosis was visible in a striatal microvessel close to the haematoma. This volume (50μl) was used for the main experimental procedures. Of the diabetic rats used in this study, 20% died after the induction of the haematoma, and before the LCBF or BBB permeability experiment for reasons unrelated to the anaesthesia. A seizure was witnessed in one of these animals just before it died, but most of them were found dead in cages the morning after the experimental ICH.

The experimental ICH studies in the SHR and WKY rats posed certain problems. The silicon oil injections in the SHRs were invariably associated with endogenous bleeding, and it was impossible to differentiate the differential influence of the blood constituents from that of the mass effect.

3. 1. Physiological studies with pharmacological manipulation in diabetic and control animals

3. 1. 1. Physiological variables

In the control (saline or sesame oil-treated) animals, there were no significant differences in any of the physiological parameters measured, including plasma glucose, between non-diabetic and insulin-treated diabetic rats (Tables 1, 2 and 3). Although there were some effects of the drug treatments, physiological variables (with the exception of MABP) were within the physiological range for rat. Body weights were not significantly different between non-diabetic and diabetic groups in any of the studies presented in this thesis (mean weight in both sub-strains was ~400g, data not shown).

The physiological variables of the CSII-treated diabetic rats and the conventionally-treated diabetic animals used as controls are presented in Table 4. No significant differences were found in any of the parameters measured, including plasma glucose. Glycosylated haemoglobin and haematocrit values were measured only in the CSII-treated rats.

In the animals treated acutely with L-NAME, there were no differences in the physiological parameters between non-diabetic and diabetic rats (Table 1). MABP was significantly higher in both non-diabetic (+24%) and diabetic (+37%) groups, compared to the saline-treated animals of the same sub-strain. The pattern of rise in MABP following i.v. administration of L-NAME differed between individual animals, being sometimes abrupt and more gradual in other occasions, but always lasting between 2 and 5min before reaching a plateau, which was invariably sustained until the measurement of LCBF, 20min after the injection. Heart rate was lower in the L-NAME treated non-diabetic (-26%) and diabetic rats (-19%), compared to the saline-treated animals of the same sub-strain, although this was not significant (Table 1).

In the groups treated with 7-NI, heart rate was significantly lower in the diabetic animals (-29%), compared to the non-diabetic rats (Table 2). In the control groups,

heart rate was again lower (-15%), although not significantly, in the diabetic animals. Heart rate in the 7-NI - treated diabetic group was also significantly lower (-40%) compared to the diabetics treated with sesame oil (Table 2). In the non-diabetic rats, there was also a trend, though not significant for the 7-NI - treated rats to have lower heart rate (-30%) compared to controls. In contrast to the L-NAME findings, MABP was not different between 7-NI treated and control animals of any sub-strain.

Following infusion with SIN-1, arterial pO_2 and body temperature were significantly lower and pH, plasma bicarbonate and base excess significantly higher in the diabetic group, compared to the non-diabetic animals (Table 3). In the non-diabetic animals treated with SIN-1, temperature was significantly higher compared to the animals treated with saline (Table 3). In the diabetic groups, pH and base excess were significantly higher and pCO_2 significantly lower in the SIN-1 - treated rats, compared to those treated with saline (Table 3). MABP was lower in both non-diabetic (-20%) and diabetic (-16%) rats treated with SIN-1, compared to the saline-treated animals of the same sub-strain, although this difference was not statistically significant.

There were no significant differences in the physiological variables between the saline and SB209670-treated diabetic animals (Table 1). A modest (+11%) non-significant increase in heart rate was observed in the SB209670-treated rats (Table 1).

Whole blood viscosity was significantly higher in the diabetic animals (4.622 ± 0.174 mPa.s, n = 5) compared to the non-diabetic group (3.840 ± 0.132 mPa.s, n = 6). Haematocrit did not differ between the same groups (45.4 ± 2.7 % in the diabetics *vs* 45.2 ± 1.3 % in the non-diabetics).

Plasma L-arginine levels were similar between groups (94.0 ± 2.4 $\mu mol.l^{-1}$ in the non-diabetic and 93.8 ± 6.7 $\mu mol.l^{-1}$ in the diabetic, n = 4 in each group).

3. 1. 2. LCBF measurements with pharmacological manipulation

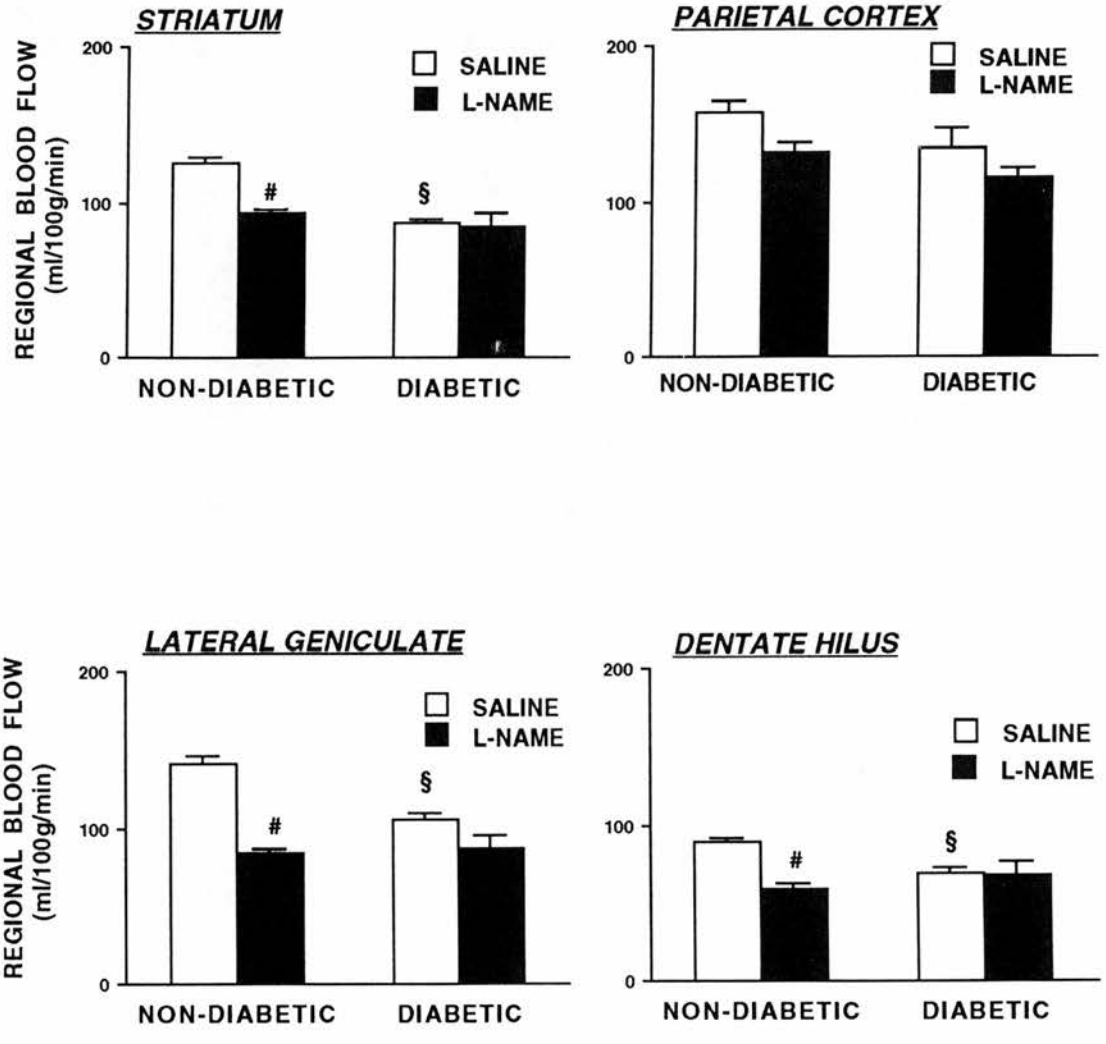
LCBF was measured in areas chosen to represent brain regions in the vascular territories of the anterior, middle and posterior cerebral arteries. In control saline or sesame oil-injected diabetic rats, mean LCBF was reduced in all 10 brain areas measured when compared to non-diabetic controls (Tables 5, 6 & 7). However, the extent of these reductions in LCBF were regionally heterogeneous, ranging from -14% in parietal cortex (not significant) to -31% in striatum ($P < 0.05$). The reductions were statistically significant in six of the areas examined, and these were predominantly sub-cortical (striatum, globus pallidus, lateral geniculate, corpus callosum, hippocampal fields CA 2&3 and dentate hilus; Tables 5, 6 & 7; Figures 1, 2 & 3). Taking each diabetic animal individually, there was no correlation between the extent of LCBF reduction and either duration of diabetes or plasma glucose status at the time of the experiment.

In the intensively treated (with CSII) diabetic rats, LCBF measurements (Table 8) were restricted to those grey matter areas where significant differences were previously encountered between saline-treated non-diabetic and diabetic animals. No significant differences were observed between the two groups (conventionally-treated *vs* CSII-treated), but LCBF was higher in 3 out of the 5 areas examined in the CSII-treated group (striatum, +17%; lateral geniculate, +12%; globus pallidus, +9%; *vs* conventionally-treated animals). No differences were found in the hippocampal fields CA 2&3 and dentate hilus (Table 8). Within the CSII-treated group, no correlation was found between glycosylated Hb (8.8 ± 0.6%), or plasma glucose and LCBF in any of the brain areas examined (Figure 4).

L-NAME treatment produced reductions in LCBF throughout the brain in non-diabetic animals (Table 5). Only in parietal (-16%) and cingulate areas of cortex (-18%) and in nucleus accumbens (-25%) did the effects of L-NAME fail to reach statistical significance. Elsewhere, significant ($P < 0.05$) reductions in LCBF were measured, ranging from -26% in the striatum to -40% in lateral geniculate (Table 5). In contrast, L-NAME treatment had no significant effect upon LCBF in diabetic rats,

FIGURE 1

Local cerebral blood flow ($ml.100g^{-1}.min^{-1}$) measured with quantitative [^{14}C]-iodoantipyrine autoradiography, in four functionally diverse brain regions of non-diabetic and diabetic BB rats, 20min after i.v. injection of saline (0.5ml) or L-NAME ($30mg.kg^{-1}$)



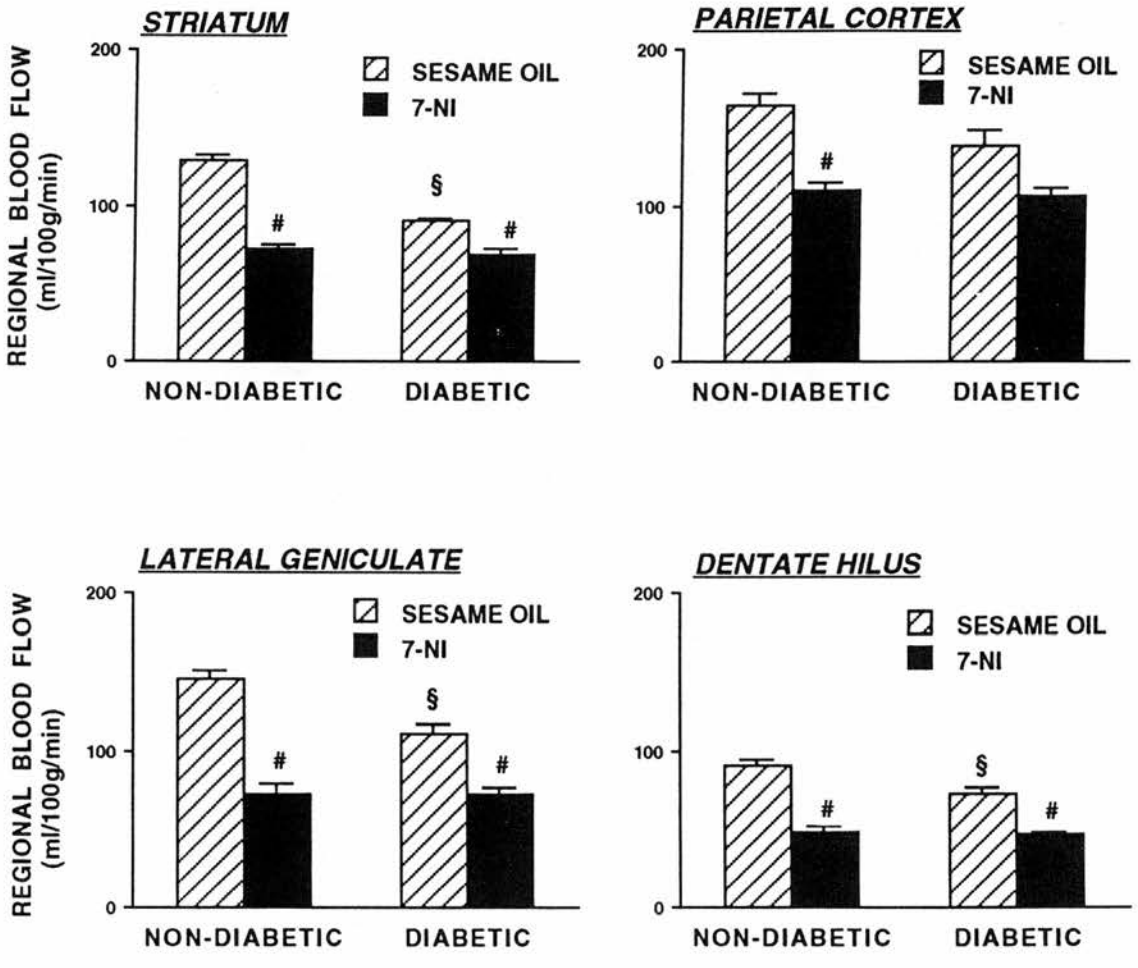
Data are presented as mean ± s.e. mean (n = 6 in saline-treated groups, n = 4 in L-NAME treated groups)

§: significant difference between non-diabetic and diabetic rats receiving the same treatment ($P < 0.05$)

#: significant difference between saline and L-NAME treated rats of the same sub-strain ($P < 0.05$)

FIGURE 2

Local cerebral blood flow (ml.100g^{-1}.min^{-1}) measured with quantitative [^{14}C]-iodoantipyrine autoradiography, in four functionally diverse brain regions of non-diabetic and diabetic BB rats, 40min after i.p. injection of sesame oil (0.6ml) or 7-NI (25mg.kg^{-1})



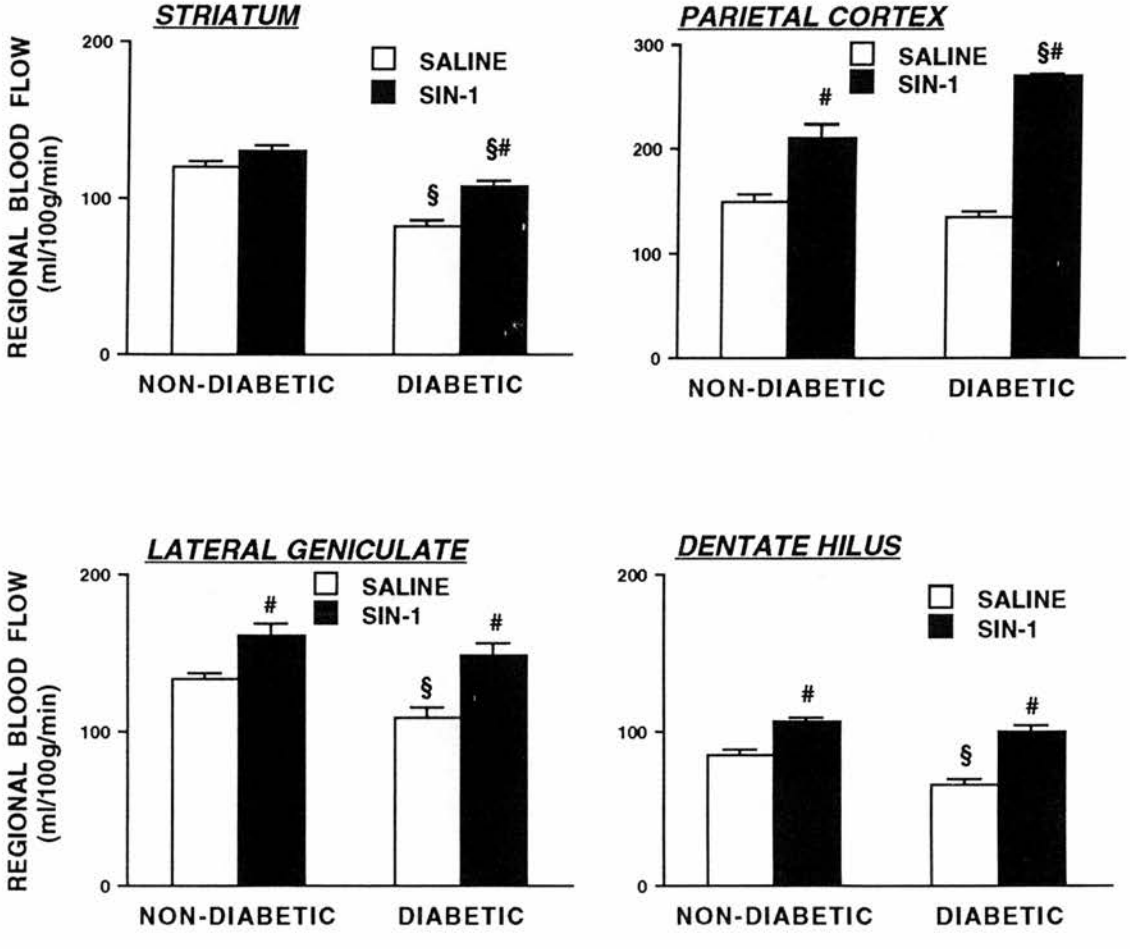
Data are presented as mean ± s.e. mean (n = 4 in each group)

§: significant difference between non-diabetic and diabetic rats receiving the same treatment ($P < 0.05$)

#: significant difference between sesame oil and 7-NI treated rats of the same sub-strain ($P < 0.05$)

FIGURE 3

Local cerebral blood flow (ml.100g^{-1}.min^{-1}) measured with quantitative [^{14}C]-iodoantipyrine autoradiography, in four functionally diverse brain regions of non-diabetic and diabetic BB rats, 20min after the onset of i.v. infusion of saline (40µl.min^{-1}) or SIN-1 (1.8mg.kg^{-1}.hr^{-1})



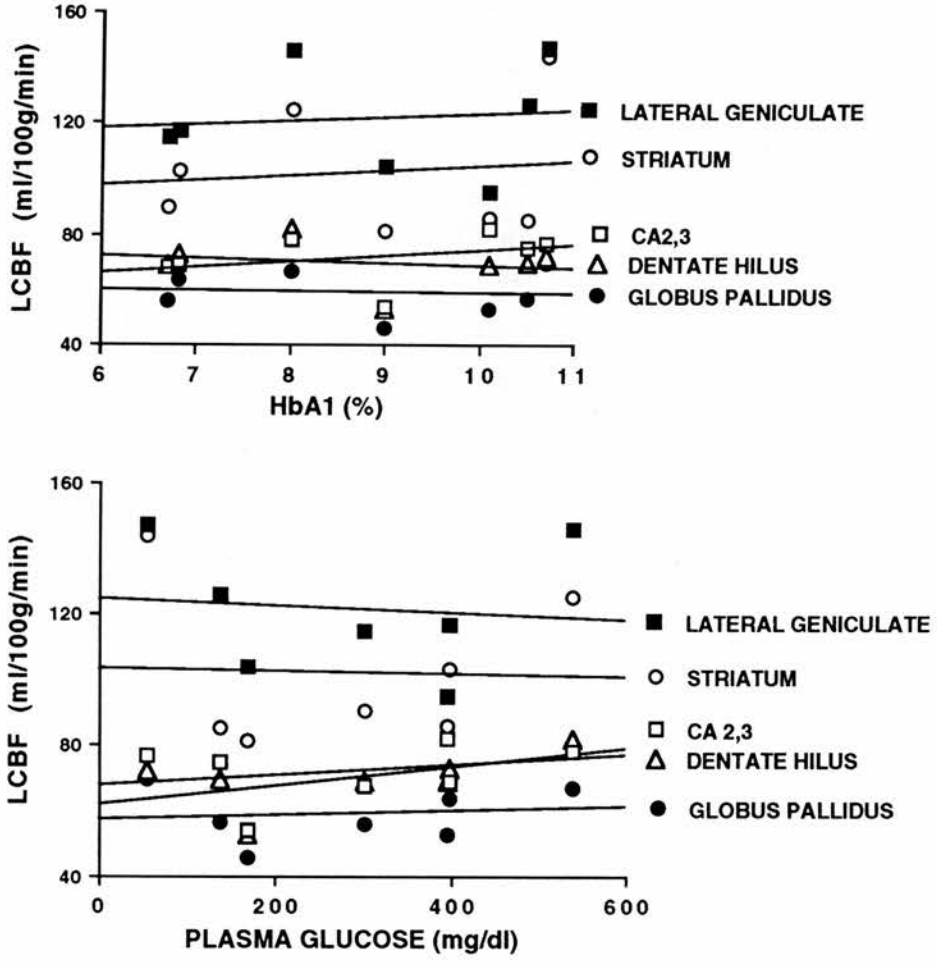
Data are presented as mean ± s.e. mean (n = 4 in each group). Note the differences in scale along Y axis between the top right (parietal cortex) and the rest of the graphs.

§: significant difference between non-diabetic and diabetic rats receiving the same treatment ($P < 0.05$)

#: significant difference between saline and SIN-1 treated rats of the same sub-strain ($P < 0.05$)

FIGURE 4

Individual local cerebral blood flow (LCBF, ml.100g^{-1}.min^{-1}) values in CSII-treated diabetic rats (n = 7), measured in the brain areas where significant reductions were observed under basal conditions in the conventionally-treated diabetic animals of the same sub-strain.



LCBF was measured with quantitative [^{14}C]-iodoantipyrine autoradiography, is presented on the Y axis of both graphs and plotted against glycosylated haemoglobin values (HbA_1) from each individual animal (X axis, top graph) or plasma glucose values measured in each individual animal at the time of the LCBF experiment (X axis, bottom graph). No correlation between LCBF in any of the five brain areas measured and HbA_1 or plasma glucose was found. The lines in each graph are computer-generated simple curve fits (Cricket Graph, Macintosh) for each individual brain region.

with the exception of hypothalamus (Table 5, Figure 1), when compared to the saline-treated (diabetic) group. Modest non-significant decreases in LCBF were observed, between -2% in striatum and dentate hilus of hippocampus and -20% in lateral geniculate and corpus callosum. A comparison of the L-NAME effects between non-diabetic and diabetic rats revealed a significant difference in the cerebrovascular response to this non-selective NOS inhibitor. The median reduction in LCBF was -31.5% in the non-diabetic and -12.5% in the diabetic group ($P = 0.004$, Mann-Whitney U-test). In contrast to the significant differences in blood flow between the saline treated non-diabetic and diabetic groups, there were no significant differences in LCBF between the groups treated with L-NAME, possibly reflecting an already reduced basal LCBF in the diabetic rats.

Following treatment with 7-NI there were significant reductions in LCBF in all areas examined in the non-diabetic animals, compared to sesame oil-treated rats (Table 6, Figure 2). This reduction in LCBF ranged between -33% in parietal cortex and -55% in hypothalamus. In contrast to the effects of L-NAME, 7-NI treatment in the diabetic animals resulted in significant reductions in LCBF in 9 out of the 10 brain areas examined (Table 6, Figure 2). The reductions ranged between -24% in striatum and -53% in hypothalamus. The median reduction in LCBF was -50% in the non-diabetic and -35.5% in the diabetic animals ($P = 0.014$, Mann-Whitney U-test).

In one of the diabetic animals treated with 7-NI, focal hyperaemia was observed unilaterally in the cortex, and in the hippocampus and thalamus on the opposite side (Figures 5 and 6). This phenomenon has been described previously in normal (non-diabetic) rats, but only at higher 7-NI doses (Kelly *et al.*, 1995b).

SIN-1 infusion resulted in increases in LCBF in the non-diabetic animals in all but one (corpus callosum) brain areas, compared to the saline-infused rats (Table 7, Figure 3). However, these increases were statistically significant only in parietal cortex (+42%), nucleus accumbens (+33%), lateral geniculate (+20%) and dentate hilus of hippocampus (+26%). In the rest of the areas examined, the increases in LCBF ranged

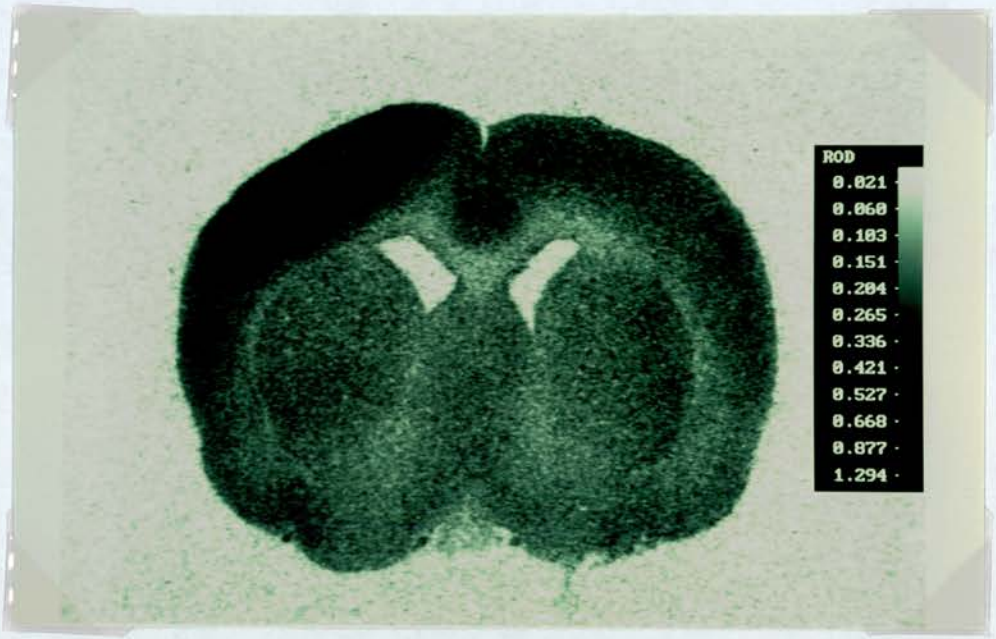


Figure 5: Blood flow (IAP) autoradiogram from a diabetic animal injected acutely with 7-NI ($25mg.kg^{-1}$). Coronal section obtained at the level of caudate nucleus. Note focal hyperaemia in the cortex on the left side. Grey scale on the right represents relative optical densities, which can be used to calculate blood flow (see Methods).

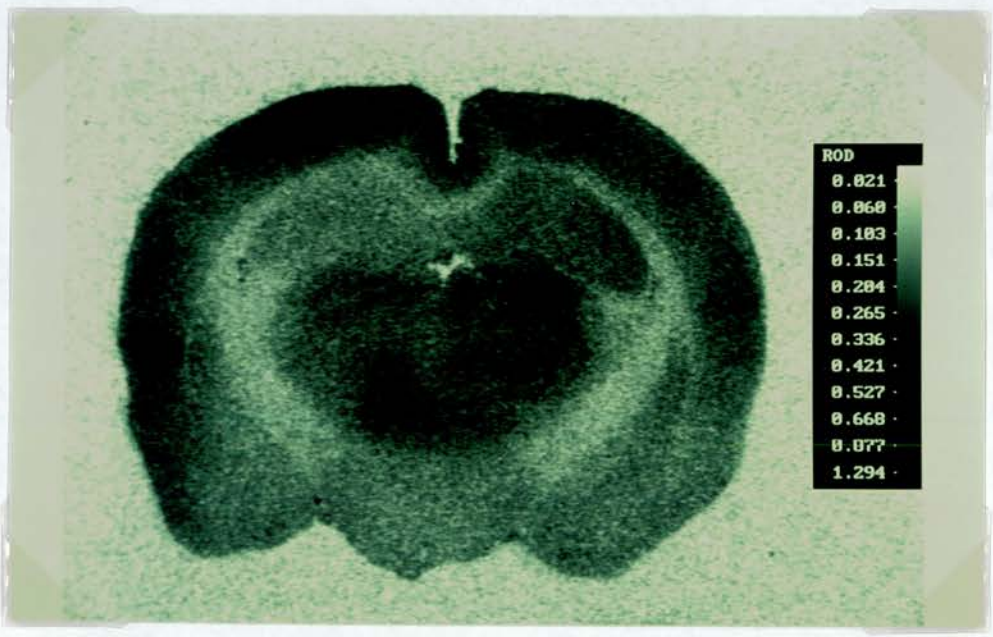


Figure 6: Blood flow (IAP) autoradiogram from the same animal shown in Figure 5. Coronal section taken at the level of thalamus. Note contralateral hyperaemia in the hippocampus (mainly CA2 and CA3 layers) and thalamus, in relation to the left sided cortical hyperaemia.

between +8% in striatum and +21% in cingulate cortex and hypothalamus (median increase: +20.5%). In the diabetic animals SIN-1 produced increases in LCBF in all but one (hypothalamus) area (Table 7, Figure 3). These increases reached statistical significance in seven brain areas (parietal cortex, +100%; cingulate cortex, +31%; striatum, +31%; globus pallidus, +24%; nucleus accumbens, +39%; lateral geniculate, +37%; and dentate hilus of the hippocampus, +52%; Table 7). The median increase in LCBF in the diabetic group was +31% (P = 0.0413, Mann-Whittney U-test *vs* non-diabetic). Although globally the response to SIN-1 was greater in diabetics, LCBF was significantly higher in the striatum and globus pallidus of the SIN-1 treated non-diabetic rats, compared to the diabetic animals (Table 7, Figure 3).

SB209670 treatment was confined to the diabetic rats, in order to test whether an increased ET activity was responsible for the reduced basal LCBF in these animals. However, non-significant changes in LCBF were observed in the SB209670-treated rats, compared to the saline-treated diabetic rats (Table 5, Figure 7). Modest, non-significant increases in LCBF were observed in 5 out of the ten areas examined, compared to the saline-treated animals. These ranged between +6% in parietal cortex, globus pallidus and nucleus accumbens and +13% (almost significant) in the striatum. LCBF was similar in 3 areas (hypothalamus, lateral geniculate and dentate hilus of the hippocampus) and showed modest non-significant reduction in 2 areas (cingulate cortex, -16%; and corpus callosum, -9%).

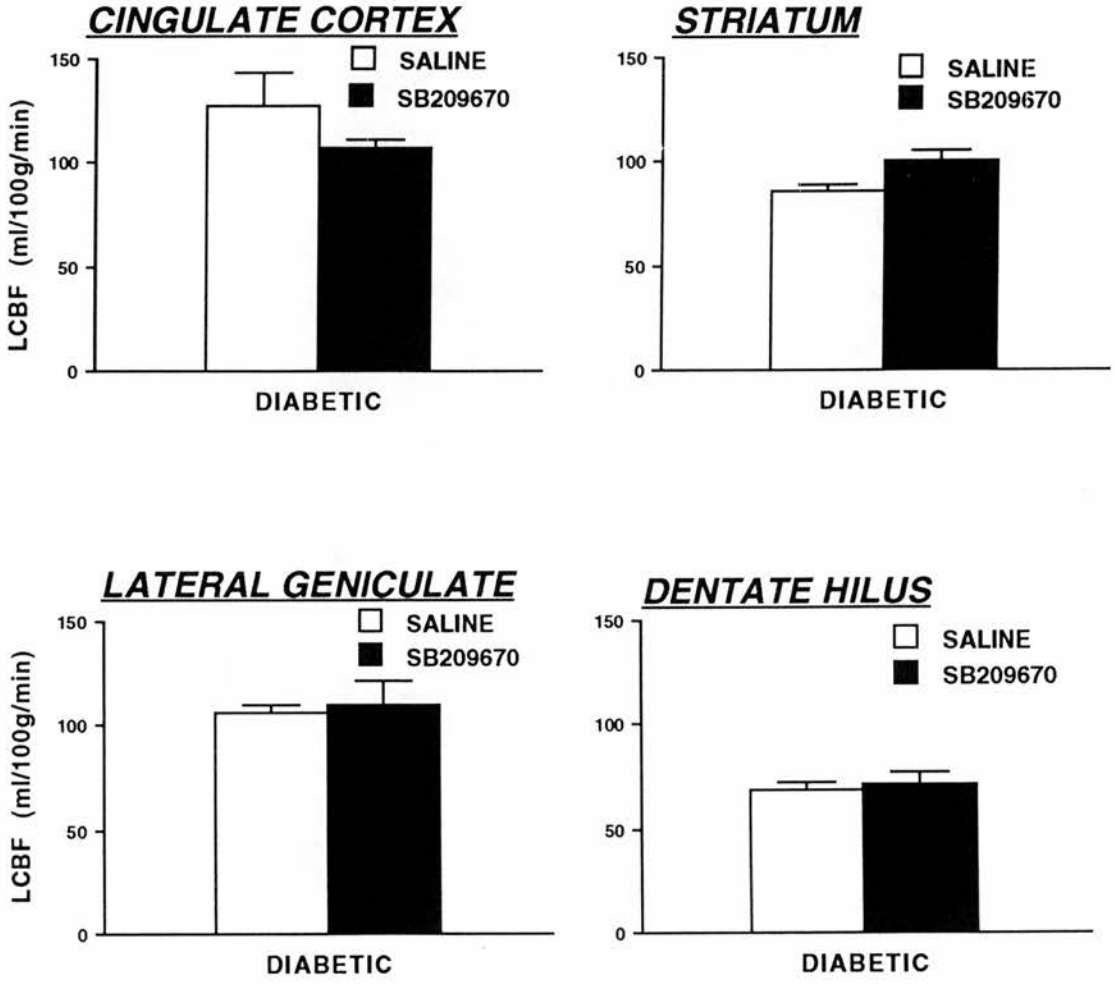
There was no correlation between LCBF increases following SB209670 administration and regions that had significant reductions in basal LCBF amongst diabetic animals (Table 5).

3. 2. Electron microscopy results

The potential presence of subendothelial glycosylation products associated with IDDM, as already mentioned in the ***Introduction***, could have contributed to the LCBF results, by quenching the endothelially derived NO and therefore reducing its vasodilatory capacity. Electron microscopy (EM) was used to study the

FIGURE 7

Local cerebral blood flow (ml.100g^{-1}.min^{-1}) measured with quantitative [^{14}C]-iodoantipyrine autoradiography, in four functionally diverse brain regions of diabetic BB rats, 20min after the i.v. injection of saline (0.5ml) or SB209670 (10mg.kg^{-1})



Data are presented as mean ± s.e. mean (n = 6 in saline-treated and n = 4 in SB209670-treated animals)

No significant differences were found between saline-treated and SB209670-treated rats

intraparenchymal microvessels predominantly in the areas where basal LCBF was significantly lower in the diabetic rats (e.g. striatum, hippocampus), compared to their non-diabetic controls. However, neither abnormal thickening nor differences in the density of the basement membranes were observed in the diabetic cerebrovasculature, when compared to vessels obtained from the non-diabetic controls (n = 2, in each group). Characteristic vessels from striatum of both diabetic and non-diabetic rats are shown in Figures 8 & 9.

3. 3. Periodic acid schiff (PAS) staining in diabetic brains

The frozen brain sections studied were obtained from the animals used for the basal LCBF measurements (section 2. 7. 1.). No differences in vessel wall PAS staining were found between the diabetic and the non-diabetic animals in any of the brain areas (n = 4, in each group). In particular, no evidence of vascular endothelial basement membrane thickening or deposition of PAS-positive material was seen in the diabetic animals in comparison with the controls. These brain sections and the EM photographs, were examined by Dr James Ironside.

In summary, these results indicate that local cerebral blood flow is compromised in a regionally heterogeneous manner in the diabetic BB rats under physiological conditions, associated with reduced endothelial NO bioactivity. This perturbation in the endothelial NO system does not result from reduced substrate (L-arginine) availability or structural microvascular abnormalities, and is possibly unrelated to development of subendothelial AGEs in the cerebrovasculature.

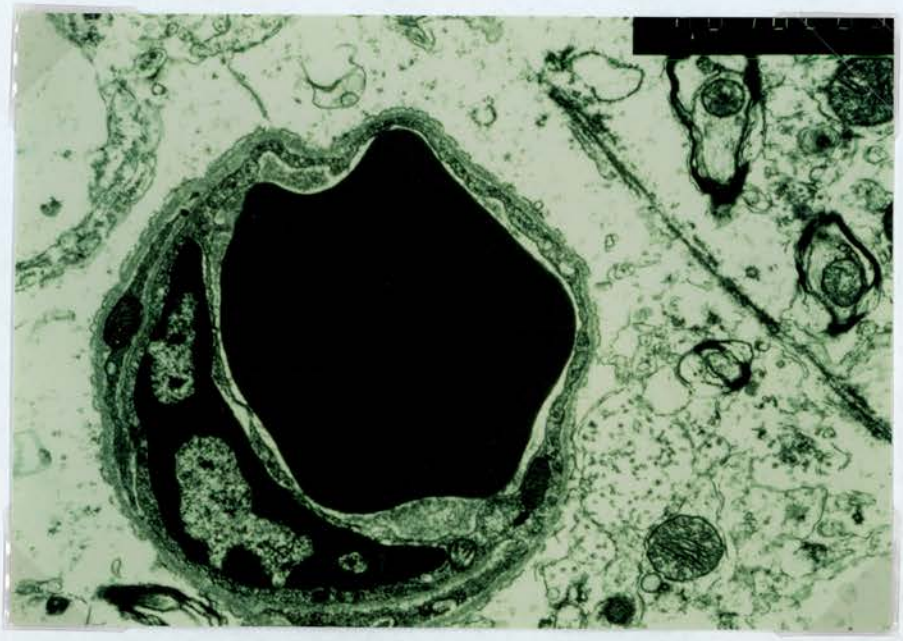


Figure 8: Electron microscopic appearance of a microvessel (capillary) in the caudate nucleus of a non-diabetic rat. There is a red cell in the lumen of the vessel. (Print magnification x16000).

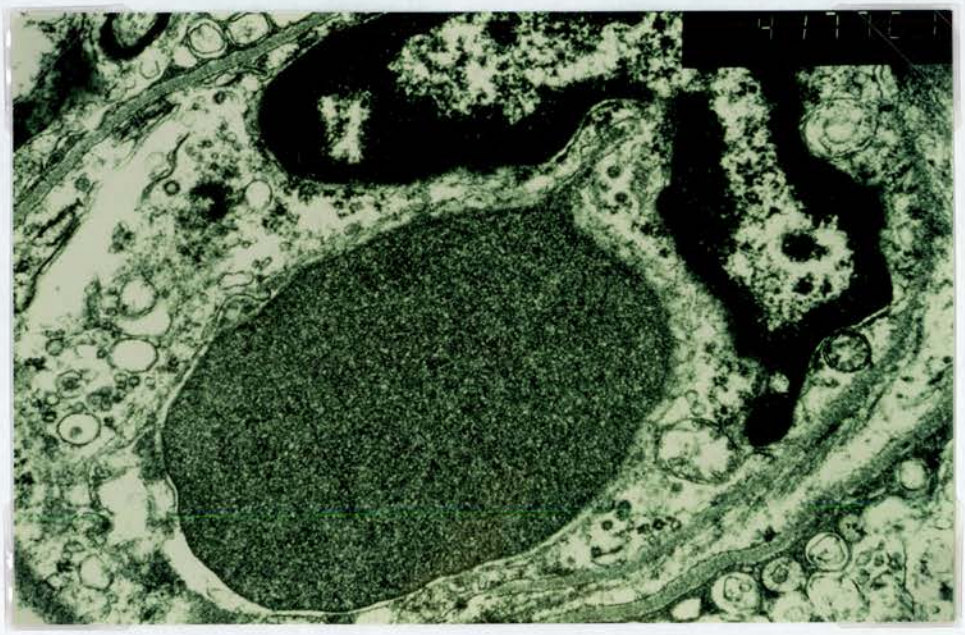


Figure 9: Electron microscopic appearance of a capillary in the caudate nucleus of a diabetic rat. Not withstanding the difference in magnification, there was no measurable difference in the thickness or density of the basement membrane of this vessel, compared to that shown in Figure 8. (Print magnification x28086). There is a platelet in the lumen of the vessel.

3. 4. Physiological studies with pharmacological manipulation in hypertensive and control animals

3. 4. 1. Physiological variables

MABP and heart rate were significantly higher in the control (saline and sesame oil-treated) SHR rats compared to the WKY groups with the same treatment (Tables 9, 10, and 11). In addition, haematocrit was also significantly higher in control SHR rats compared to WKY (Table 9). Body weights were not significantly different between WKY and SHR groups in all studies presented in this thesis (mean weight was ~290g in WKY and ~260g in SHR, data not shown).

L-NAME treatment resulted in significant increases in MABP in both WKY (+27%) and SHR (+18%)(Table 9). Despite the greater increase in MABP in WKY following L-NAME, MABP in SHR remained significantly higher than in WKY. Heart rate in SHR was not significantly affected by L-NAME (-18%), but decreased (-27%) in WKY and as a result, was significantly lower than in SHR (Table 9).

Treatment with 7-NI had no significant effect upon MABP in either WKY or SHR, and as a result significant differences in blood pressure found in control rats of these sub-strains were maintained (Table 10). In SHR treated with 7-NI, a non-significant reduction in heart rate (-20%) was observed, compared to sesame oil-treated rats (Table 10).

The lower dose of SIN-1 ($0.54 mg.kg^{-1}.h^{-1}$), resulted in a significant reduction in MABP in SHR (-18%). Although reductions of similar magnitude were also found in WKY (-20%), these were more variable and not significant (Table 11). With these parallel decreases in MABP in both sub-strains, the significant difference in blood pressure found in control rats was maintained. In addition, heart rate was significantly higher in the SIN-1 - treated SHR compared to the WKY with the same treatment (Table 11).

Following the higher dose of SIN-1 (1.8mg.kg^{-1}.h^{-1}), the reductions in MABP in WKY (-21%) were of a magnitude similar to that found with the lower dose. In contrast, the higher dose of SIN-1 produced a more marked reduction in MABP in SHR (-42%) so that the differential, evident in control rats of these sub-strains, was no longer evident (95 ± 6mmHg in WKY *vs* 104 ± 5mmHg in SHR) (Table 11).

There were no significant differences in any of the physiological variables between the saline and SB209670-treated hypertensive animals (Table 9). A modest (-7%) non-significant decrease in heart rate was observed in the SB209670-treated rats (Table 9).

3. 4. 2. LCBF measurements with pharmacological manipulation

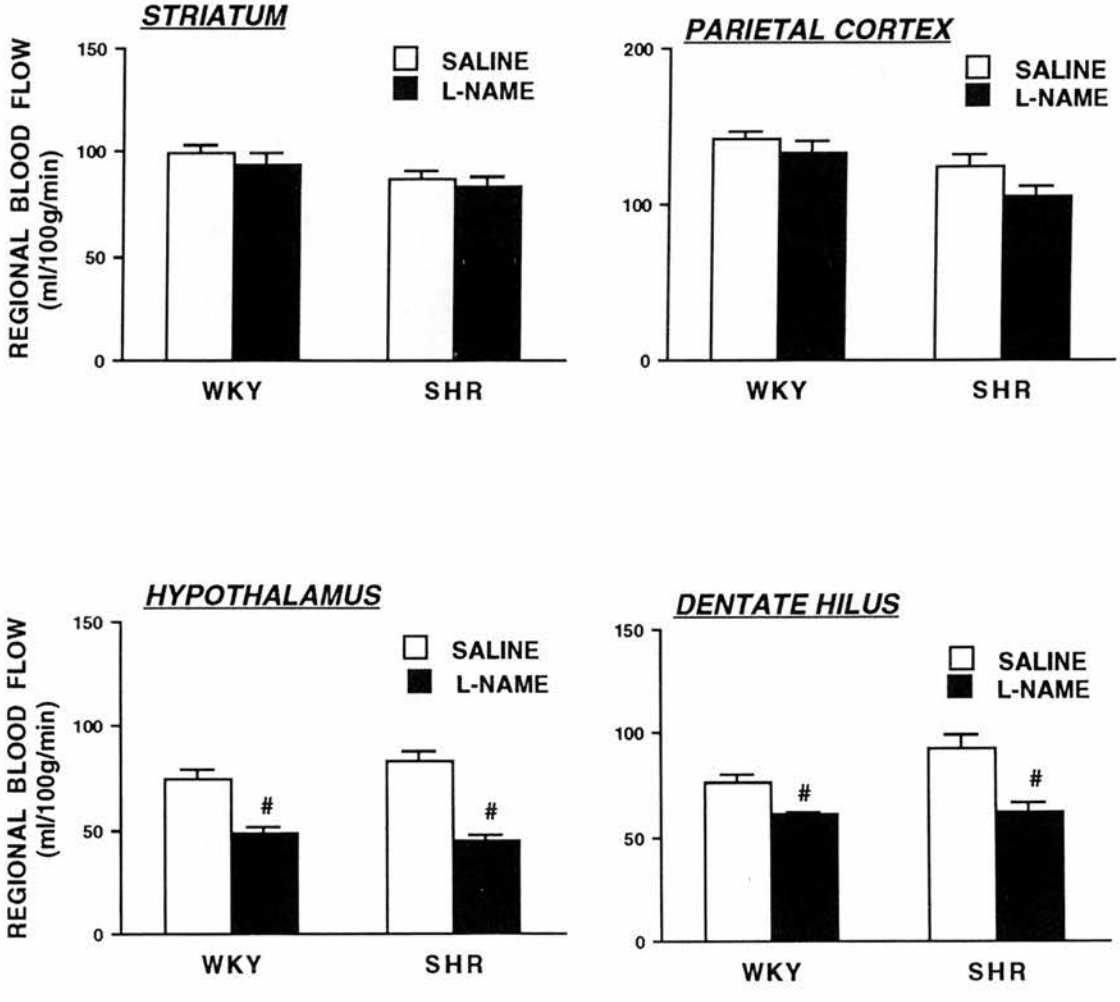
Basal LCBF in control WKY and SHR rats was broadly similar between the two sub-strains. Only nucleus accumbens had significantly higher blood flow in the control SHR. This result was consistent, whether the rats were treated with i.v. saline (Table 12) or sesame oil (Table 13).

Following injection of L-NAME in the WKY group, there was a global tendency towards decreases in LCBF (with the exception of cingulate cortex) ranging between -12 and -46% (median= -20%). LCBF was significantly reduced (compared to saline-treated animals) in seven out of the thirteen brain areas examined (corpus callosum, nucleus accumbens, hypothalamus, hippocampal fields CA2 and CA3, molecular layer and dentate hilus; Table 12 & Figure 10).

Similarly, in the L-NAME-treated SHR group there was a global trend towards reductions in LCBF ranging between -9 and -46% (median= -22%), which reached acceptable levels of significance in the cingulate cortex, nucleus accumbens, hypothalamus, hippocampal fields CA2 and CA3, molecular layer and hilus of the dentate gyrus (Table 12 & Figure 10). There were no significant differences in the LCBF between L-NAME treated WKY and SHR, apart from in the corpus callosum. (Table 12).

FIGURE 10

Local cerebral blood flow (ml.100g^{-1}.min^{-1}) measured with quantitative [^{14}C]-iodoantipyrine autoradiography, in four functionally diverse brain regions of WKY and SHR rats, 20min after i.v. injection of saline (0.5ml) or L-NAME (30mg.kg^{-1})



Data are presented as mean ± s.e. mean (n = 6 in saline-treated WKY group, n = 5 in saline-treated SHR group, n = 4 in L-NAME treated groups). Note the differences in scale along Y axis between the top right (parietal cortex) and the rest of the graphs.

#: significant difference between saline and L-NAME treated rats of the same sub-strain ($P < 0.05$)

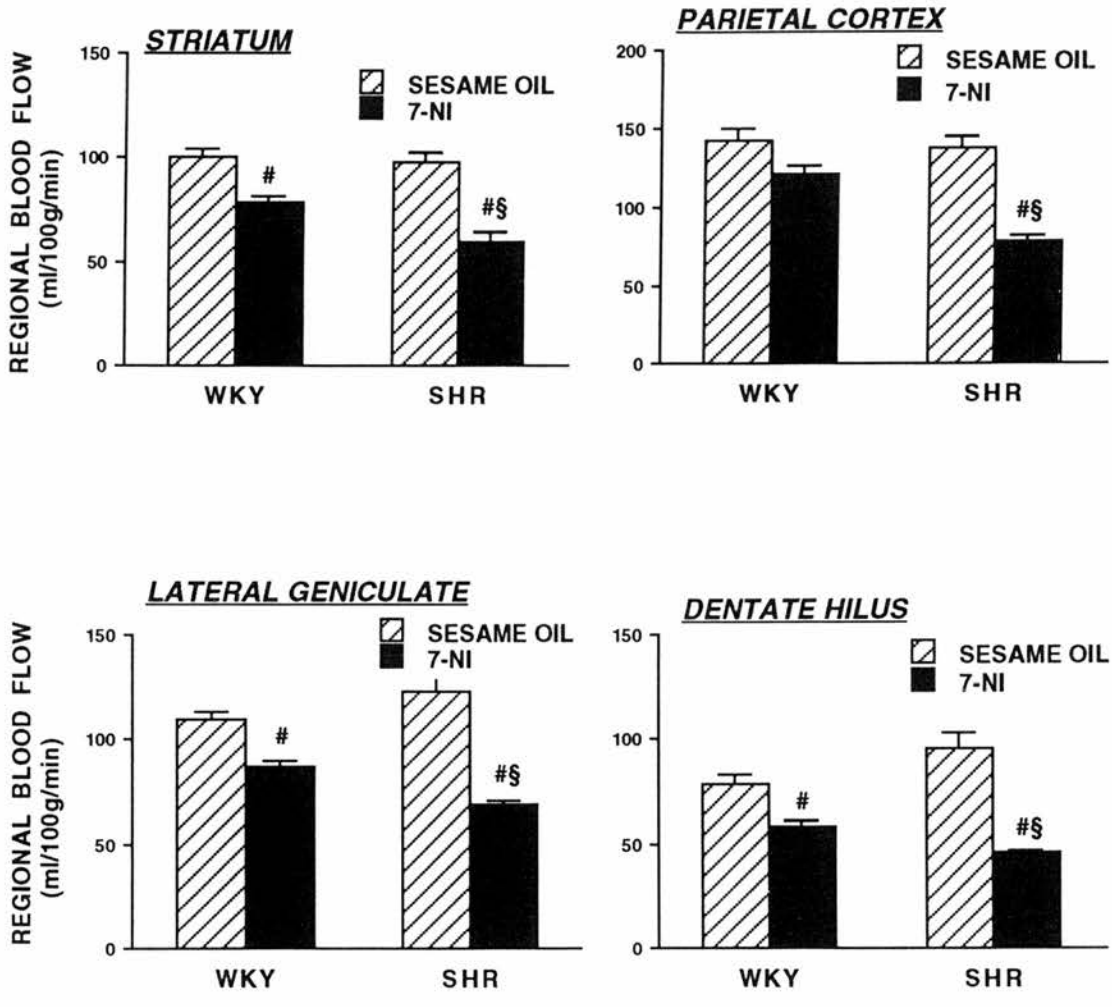
Following the intraperitoneal injection of 7-NI in SHR (Table 13), significant reductions in LCBF (ranging between -36 and -59%; median= -47%) were found in all brain areas examined, when compared to the appropriate sesame oil-treated group (Table 13 & Figure 11). Although the response to 7-NI in the WKY group was qualitatively similar, significant reductions in LCBF were limited to eleven out of the thirteen brain areas examined (Table 13 & Figure 11). However, throughout the brain, the response to 7-NI was less marked in the WKY group (ranging between -15 and -45%; median= -26%; P = 0.0001 Mann-Whitney U-test) when compared to SHR, and in seven brain areas (parietal cortex, striatum, globus pallidus, nucleus accumbens, hypothalamus, lateral geniculate and hilus of the hippocampal dentate gyrus) the decreases in LCBF were significantly greater in the SHR group (Table 13 & Figure 11).

Following treatment with the lower dose of SIN-1 ($0.54mg.kg^{-1}.h^{-1}$) there were significant increases in LCBF in all but five brain areas (parietal cortex, corpus callosum, hypothalamus, hippocampal fields CA1 and CA2) in the WKY group (Table 14 & Figure 12). Significant effects ranged from +22% in striatum, to +60% in cingulate cortex (median= +29%). In contrast, in the SHR group there were significant effects upon LCBF in only two brain regions (cingulate cortex and globus pallidus; Table 14 & Figure 12). In the rest of the brain areas examined, the effects of this lower dose of SIN-1 upon LCBF were less marked than in the WKY (median increase = +12%, P = 0.0378 *vs* WKY, Mann-Whitney U-test).

Following intravenous infusion of the higher dose of SIN-1 ($1.8mg.kg^{-1}.h^{-1}$) there were significant increases in LCBF in all but three brain areas (corpus callosum, globus pallidus and hypothalamus) in the WKY group (Table 14 & Figure 12). Significant effects ranged from +34% in hippocampal field CA2 and striatum, to +80% in cingulate cortex (median increase was 45%, in comparison to the saline-infused WKY). The CBF values in most of the brain areas examined were higher compared to those obtained following treatment with the lower dose of SIN-1 (Table 14 & Figure 12). In contrast, in the SHR group there were no significant effects of

FIGURE 11

Local cerebral blood flow (ml.100g^{-1}.min^{-1}) measured with quantitative [^{14}C]-iodoantipyrine autoradiography, in four functionally diverse brain regions of WKY and SHR rats, 40min after i.p. injection of sesame oil (0.6ml) or 7-NI (25mg.kg^{-1})



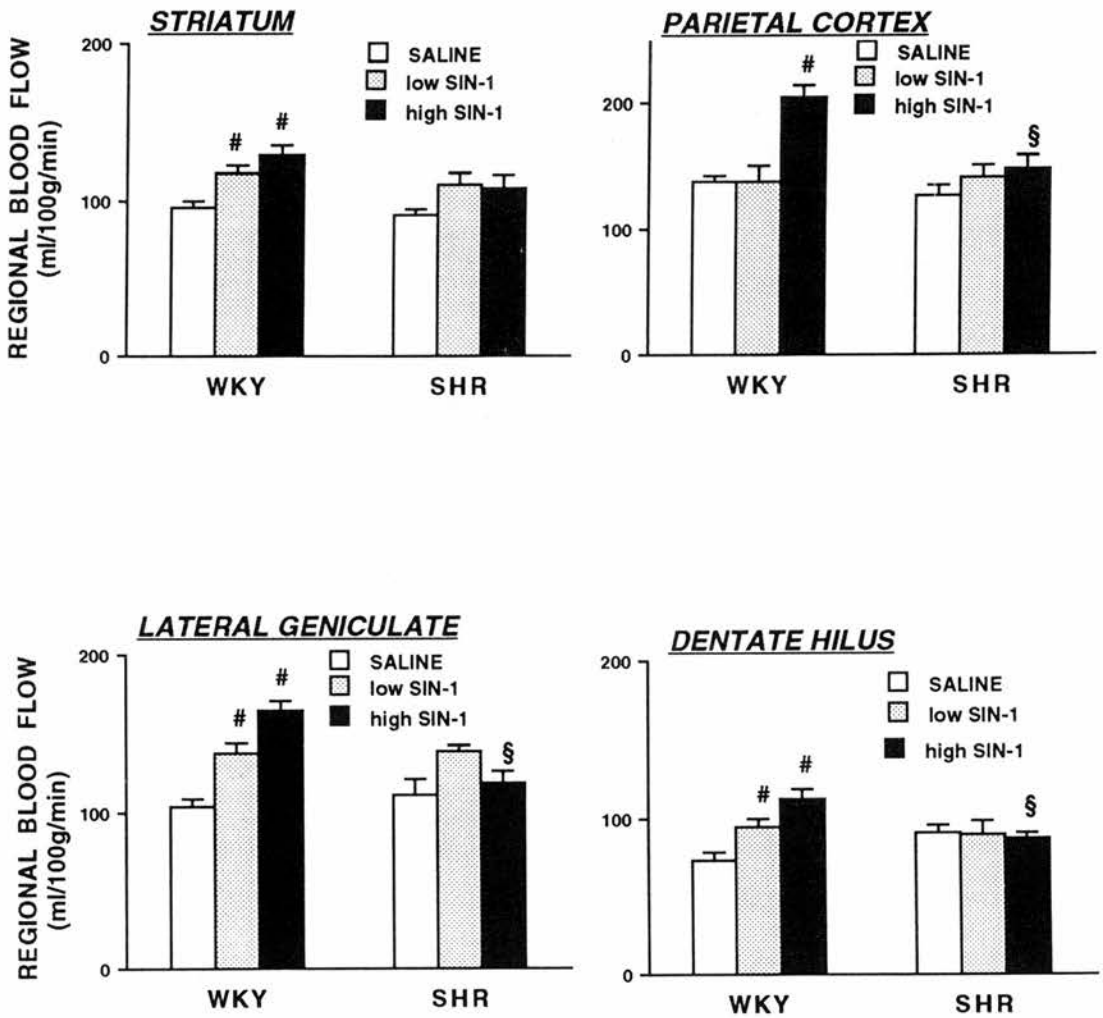
Data are presented as mean ± s.e. mean (n = 5 in WKY treated with sesame oil, n = 4 in all other groups). Note the differences in scale along Y axis between the top right (parietal cortex) and the rest of the graphs.

§: significant difference between WKY and SHR rats receiving the same treatment ($P < 0.05$)

#: significant difference between sesame oil and 7-NI treated rats of the same sub-strain ($P < 0.05$)

FIGURE 12

Local cerebral blood flow (ml.100g^{-1}.min^{-1}) measured with quantitative [^{14}C]-iodoantipyrine autoradiography, in four functionally diverse brain regions of WKY and SHR rats, 20min after the onset of i.v. infusion of saline (40µl.min^{-1}), low SIN-1 dose (0.54mg.kg^{-1}.hr^{-1}) or high SIN-1 dose (1.8mg.kg^{-1}.hr^{-1})



Data are presented as mean ± s.e. mean (n = 5 in WKY rats treated with saline, n = 4 in all other groups) Note the differences in scale along Y axis between the top right (parietal cortex) and the rest of the graphs.

§: significant difference between WKY and SHR rats receiving the same treatment ($P < 0.05$)

#: significant difference between saline and SIN-1 treated rats of the same sub-strain ($P < 0.05$)

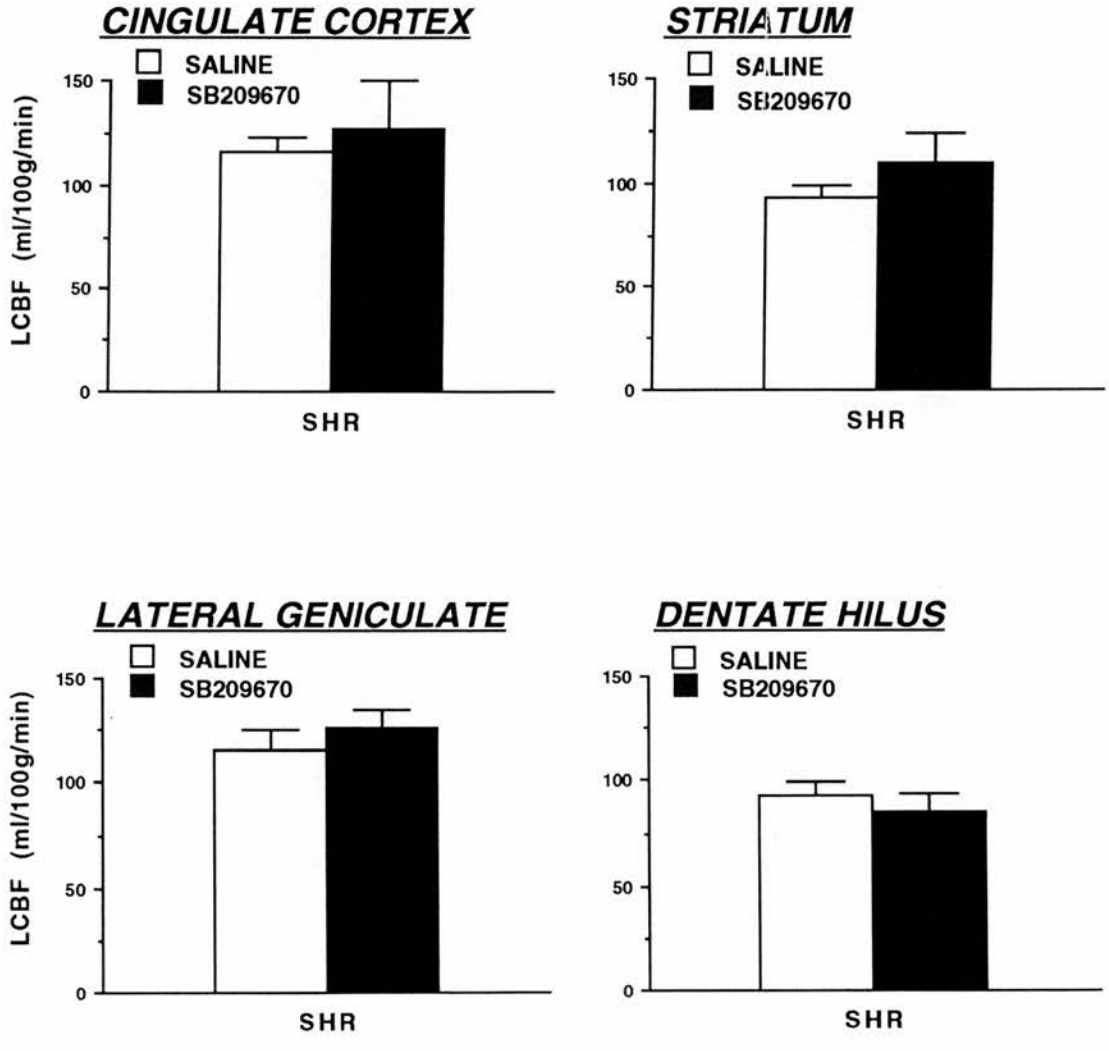
this higher dose of SIN-1 upon LCBF in any region of the brain. In this group, LCBF ranged from -13% in hypothalamus to +28% in corpus callosum (median increase = 6%; P = 0.0001 *vs* WKY, Mann-Whitney U-test; Table 14 & Figure 12). A comparison of LCBF between WKY and SHR groups treated with the higher dose of SIN-1 revealed significant differences in six of the thirteen brain areas examined (parietal cortex, cingulate cortex, lateral geniculate, hippocampal field CA1, molecular layer and hilus of the dentate gyrus) (Table 14 & Figure 12). Although there were no significant differences between the effects upon LCBF of the two doses of SIN-1 in SHR, the values obtained following treatment with the higher dose of SIN-1 were generally lower in most of the areas examined (Table 14 & Figure 12).

SB209670 treatment resulted in non-significant changes in LCBF, compared to the saline-treated hypertensive rats (Table 12 & Figure 13). Modest, non-significant increases in LCBF were observed in seven out of the thirteen areas examined, compared to the saline-treated animals. These ranged between +20% in parietal cortex and CA1 layer of the hippocampus, +15% in striatum and globus pallidus and +9% in lateral geniculate, cingulate cortex and corpus callosum. Finally, modest non-significant reductions in LCBF were observed in 2 areas (hypothalamus -22% and dentate hilus of the hippocampus -9%) associated with SB209670 treatment (Table 12 & Figure 13).

In summary, these results indicate that although local cerebral blood flow is not compromised in the SHR under physiological conditions, the NO-dependent vasodilatory reserve appears to be attenuated, possibly associated with an upregulated neuronal NO system.

FIGURE 13

Local cerebral blood flow (ml.100g^{-1}.min^{-1}) measured with quantitative [^{14}C]-iodoantipyrine autoradiography, in four functionally diverse brain regions of SHR rats, 20min after the i.v. injection of saline (0.5ml) or SB209670 (10mg. kg^{-1})



Data are presented as mean ± s.e. mean (n = 5 in saline-treated and n = 4 in SB209670-treated animals)

No significant differences were found between saline-treated and SB209670-treated rats

3. 5. LCBF 24h after induction of experimental ICH in diabetic and control animals

3. 5. 1. Physiological variables

The physiological variables of the animals used to assess the effect of the anaesthetic during the induction of the haematoma are outlined in Table 15. There were no pathological derrangements of arterial blood gases or MABP measurements in either the diabetic or non-diabetic animals. Interestingly, MABP was elevated post-induction of haematoma, not significantly though, in both groups, compared to pre-induction of the mass lesion (Table 15).

Heart rate was significantly lower in the diabetic animals injected with blood or silicon oil, compared to the non-diabetic rats receiving the same insult (Table 16). Arterial pH, body temperature, plasma bicarbonate and base excess were also significantly lower in the diabetic animals injected with silicon oil compared to the non-diabetic animals with the same mass lesion (Table 16). The temperature of the diabetic animals injected with silicon oil was also significantly lower to that of the diabetic animals injected with blood (Table 16).

3. 5. 2. Volume of haematoma/silicon oil

The volume occupied by the mass of blood or silicon oil (measured exclusively in the striatum) and estimated *post mortem* by planimetry, was not significantly different between groups. In non-diabetic rats, 10 ± 2.5% of total striatal volume was occupied by blood *post mortem* (Figure 15), compared to 8.5 ± 2.5% in diabetic rats (Figure 14). In non-diabetic rats injected with silicon oil, 7.5 ± 3% of total striatal volume was occupied by the injectate, compared to 7.0 ± 1.5% in diabetic animals.

3. 5. 3. Volume of striatal oligaemia

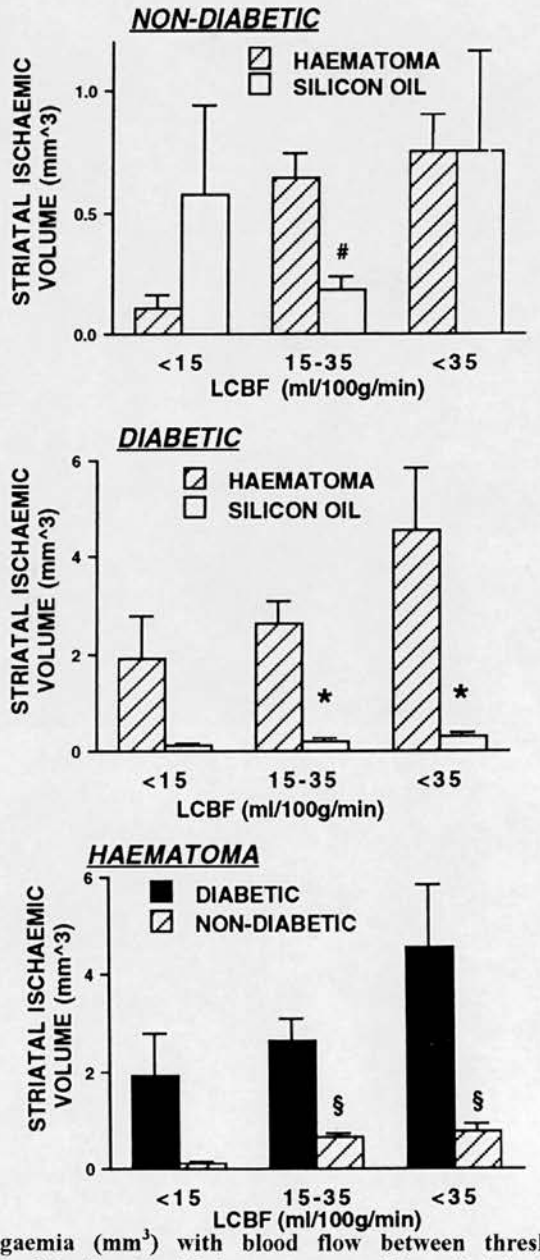
The volume of striatal tissue with LCBF less than $15ml.100g^{-1}.min^{-1}$ was not significantly different between groups, whether injected with blood or silicon oil (Table 17 & Figure 16). Although the mean value for diabetic animals injected with blood (1.91 ± 0.90 mm^3) was considerably higher than in either the same sub-strain

Figure 14: Unstained coronal section from an individual diabetic rat brain showing presence of blood in the right caudate nucleus. Arterial blood (50µl) was injected 24h prior to the measurement of local cerebral blood flow (see Methods). Figures 17 & 19 are from sections obtained from the same animal.

Figure 15: Unstained coronal section from an individual non-diabetic rat brain showing presence of blood in the right caudate nucleus. Arterial blood (50µl) was injected 24h prior to the measurement of local cerebral blood flow. Figures 18 & 20 are from sections obtained from the same animal.

FIGURE 16

Volumes of striatal oligaemia following injection of blood (50µl) or silicon oil (50µl) in non-diabetic and diabetic rats



Volumes of striatal oligaemia (mm^3) with blood flow between thresholds of 0-15, 15-35 and 0-35ml.$100g^{-1}.min^{-1}$, in non-diabetic rats with haematoma or silicon oil injection, top graph; and diabetic rats with haematoma or silicon oil injection, middle graph. The response of non-diabetic and diabetic rats to haematoma alone is summarized in the bottom graph. LCBF was measured with quantitative [^{14}C]-iodoantipyrine autoradiography, 24h after induction of haematoma or silicon oil injection. Data are presented as mean ± s.e.mean (n=6 in groups with haematoma, n=5 in groups with silicon oil injection). Note the differences in scale along Y axis of non-diabetic and diabetic animals (top and middle graphs)

#: significant difference between non-diabetic rats with haematoma or silicon oil injection ($P < 0.05$)

*: significant difference between diabetic rats with haematoma or silicon oil injection ($P < 0.05$)

§: significant difference between non-diabetic and diabetic rats with haematoma ($P < 0.05$)

injected with silicon oil (0.12 ± 0.04 mm^3) or in non-diabetic rats injected with either blood (0.11 ± 0.05 mm^3) or silicon oil (0.57 ± 0.37 mm^3), the co-efficient of variation [*standard deviation / mean*] was close to 100%. It is also likely that part, or in the case of silicon oil all (Figures 21 & 22) of the tissue with this level of ischaemia is occupied by the mass lesion itself. As this is largely similar between groups, differences in the effects of the mass lesion upon the perilesional flow are likely to be obscured. Another consideration is that in the case of silicon oil the mass was typically spherical, whereas in the case of blood ,it traversed along those tissue planes with least resistance resulting in interdigitations of parenchyma (Figures 14 & 15).

The volume of striatal tissue with LCBF less than 35ml.$100g^{-1}$.min^{-1} was significantly larger in diabetic animals injected with blood (4.55 ± 1.30mm^3) than in either diabetic animals injected with silicon oil (0.31 ± 0.08mm^3) or non-diabetic animals injected with blood (0.75 ± 0.15 mm^3; Table 17 & Figure 16). Upon comparison of the iodoantipyrine autoradiograms with the neuropathological slides from the adjacent brain sections, it was evident that these areas of severe oligaemia correlated closely to areas with evidence of tissue damage (pallor and cell loss; Figures 17, 18, 19 & 20). No difference in the volume of oligaemia was observed between non-diabetic animals injected with blood or silicon oil (0.75 ± 0.41mm^3).

For reasons explained above striatal tissue with blood flow thresholds between 15 and 35ml.$100g^{-1}$.min^{-1} was also measured, in an attempt to identify presence of significant hypoperfusion in an area *purely* perilesional, since part of the tissue with LCBF values less than 35ml.$100g^{-1}$.min^{-1} would have been occupied by the haematoma or silicon oil. Striatal tissue with blood flow between 15 and 35ml.$100g^{-1}$.min^{-1} was significantly larger in the diabetic animals injected with blood (2.64 ± 0.44 mm^3) compared to non-diabetic animals injected with blood (0.64 ± 0.10 mm^3) or diabetic animals injected with silicon oil (0.19 ± 0.06 mm^3; Table 17 & Figure 16). Interestingly, the volume of tissue within these thresholds was significantly larger in the non-diabetic animals injected with blood compared to those injected with silicon oil (0.18 ± 0.06 mm^3).

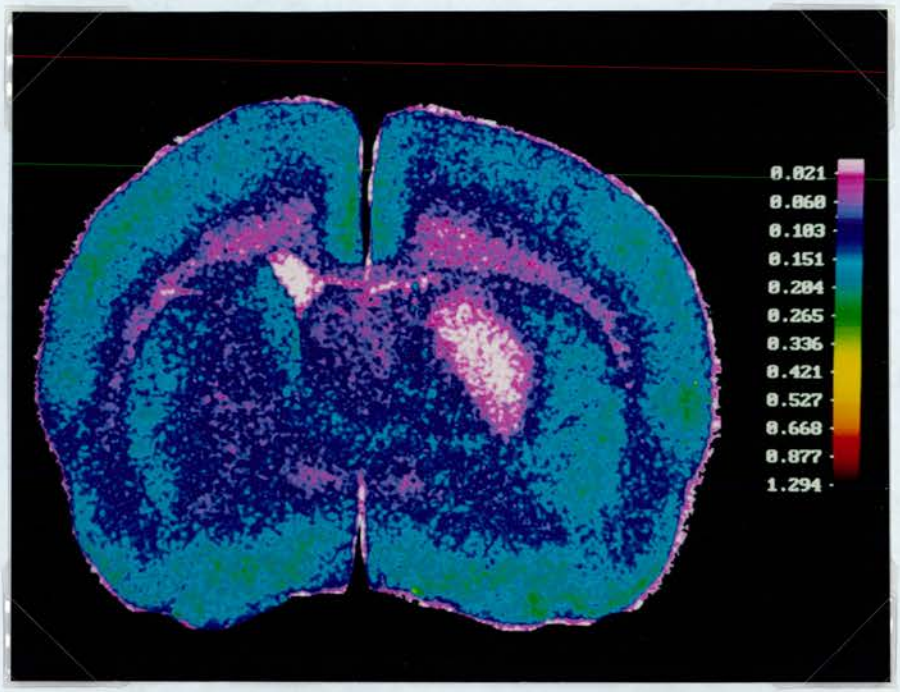


Figure 17: Colour-coded IAP autoradiogram from a diabetic animal injected with blood (50µl) in the right caudate nucleus. Note the area of dense striatal ischaemia (white colour) surrounded by a zone of significant oligaemia (purple and dark blue colour). The caudate lateral to the oligaemic rim has hyperaemia (green colour) relative to the contralateral non-lesioned striatum. The colour scale on the right represents relative optical densities.

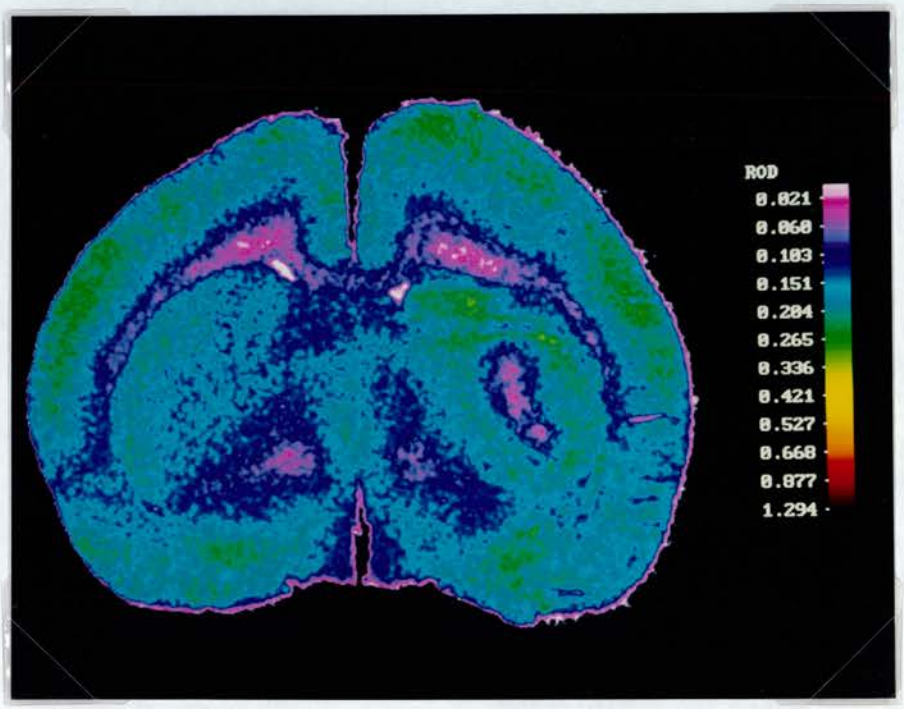


Figure 18: IAP autoradiogram from a non-diabetic animal injected with 50µl blood in right striatum. Note that the area of the caudate with significant hypoperfusion is smaller than that of the animal in Figure 17. There is presence of hyperaemia in the lesioned striatum (green colour) relative to the contralateral side.

Figure 19: Coronal brain section stained with haematoxylin and eosin, obtained from the same animal and adjacent to that shown previously in Figure 17. Note an area of tissue damage in the right striatum clearly demarcated from the surrounding normal parenchyma, corresponding closely to the area of oligaemia evident on the autoradiogram of Figure 17.

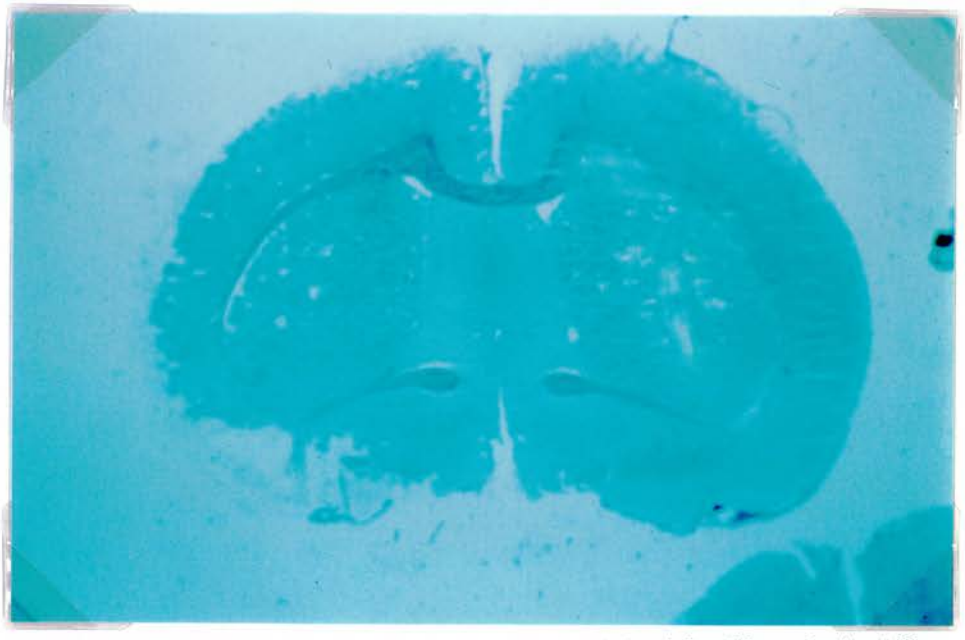


Figure 20: Coronal brain section stained with cresyl violet and luxol-fast blue, obtained from the same animal and adjacent to that shown in Figure 18. Note an area of tissue pallor in the right striatum clearly demarcated from the surrounding normal parenchyma, corresponding closely to the area of hypoperfusion evident on the autoradiogram of Figure 18.

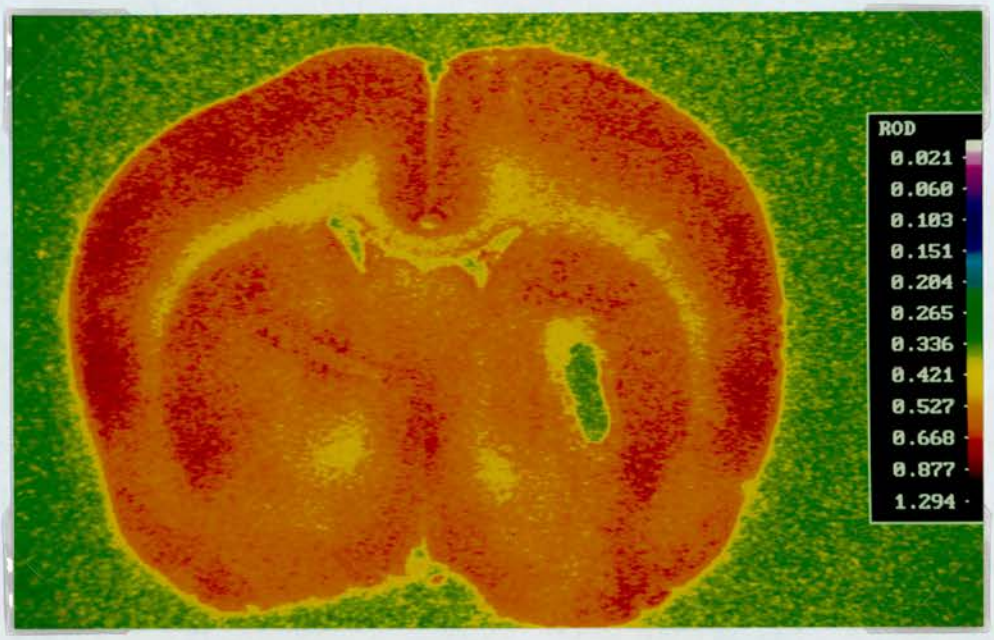


Figure 21: Colour-coded IAP autoradiogram from an animal with right-sided intrastriatal injection of 50µl silicon oil. The area occupied by the silicon oil has no detectable blood flow (green colour in the striatum similar to the colour of the background) and is surrounded by a thin zone of hypoperfusion (yellow colour) located mainly in the superomedial aspect of the striatum. The colour scale on the right side represents relative optical densities.

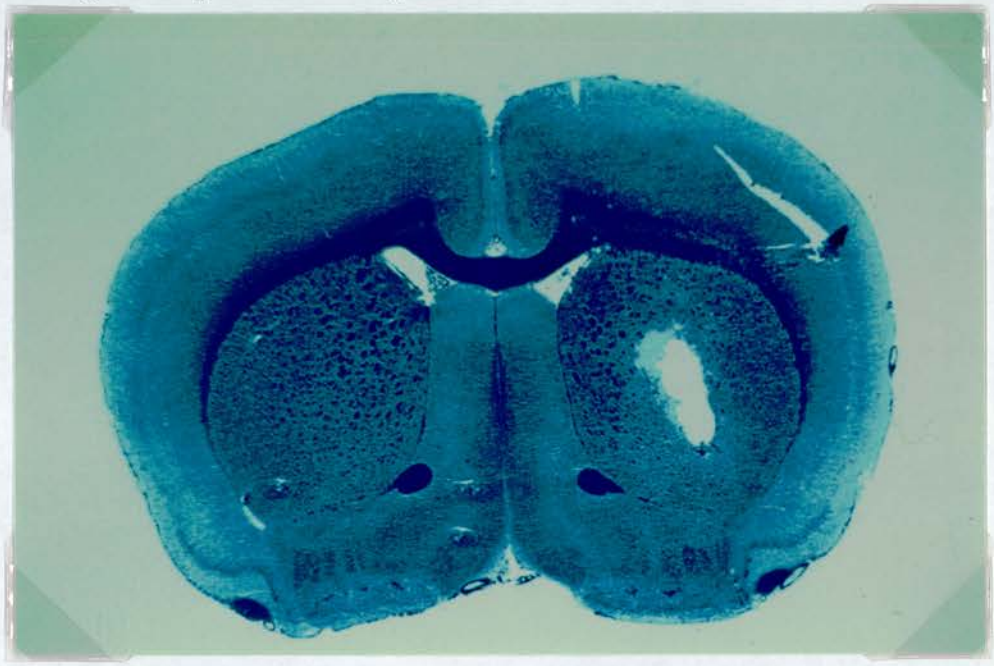


Figure 22: Coronal section obtained from the same animal and adjacent to that shown in Figure 21, stained with cresyl violet and luxol-fast blue. Note the area of the right striatum occupied by the silicon oil, surrounded by an area of pallor, clearly demarcated from the surrounding normal tissue, and corresponding to the area of hypoperfusion visible in Figure 21.

Although it is appreciated that the selection of blood flow threshold at 15ml. $100g^{-1}.min^{-1}$ representing tissue potentially occupied by the haematoma or silicon oil was somehow arbitrary, the results obtained with flow between 15 and 35ml.$100g^{-1}.min^{-1}$ were rather robust and reinforced by the manifestation of similar differences between groups when thresholds between 15 and 25ml.$100g^{-1}.min^{-1}$ or between 25 and 35ml.$100g^{-1}.min^{-1}$ were estimated (Table 17). In one diabetic animal there was clear evidence of striatal oligaemia and tissue damage, despite the fact that the haematoma was contained within ventrolateral white matter, with no evidence of blood within the striatum itself. This animal was excluded from the quantitative analysis of striatal oligaemia described above.

In summary, these results show that the volume of perilesional striatal oligaemia, 24h after experimental ICH, was influenced by the presence of intraparenchymal blood and that the presence of diabetes mellitus exacerbated the extent of this oligaemia.

3. 5. 4. Presence of hyperaemia

Presence of hyperaemia was observed in the lesioned striatum just lateral to the area of oligaemia (Figures 17 & 18), when compared to blood flow in the contralateral striatum (ranging between +27 and +116 % in the diabetics injected with blood, +25 and +113 % in the non-diabetics injected with blood and +23 and +82 % in the silicon oil-injected groups). Hyperaemia was also observed in the overlying cortex in 2 out of the 6 diabetic animals injected with blood (Figures 23 & 24). The extent of this hyperaemia was not influenced by the presence or not of diabetes (with the exception of cortical hyperaemia) or by the presence of blood or silicon oil.

3. 5. 5. LCBF remote from the haematoma or silicon oil

LCBF was measured in structures contralateral to the haematoma or silicon oil. The blood flow in the contralateral striatum of the diabetic animals injected with blood was significantly lower compared to the non-diabetic rats with the same mass lesion (89 ± 7 *vs* 120 ± 3ml.$100g^{-1}.min^{-1}$). Similarly, blood flow in the contralateral globus pallidus

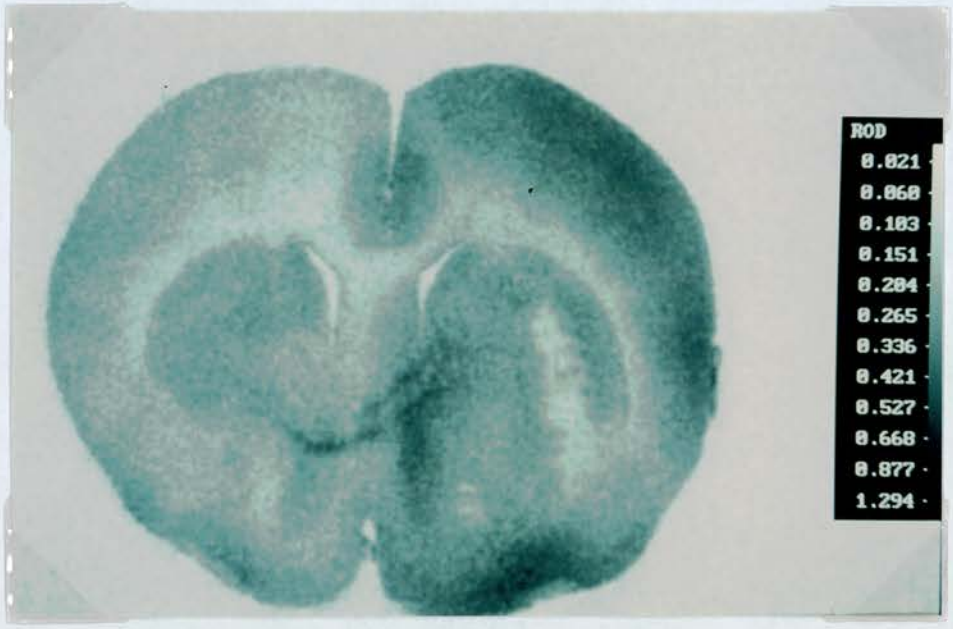


Figure 23: IAP autoradiogram from a single diabetic rat with right-sided intrastriatal injection of blood. Ipsilateral striatal and cortical hyperaemia were observed, surrounding the hypoperfused area in the striatum. This phenomenon was observed in only 2 out of 6 rats in this group. Grey scale in the right side represents relative optical densities, which can be used to calculate blood flow (see Methods).

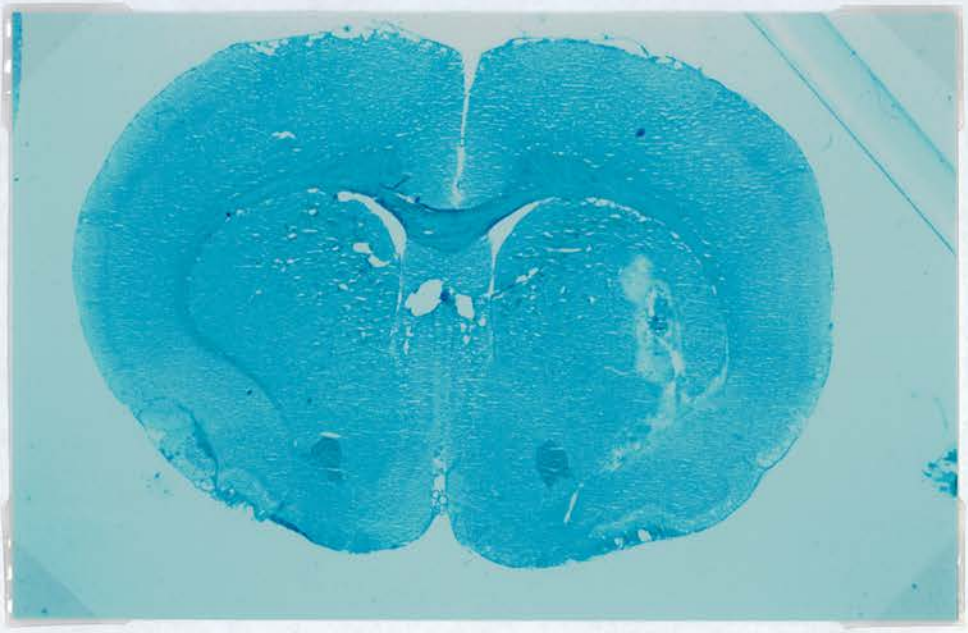


Figure 24: Coronal brain section stained with cresyl violet and luxol-fast blue, obtained from the same animal and adjacent to that shown in Figure 23. No evidence of structural abnormality in the hyperaemic areas seen in Figure 23.

was significantly lower in the diabetic group (64 ± 4 *vs* 77 ± 2ml.100g^{-1}.min^{-1} in the non-diabetic animals injected with blood).

In the silicon oil injected groups, similar patterns of significant differences were obtained. Striatal blood flow was 92 ± 3ml.100g^{-1}.min^{-1} in the diabetics and 123 ± 3ml.100g^{-1}.min^{-1} in the non-diabetics. Blood flow in the globus pallidus was 49 ± 5ml.100g^{-1}.min^{-1} in the diabetics and 78 ± 2ml.100g^{-1}.min^{-1} in the non-diabetics. Obviously, these LCBF findings were expected on the basis of the earlier presented results in the studies of cerebrovascular physiology.

3. 6. Effects of haematoma upon BBB permeability

3. 6. 1. Physiological variables and volume of haematoma

Physiological parameters did not differ between groups, apart from the temperature being significantly reduced in the diabetic group compared to the non-diabetic animals (Table 18).

Haematoma volume lying exclusively in the striatum and measured *post mortem* by planimetry was not significantly different between groups (9 ± 2.5% of total striatal volume in both non-diabetic and diabetic groups) and was similar to that found in the LCBF studies.

3. 6. 2. Unidirectional transfer constant for AIB

Visual inspection of the autoradiograms showed that extravasation of the isotope was limited to the tissue surrounding the haematoma. Quantification of the films revealed that there was no significant difference in the calculated K_i for AIB between diabetic and non-diabetic animals in either the lesioned or contralateral striatum (7.66 ± 1.29ml.g^{-1}.min^{-1}.10^{-3} in the diabetic and 6.00 ± 0.55ml.g^{-1}.min^{-1}.10^{-3} in the non-diabetic animals in the lesioned side and 2.17 ± 0.35ml.g^{-1}.min^{-1}.10^{-3} in the diabetic and 2.05 ± 0.29ml.g^{-1}.min^{-1}.10^{-3} in the non-diabetic animals in the contralateral side; Figures 25 & 26). The area of tissue pallor in the neuropathological slides taken from adjacent sections was generally larger than the area of isotope accumulation (Figures 27 & 28).

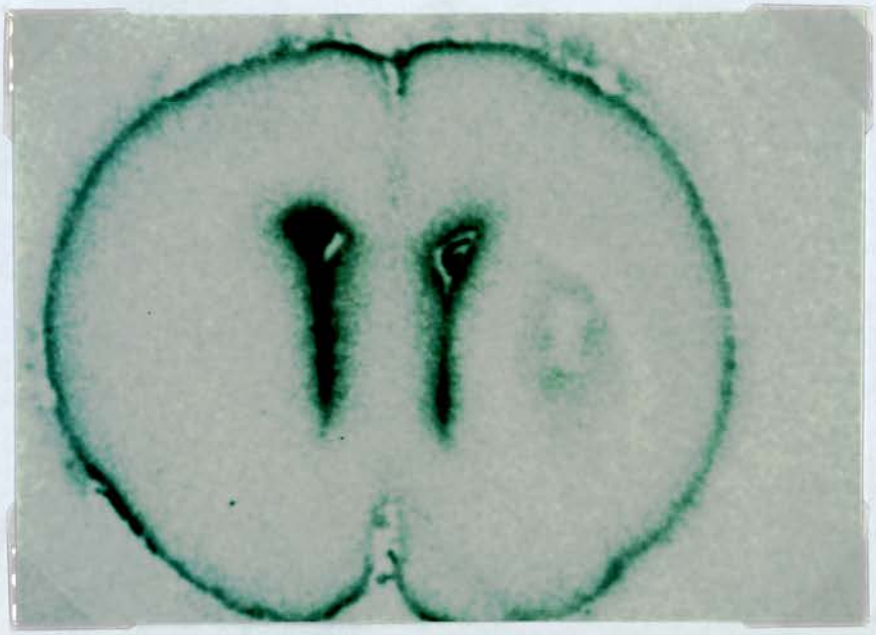


Figure 25: AIB autoradiogram from a non-diabetic animal with intrastriatal injection of 50μl blood. Isotope extravasation was confined to the area surrounding the blood clot. There is also isotope extravasation in the pial surface and the choroidal ventricular surface.

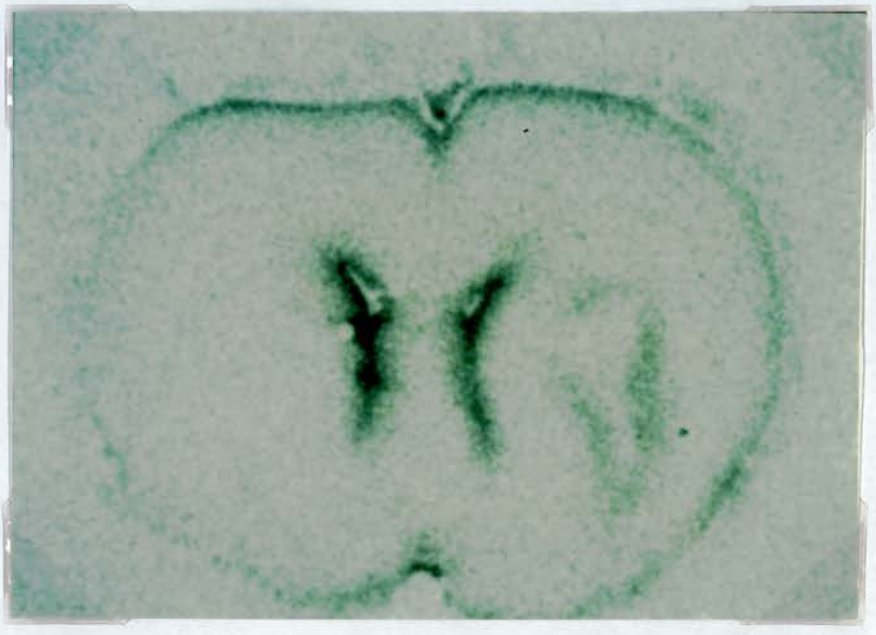


Figure 26: AIB autoradiogram from a diabetic rat injected with 50μl blood. Note the similar pattern and intensity of isotope extravasation to that shown in Figure 25.

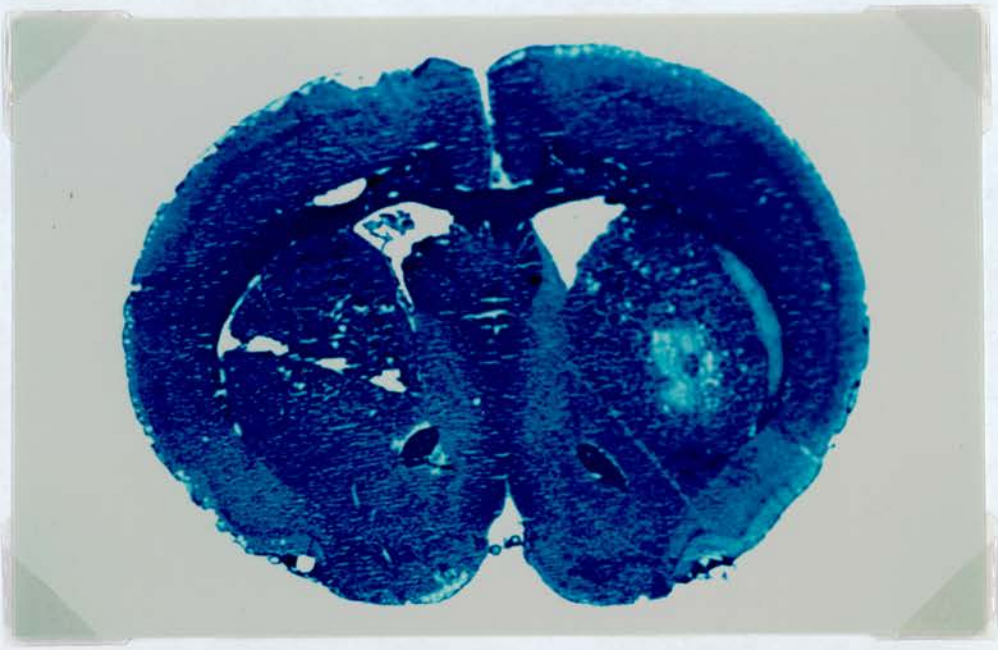


Figure 27: Coronal brain section stained with cresyl violet and luxol-fast blue, from the same animal and adjacent to that shown in Figure 25.

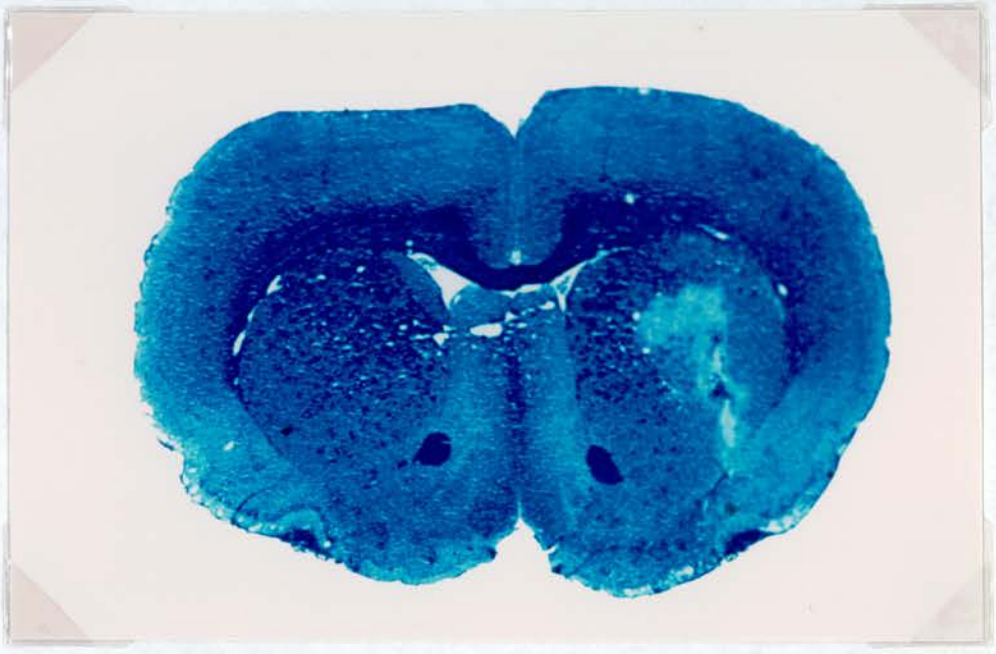


Figure 28: Coronal brain section stained with cresyl violet and luxol-fast blue, from the same animal and adjacent to that shown in Figure 26.

3. 7. Haemodynamic effects of the endothelin antagonist SB209670

3. 7. 1. Physiological variables and volume of haematoma

In SB209670-treated non-diabetic and diabetic groups, plasma bicarbonate and base excess were significantly higher compared to the saline-treated animals (Table 19). Arterial pH was also significantly higher in the non-diabetic animals treated with SB209670 compared to the saline-treated animals of the same sub-strain.

MABP, measured at the time of the iodoantipyrine experiment, was considerably lower in the diabetic animals treated with SB209670 (98 ± 6mmHg) compared to the MABP of the diabetic animals with saline treatment (116 ± 9mmHg) (Table 19). This 16% difference did not reach statistical significance though. There were no differences in MABP between non-diabetic rats with saline (117 ± 2mmHg) or SB209670 treatment (115 ± 4mmHg).

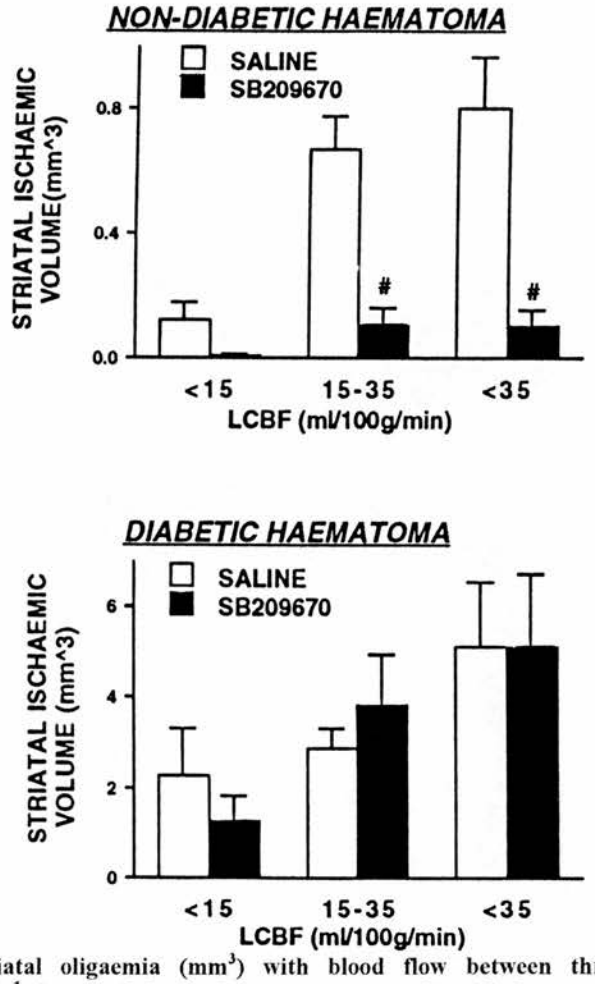
The volume occupied by the haematoma in the striatum, measured *post mortem* by planimetry, was not significantly different between groups. In the non-diabetic animals, it occupied 11 ± 2.5% of the striatum in the saline-treated and 10 ± 2.5% in the SB209670-treated groups, whereas in the diabetic animals it occupied 9 ± 2.5% of the striatum in the saline-treated and 10 ± 2.5% in the SB209670-treated animals.

3. 7. 2. Volume of striatal oligaemia

The volume of striatal tissue with LCBF less than $15ml.100g^{-1}.min^{-1}$ was not significantly different between groups, treated with saline or SB209670 (Table 20 & Figure 29). Although the mean value for non-diabetic animals treated with SB209670 ($0.0015 \pm 0.0001mm^3$) was considerably lower than that of saline-treated animals of the same sub-strain ($0.13 \pm 0.06mm^3$), the co-efficient of variation was close to 100% in the saline-treated rats. In the diabetic rats, there was no difference between saline ($2.27 \pm 1.03mm^3$) or SB209670-treated ($1.30 \pm 0.53mm^3$) groups (Figure 29 & Table 20).

FIGURE 29

Volumes of striatal oligaemia following experimental ICH in non-diabetic and diabetic rats treated with saline or SB209670



Volumes of striatal oligaemia (mm^3) with blood flow between thresholds of 0-15, 15-35 and 0-35ml.100g^{-1}.min^{-1}, in non-diabetic (DR) rats (top graph) with haematoma (50µl) treated with either saline (0.5ml i.p. every 6h, starting 30min prior to induction of haematoma and continuing for 24h) or SB209670 (10mg.kg^{-1} i.p. every 6h, starting 30min prior to induction of haematoma and continuing for 24h), and diabetic BB rats (bottom graph) with haematoma (50µl), treated with either saline or SB209670 employing the same protocol used in the non-diabetic animals. Blood flow was measured with quantitative [^{14}C]-iodoantipyrine autoradiography, 24h after the induction of haematoma. Note the differences in scale along Y axis of non-diabetic and diabetic animals (top and bottom graphs)

Data are presented as mean ± s.e.mean (n = 6 in SB209670-treated non-diabetic group, n = 5 in all other groups)

#: significant difference between saline and SB209670-treated non-diabetic rats ($P < 0.05$)

In diabetic animals, SB209670 treatment had no significant effect upon the volume of striatal tissue with LCBF less than 35ml.100g^{-1}.min^{-1} (5.14 ± 1.58mm^3 *vs* 5.11 ± 1.44mm^3 following saline treatment). Interestingly, the non-diabetic animals treated with SB209670 had a significantly smaller volume (0.10 ± 0.05mm^3) compared to the non-diabetic with saline treatment (0.80 ± 0.17mm^3; Figure 29 & Table 20).

Striatal tissue with flow thresholds between 15 and 35ml.100g^{-1}.min^{-1} was significantly larger in the non-diabetic animals with saline (0.67 ± 0.11mm^3) compared to non-diabetic animals with SB209670 (0.10 ± 0.06mm^3). In the diabetic animals, SB209670 had no effect (2.85 ± 0.47 mm^3 in the group with saline and 3.85 ± 1.10mm^3 in the group with SB209670; Table 20 & Figure 29).

Similar patterns of differences between groups were observed in the blood flow thresholds between 25 and 35ml.100g^{-1}.min^{-1}, and between 15 and 25ml.100g^{-1}.min^{-1} (Table 20).

These results show that the treatment with the non-peptide ET_A/ET_B receptor antagonist SB209670 resulted in some haemodynamic improvement in the perilesional area of non-diseased rats 24h after experimental ICH, whereas it had no effect in the diabetic group.

3. 7. 3. Effects of SB209670 upon LCBF remote from the haematoma

Blood flow in the contralateral to the haematoma striatum was significantly increased (+41%) in the diabetic rats treated with SB209670 (121 ± 6ml.100g^{-1}.min^{-1}) compared to the diabetic animals with saline treatment (86 ± 7ml.100g^{-1}.min^{-1}). In the non-diabetic groups there was a small (-5%) non-significant decrease in flow (123 ± 3ml.100g^{-1}.min^{-1} in the SB209670-treated group and 129 ± 5ml.100g^{-1}.min^{-1} in the saline-treated group).

In the contralateral globus pallidus, flow in the SB209670-treated diabetic rats was non-significantly increased (+13%) compared to the saline-treated animals (70 ±

5ml.100g^{-1}.min^{-1} and 62 ± 4ml.100g^{-1}.min^{-1} respectively). In the non-diabetic animals, SB209670 treatment resulted in a +6% (non-significant) increase in flow (83 ± 4ml.100g^{-1}.min^{-1} *vs* 78 ± 2ml.100g^{-1}.min^{-1}).

3. 8. LCBF 24h after induction of experimental ICH in spontaneously hypertensive and control animals

3. 8. 1. Physiological variables

The physiological variables of the animals used to assess the effect of the anaesthetic during the induction of the haematoma are outlined in Table 21. There were no pathological de-arrangements of arterial blood gases or MABP measurements in either the WKY or SHR animals. Again, as found in the diabetic studies, MABP post-induction of haematoma was elevated, not significantly though, in both groups, compared to pre-induction of the mass lesion (Table 21).

As expected, MABP measured at the time of the iodoantipyrine experiment was significantly higher in the hypertensive animals injected with blood or silicon oil, compared to the normotensive animals injected with the same mass lesion (Table 22). Heart rate in the SHR injected with blood was significantly higher compared to the WKY injected with blood and also compared to the SHR injected with silicon oil (Table 22).

3. 8. 2. Volume of haematoma/silicon oil

The volume occupied by the mass of blood or silicon oil, measured exclusively in the striatum and estimated *post mortem* by planimetry, was not significantly different between SHR and WKY groups. In WKY, 11 ± 3% of total striatal volume was occupied by blood *post mortem*, compared to 10 ± 3% in SHR. In WKY rats injected with silicon oil, 9 ± 3% of total striatal volume was occupied by the injectate, compared to 7 ± 3% in SHR.

3. 8. 3. Volume of striatal oligaemia

The volume of striatal tissue with LCBF less than $15ml.100g^{-1}.min^{-1}$ was not significantly different between groups. Its mean value was 1.25 ± $0.63mm^3$ for the hypertensive animals injected with blood, 1.48 ± $1.32mm^3$ for the normotensive animals with blood, 0.51 ± $0.24mm^3$ for the hypertensive animals injected with silicon

oil and 1.57 ± 1.14mm^3 for the normotensive animals injected with silicon oil (Table 23 & Figure 30).

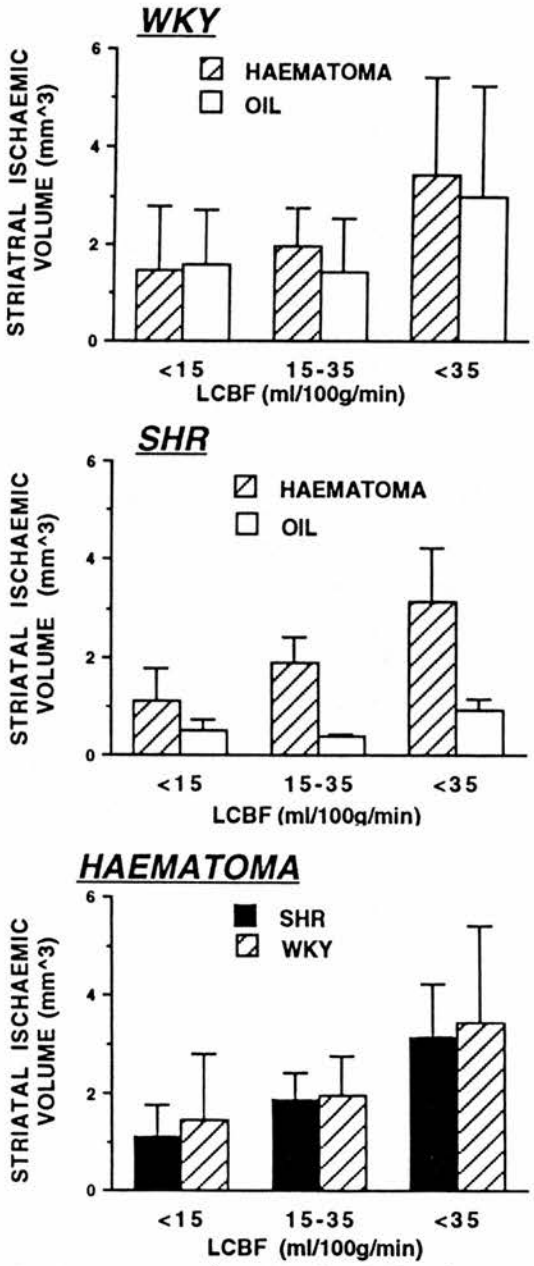
The volume of striatal tissue with LCBF less than 35ml.100g^{-1}.min^{-1} was not significantly different between groups. It was 3.14 ± 1.09mm^3 in the hypertensive animals injected with blood, 0.90 ± 0.24mm^3 in the hypertensive animals injected with silicon oil, 3.45 ± 1.97mm^3 in the normotensive animals injected with blood and 2.99 ± 2.24mm^3 in the normotensive animals injected with silicon oil (Table 23 & Figure 30). Upon comparison of the iodoantipyrine autoradiograms with the neuropathological slides from adjacent brain sections, it was evident that the areas within the above stated blood flow threshold correlated closely with areas showing evidence of tissue damage (pallor and cell loss).

Striatal tissue with flow thresholds between 15 and 35ml.100g^{-1}.min^{-1} was not significantly different between hypertensive and normotensive animals injected with blood (1.89 ± 0.50mm^3 *vs* 1.96 ± 0.78mm^3). The normotensive animals injected with silicon oil had a similar volume of significant oligaemia (1.42 ± 1.11mm^3), whereas the hypertensive animals injected with the same agent had a volume almost significantly smaller (0.40 ± 0.02mm^3) compared to those injected with blood (Table 23 & Figure 30). There was invariably a degree of bleeding in the striatal parenchyma upon injection of the silicon oil in the SHR (Figure 31), so it is impossible to isolate the cerebrovascular effects of the mass lesion from those of the blood itself.

Similar patterns of differences were observed in the blood flow threshold between 25 and 35ml.100g^{-1}.min^{-1} the volumes being 0.82 ± 0.18mm^3 for the hypertensive animals injected with blood, 0.82 ± 0.28mm^3 for the normotensive animals injected with blood, 0.19 ± 0.004mm^3 for the hypertensives injected with oil and 0.65 ± 0.48mm^3 for the normotensive animals injected with oil (Table 23). Again, at these flow thresholds the hypertensive animals injected with silicon oil had an almost significantly lower volume of oligaemia compared to those injected with blood, but for the same

FIGURE 30

Volumes of striatal oligaemia following injection of blood or silicon oil in WKY and SHR rats



Volumes of striatal oligaemia (mm^3) with blood flow between thresholds of 0-15, 15-35 and 0-35ml.100g^{-1}.min^{-1}, in WKY rats with haematoma or silicon oil injection (50µl), top graph; and SHR rats with haematoma or silicon oil injection (50µl) middle graph.The response of SHR and WKY to haematoma alone is summarized in bottom graph. LCBF was measured with quantitative [^{14}C]-iodoantipyrine autoradiography, 24h after the induction of haematoma or silicon oil injection. Data are presented as mean ± s.e.mean (n = 6 in groups with haematoma, n = 4 in groups with silicon oil injection) No significant differences were found between groups

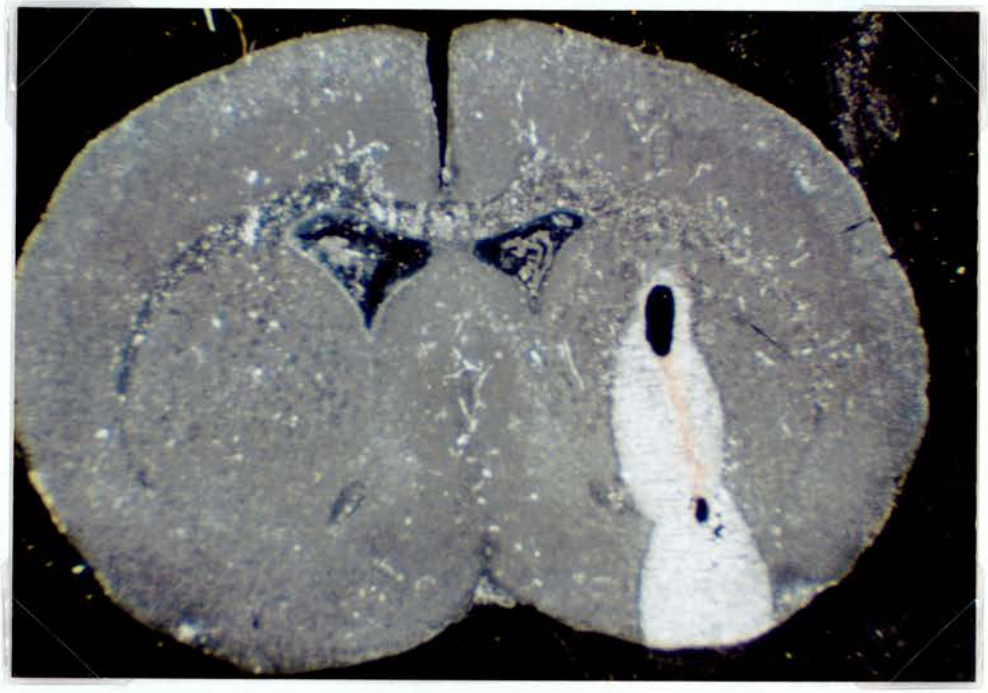


Figure 31: Unstained coronal brain section from a hypertensive rat injected with 50µl silicon oil in the right striatum. Note the presence of endogenous bleeding associated with the needle injection. The appearance of the tissue surrounding the silicon oil cavity and the clot is different from the rest of the brain, because during brain sectioning (see Methods), the silicon oil was smeared along the direction of cutting.

reasons outlined above this could not be verified. Similar patterns were observed in flow threshold between 15 and 25ml.100g^{-1}.min^{-1} (Table 23).

These results indicate that the volume of perilesional striatal oligaemia, 24h after experimental ICH, was probably influenced by the presence of intraparenchymal blood in the hypertensive animals but not in the normotensive rats. The presence of chronic hypertension did not exacerbate the extent of this oligaemia.

3. 8. 4. LCBF remote from the haematoma/silicon oil

LCBF was measured in structures contralateral to the mass lesion (haematoma or silicon oil). The blood flow in the striatum of the hypertensive animals injected with blood was 100 ± 6ml.100g^{-1}.min^{-1}, and in those injected with silicon oil was 112 ± 13ml.100g^{-1}.min^{-1}. The normotensive animals injected with blood had a flow of 120 ± 4ml.100g^{-1}.min^{-1}, almost significantly higher compared to the hypertensive rats with the same mass lesion and the normotensive animals with silicon oil injection had a flow of 139 ± 4ml.100g^{-1}.min^{-1}, which was significantly increased compared to the normotensive animals injected with blood. Upon comparison of these LCBF findings with those observed in the studies of cerebrovascular physiology (Tables 12, 13 & 14), WKY rats subjected to ICH or silicon oil injection had higher LCBF values compared to the non-lesioned animals of the same sub-strain. This was not the case in the SHR rats, where LCBF values following ICH or silicon oil injection were similar to those obtained in the studies of cerebrovascular physiology.

Blood flow in the globus pallidus of the hypertensive group injected with blood was 66 ± 3ml.100g^{-1}.min^{-1} and in the normotensive group was 74 ± 3ml.100g^{-1}.min^{-1}. In the animals injected with silicon oil, blood flow was 66 ± 5ml.100g^{-1}.min^{-1}. in the hypertensives and 90 ± 2ml.100g^{-1}.min^{-1} in the normotensive animals which was significantly increased compared to both the normotensives injected with blood and the hypertensives injected with silicon oil. Again, WKY animals subjected to an intracranial insult had higher LCBF values compared to non-lesioned animals of the same sub-strain, whereas this was not the case with SHR.

3. 9. Effects of haematoma upon BBB permeability

3. 9. 1. Physiological variables and volume of haematoma

Arterial pH, plasma bicarbonate and base excess were significantly lower and MABP significantly higher in the SHR compared to the WKY rats (Table 24). No other significant differences in physiological parameters were observed between groups.

Haematoma volume lying exclusively in the striatum, and measured *post mortem* by planimetry, was not significantly different between groups, (10.5 ± 2.5% of the total striatal volume in both WKY and SHR groups) and was similar to that found in the LCBF studies.

3. 9. 2. Unidirectional transfer constant for AIB

There was intraparenchymal extravasation of the isotope, limited to the tissue surrounding the haematoma. There were no significant differences in the intensity of K_i for AIB between WKY and SHR animals in the lesioned or contralateral striatum (6.95 ± 0.64ml.g^{-1}.min^{-1}.10^{-3} in the WKY and 6.66 ± 0.82ml.g^{-1}.min^{-1}.10^{-3} in the SHR animals in the lesioned side and 2.38 ± 0.36ml.g^{-1}.min^{-1}.10^{-3} in the WKY and 1.79 ± 0.34ml.g^{-1}.min^{-1}.10^{-3} in the SHR animals in the contralateral side).

3. 10. Haemodynamic effects of 7-NI

3. 10. 1. Physiological variables and volume of haematoma

MABP was significantly higher and heart rate significantly lower in the hypertensive rats treated with 7-NI compared to the hypertensive animals with sesame oil (Table 25).

The volume occupied by the haematoma in the striatum, measured *post mortem* by planimetry, was not significantly different between groups. It occupied 10 ± 2% of the striatum in the SHR treated with sesame oil, 11 ± 2% in the WKY treated with sesame oil, 8 ± 1.5% in the SHR treated with 7-NI and 10 ± 3% in the WKY treated with 7-NI.

3. 10. 2. Volume of striatal oligaemia

The volume of striatal tissue with LCBF less than 15ml.100g^{-1}.min^{-1} was not significantly different between groups. Its mean value was 0.09 ± 0.05mm^3 for the hypertensive animals injected with 7-NI, 1.23 ± 0.75mm^3 for the hypertensive animals with sesame oil, 0.095 ± 0.058mm^3 for the normotensive animals with 7-NI and 1.78 ± 1.57mm^3 for the normotensive animals with sesame oil (Table 26 & Figure 32). Presumably, the volume was smaller in the hypertensive animals with 7-NI compared with the sesame oil-treated group partially because the mean volume of the haematoma was smaller in the latter group.

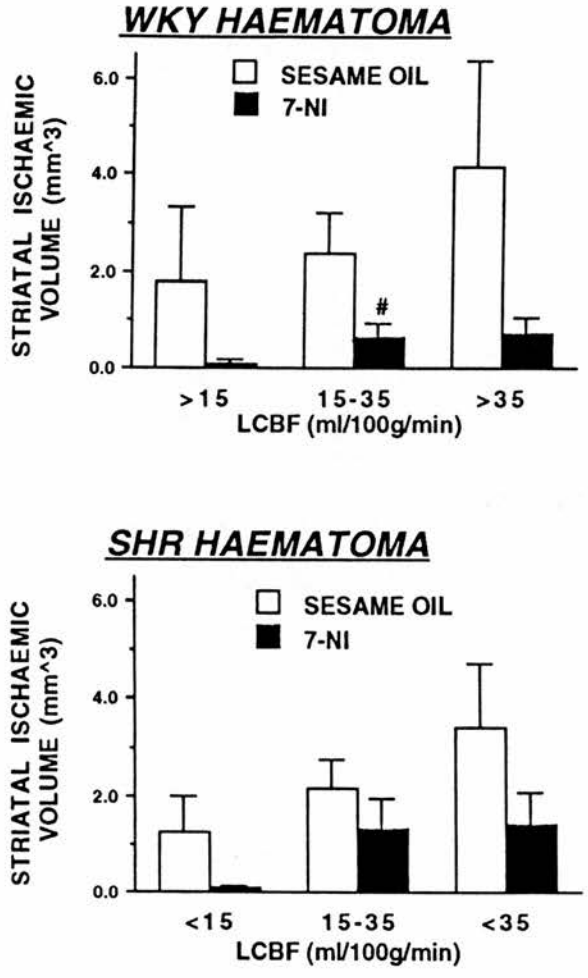
The volume of striatal tissue with LCBF less than 35ml.100g^{-1}.min^{-1} was smaller in the hypertensive animals with 7-NI (1.41 ± 0.66mm^3) compared to the hypertensive animals with sesame oil (3.4 ± 1.3mm^3), although this difference was not significant. Similarly, the normotensive animals treated with 7-NI had a non-significantly smaller volume (0.71 ± 0.33mm^3) compared to the normotensives with sesame oil treatment (4.13 ± 2.26mm^3; Figure 32 & Table 26).

Striatal tissue with blood flow between 15 and 35ml.100g^{-1}.min^{-1} was significantly larger in the normotensive animals with sesame oil (2.35 ± 0.83mm^3) compared to normotensive animals with 7-NI (0.61 ± 0.28mm^3). In the hypertensive animals, 7-NI had no significant effect (1.32 ± 0.62mm^3) compared to the group with sesame oil treatment (2.17 ± 0.55mm^3), although the volume of oligaemia was considerably smaller in the 7-NI - treated group (Table 26 & Figure 32).

Similar patterns of differences between groups were observed in the blood flow thresholds between 15 and 25ml.100g^{-1}.min^{-1} or between 25 and 35ml.100g^{-1}.min^{-1} (Table 26). However, the differences between WKY rats treated with 7-NI or sesame oil did not reach statistical significance, probably as a result of the large variability within treatment groups (Table 26).

FIGURE 32

Volumes of striatal oligaemia following experimental ICH in WKY and SHR rats treated with sesame oil or 7-NI



Volumes of striatal oligaemia (mm^3) with blood flow between thresholds of 0-15, 15-35 and 0-35ml.100g^{-1}.min^{-1}, in WKY rats (top graph) with haematoma (50µl) treated with either sesame oil (0.5ml i.p. 30min after induction of haematoma) or 7-NI (25mg.kg^{-1} i.p. 30min after induction of haematoma), and SHR rats (bottom graph) with haematoma (50µl), treated with either sesame oil or 7-NI employing the same protocol used in the WKY animals. Blood flow was measured with quantitative [^{14}C]-iodoantipyrine autoradiography, 24h after the induction of haematoma.

Data are presented as mean ± s.e.mean (n = 7 in 7-NI treated WKY group, n = 5 in all sesame oil-treated groups, n = 4 in SHR group with 7-NI treatment)

#: significant difference between sesame oil and 7-NI treated WKY rats ($P < 0.05$)

These results indicate that the treatment with the selective neuronal NOS inhibitor 7-NI resulted in haemodynamic improvement in the perilesional area of normotensive rats 24h after experimental ICH, whereas the same treatment had no significant effect in the hypertensive animals.

3. 10. 3. LCBF remote from the haematoma

LCBF in striatum opposite the ICH was 100 ± 4ml.100g^{-1}.min^{-1} in SHR with 7-NI, compared to 96 ± 6ml.100g^{-1}.min^{-1} in hypertensive animals with sesame oil treatment. In the normotensive animals treated with the 7-NI there was a small (+5%) non-significant increase in blood flow (127 ± 5ml.100g^{-1}.min^{-1} in the 7-NI - treated group and 121 ± 5ml.100g^{-1}.min^{-1} in the sesame oil-treated group). The 7-NI and sesame oil-treated WKY rats had significantly higher striatal blood flow compared to the 7-NI and sesame oil-treated SHR. The failure to observe reductions in LCBF associated with 7-NI treatment was not surprising, in contrast to the findings of the studies of cerebrovascular physiology (Table 13), because the time interval of 24h between 7-NI administration and LCBF measurement would have obscured its direct cerebrovascular effects.

In the contralateral globus pallidus, blood flow in the hypertensive rats with 7-NI treatment was non-significantly decreased (-12%) compared to sesame oil-treated SHR animals (58 ± 2ml.100g^{-1}.min^{-1} and 66 ± 4ml.100g^{-1}.min^{-1} respectively). In the normotensive animals, 7-NI treatment had no effect in flow (77 ± 4ml.100g^{-1}.min^{-1} *vs* 75 ± 4ml.100g^{-1}.min^{-1} in the sesame oil-treated animals). Finally, the WKY animals treated with 7-NI had significantly higher blood flow compared to the SHR rats with the same treatment.

Summary of Results

Following pharmacological manipulation in diabetic and non-diabetic BB rats, local cerebral blood flow is compromised in a regionally heterogeneous manner in the diabetic BB rats under physiological conditions, associated with reduced endothelial NO bioactivity. This perturbation in the endothelial NO system does not result from reduced substrate (L-arginine) availability or structural microvascular abnormalities, and is possibly unrelated to development of subendothelial AGEs in the cerebrovasculature. Increased whole blood viscosity was found in the diabetic animals, but a fundamental role for chronic glycaemic control in association with the observed phenomena was not established.

Following pharmacological manipulation in SHR and WKY rats, local cerebral blood flow is not compromised in the SHR under physiological conditions, but the NO-dependent vasodilatory reserve appears to be attenuated, possibly associated with an upregulated neuronal NO system.

Experimental ICH studies in the diabetic animals show that the volume of perilesional striatal oligaemia, 24h after induction of ICH, is influenced by the presence of intraparenchymal blood. The presence of diabetes mellitus exacerbated the extent of this oligaemia, but not the extent of BBB disruption. Treatment with the non-peptide ET_A/ET_B receptor antagonist SB209670 resulted in some haemodynamic improvement in the perilesional area of non-diseased rats, whereas it had no effect in the diabetic group.

Experimental ICH studies in the hypertensive animals indicate that the volume of perilesional striatal oligaemia, 24h after induction of ICH, is probably influenced by the presence of intraparenchymal blood in the hypertensive animals, but not in normotensive controls. Chronic hypertension did not exacerbate the extent of this oligaemia or the extent of BBB disruption. Treatment with the selective neuronal NOS inhibitor 7-NI resulted in haemodynamic improvement in the perilesional area of

normotensive rats, whereas the same treatment had no significant effect in the hypertensive animals.

In conclusion, there is abnormal cerebrovascular homeostasis associated with IDDM and chronic hypertension resulting in increased volume of delayed perilesional oligaemia following experimental haemorrhagic stroke. This cerebrovascular pathology results in resistance to neuroprotection with endothelin antagonists or neuronal NOS inhibitors which are effective in otherwise healthy animals. These results are of particular importance since stroke commonly occurs in patients with assorted "risk" factors that can cause underlying alterations in cerebrovascular regulation. Extrapolation of the neuroprotective strategies that have evolved from tests in young healthy animals may therefore be inappropriate.

CHAPTER 4

DISCUSSION

4. 1. Physiological studies in the diabetic animals

4. 1. 1. Basal LCBF

This is the first study of local cerebral blood flow in spontaneously diabetic insulin-dependent BB rats. The global tendency towards reduced cerebral blood flow which was observed in these animals parallels to some extent that found originally in human diabetic patients (Kety *et al.*, 1948). With the greater spatial resolution afforded by the use of quantitative autoradiography, it has been feasible to identify a degree of regional heterogeneity in the effects of diabetes upon cerebral blood flow. Whether this apparent differential susceptibility to the disease processes in different parts of the cerebrovascular bed reflects regional variations in vascular pathology, is a matter of speculation. There is certainly evidence from published reports of a regional heterogeneity in the mechanisms responsible for cerebrovascular dysfunction associated with IDDM (Mayhan *et al.*, 1991; Mayhan, 1992a). Regional differences in LCBF have also been described recently in human diabetics when compared to healthy control subjects (Grill *et al.*, 1990; Macleod *et al.*, 1994). In these human studies a relative sparing of flow in fronto-parietal cortex and large decreases in the caudate nucleus show remarkable similarities to the results described here. However, even the most sophisticated imaging techniques currently available in man do not have the spatial resolution of animal brain autoradiography, nor can the experimental conditions be as rigorously controlled. It may, therefore, be impossible to find exact parallels between the effects of diabetes upon LCBF described in this study and those in diabetic humans.

A number of studies have examined cerebral blood flow in untreated streptozotocin-induced diabetic rats, with varying results (Duckrow *et al.*, 1987; Harik & LaManna, 1988; Jakobsen *et al.*, 1990; Pelligrino *et al.*, 1991). Although in general terms reductions in LCBF were found when streptozotocin diabetic rats were compared to controls, the results were often too variable to reach statistical significance, and no clear consensus emerged on the susceptibility of particular regions of the brain to the condition. Interestingly however, if the rats were treated with insulin to normalize glycaemia at the time of the measurement, any differences in LCBF between diabetics

and controls were eliminated (Pelligrino *et al.*, 1991). A similar effect has also been described in peripheral nerve blood flow (Kihara & Low, 1995). In contrast, in the present study of spontaneously diabetic insulin-dependent rats, significant decreases in LCBF were evident despite the fact that there was no difference in plasma glucose levels between diabetic and non-diabetic animals at the time of the study. This is not to say, however, that the BB rats have not experienced periods of hyperglycaemia. The measurement of LCBF in the diabetic animals was conducted around 4h after the injection (s.c.) of medium-acting insulin, and the evidence from the physiological data (Tables 1, 2 & 3) suggests that the exogenous hormone was acting to normalize plasma glucose. Over a longer time scale, plasma glucose concentrations in BB/E rats are quite unstable and fluctuate in the course of any 24h cycle between 54 and 460mg.dl^{-1} (unpublished data). Glycosylated haemoglobin (HbA_1) values are elevated in the diabetic animals compared to non-diabetic controls in the Edinburgh BB colony (Lindsay *et al.*, 1997). There is no doubt therefore, that the diabetic animals used in this study are hyperglycaemic for a large part of the time, but maximum plasma glucose levels are unlikely to reach those found in streptozotocin-treated rats.

I chose throughout these studies to measure LCBF in conscious animals. Apart from the fact that anaesthesia reduces cortical blood flow, there is also direct evidence that it compromises the responsiveness of cerebral vessels (Edvinsson & McCulloch, 1981) in a rather unpredictable mode. It has also been shown that the type of anaesthesia used during the induction of focal cerebral ischaemia (halothane or barbiturate) influences the functional and pathological responses to the ischaemic insult (Kawai *et al.*, 1997), depending also on the species examined (Michenfelder & Milde, 1975). On the other hand, the restrained animals used here may be stressed. However, there was no evidence of stress in these animals. Plasma catecholamine levels have been measured in previous experiments and no elevation was found (Bryan *et al.*, 1983; Seckl *et al.*, 1991). Moreover, no evidence of hyperglycaemia was found in any of the experimental groups presented in this thesis, or evidence of stress-induced gastric ulcers in animals examined *post mortem*.

4. 1. 2. Influence of chronic glycaemic control upon LCBF

Recent reports from human sufferers of IDDM show a fundamental influence of glycaemic control upon the progress of extracranial complications (DCCT Research Group, 1993). An attempt was made to improve glycaemic control in a subgroup of diabetic animals, in order to establish whether glucose *per se* was directly responsible for the observed LCBF perturbations. This exercise was rather complicated, with one third of the animals treated by the continuous subcutaneous insulin infusion dying prematurely, presumably due to hypoglycaemia. This is actually an equally important complicating factor of treatment in intensively treated diabetics, where 3 times higher incidence of severe hypoglycaemia is encountered (DCCT Research group, 1996), associated with lack of preservation of higher brain function (Maran *et al.*, 1995).

In the animals that were used, this intensified insulin treatment protocol failed to alter LCBF in the regions where significant flow reductions were found in rats subjected only to a single daily dose of insulin. However, there was a trend (non-significant) towards blood flow increase in some areas. Since the concentration of HbA_1 was measured in this group, an attempt was made to correlate HbA_1 as a marker of long-term glycaemic control and blood flow, but no influence could be found in this small group of animals. Although HbA_1 was not measured in the conventionally treated rats, the values obtained in the intensively treated rats were not different from those reported from conventionally treated BB rats from the same colony (Lindsay *et al.*, 1997). Therefore, although a direct *cause and effect* relationship between level of chronic glycaemic control and LCBF could not be established from these experiments, this possibility cannot be discounted either.

4. 1. 3. Effects of L-NAME and SB209670 upon LCBF

In the present study there appeared to be an attenuation of the effects of L-NAME upon LCBF in diabetic rats. It is tempting to speculate that the decreases in LCBF apparent in saline-treated diabetics may be the result of reduced dilatatory influence of endogenous NO in determining basal cerebral blood flow. There is certainly evidence from the peripheral circulation that endothelial NO systems are disrupted in diabetes

(Cohen, 1993; Poston & Taylor, 1995; Steinberg & Baron, 1997), although there is clear evidence of variation in the defect between different vascular beds (Kiff *et al.*, 1991a). In the cerebral circulation the data are equally complex. Indirect evidence for reduced NO activity comes from studies of streptozotocin-induced diabetic rats where a significant, but regionally variable, impairment of endothelium-dependent vascular relaxation was observed following injection of a muscarinic receptor agonist (Pelligrino *et al.*, 1992). More direct evidence of an impairment of cerebrovascular NO systems in this diabetic model are however lacking, in that the effects of L-NAME upon cerebral blood flow (Pelligrino *et al.*, 1992) and pial vessel diameter (Mayhan *et al.*, 1991) were reported to be similar in both non-diabetic and diabetic rats. Whilst in this study no difference in LCBF values between diabetic and non-diabetic rats following L-NAME were found, with the exception of the hypothalamus, this did not represent a significant decrease in flow from saline-treated diabetic rats in which blood flow was already depressed. This could be interpreted as an attenuation of the cerebrovascular response to L-NAME.

Since continuous production of NO from the endothelial cells inhibits the vasoconstrictor effects of ET, as outlined in the ***Introduction***, inhibition of NO synthesis may unmask a continuous endothelin release in extracranial (Gardiner *et al.*, 1995; Richard *et al.*, 1995) and intracranial (Kelly *et al.*, 1995a) vessels. There is certainly indirect evidence from *in vitro* studies in cultured bovine aortic endothelial cells of an increased ET production associated with hyperglycaemia (Yamaguchi *et al.*, 1990). The possibility that an increased endogenous release of ET is responsible for the observed LCBF in diabetes was tested with the administration of a potent non-peptide ET_A/ET_B-antagonist (SB209670), but the finding of no alteration in LCBF in the SB209760-treated diabetic rats is consistent with only a minor, if any, role of ET in determining basal CBF in the diabetic rats. However, the findings by others (Haynes & Webb, 1994) and also in the haematoma studies presented in this thesis, that during ET inhibition more than 20min drug exposure may be required for an effect to be observed, raises also the possibility that ET may play a more substantial role in the regulation of LCBF in the diabetic BB rats. Obviously, this possibility

needs further clarification employing a longer therapeutic experimental design and is outwith the basic hypothesis of this thesis. It is no doubt a starting point for further studies. The experimental protocol, that only 20min elapsed from the i.v. injection of SB209670 to the measurement of LCBF, was based on the report by Ohlstein and his colleagues (1994) that this ET-antagonist has almost immediate effects following i.v. administration. In fact, recent *in vitro* studies in support of no basal ET influence upon LCBF in the diabetic rats, report that ET-1 and ET_A receptors do not contribute to the sustained hypertension induced by chronic NO synthesis blockade (Fujita *et al.*, 1995).

It is interesting that whilst reduced basal LCBF and an attenuated cerebrovascular response to L-NAME were found in the diabetic rats, the saline-treated diabetic animals were not hypertensive and L-NAME-treated diabetics displayed the normal blood pressure response, i.e. hypertension (Table 1). This might suggest that the disease process is more pronounced in the cerebral circulation than it is in other vascular beds. However, the aortas of diabetic BB rats do develop morphological defects in endothelial cells and abnormal endothelium-dependent responses to acetylcholine (Meraji *et al.*, 1987). Moreover, the hypertensive response to chronic L-NAME administration is attenuated in diabetic BB/E rats (Lindsay *et al.*, 1995). Thus it is possible that the mechanisms of NO dysfunction associated with diabetes develop differentially in different vascular beds. In line with the finding of a preserved effect of L-NAME upon peripheral resistance in the diabetic BB animals used in this study, basal mesenteric blood flow and flow in response to L-NNA were also preserved in STZ-diabetics compared to non-diabetic controls (Olbrich *et al.*, 1996).

No significant differences were found in the physiological parameters between L-NAME treated non-diabetic and diabetic animals, and the values of arterial blood gases and pH were within normal range in all experimental groups (Table 1). No effort was made to reduce MABP in the L-NAME treated rats to values similar to those recorded in the saline-treated animals because, within the autoregulatory range, LCBF is not dependent on MABP (Paulson *et al.*, 1990; Strandgaard, 1978).

Previous experimental paradigms have shown that reduction of MABP in L-NAME treated animals with controlled haemorrhage had no effect upon LCBF (Yang, 1996). Moreover, MABP in the L-NAME treated diabetic BB rats was within the autoregulatory range reported for both normotensive and diabetic (Rubin & Bohlen, 1985) animals, the upper limit of which would have probably shifted to a higher level following NOS inhibition (Kelly *et al.*, 1994b). Recent studies have also reported that endothelin release is enhanced during haemorrhagic hypotension, presumably as a homeostatic mechanism (Zimmerman *et al.*, 1994), so any effort to reduce MABP in the L-NAME treated rats with controlled haemorrhage could have potentially triggered compensatory mechanisms, including the release of endothelin.

4. 1. 4. Role of L-arginine availability in cerebrovascular responses in IDDM

Although there is evidence that the effectiveness of endogenous NO in influencing basal vascular tone may be altered by diabetes (Bucala & Cerami, 1992; Wascher *et al.*, 1994), it is not clear whether this represents a change in synthesis and release, or in activity. There is evidence for reduced levels of L-arginine in the plasma of STZ-induced diabetic rats (Mans *et al.*, 1987) which might indirectly explain altered NO synthesis due to reduction of substrate availability. Recent studies have also reported that extracranial endothelial dysfunction associated with IDDM was corrected following administration of L-arginine (Matsunaga *et al.*, 1996; Pieper & Peltier, 1995; Wascher *et al.*, 1996).

The possibility that L-arginine availability was inadequate in the diabetic BB rats was therefore examined, but the plasma levels of L-arginine were found to be similar in non-diabetic (DR) and diabetic animals. In support of this observation, and against a pathogenetic role of reduced L-arginine availability in the diabetic cerebrovasculature, Mayhan *et al.* recently (1996) reported that the impaired responsiveness of basilar artery to acetylcholine and bradykinin was not altered following administration of exogenous L-arginine in STZ-induced diabetic rats.

4. 1. 5. Effects of 7-NI upon LCBF

The cerebrovascular responsiveness to 7-NI was preserved in the diabetic BB rats, in contrast to the L-NAME findings. This observation is consistent with a preserved non-endothelial constitutive NO system in the brain associated with IDDM, and suggests that the perturbed basal LCBF is not a result of an abnormal influence of the neuronally derived NO.

It was surprising to find that L-NAME, although being a non-selective, and presumably both endothelial and neuronal, NOS inhibitor had no effect upon LCBF in the diabetic BB rats, whereas 7-NI, which is a selective neuronal NOS inhibitor, had significant influences upon LCBF in the same group of animals. It has to be assumed therefore that L-NAME acts predominantly as an inhibitor of endothelial NOS in the cerebrovasculature, although this assumption is obviously speculative. The development of a selective eNOS inhibitor would be ideal to test this possibility.

Another observation that warrants consideration was that the percentage changes in LCBF following 7-NI administration, and in comparison to the sesame oil-treated rats, were significantly lower in the diabetic animals (median= -35.5%), compared to the non-diabetic controls (median= -50%). This most probably occurred because of the lower basal LCBF in diabetics. Since blood flow values were similar between groups (non-diabetic and diabetic) following 7-NI treatment in all areas examined, a nadir was possibly reached, beyond which any further reductions in LCBF might have been detrimental to the brain, and which could therefore have triggered other regulatory mechanisms.

A rather intriguing observation was that in one diabetic animal injected with 7-NI, anomalous hyperaemia was observed unilaterally in the cortex, the thalamus and hippocampus (Figures 5 & 6). It has been reported that L-NAME administration, presumably effecting nNOS inhibition, results in paradoxical MABP reduction and cortical microvascular vasodilation in eNOS knockout mice (Huang *et al.*, 1995 & 1996). The diabetic BB animals used in this study might be considered functionally

analogous to the eNOS knockout mice, at least in the cerebrovasculature, and exhibit a somehow similar paradoxical effect. It is attractive to hypothesize that neuronally derived NO may possess vasoconstrictive functions, or that there is an interaction between endothelial and neuronal NO for maintenance of vascular tone, the disturbance of which may unmask an otherwise obscure NO-related vasoconstrictor tone. To complicate interpretation of these observations further, similar unexplained hyperaemia in response to much higher doses of 7-NI than the one used in this study, has also been reported in normal animals (Kelly *et al.*, 1994a; 1995b).

Local tissue glucose use was not measured in these studies for several reasons. As has been reported previously (Kelly *et al.*, 1994b & 1995b; Macrae *et al.*, 1993), L-NAME and 7-NI affect LCBF without any changes in metabolic rate for glucose. Therefore, any changes in LCBF observed in the studies presented here were unlikely to have been driven by metabolic alterations. An additional consideration that applies to the diabetic animals is that diabetes mellitus and insulin treatment alter the density of glucose transporters in the brain vessels in a rather unpredictable manner, as outlined in the ***Introduction***. Therefore, the methodology for autoradiographic studies of metabolic rate for glucose would have had to be re-characterized.

A trend towards bradycardia in the diabetic BB rats compared to the non-diabetic (DR) BB rats has also been reported by other groups using BB rats from other colonies (Stevens *et al.*, 1994). Physiological variables in the 7-NI treated animals were within the normal range (Table 2). Heart rate in the 7-NI treated diabetics was significantly lower (-40%) compared to the sesame oil treated rats, an observation that has been reported previously in association to 7-NI treatment (Kelly *et al.*, 1995b). In the non-diabetic animals, the effect of 7-NI (-30% reduction in heart rate) was not statistically significant. Heart rate was also significantly different between the 7-NI treated non-diabetic and diabetic groups (-29%), in part because heart rate was also lower (-15%) -though not significantly- in the sesame oil-treated diabetic rats, compared to the non-diabetics.

4. 1. 6. Effects of SIN-1 upon LCBF

LCBF responses to i.v. SIN-1 infusion were modestly enhanced in the diabetic BB animals compared to the non-diabetic controls, in most of the brain areas examined. These results are consistent with the L-NAME and 7-NI findings in the same groups of animals, because a reduced basal production of endothelial NO may be associated with modestly increased sensitivity of VSMC to NO (Moncada *et al.*, 1991). Another important consideration is that increased inactivation of endothelium-derived NO by free radicals (Cameron & Cotter, 1995; Graier *et al.*, 1996; Pieper *et al.*, 1996) or subendothelial advanced glycosylation end-products (Bucala *et al.*, 1991; Cohen, 1993), or reduced responsiveness of the vascular smooth muscle associated with diabetes, are not the principal determinants of the cerebrovascular dysfunction associated with this model of IDDM. It is therefore possible that reduced EDNO formation, as a result of NADPH depletion following overactivity of the polyol pathway or protein kinase C associated with diabetes (Bredt *et al.*, 1992; Cohen, 1993), or some other, as yet undefined, cause could play a pathogenetic role in the evolution of cerebrovascular dysfunction associated with this disease process.

The autoradiographic blood flow findings are also supported by the electron microscopic appearance of diabetic vessels. These did not show any thickening of the sub-endothelial basement membrane (although deposition of advanced glycosylation end-products can sometimes occur in the absence of any visible vascular wall thickening), and the PAS staining that failed to reveal any excessive glucose deposition in the brains of the diabetic animals. A time period of 8 weeks from the onset of the disease is necessary for the deposition of AGE to occur, and the duration of diabetes in all the animals used was over eight weeks in order to avoid the duration of the disease being a confounding factor in this experimental design.

Finally, and although speculative, the observation that CBF in the striatum and globus pallidus of the diabetic animals following SIN-1 administration was still significantly lower than that of the non-diabetic rats with the same treatment (Table 7, Figures 33 & 34), whereas in the rest of the brain there were no similar differences between

groups, may be associated with an increased clearance of EDNO in these particular brain regions. However, there is no conclusive evidence from the rest of the studies in support of this possibility.

The finding of an attenuated cerebrovascular response to L-NAME and preserved response to SIN-1 in the diabetic BB rats is also in accordance with studies in human sufferers of IDDM complicated by microalbuminuria, where the forearm vascular response to L-NMMA was blunted but the response to sodium nitroprusside was preserved (Elliott *et al.*, 1993). Similar findings have also been reported in drug-induced diabetic rats, where nerve blood flow was manipulated with L-NAME and sodium nitroprusside (Omawari *et al.*, 1996).

The physiological variables in the SIN-1 treated animals, as seen on Table 3, could not have influenced the LCBF findings. Although body temperature was significantly reduced in the diabetic animals, which could in theory reduce LCBF in response to reduction in metabolic demand (Harper, 1990), this difference was very small in absolute terms and if anything, the observed responsiveness of the diabetics to SIN-1 was higher compared to the non-diabetics. The same applies to the pO_2 in the diabetics treated with SIN-1, which although significantly lower to that of the non-diabetics, was still well within physiological range, and in the presence of a physiological pCO_2 would not have affected LCBF (Harper, 1990) (Table 3).

4. 1. 7. Plasma viscosity in diabetic rats and its role in LCBF

Although hyperglycaemia is considered to be an important factor contributing to vascular dysfunction associated with diabetes, and would certainly account for dysfunction in endothelial NO systems (Cohen, 1993; Poston & Taylor 1995), other mechanisms may also be involved. Rheological problems such as an increase in plasma viscosity (Barnes *et al.*, 1977) and increased adhesion of platelets to endothelial cells (MacMillan *et al.*, 1978; Wautier *et al.*, 1981) may contribute to cerebrovascular dysfunction in diabetes. Alternatively, some aspects of diabetic vascular pathology, notably arteriosclerosis (Grunnet, 1963), may be related to the hypertension often

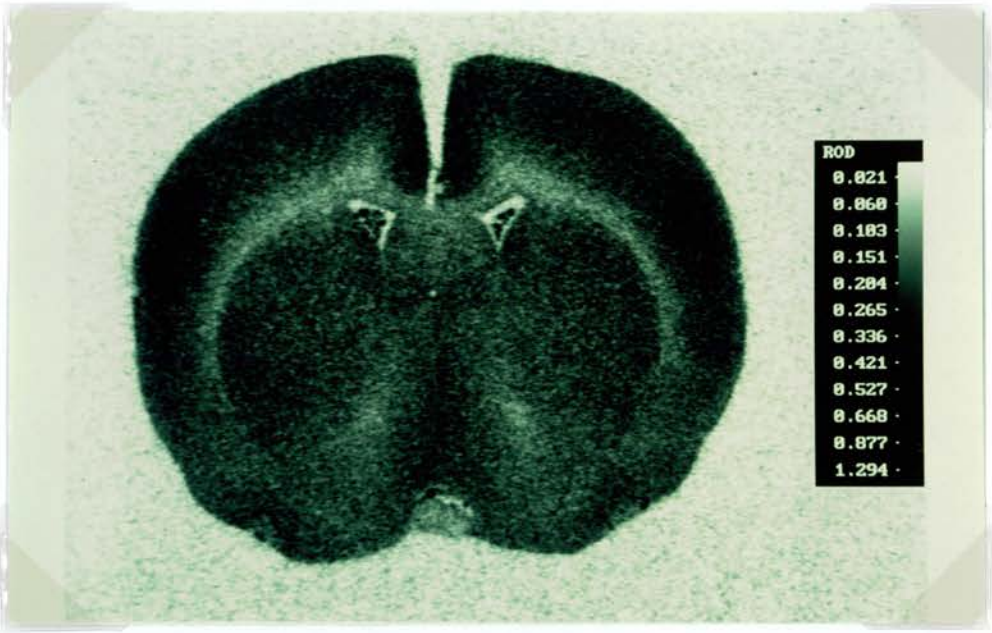


Figure 33: Blood flow (IAP) autoradiogram from a non-diabetic animal with acute SIN-1 treatment ($1.8mg.kg^{-1}.h^{-1}$ i.v. infusion). Grey scale on the right represents relative optical densities, which can be used to calculate blood flow (see Methods).

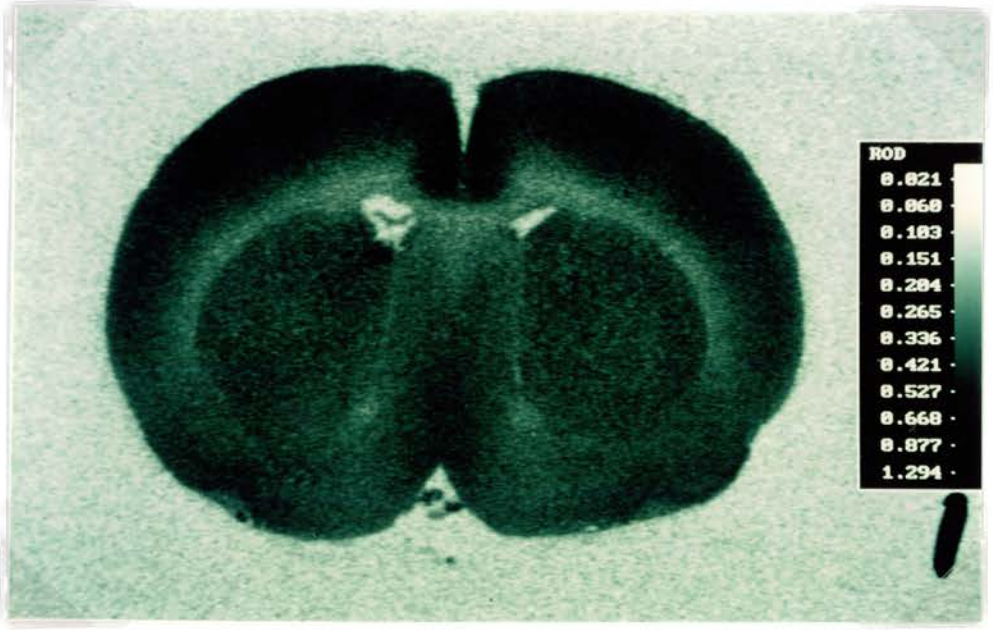


Figure 34: Blood flow (IAP) autoradiogram from a diabetic animal with the same SIN-1 treatment as above. Although cortex appears darker compared to the animal seen in Figure 35, striatum is lighter compared to the same animal.

associated with diabetes. Hypertension develops only at a later stage in BB rats and is not therefore an issue in these studies, but increased blood viscosity was found indeed in the diabetic BB rats. However, not only is the significance of blood viscosity in determining LCBF contested (Brown & Marshall, 1985; Waschke *et al.*, 1994), it is also unlikely that increased viscosity can explain the heterogeneity in the reduction of blood flow which was observed in the cerebrovasculature of these animals. There is increasing evidence that diabetes adversely affects outcome in experimental models of cerebral ischaemia (Nedergaard & Diemer, 1987; Sutherland *et al.*, 1992) and in human stroke victims (Jørgensen *et al.*, 1994) and obviously, in brain areas with critically compromised blood flow the presence of increased viscosity could present an important factor for worse outcome (Marshall, 1982).

4. 2. Physiological studies in the hypertensive animals

4. 2. 1. Basal LCBF

There was little evidence of any fundamental difference in basal LCBF between WKY and SHR groups in these studies, although in one area of the brain, the nucleus accumbens, blood flow was significantly higher in SHR. These observations are largely in keeping with previous reports, where either similar quantitative autoradiographic techniques were used to measure LCBF in conscious rats of these two sub-strains (Wei *et al.*, 1992), or where rats of a similar age to those used in this study were examined using different measurement protocols (Grabowski & Johansson, 1985). Moreover, the results of these physiological studies are in keeping with morphological examination of cerebral capillary bed structure (Lin *et al.*, 1990a) and precapillary arterioles on the pial surface (Harper & Bohlen, 1984) which show no differences between SHR and WKY. Although structural changes have been described in larger blood vessels of the cerebrovascular bed in SHR (Folkow, 1990), it is not these vessels which regulate LCBF.

Age may be another factor to be considered as CBF reduces with advancing age (Naritomi *et al.*, 1979). All animals used for the studies presented in this thesis were therefore young adults, and the DR and WKY controls were always age-matched to the diabetic and SHR, so age was not a variable that could influence LCBF.

Physiological parameters, as seen on Tables 9, 10 and 11 were similar between groups (with the exception of MABP, heart rate and haematocrit) and could not have influenced the LCBF data. Although haematocrit was significantly elevated in the SHR rats treated with saline, as compared to the WKY animals with the same treatment (Table 9), it is unlikely that the actual difference (4%) could have had any fundamental influence upon LCBF.

4. 2. 2. Effects of L-NAME and SB209670 upon LCBF

Early *in vitro* investigations identified a decrease in NO-mediated activity in cerebral blood vessels taken from SHR (Miyata *et al.*, 1990; Malinski *et al.*, 1993b).

Subsequent examination of the basilar artery *in situ* showed that L-NAME induced greater constriction in hypertensive rats (Kitazono *et al.*, 1995). These authors suggested that basal release of NO might be somewhat enhanced in SHR over that in WKY, and *in vivo* studies confirmed that the cerebrovascular response to NO inhibition with L-NMMA was greater in SHR (Izuta *et al.*, 1995), although no significant difference in LCBF was found in SHR and WKY treated with L-NA. In the studies presented in this thesis, acute treatment with L-NAME had broadly similar effects upon LCBF in the WKY and SHR groups and were in keeping with previously published results (Izuta *et al.*, 1995; Yang, 1996). The dose of L-NAME used in the present studies has previously been found in Sprague-Dawley (SD) rats to produce significant reductions in LCBF at 15min post-injection (Kelly *et al.*, 1994b) which are maintained for at least 3h (Macrae *et al.*, 1993), but in general it appears that L-NAME is not as efficacious in reducing LCBF in WKY and SHR as it is in SD rats. Although it was outwith the present experimental design to make such inter-strain comparisons, it is also noteworthy that differences in vascular structure have been found between normotensive WKY and SD in the cerebral capillary bed (Lin *et al.*, 1990a).

The pressor effect of L-NAME treatment was broadly similar in both groups (+27% in WKY and +18% in SHR, Table 9), but MABP was within the autoregulatory range reported previously (Barry *et al.*, 1982; Kelly *et al.*, 1994b). Moreover, the significant reductions of LCBF that resulted from this treatment, would have been obscured if the upper limit of autoregulation had been exceeded.

Treatment with SB209670 had no significant effects upon LCBF in the SHR. This observation is in accordance to the general impression from previous studies in humans and animals, that ET may have a more substantial role in fulminant forms or stages of the disease (Rubanyi & Polokoff, 1994) and also with reports where, in the spontaneously hypertensive rats (SHR) chronic blockade of ET-1 did not alter MABP (Li & Schiffrin, 1995).

4. 2. 3. Effects of 7-NI upon LCBF

Whilst there was no evidence of any difference between WKY and SHR in the response to the non-selective NOS inhibitor L-NAME, the response to the intraperitoneal injection of 7-NI, which *in vivo* is a selective inhibitor of the neuronal isoform of NOS (nNOS; Moore *et al.*, 1993a & b), was significantly greater in the majority of brain areas of hypertensive animals. Although previous studies have suggested that there may be an upregulation of cerebrovascular NO systems in hypertension (Kitazono *et al.*, 1995), the current observations point to there being a more specific upregulation of neuronal NOS. Studies specifically addressing the role of neuronal NOS in the brains of SHRs are lacking, although it does seem that nNOS expression is normal in the cerebellum and brain stem of 4-, 16-, and 24-week-old SHR, compared to age-matched WKY (Iwai *et al.*, 1995). It is interesting to note that the activity of nNOS in cerebral ischaemia is potentially detrimental (Huang *et al.*, 1994) and although perhaps speculative at this stage, the current findings, consistent with an upregulated nNOS system in hypertension, could offer one explanation for the predisposition of hypertensives to ischaemia following stroke (Coyle, 1984). These findings, of comparable cerebrovascular responses to L-NAME but enhanced responses to 7-NI in the SHR could also be compatible with a down-regulation of endothelial NOS in these animals together with an up-regulation of the neuronal NOS. To test this hypothesis awaits the development of specific endothelial NOS inhibitors.

4. 2. 4. Effects of SIN-1 upon LCBF

There is growing evidence that endothelium-dependent vascular dilatation is heterogeneously affected in hypertension (Nava *et al.*, 1995) with both regional and species differences (Deng *et al.*, 1995). Studies in hypertensive humans and animals have reported preserved dilatatory responses to sodium nitroprusside in peripheral vascular beds (Taddei *et al.*, 1993; Küng & Lüscher, 1995). In contrast to the qualitatively similar effects of SIN-1 upon blood pressure in both WKY and SHR reported here, the effects of SIN-1 upon LCBF were attenuated in the SHR group when compared with WKY. These apparent differences in the response to SIN-1 between vascular beds in the SHR group suggest that there may be regional

perturbations of NO-specific vasodilatatory reserve, and would be consistent with an up-regulation of endogenous cerebral NOS activity. In support of these observations, L-arginine was found to have no effect upon LCBF in the contralateral hemisphere of SHR subjected to MCA occlusion (Prado *et al.*, 1996), and SIN-1 had no significant effect upon cortical blood flow in SHR subjected to sham-occlusion of the MCA (Zhang *et al.*, 1994).

Contradictory results have also been reported using *in situ* methodology to measure responsiveness of pial arteries, or the basilar artery, to superfusion of NO donors in the stroke-prone substrain of the SHR (Kitazono *et al.*, 1993; Mayhan *et al.*, 1988; Yang *et al.*, 1991a & b; Yang *et al.*, 1993) and also in stroke-resistant SHR (Mayhan *et al.*, 1987; Mayhan, 1991). There are several potential explanations for the apparent differences between these results and the data reported in this thesis, including the fact that the previous experiments were performed in anaesthetized animals, with the potential influence of anaesthetic agents on cerebrovascular responsiveness (Edvinsson & McCulloch, 1981); the possibility that NO bioactivity might differ markedly between the stroke-prone substrain of SHR and the SHR used in the current studies (Dominiczak & Bohr, 1995); the fact that the animals used for the current studies were considerably younger than those used previously, and NO responsiveness has been shown to change - at least in renovascular hypertension - as the duration of hypertension progresses (Dubey *et al.*, 1996); most importantly, neither the basilar artery nor the pial vessels constitute the principal source of resistance to flow in the cerebrovascular bed, and are therefore not responsible for the control of LCBF (Harper, 1990). Recent investigations in conscious patients with arterial hypertension have also confirmed impaired responsiveness to NO-donors (Preik *et al.*, 1996), although once again other, earlier reports have reached contradictory conclusions (Creager & Roddy, 1994; Panza *et al.*, 1995).

The vessels of the cerebrovascular bed are normally endowed with the ability to alter their calibre in response to fluctuations in perfusion pressure. The resulting changes in vascular resistance ensure the maintenance of constant cerebral blood flow over a

wide range of arterial blood pressures, a phenomenon known as autoregulation (Paulson *et al.*, 1990). The lower limit of autoregulation below which the relationship between cerebral blood flow and perfusion pressure becomes linear is not a fixed point, and chronic hypertension is known to raise the arterial pressure threshold at the lower limit of the autoregulatory range (Paulson *et al.*, 1990; Strandgaard, 1978). Although the levels of MABP measured in the SHR in response to the higher dose of SIN-1 (104mmHg) were above the lower limit of autoregulation (90mmHg) reported for this strain (Barry *et al.*, 1982; Harper & Bohlen, 1984), the observation that LCBF was higher - though not significantly so - in most of the areas examined in the SHR treated with the lower SIN-1 dose compared to those treated with the higher dose, could be interpreted as indicating pressure-dependency in flow to some extent at least. It is known that NO inhibition shifts the upper limit of cerebrovascular autoregulation to higher pressure levels in normal animals (Kelly *et al.*, 1994b) so it is conceivable that NO could have a role in determining the lower limit of autoregulation. A definitive conclusion regarding this possibility cannot be reached however.

SIN-1 generates NO and superoxide, which can potentially react with NO to form peroxynitrite (Moncada & Higgs, 1995; Plane *et al.*, 1997). The dilator actions of SIN-1 in extracranial tissues are also modulated by the basal production of endothelium-derived NO (Plane *et al.*, 1997). Studies performed *in vitro* with endothelial cells from peripheral tissues of 5 week old SHR showed that superoxide may be responsible for the decreased activity of NO (Grunfeld *et al.*, 1995). There is also growing speculation of an enhanced oxidative stress in the pathogenesis of hypertensive complications (Alexander, 1995). It is clearly possible that the effects of SIN-1 upon LCBF could be related to generation of superoxide, complicated further by the potential involvement of free radicals in the evolution of hypertensive complications. The exact mechanism by which SIN-1 effects changes in cerebral blood flow may therefore be quite complex, but whatever the underlying mechanism, these studies clearly identify differences in the cerebrovascular response to SIN-1 in WKY and SHR.

4. 2. 5. General discussion of the LCBF data

Morphological analysis of those cerebral blood vessels which are largely responsible for the control of LCBF, have revealed no structural differences between WKY and SHR (Lin *et al.*, 1990a). Whilst it is possible that subtle changes might go undetected in these rather small vessels, the method of analysis did prove sufficiently sensitive to detect differences between vessels from both WKY and SHR, when compared to those from SD rats (Lin *et al.*, 1990a). It has been argued that structural changes in the vessel wall associated with chronic hypertension could explain altered responses to vasoactive agents (constrictor and dilator) (Calver *et al.*, 1992), but it might be expected that the response to all vasoactive agents would be affected in a non-specific manner (Folkow, 1990; Harper & Bohlen, 1984). It is possible that structural changes might have been present in the relatively young (~14 weeks old) animals presented in this thesis, particularly as structural changes appear even before frank hypertension has been established (Folkow, 1990), but structural differences cannot readily provide an explanation for the differential response to the two NOS inhibitors (L-NAME and 7-NI) and nor could they explain the attenuated vasodilatory response to SIN-1. Thus the current results would appear to support the concept of hypertension-induced functional changes (Winquist & Bohr, 1983) in the cerebrovasculature.

Cerebrovascular dysfunction associated with hypertension is most probably multifactorial. Studies using pial arteries from hypertensive rats have identified altered dilator responses which appear to involve vasoconstrictor prostanoids (Mayhan, 1992b; Yang *et al.*, 1991a), and *in situ* studies have shown that the prostaglandin-induced pial arterial vasodilatation is related to NO production (Armstead, 1995). Interestingly, enhanced responses of the basilar artery to activation of ET_B receptors in hypertensive rats, independent of NO or prostanoid pathways, have also been observed (Kitazono *et al.*, 1995). The same group also found that the mechanisms responsible for the impaired responses of the basilar artery in SHRs (Mayhan, 1990) are not the same as those responsible for the attenuated responses of the pial vessels.

The results of these studies provide further evidence for heterogeneous perturbation of NO systems in the cerebrovasculature of the SHR strain, with a reduced vasodilatory reserve and possibly an up-regulation of neuronally derived NO systems. The importance of these findings may become pronounced in situations of cerebral ischaemia, which is one of the commonest complications of chronic hypertension.

4. 3. Haematoma studies in the diabetic animals

4. 3. 1. Consideration of haematoma model

The reproducibility of ICH models is variable, and this is reflected by the fact that there are several models with certain particular advantages and disadvantages, as outlined in the ***Introduction***.

Whenever blood is injected into the striatum, there is always variable reflux into the white matter and subarachnoid space (Deinsberger *et al.*, 1996). The only model in which reflux does not occur is that with the inflation of a microballoon (Kingman *et al.*, 1988; Nehls *et al.*, 1990). It was not appropriate to use the microballoon model in these experiments, because the principal hypothesis that blood is responsible for the development of delayed perilesional ischaemia, and not the mass lesion itself, could not have been tested with that particular model. The important issue is that the volume of *striatal* blood load was measured post mortem in each animal with tissue planimetry, so that any differences in ICH volume between different groups could be detected. The volume of striatal blood or silicon oil load was similar in all groups studied (~10% of whole caudate). Volume of *purely* striatal blood is of importance in the LCBF studies, because the surrounding white matter has very low basal LCBF, with values similar to the ischaemic levels in grey matter. Therefore, inclusion of this area in the results would have resulted in overestimation of the actual ischaemic area.

The actual value of the ischaemic threshold ($35ml.100g^{-1}.min^{-1}$), was selected for reasons clearly defined in the ***Materials and Methods***. In addition, in models of focal ischaemia studying MCAO with perivascular application of ET-1 in the rat, LCBF in the ischaemic caudate nucleus had a mean value of $31ml.100g^{-1}.min^{-1}$, associated with neuropathological evidence of ischaemic cell injury (Gartshore *et al.*, 1996). In addition, following MCA occlusion in SHRSP, mean LCBF in the peripheral area of the infarct was reported to be $\sim 27ml.100g^{-1}.min^{-1}$ (Shima *et al.*, 1994), whereas following MCA occlusion in normotensive rats the ischaemic border had a CBF of $\sim 30ml.100g^{-1}.min^{-1}$ (Nedergaard *et al.*, 1986). Another point for consideration was the reason for calculating blood flow between 15 and $35ml.100g^{-1}.min^{-1}$. Since part of

the tissue with LCBF less than 35ml.100g^{-1}.min^{-1} is occupied by blood or silicon oil, the threshold of 15ml.100g^{-1}.min^{-1} was selected, above which the tissue studied would have most likely been perilesional.

The volume of blood that was injected is unlikely to have caused any significant influence upon ICP, which has been observed in association with volumes of 100µl (Kingman *et al.*, 1987; 1988), where ICP has been found to raise sufficiently enough to compromise CPP. In the pilot experiments that were performed in advance of the rest of the studies, a very modest increase in ICP (never exceeding 16mmHg) was observed, as has been reported previously (Jenkins *et al.*, 1990; Kingman *et al.*, 1988). Observational studies in humans have identified volume of ICH of at least 40ml (equivalent to a 50µl clot in the rat brain) to be associated with delayed neurological deterioration (Mayer *et al.*, 1994).

Although it has been claimed that with slower infusion rates the rate of backflux is lower (Yang *et al.*, 1994), it was found in the preliminary experiments already mentioned in the ***Materials and Methods*** section that if obvious backflux from the burr-hole was present at the onset of the injection, this continued even if the rate of the injection was reduced. It should also be mentioned that in the clinical setting, active bleeding lasts for less than 1min in most ICHs (Kingman *et al.*, 1988). Therefore, a slower injection rate is more akin to an *infusion* of blood, where the physical disruption of brain parenchyma by the blood may have rather different characteristics.

Inbred rats are genetically very similar, so differences in immunological reaction to donor blood as a factor for the autoradiographic findings is unlikely. In addition, there were no differences in BBB permeability in any of the groups, excluding the possibility that an exacerbated inflammatory reaction of the diabetic animals to the blood was present. The blood that was injected was derived from non-diseased animals, because the purpose of these studies, as outlined in the ***Materials and Methods***, was to study the responses of *host* cerebrovasculature to the presence of

intraparenchymal blood, independent of the potential effects that diabetic blood may have upon this responsiveness, in terms of clotting, white and red cell abnormalities, as outlined in the ***Introduction***. Other groups studying pressure autoregulation have injected intravenously blood withdrawn from syngeneic animals with no ill-effects (Barry *et al.*, 1982). Another potential problem might have arisen if blood was withdrawn from the animal's own femoral artery and then the vessel was to be ligated, that is the ischaemic inflammatory sequelae in the limb could have induced secondary cerebral responses (Bileviciute *et al.*, 1995).

Extensive surgical manipulation and instrumentation for monitoring of physiological parameters during the induction of the haematoma was avoided. An alternative approach was followed, with the use of parallel groups of animals, in which arterial blood gases, pH and MABP were monitored during induction of the haematoma, to ensure that the anaesthetic regime (pentobarbitone) did not induce any cardiovascular or respiratory effects that could account for the observed phenomena at 24h. It is of note that other groups who study experimental intracerebral haematoma models associated with animal recovery (Del Bigio *et al.*, 1996; Lee *et al.*, 1997; Lyden *et al.*, 1997) acknowledge the difficulties and avoid cannulation of vessels for arterial blood gases and pH monitoring at the time of haematoma induction.

Fully quantitative autoradiography was employed for measurements of LCBF and BBB permeability, because it provides the gold standard for *spatial* measurements of these parameters (Hossmann, 1994). This approach lacks the same advantages when *temporal* evolution of the above mentioned processes are to be measured. Therefore, the exact time point that was selected for the identification of the pathophysiological events (24h post induction of the haematoma) corresponded to that reported by other experimental groups to represent peak for oedema formation and blood flow decrease (Yang *et al.*, 1994). There is no doubt that an evolving phenomenon was studied and therefore blood flow and permeability changes continue after 24h, together with the possibility that diabetes and hypertension could alter the temporal characteristics of these events. However, most groups who study experimental focal cerebral ischaemia

use 24h as an acceptable endpoint for the study blood flow as well as neuropathological evidence of stroke.

Detailed neuropathological analysis was not undertaken in these studies. The limited histopathological data presented were used in support of the LCBF findings and particularly the study of ischaemic thresholds. Since most of the histopathological specimens were from frozen brains, which was inevitable because they had to be from the same animals used for LCBF and BBB permeability studies, subtle histopathological evidence of hypoxic-ischaemic damage could not be reliably assessed (Brown and Brierley, 1968). However, a clearly demarcated area of abnormality was always present in the striatum in sections stained with conventional methods, the size of which could be compared to the size of striatal tissue within the preset flow thresholds. For these reasons volumetric data of actual stroke are not presented. However, in all groups the volume of tissue pallor corresponded closely to the ischaemic regions, as defined.

4. 3. 2. Influence of blood constituents upon perilesional LCBF following experimental ICH

The results of the silicon oil injections compared to the injection of blood, in both non-diabetic and diabetic groups, support the hypothesis that factors released from the blood influence LCBF regulation in the perilesional cerebrovasculature (Mendelow, 1993). In addition, the presence of diabetes mellitus exacerbates the oligaemic tendency in this animal model of the disease process. The presence of an oligaemic area surrounding an intracerebral haematoma, analogous to the ischaemic penumbra described in occlusive stroke, has also been demonstrated recently in human sufferers of ICH using PET for measuring CBF (Villar-Cordova *et al.*, 1997).

The small differences in baseline physiological variables in these experimental groups, as shown on Table 16, should not have significantly influenced the LCBF or histopathological findings. Although body temperature was significantly lower in the diabetic animals injected with silicon oil, the actual difference was not great enough

(less than 1°C) to have exerted any neuroprotective influence (Busto *et al.*, 1989; Zhang *et al.*, 1993). The pH in the same group was lower than that of the non-diabetic rats with silicon oil injection, but if anything, its effect would have been detrimental, and again, the difference was very small in actual terms (~1.5%).

4. 3. 3. Haemodynamic compromise vs glucotoxicity

The effect of IDDM upon LCBF following experimental ICH has not been studied before. Experimental information is available from studies of focal (Duverger & MacKenzie, 1988; Nedergaard *et al.*, 1988; Wagner *et al.*, 1992) or global (Sutherland *et al.*, 1992) cerebral ischaemia associated with either STZ-induced diabetes or acute hyperglycaemia. There is substantial evidence that drug-induced diabetes increases the volume of infarction following experimental focal ischaemia, but whether this is related to altered haemodynamic responses or direct glucotoxicity is not clear. Studies of MCA occlusion in STZ-induced diabetic rats have revealed no differences in the patterns of LCBF changes, measured with fully quantitative autoradiography acutely after the onset of the occlusion, despite the larger final stroke (Nedergaard & Diemer, 1987; Nedergaard *et al.*, 1988). Other groups however, have reported delayed cortical CBF recovery, measured with laser-doppler flow in cats (Wagner *et al.*, 1992) and more recently in rats (Kittaka *et al.*, 1996), following reperfusion.

It should be pointed out that all the above mentioned studies address purely the effect of chronic hyperglycaemia, rather than diabetes *per se* in cerebral ischaemia, and that insulin treatment reversed the deleterious effects of hyperglycaemia upon stroke volume in one of the studies (Bômont & MacKenzie, 1995). In the experiments reported in this thesis, due to insulin treatment, there were no significant differences in plasma glucose between groups when LCBF was measured. The results obtained therefore, represent more the effects of the total diabetic condition. There is obviously substantial indirect evidence that chronic glucose control was far from optimal in the diabetic animals used in this study. However, any potential influence of acute hyperglycaemia upon CBF was eliminated. Previous studies addressing the

vulnerability of the BB rat brain to ischaemia have not been performed. There is a single published report of impaired metabolic recovery, measured with SPECT, at reperfusion following transient forebrain ischaemia in diabetic BB rats (Sutherland *et al.*, 1992).

Although there is evidence for an alteration in cerebrovascular responsiveness following occlusive stroke associated with diabetes (Kittaka *et al.*, 1996), the situation in relation to haemorrhagic stroke is rather more complicated. The presence of intraparenchymal blood can potentially induce effects in the perilesional vasculature by several mechanisms (Martin *et al.*, 1986; Ohlstein *et al.*, 1991; Sinar *et al.*, 1988). As evidenced by the differences in the volumes of striatal oligaemia following silicon oil injections in both diabetic and non-diabetic animals, factors related to the presence of blood do influence the extent of delayed perilesional haemodynamic compromise following experimental ICH. Further indirect evidence that this is true was provided with the appearance of fibrinoid necrosis in a perilesional striatal vessel from a diabetic animal injected with blood (Figures 35 & 36). Such reactive small vessel changes are a common finding around acute human ICH at autopsy, and may represent a toxic effect of blood constituents, possibly mediating intense vasoconstriction. Also of interest was the presence of extensive striatal ischaemia in a diabetic animal in which the blood was lying exclusively in the white matter (Figures 37, 38, & 39), presumably as a result of significant vasoconstriction of the penetrating vessels derived from the cortex and traversing the white matter to supply the striatum.

The presence of intermittent hyperglycaemia in the diabetic BB rats could have additional confounding effects in terms of endothelin (Yamaguchi *et al.*, 1990) and adenosine (Hsu *et al.*, 1991) production. Obviously, the presence of increased viscosity found in the diabetic rats in line to that reported in humans (Barnes *et al.*, 1977), could have possibly created a further rheological burden, but whether it would have directly influenced blood flow in the perilesional area is a matter of speculation (Brown & Marshall, 1985; Waschke *et al.*, 1994).

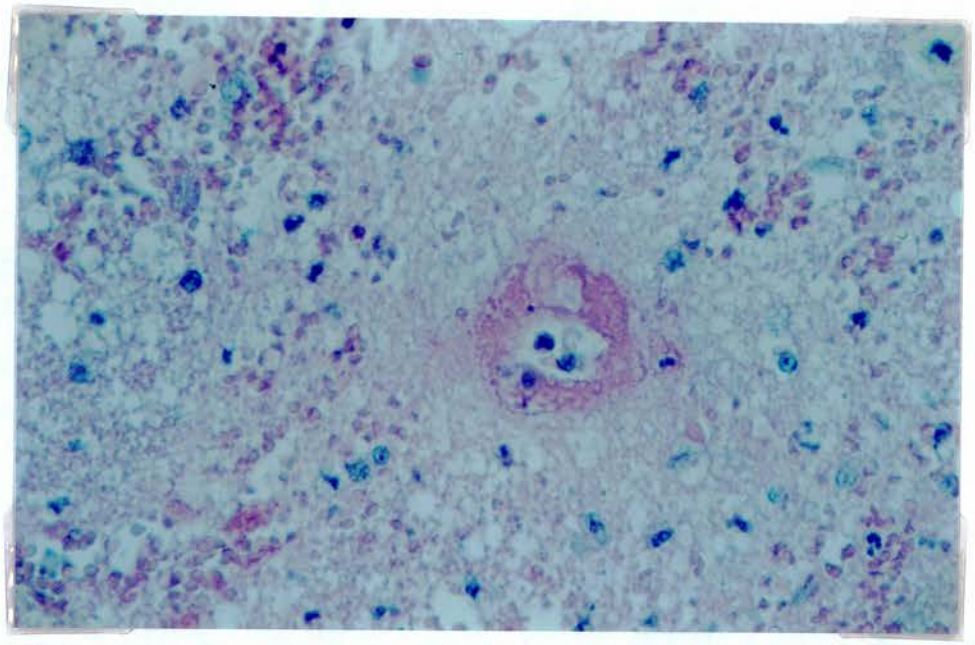


Figure 35: Section from the caudate nucleus of a diabetic rat injected with 50μl blood, stained with haematoxylin and eosin. This is a paraffin section, as opposed to the frozen sections shown in previous figures, and was obtained from one of the animals used in the preliminary experiments (section 2. 2. 1.). Note the presence of fibrinoid necrosis in the capillary shown in the centre of the picture, which is adjacent to the haematoma, as evidenced by the presence of red cells in the periphery of the picture. Magnification x600.

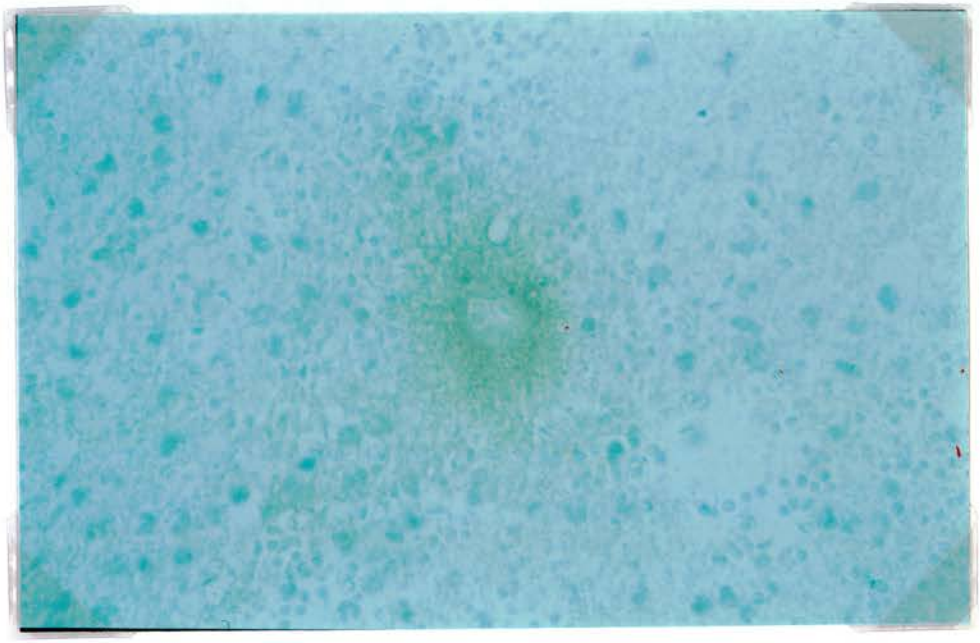


Figure 36: Immunohistochemistry for fibrinogen, from an adjacent section to that shown in Figure 35, shows positive staining (brown) in the neuropil around the capillary, confirming that breakdown of BBB has occurred as a consequence of fibrinoid necrosis. Magnification x600. For Method see page 193.

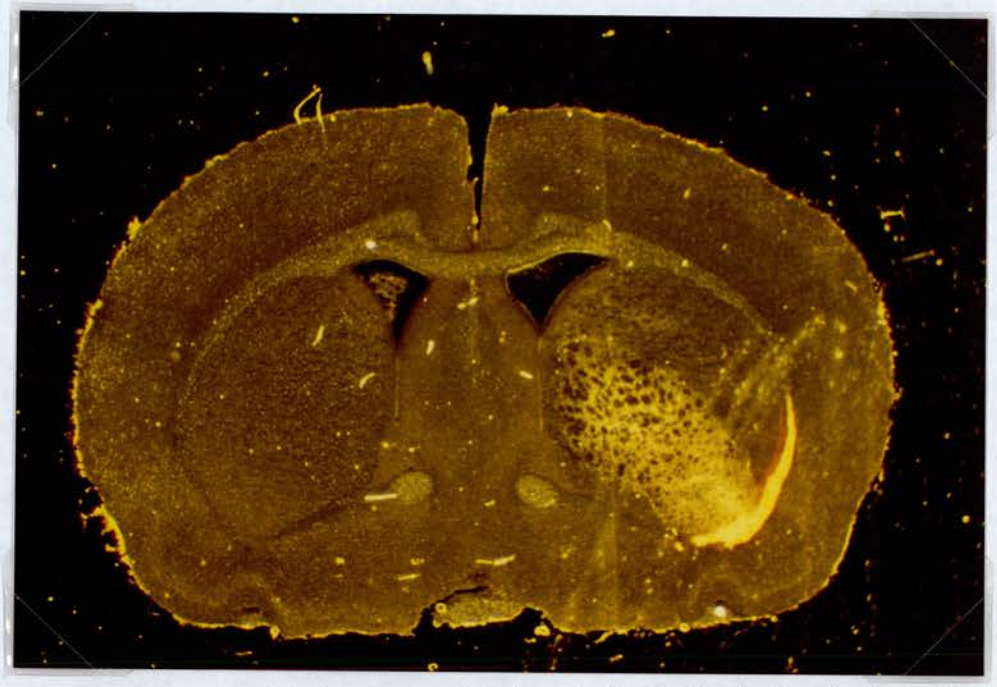


Figure 37: Unstained coronal section from a diabetic rat brain. In this single animal, the blood was confined exclusively to the white matter. Note that the tissue appearance of the caudate medial to the blood is abnormal.

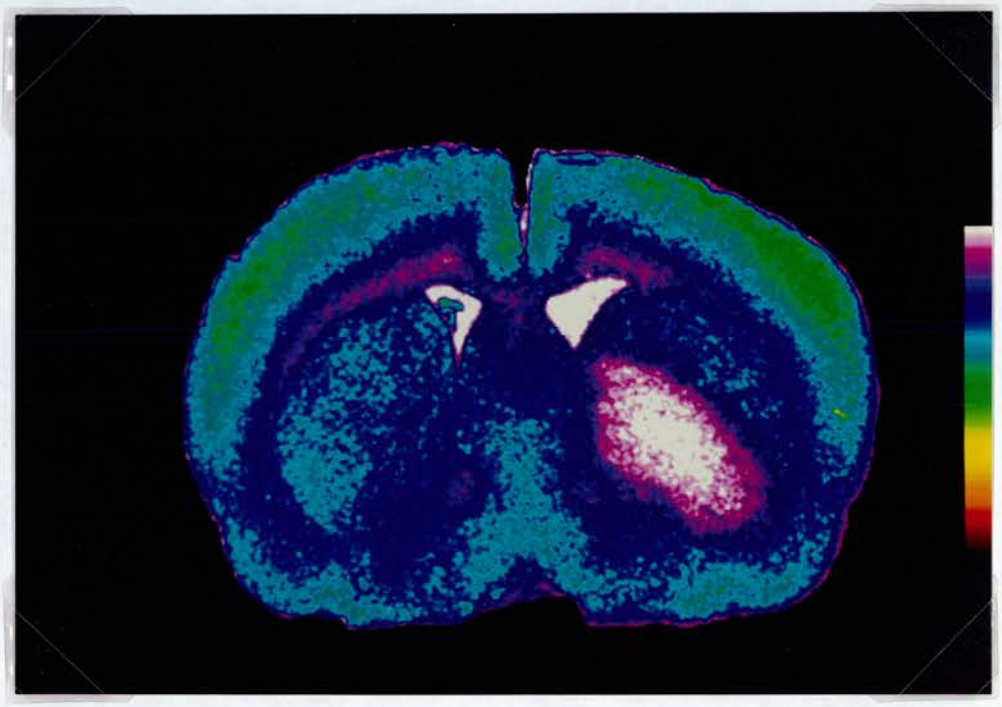


Figure 38: Blood flow (IAP) autoradiogram from a section adjacent to that shown in Figure 37. Note extensive striatal ischaemia (white, pink and purple colour), possibly as a result of vasospasm in the penetrating vessels arising from the pial surface, and traversing the white matter containing the haematoma, to supply the infero-lateral area of the striatum.

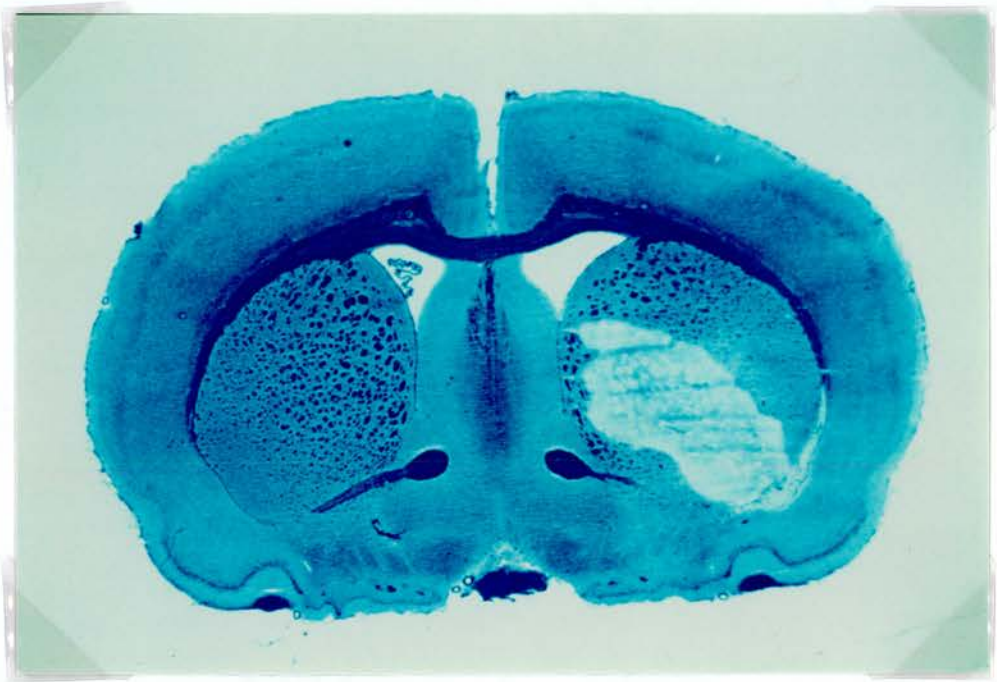


Figure 39: Coronal brain section from the same animal and adjacent to the section shown in Figure 38, stained with cresyl violet and luxol-fast blue. Note the presence of striatal tissue pallor sharply demarcated from the surrounding normal parenchyma and corresponding closely to the area of striatal ischaemia present in the IAP autoradiogram of Figure 38.

As already mentioned, 15-20% of the diabetic animals died after regaining consciousness from the anaesthesia used during haematoma induction but before the LCBF experiment. A seizure, which was witnessed in one of these animals just before it died, could have possibly resulted from hyperglycaemia at the time of the event, since an association between hyperglycaemia and seizures has been reported previously in experimental cerebral ischaemia (Warner *et al.*, 1987).

The diabetic animals used in these studies were treated with insulin to prevent death. Insulin administration is associated with improved outcome in experimental ischaemia probably due to its anti-hyperglycaemic effects, together with its directly vasodilatory influence (Fukuoka *et al.*, 1989; LeMay *et al.*, 1988). However, in this study although its administration resulted in comparable plasma glucose levels between non-diabetic and diabetic rats at the time of the final experiment, it did not affect the detrimental influence of IDDM upon haemorrhagic stroke.

4. 3. 4. Volume of striatal oligaemia following ICH

The volume of significant oligaemia was ~4% of the striatum in the non-diabetic and ~17% of the striatum in the diabetic rats. The group from Glasgow has reported significant oligaemia in 7.5% of the caudate nucleus at 4h post-injection of 100μl blood (Sinar *et al.*, 1988). Following injection of 25μl of blood, the same group reported significant oligaemia in 12% of caudate nucleus at 1min, 5% at 10min and 0.2% at 3h (Nath *et al.*, 1987). The same group, using the microballoon model reported significant oligaemia in ~12% of caudate at 5min and ~35% at 4h, together with a haemodynamic improvement associated with temporary inflation of the microballoon (Kingman *et al.*, 1987; Nehls *et al.*, 1988 & 1990). The microballoon model has two fundamental differences in comparison to the model used in these studies: the effects of blood could not be addressed with the microballoon, and the volume of the actual striatal mass was much larger with the microballoon, because no backflux along the white matter or the sub-arachnoid space could have occurred.

The group from Michigan has also used a similar experimental ICH model, and has addressed the temporal evolution of pathophysiological sequelae (Yang *et al.*, 1994). This group failed to identify any presence of striatal oligaemia at 24h, even with volumes of blood double to that used in the studies presented in this thesis (Lee *et al.*, 1997; Yang *et al.*, 1994). The most likely explanation is that, although quantitative methodology was employed, it was actually global striatal blood flow rather than local (including the perilesional area) CBF that was measured by the Michigan group. Therefore, small areas of oligaemia would have been obscured, especially in the presence of simultaneous hyperaemia in the lesioned striatum. It should however be noted that, the temporal evolution of CBF changes reported by this group parallel those described by the Glasgow group, with an immediate reduction in blood flow at induction of the haematoma, which returns to baseline values at 4h and then starts declining to reach a nadir at 24h.

Finally, Rosenberg reported dense striatal ischaemia at 24h in his collagenase ICH model (Rosenberg *et al.*, 1992). The volume of the haematoma is very large in this model, occupying virtually the entire caudate nucleus. It is impossible to know whether this ischaemia represents tissue occupied by the actual haemorrhagic lesion or the perilesional area and therefore, direct comparisons with the findings of the current studies cannot be made.

The manifestation of hyperaemia in the lesioned striatum peripheral to the oligaemic zone in all experimental groups, including those injected with silicon oil, was a rather interesting observation. It would have been attractive to hypothesize that it was a manifestation of reactive hyperaemia, following reperfusion of a previously oligaemic area, upon exhaustion of the vasospastic influences by the putative mediators in the blood. However, the presence of similar striatal hyperaemia in the silicon oil injected animals makes this possibility unlikely. The other potential explanation would have been for this hyperaemia to manifest not regional cerebrovascular dysregulation, but a homeostatic response in an attempt to increase flow in the inadequately perfused perilesional tissue. This explanation is again inadequate, because it cannot provide an

acceptable explanation for the hyperaemia observed in the cortex ipsilateral to the haematoma in a minority of the diabetic animals (Figure 23). Whatever the cause of this hyperaemia, it was not associated with evidence of histopathological abnormality in any animal. Obviously, the pathogenetic mechanisms underlying this phenomenon can only be studied adequately with methods, such as diffusion weighted MRI, that can measure the temporal evolution of LCBF changes following experimental ICH. Interestingly, Ackerman *et al.* (1983) reported the presence of increased cortical blood flow, measured with PET, adjacent to the haematoma in a patient with ICH. Another study in human sufferers of ICH reported recently that, an initially oligaemic zone (measured with SPECT) around an intracerebral haematoma may become hyperaemic subacutely, resulting in reperfusion injury (Mayer *et al.*, 1997).

4. 3. 5. BBB permeability findings

Transport of AIB across the capillary endothelium is the rate-limiting step for the accumulation of this inert, neutral amino acid in normal brain tissue. The back-flux of AIB is minimized by active cellular uptake, and therefore unidirectional transport can be quantified from tissue concentrations of radiolabelled AIB at the end of an experiment (Blasberg *et al.*, 1983). The degree of spatial resolution afforded by this technique, when used in combination with quantitative autoradiography, makes it a particularly useful approach for the study of cerebrovascular permeability following experimental ICH.

No differences were observed in the blood-to-brain transfer constant (k_i) to AIB, in the tissue surrounding the experimental ICH or the contralateral caudate, between non-diabetic and diabetic animals. The presence therefore, of increased oligaemia in the diabetic BB rats in the area surrounding the experimental ICH was not associated with an exacerbated disruption of BBB in the same region. Previous studies in diabetic BB rats have also revealed a relative resistance of the brain to an increased vascular permeability, which was present in extracranial tissues (Williamson *et al.*, 1987). Interestingly also, although acute hyperglycaemia is associated with increased BBB permeability following experimental cerebral ischaemia (Dietrich *et al.*, 1993;

Warner *et al.*, 1987), the presence of chronic hyperglycaemia is not (Sieber *et al.*, 1994).

The absence of any differences in BBB permeability between diabetic and non-diabetic rats does not necessarily prove that there were no differences in oedema surrounding the experimental ICH between those two groups of rats. Since oedema has vasogenic and cytotoxic elements, it is quite possible that the cytotoxic element of oedema was exacerbated in the diabetic animals subjected to ICH. Cell swelling can certainly occur in the presence of hyperglycaemia, which is associated with increased intracellular acidosis when blood flow reaches ischaemic levels to result in anaerobic glycolysis. The reason for this phenomenon is that accumulation of intracellular H^+ activates the membrane-bound Na^+/H^+ transporter. H^+ is extruded but Na^+ accumulates inside the cell in exchange. The sodium pump being out of energy cannot evacuate this excess Na^+ and the cell swells with osmotically attracted water (Siesjö, 1988). Ischaemic blood flow levels were found in the surrounding the experimental ICH tissue in the diabetic rats, and hyperglycaemia occurs frequently in the same animals. The possibility therefore for an increased cellular oedema in the diabetic BB rats cannot be excluded.

There are two methodological considerations applicable to the AIB method used for estimating the intensity of BBB permeability in these studies. First, the k_i of AIB obtained in these studies is dependent upon the perfused capillary vessel surface area. Whether differences were present between non-diabetic and diabetic animals in the perfused surface area on the side of the haematoma could not be established directly. There is certainly evidence arising from the studies of cerebrovascular physiology presented earlier in this thesis that perfusion was lower in the striatum of the diabetic BB rats under physiological conditions. However, perfusion was altered in the lesioned side in both non-diabetic and diabetic rats, according to the results of the LCBF experiments associated with ICH. Therefore, whether perfusion was different in the lesioned side between groups at the area of k_i estimation is not known. Second, the tracer is trapped into functional cells, and this is the reason for no isotope uptake

in the core of the lesion. Whether differences in the intensity of ischaemic cell damage were responsible for a reduced AIB uptake by cells in the diabetic animals is possible. However, upon comparison of the AIB autoradiograms with the neuropathological slides from adjacent sections, it was evident that the tracer accumulated mainly in the border zone between the structurally abnormal area and the normal striatal tissue, where the cells were expected to be equally functional in both groups.

4. 3. 6. The role of endothelin in the development of delayed ischaemia following experimental ICH

ET is a potent vasoconstrictor of cerebral arteries both *in vivo* and *in vitro*, with long-lasting effects (Robinson *et al.*, 1991). Previously published studies reported that factors released from blood promote the bioavailability of ET in a time-dependent manner *in vitro,* reaching a peak at 24h (Ohlstein *et al.*, 1991). This *in vitro* finding was re-enforced by the current studies performed *in vivo*, where in both non-diabetic and diabetic rats intrastriatal injection of blood resulted in an increased volume of hypoperfusion when compared to intrastriatal silicon oil injection. Additional studies in models of focal cerebral ischaemia found that endogenous tissue levels of ET started to rise after 4 hours, and were significantly increased by 24 hours (Barone *et al.*, 1994), and the use of ET-antagonists reduced infarct volume following middle cerebral artery occlusion in normal animals (Barone *et al.*, 1995). These observations led to the testing of the hypotheses that ET was involved in the evolution of the observed delayed perilesional ischaemia following experimental ICH in the rat and was also implicated in the potentiated ischaemic insult observed in the diabetic BB animals. These hypotheses were tested using the potent non-peptide ET-antagonist SB209670 (Ohlstein *et al.*, 1994), employing a treatment protocol which was associated with reduced infarct volume following middle cerebral artery occlusion in normal animals (Barone *et al.*, 1995).

The finding that treatment with SB209670 was associated with significant haemodynamic improvement in the striatum of normal rats subjected to experimental ICH was consistent with an important role for ET in the development of delayed

ischaemia following haemorrhagic stroke. This compound has obviously neuroprotective potential, the testing of which was outwith the purpose of the current experimental design, in which potential mechanisms underlying the pathophysiological sequelae following ICH were studied. In contrast, treatment with SB209670 failed to alter the striatal oligaemia in the diabetic animals subjected to experimental ICH. There are important implications associated with this observation. A large proportion of patients that sustain strokes, both occlusive and haemorrhagic, suffer with diabetes mellitus (Juvela, 1996). The outcome following stroke is also worse in these patients (Jørgensen *et al.*, 1994). Since human trials of potential neuroprotective agents have failed to provide a single agent with proven therapeutic potential (Hsu, 1993), despite the encouraging results obtained from various experimental models of stroke conducted almost exclusively in young healthy animals without background pathology, a potential explanation for this discrepancy may be based on the failure of these experimental strategies to address the potential influence of host diseases upon the pathophysiology of cerebral ischaemia.

There are two points that warrant further consideration in relation to the findings associated with the SB209670 treatment in the diabetic BB rats subjected to experimental ICH. First, MABP in the SB209670-treated diabetic rats at the time of the LCBF experiment (98mmHg) was considerably lower than that of the diabetic animals treated with saline (116mmHg). A similar trend towards MABP reduction following SB209670 treatment was not evident in the non-diabetic rats (Table 19). Although the differences in MABP were not significant, it is impossible to be certain whether MABP had fluctuated during the 24h period from the induction of the haematoma until the LCBF measurement to an extent which might have adversely influenced the outcome. The recorded MABP value in the SB209670-treated rats (98mmHg) was within the normal range but, in the presence of significant oligaemia, cerebral blood flow becomes pressure dependent due to exhaustion of collateral vasodilatory reserve. It is therefore conceivable that even this modest MABP reduction was important in obscuring any beneficial haemodynamic effect of SB209670 in the diabetic rats. Interestingly, in a recent study an endothelin antagonist

failed to alter postischaemic cerebral hypoperfusion following experimental global ischaemia, reportedly because it was associated with decreased MABP (Yasuma *et al.*, 1997). It is unlikely that the rest of the physiological variables (Table 19) could have influenced the LCBF findings. Although pH was elevated in the non-diabetic animals treated with SB209670 in relation to the saline-treated animals, both values were within physiological range, and in addition, a trend of pH towards increase was similarly evident in the diabetic animals, in which no significant effect was achieved with SB209670 treatment upon striatal oligaemia.

The second point for consideration relates to the elevated LCBF in the hemisphere opposite to the haematoma in the diabetic rats treated with SB209670, as compared to the saline-treated animals. The same treatment had no significant effect upon LCBF in the non-diabetic rats subjected to experimental ICH. This finding could be consistent with an increased basal production of ET in the diabetic brain, and therefore, the dose of SB209670 used might have been insufficient to block its effect. However, in view of the MABP findings, a higher dose of this ET-antagonist might have resulted in further decrease in MABP and possibly masked any beneficial effect. It should also be stressed that the acute SB209670 injection had no effect upon LCBF in any of the brain areas examined in the diabetic animals during the pharmacological manipulation of the cerebrovascular endothelium (section 4. 1. 3.), providing evidence against an increased bioavailability of endothelin associated with diabetes mellitus. However, firm conclusions in relation to the bioactivity of ET during IDDM cannot be drawn with this experimental design.

4. 4. Haematoma studies in the hypertensive animals

4. 4. 1. Influence of blood constituents upon perilesional LCBF following experimental ICH

In keeping with the findings in the non-diabetic and diabetic animals, it appeared to be the presence of blood and not the mass effect alone which was responsible for the delayed striatal oligaemia in the SHR rats (Figure 30, Table 23). It is impossible to be absolutely certain however, because invariably endogenous bleeding occurred in all the SHR animals subjected to silicon oil injection (Figure 31) and it was therefore not feasible to investigate adequately the isolated effect of the ICH mass. However, there was a trend in the SHR animals injected with silicon oil to have smaller volumes of striatal hypoperfusion, compared to the animals of the same sub-strain with ICH.

The finding in the WKY animals that silicon oil injections were associated with similar haemodynamic compromise at 24h, compared to the experimental ICHs was rather surprising, in relation to the rest of the experimental groups. An explanation other than that in WKY the isolated mass effect of an ICH results in significant and long-lasting oligaemia cannot be offered. It was surprising to observe that the blood itself had probably no deleterious influences in WKY, contrary to what was found in the SHR and the rest of the experimental groups. One important point that warrants discussion is that the responses to the same insult (intrastriatal silicon oil injection) can be variable between different experimental groups, even in animal strains derived from the same species. Along the same lines, it is equally important to appreciate that control animals (in our case WKY and DR rats of the BB strain) can differ widely upon their vulnerability following an intracranial insult, such as experimental ICH. It is therefore essential to employ the appropriate control animals for each rat strain used for experimental purposes.

4. 4. 2. Influence of chronic hypertension upon LCBF following experimental ICH

The experiments with WKY and SHR rats did not provide evidence in favor of the hypothesis that chronic hypertension is associated with altered pathophysiolgy, in terms of local cerebral perfusion, following experimental ICH. This was another

surprising observation, since SHR rats have been reported to sustain an increased ischaemic burden following experimental occlusive stroke (Coyle, 1984; Grabowski *et al.*, 1988). The single experimental ICH study which was conducted in SHRs reported that the hypertensive animals had greater mortality associated with the haematoma (Gonzàlez-Darder & Duràn-Cabral, 1990), but the volume of blood injected into the animals (400µl, equivalent to almost 25% of the rat's brain) makes it prohibitive for any clinically applicable conclusions to be drawn from it. However, studies in human sufferers of ICH have concluded that hypertensive patients do not have worse outcome *per se*, but because the strokes they sustain are more extensive than in the population at large, there is a misconception that chronic hypertension is associated with worse outcome (Jørgensen *et al.*, 1995). Mizukami and Tawaza measured regional CBF with intracarotid xenon-133 injections in 14 hypertensive patients with ICH in 1983. In areas adjacent to the haematoma CBF was depressed in 13 out of the 14 patients, despite a normal ICP. Studies in human sufferers of ICH however, do not provide clear information whether hypertension predisposes to a larger area of ischaemia following haemorrhagic stroke or whether hypertensive subjects, as stated earlier, have worse outcome because they have larger haemorrhages. Epidemiological studies, in which diabetes mellitus but not chronic hypertension was associated with poor outcome following ICH (Juvela, 1995), support the findings of these experiments, in which delayed perilesional ischaemia following experimental ICH was increased in spontaneously diabetic BB rats but not in spontaneously hypertensive SHR rats.

The susceptibility to stroke in association with chronic hypertension has received attention recently, especially in relation to a presence of a potential gene which is responsible for this susceptibility to stroke (Jacewitz, 1992). The relevance of this information is based upon claims that the susceptibility to infarction in SHR is determined by an unidentified autosomal recessive gene, unrelated to the hypertension itself and sometimes present in the minority of normotensive rats (Jacewitz, 1992). In addition, recent studies from groups supporting this hypothesis, that a specific gene specific for stroke cosegregates with that for hypertension, have shown that such a

gene exists in the SHRSP but not in the SHR (Rubattu *et al.*, 1997). It is also suggested that the impaired endothelium-dependent vasorelaxation in stroke-prone rats is genetically determined and again cosegregates with the stroke-prone phenotype (Volpe *et al.*, 1996). This information is obviously of peripheral importance to the studies presented in this thesis, but might, nevertheless provide an indirect explanation for the relatively large volumes of hypoperfusion in the WKY animals subjected to ICH, as well as the reason that no differences in the LCBF findings were observed between WKY and SHR animals with ICH.

The SHR rat was used as the model of chronic hypertension in these studies, in spite of the above-mentioned genetic findings, since it is the most commonly used model of the disease, and in the absence of a superior model. The SHRSP sub-strain was not used for two reasons. First, the lesions developing in this model are occlusive rather than haemorrhagic and second it would have been rather complicated to define an appropriate control (SHR or WKY) for it. In fact, there are reports in which both the WKY and SHR have been used as controls in the same study (Izuta *et al.*, 1995), so a similar design in the current studies would have been excessively complicated and difficult to analyze statistically. Finally the genetic speculations discussed above would have applied to any findings obtained with this model of hypertension.

4. 4. 3. BBB permeability findings

BBB permeability to AIB was not increased in SHR following ICH. It should be mentioned that, as was the case with the same experimental paradigm in the diabetic groups, oedema *per se* was not addressed in these experiments. Certainly, in experimentally induced occlusive stroke, hypertensive animals have been reported to develop significantly more brain edema, as compared to normotensive ones (Olsson *et al.*, 1989). Whether this is the case following haemorrhagic stroke needs further elucidation, but, it is clear that the vasogenic element of oedema certainly is not different between WKY and SHR.

4. 4. 4. Protective effects of 7-NI

There was clear evidence from the experimental ICH studies conducted in the diabetic groups, that blood had deleterious effects upon the parenchymal cerebrovasculature, in comparison to silicon oil. Similar definitive conclusions could not be reached in the experiments performed in SHR and WKY rats. Although there was a trend in the SHR group for the haematoma to be associated with increased striatal oligaemia, the results obtained from the WKY animals were more inconclusive. There was therefore no factual evidence upon which to base the same hypothesis, in relation to the role of endothelin upon the evolution of ischaemia following ICH, because an equally deleterious effect of blood was not observed. The striking observation during pharmacological manipulation of the cerebrovascular control mechanisms in WKY and SHR was the finding of an upregulated neuronal NO system in the SHR (section 4. 2. 3.). There is extensive recent speculation for a potentially detrimental role of neuronally derived NO, linked to excitotoxicity, following various types of brain insult, including ischaemia (Huang *et al.*, 1994). The role of neuronally-derived NO is rather complex, because following glutamate receptor activation its haemodynamic vasodilatory influence is probably beneficial, whereas its effects upon non-vascular cells are detrimental (Globus *et al.*, 1995).

The experimental protocol with 7-NI treatment following ICH was selected after consideration of the above mentioned aspects. The dose ($25mg.kg^{-1}$) has been shown to be neuroprotective in a model of photothrombotic stroke (Kelly *et al.*, 1994a), which has certain similarities with ICH in view of the presence of blood cell exudates into the brain tissue in areas of thrombosis (Prado *et al.*, 1996). The timing of the administration was also based on the same protocol. In contrast to the findings with the diabetic groups, the WKY (mainly) and the SHR exhibited vulnerability to the mass effect of the haematoma, which obviously presents at the induction of the lesion rather than as a maturation phenomenon, so it was felt that the timing of the injection could be the same with those published protocols.

This treatment protocol was associated with a significant reduction in the volume of presumed perilesional oligaemia in the WKY, at 24h. This finding indirectly re-enforces the observations with the silicon oil injections in the same sub-strain, where the mass effect of the ICH induced hypoperfusion to the surrounding tissue, the onset of which coincides with the induction of the lesion.

The 7-NI treatment was associated with a trend towards haemodynamic improvement in the SHR animals, but the response was less robust and more variable compared to the WKY group. Several potential explanations can be offered for this observation. As was discussed earlier, SHR rats responded to the ICH and silicon oil injections in a way that was probably compatible with the blood *per se* being deleterious to the cerebrovasculature. This influence of the blood upon the cerebrovasculature is a delayed event on the basis of all available scientific information, and therefore the timing of the 7-NI treatment would have been too early to have an effect. Secondly, since nNO is probably overactive in the SHR, this dose of 7-NI might have been ineffective. A higher dose was not used because, as discussed earlier, it had detrimental effects in normal rats (Kelly *et al.*, 1994a), and is sometimes associated with bizarre cerebrovascular phenomena (Kelly *et al.*, 1995b; Wang *et al.*, 1995) so appropriate controls for the SHR would have been difficult to obtain.

Finally, a point that applies to all the results of the ICH experiments is the variability of both the haematoma volumes and the volumes of striatal hypoperfusion. In the diabetic groups, the differences between the volumes of oligaemia in various groups were so large, that despite the variability they were not obscured. Obviously, part of the variability in the responses to the ICH in the diabetic animals can be attributed to the apparent differences in the disease process between individual animals. Even in the non-diabetic animals, where the volumes of hypoperfusion were rather small, a significant difference with the SB209670 treatment was evident. However, in the WKY and SHR groups, where the differences between groups were not so large, the limitations of the model became more obvious.

4. 5. Concluding remarks - Future studies

The results of these studies provided substantial evidence for the presence of cerebrovascular dysfunction, in the form of regionally heterogeneous reduction of basal LCBF linked to an attenuated eNOS activity, in the diabetic BB rats. This alteration in cerebrovascular physiology was associated with increased haemodynamic compromise in the tissue surrounding experimental ICH in this model of IDDM. This information can provide the basis of future studies, in which treatment protocols for correction of the eNOS dysfunction in the BB rats can be tested in relation to their capability to alter basal LCBF and the pathophysiological response to an ICH. Although the reproducibility of the ICH model was not optimal, the diabetic BB rats bore an ischaemic burden more than 3 times larger compared to the non-diabetic DR rats of the same strain. This difference is so large, that the therapeutic potential of future strategies can be tested. Along these lines recent *in vitro* studies have located the deficit in the eNOS activity in extracranial vessels of experimental IDDM, in depletion of tetrahydrobiopterin, a cofactor of eNOS (Pieper, 1997). It will be very interesting to study whether chronic treatment of the BB rats with an exogenous cofactor can reverse *in vivo* the observed cerebrovascular dysfunction. Additionally, if any improvement of this dysfunction was to be established, it would have been possible to investigate whether this was associated with a reduction in the extent of hypoperfusion following ICH. In relation to the perturbation of eNOS in diabetes, chronic inhibition of NOS in the BB rats, similar to that investigated in normotensive animals and in which a selective upregulation of NOS was observed in the brain (Kelly *et al.*, 1995c), could delineate further the extent of dysfunction in cerebrovascular homeostatic reserve of the diabetic animals.

Along the same lines, further insight in a possible pathogenetic role of ET in the vascular dysfunction associated with IDDM could be gained with a modified SB209670 treatment protocol in the studies of cerebrovascular physiology, similar to that used in the ICH studies. Caution ought to be exercised regarding the neuroprotective potential of ET antagonists following haemorrhagic stroke in the rat however. Although treatment with SB209670 was associated with significant

reduction in striatal hypoperfusion in young healthy animals without background pathology, certain points of concern were raised following its use in the diabetic rodents, as discussed earlier. Additionally, other researchers in the field of cerebral ischaemia have recently commented on the relative resistance of rats, in comparison to other species, to the neuroprotective effects of ET antagonists (McCulloch *et al.*, 1996).

The attempt to create a model of controlled diabetes with intensified insulin treatment was not successful. It would have been interesting to ascertain whether such a treatment reversed the observed perturbations in cerebrovascular physiology and pathology. Unfortunately, the only indication provided from this experimental design was that this treatment is associated with increased mortality in this rat model of IDDM, probably resulting from insulin-induced hypoglycaemia.

There is considerable scope for further pathology studies. Firstly, the microvascular neuropathology of the BB rat has not been documented. This requires clarification at ultrastructural and light microscopic levels, using quantitative morphometric techniques to assess thickening of cerebral small vessels. The use of specific antibodies to advanced glycosylation end-products should extend the less specific, traditional histochemical observations. Analysis of a variety of protein and mRNA moieties (for example eNOS, endothelin and various markers of blood-brain barrier integrity) by immunohistochemical and in-situ hybridization techniques, might provide pathological substrates for the observed functional abnormalities of diabetic vessels. Secondly, parallel pathology studies of perfusion-fixed brains in the ICH model are required to better define the nature and extent of perilesional hypoxic-ischaemic brain injury in each group. Traditional morphological criteria could usefully be supplemented by newer methods of assessing early hypoxic-ischaemic neuronal damage. Promising avenues would be analyzing heat-shock and immediate-early gene expression in neurones, as well as loss of dendritic microtubule-associated protein 2 (MAP 2) immunoreactivity (Kitagawa *et al.*, 1989).

IDDM is a risk factor for haemorrhagic stroke, but occlusive strokes are even commoner in diabetic individuals. Although it would require preliminary characterization, a model of occlusive stroke in the BB rats would have been relevant for the investigation of the mechanisms underlying the responses of the spontaneously diabetic rats to focal ischaemia.

The studies of cerebrovascular physiology in the SHR rats provided evidence for presence of cerebrovascular dysfunction in this animal model of chronic hypertension. However, the perturbations were not of the same fundamental nature, as in the diabetic BB rats, to cause alterations in basal LCBF. The likelihood of an upregulated neuronally-derived NO system under physiological conditions having pathological implications was investigated further with the ICH model, but considerable problems were encountered. Endogenous bleeding was invariably present in the SHR rats as a result of trauma from the needle used for the induction of the haematoma. The haemodynamic responses of the control WKY rats were rather surprising for reasons already discussed. Finally, problems associated with variability in both the burden of haemorrhagic insult and the responses to ICH in WKY and SHR became more obvious. It is appreciated that chronic hypertension is the commonest risk factor for ICH, but the limitations of this model of haemorrhagic stroke that were outlined above make design of future studies based on this approach rather problematic.

APPENDIX

Table 1

Physiological variables of non-diabetic and diabetic rats treated acutely with saline (0.5ml i.v.), L-NAME (30mg.kg^{-1}, i.v.) or SB209670 (10mg.kg^{-1}, i.v.), just before the LCBF measurement (20min after the injection)

	Non-diabetic			***Diabetic***			
	Saline	*L-NAME*		*Saline*	*L-NAME*		*SB209670*
pCO_2 (mmHg)	41.1 ± 0.7	35.3 ± 1.7		42.9 ± 0.6	40.8 ± 0.9		43.2 ± 1.7
pO_2 (mmHg)	88.5 ± 2.5	100.1 ± 1.9		88.5 ± 1.4	92.1 ± 4.4		90.9 ± 0.8
pH	7.425 ± 0.005	7.379 ± 0.018		7.413 ± 0.026	7.393 ± 0.009		7.462 ± 0.005
MABP (mmHg)	115 ± 5	142 ± 3	#	109 ± 3	149 ± 4	#	118 ± 4
Heart Rate (bpm)	395 ± 29	293 ± 8		315 ± 25	255 ± 15		350 ± 26
Plasma glucose (mg.dl^{-1})	144 ± 8	251 ± 87		284 ± 65	131 ± 56		222 ± 40
Temperature (°C)	36.9 ± 0.4	36.5 ± 0.2		36.6 ± 0.3	36.4 ± 0.2		36.0 ± 0.3
Haematocrit (%)	n.d.	49 ± 1.7		n.d.	52.5 ± 1.0		54.7 ± 1.3
Plasma bicarbonate	27.0 ± 0.3	21.0 ± 1.0		27.3 ± 1.4	25.0 ± 0.8		29.4 ± 0.6
Base excess	3.0 ± 0.2	-3.1 ± 1.1		2.8 ± 1.8	0.3 ± 0.8		5.5 ± 0.7

Data are presented as mean ± s.e.mean (n = 6 in saline-treated groups, n = 4 in L-NAME and SB209670-treated groups)

n.d.: not determined

#: significant difference between saline and L-NAME or SB209670-treated animals of the same sub-strain.

Table 2

Physiological variables in non-diabetic and diabetic rats injected acutely with sesame oil (0.6ml, i.p.) or 7-NI (25mg.kg^{-1} i.p.), just before the LCBF experiment (40min after the injection)

	Non-diabetic		***Diabetic***		
	Sesame oil	*7-NI*	*Sesame oil*	*7-NI*	
pCO_2 (mmHg)	40.8 ± 0.4	41.3 ± 0.6	42.7 ± 1.0	40.1 ± 0.9	
pO_2 (mmHg)	91.0 ± 2.0	93.9 ± 1.8	89.9 ± 1.2	97.4 ± 4.6	
pH	7.427 ± 0.004	7.415 ± 0.005	7.393 ± 0.030	7.455 ± 0.008	
MABP (mmHg)	117 ± 7	122 ± 2	109 ± 2	115 ± 1	
Heart Rate (bpm)	413 ± 43	291 ± 12	349 ± 30	208 ± 8	#*
Plasma glucose (mg.dl^{-1})	152 ± 9	167 ± 6	283 ± 92	254 ± 62	
Temperature (°C)	37.2 ± 0.5	36.8 ± 0.4	36.6 ± 0.2	35.9 ± 0.3	
Haematocrit (%)	n.d.	52.6 ± 0.7	n.d.	52.3 ± 1.6	
Plasma bicarbonate	26.8 ± 0.5	26.5 ± 0.6	26.2 ± 1.3	28.4 ± 0.8	
Base excess	3.0 ± 0.3	2.3 ± 0.5	1.4 ± 1.8	4.5 ± 0.7	

Data are presented as mean ± s.e.mean (n = 4 in each group)

n.d.: not determined

#: significant difference between sesame oil and 7-NI-treated rats of the same sub-strain.

*: significant difference between non-diabetic and diabetic rats with the same treatment

Table 3

Physiological variables in non-diabetic and diabetic rats treated acutely with saline ($40\mu l.min^{-1}$, i.v. infusion) or SIN-1 ($1.8mg.kg^{-1}.h^{-1}$, i.v. infusion), just before the LCBF experiment (20min after the onset of the infusion)

	Non-diabetic		***Diabetic***		
	Saline	*SIN-1*	*Saline*	*SIN-1*	
pCO_2 (mmHg)	41.4 ± 1.0	41.5 ± 1.9	44.0 ± 0.5	38.1 ± 0.7	#
pO_2 (mmHg)	88.8 ± 4.2	98.6 ± 3.5	88.0 ± 1.8	80.1 ± 3.1	*
pH	7.424 ± 0.008	7.425 ± 0.012	7.396 ± 0.038	7.557 ± 0.016	#*
MABP (mmHg)	125 ± 9	100 ± 5	115 ± 7	97 ± 4	
Heart Rate (bpm)	383 ± 23	389 ± 33	319 ± 36	377 ± 27	
Plasma glucose ($mg.dl^{-1}$)	198 ± 58	204 ± 19	252 ± 61	512 ± 101	
Temperature (°C)	36.6 ± 0.1	37.5 ± 0.2 #	36.6 ± 0.4	35.8 ± 0.3	*
Haematocrit (%)	n.d.	50.2 ± 0.2	n.d.	51.8 ± 0.7	
Plasma bicarbonate	27.2 ± 0.4	26.6 ± 0.7	26.9 ± 2.1	34.3 ± 1.1	*
Base excess	3.0 ± 0.4	2.6 ± 0.4	2.0 ± 2.7	11.8 ± 1.3	#*

Data are presented as mean ± s.e.mean (n = 4 in each group)

n.d.: not determined

#: significant difference between saline and SIN-1-treated animals of the same sub-strain.

*: significant difference between non-diabetic and diabetic animals with the same treatment.

Table 4

Physiological variables in conventionally treated diabetic rats (with a single subcutaneous injection of insulin) and in rats with implanted insulin rods, providing continuous subcutaneous insulin delivery, just before the LCBF measurement

	Conventional insulin treatment	*CSII*
pCO_2 (mmHg)	42.7 ± 0.9	44.0 ± 1.2
pO_2 (mmHg)	88.8 ± 1.6	89.4 ± 2.1
pH	7.414 ±0.027	7.404 ± 0.030
MABP (mmHg)	109 ± 1	112 ± 4
Heart Rate (bpm)	323 ± 33	327 ± 20
Plasma glucose ($mg.dl^{-1}$)	284 ± 92	285 ± 65
Temperature (°C)	36.8 ± 0.3	36.4 ± 0.3
Haematocrit (%)	n.d.	51.3 ± 1.2
Plasma bicarbonate	27.4 ± 1.4	27.3 ± 1.4
Base excess	3.0 ± 1.8	2.3 ± 1.8
Glycosylated Hb	n.d.	8.8 ± 0.6

Data are presented as mean ± s.e.mean, n = 4 in conventional insulin treatment and n = 7 in CSII.

n.d.: not determined

Table 5

LCBF ($ml.100g^{-1}.min^{-1}$) data in non-diabetic and diabetic rats treated acutely with saline (0.5ml, i.v.), L-NAME ($30mg.kg^{-1}$, i.v.) or SB209670 ($10mg.kg^{-1}$, i.v.)

	Non-diabetic		***Diabetic***		
	Saline	*L-NAME*	*Saline*	*L-NAME*	*SB209670*
Neocortex					
Parietal	156 ± 7	131 ± 7	134 ± 12	115 ± 6	140 ± 12
Cingulate	147 ±7	120 ± 8	127 ± 16	113 ± 13	107 ± 4
Corpus Callosum	39 ± 2	27 ± 1 #	32 ± 1 *	25 ± 4	29 ± 1
Basal Ganglia					
Striatum	125 ± 4	93 ± 2 #	86 ± 3 *	84 ± 9	100 ± 5
Globus Pallidus	73 ± 1	50 ± 2 #	53 ± 1 *	47 ± 5	57 ± 3
Accumbens	130 ± 9	98 ± 4	107 ± 6	89 ± 10	113 ± 9
Thalamus					
Hypothalamus	94 ± 3	59 ± 5 #	80 ± 6	54 ± 5 #	79 ± 9
Lateral geniculate	141 ± 5	84 ± 2 #	106 ± 4 *	86 ± 10	110 ± 11
Hippocampus					
CA 2,3	93 ± 3	60 ± 3 #	72 ± 6 *	66 ± 9	82 ± 6
Dentate Hilus	89 ± 3	59 ± 2 #	69 ± 4 *	67 ± 10	72 ± 5

Data are presented as mean ± s.e.mean (n = 6 in each saline-treated group, n = 4 in L-NAME and SB209670-treated groups)

*: significant difference between non-diabetic and diabetic animals treated with saline

#: significant difference between saline and L-NAME or SB209670-treated animals of the same sub-strain

Table 6

LCBF ($ml.100g^{-1}.min^{-1}$) data in non-diabetic and diabetic rats injected acutely with sesame oil (0.6ml, i.p.) or 7-NI ($25mg.kg^{-1}$ i.p.)

	Non-diabetic			***Diabetic***			
	Sesame oil	*7-NI*		*Sesame oil*		*7-NI*	
Neocortex							
Parietal	164 ± 8	110 ± 5	#	137 ± 11		106 ± 5	
Cingulate	150 ±10	72 ± 4	#	131 ± 15		77 ± 5	#
Corpus Callosum	41 ± 3	19 ± 3	#	33 ± 1		22 ± 2	#
Basal Ganglia							
Striatum	129 ± 4	72 ± 3	#	91 ± 1	*	69 ± 4	#
Globus Pallidus	73 ± 1	45 ± 4	#	56 ± 1	*	42 ± 4	#
Accumbens	135 ± 13	64 ± 5	#	110 ± 4		60 ± 4	#
Thalamus							
Hypothalamus	92 ± 3	41 ± 3	#	81 ± 6		38 ± 2	#
Lateral geniculate	146 ± 6	73 ± 6	#	111 ± 6	*	72 ± 5	#
Hippocampus							
CA 2,3	91 ± 5	46 ± 5	#	74 ± 7		46 ± 3	#
Dentate Hilus	90 ± 4	49 ± 5	#	73 ± 3	*	47 ± 2	#

Data are presented as mean ± s.e.mean (n = 4 in each group)

*: significant difference between diabetic and non-diabetic treated with sesame oil

#: significant difference between sesame oil and 7-NI-treated animals of the same sub-strain

Table 7

LCBF (ml.100g^{-1}.min^{-1}) data in non-diabetic and diabetic rats treated acutely with saline (40µl.min^{-1}, i.v. infusion) or SIN-1 (1.8mg.kg^{-1} .h^{-1}, i.v. infusion)

	Non-diabetic		***Diabetic***	
	Saline	*SIN-1*	*Saline*	*SIN-1*
Neocortex				
Parietal	149 ± 7	211 ± 13 #	134 ± 5	269 ± 3 #*
Cingulate	135 ±7	164 ± 14	121 ± 7	159 ± 1 #
Corpus Callosum	40 ± 3	40 ± 5	32 ± 1	42 ± 4
Basal Ganglia				
Striatum	120 ± 3	130 ± 4	82 ± 3 *	107 ± 4 #*
Globus Pallidus	73 ± 2	80 ± 5	51 ± 3 *	63 ± 2 #*
Accumbens	117 ± 2	156 ± 12 #	103 ± 5	143 ± 1 #
Thalamus				
Hypothalamus	92 ± 5	111 ± 15	80 ± 3	78 ± 4
Lateral geniculate	133 ± 3	160 ± 8 #	108 ± 7 *	148 ± 7 #
Hippocampus				
CA 2,3	91 ± 4	103 ± 2	70 ± 6	91 ± 5
Dentate Hilus	84 ± 4	106 ± 2 #	65 ± 4 *	99 ± 4 #

Data are presented as mean ± s.e.mean (n = 4 in each group)

*: significant difference between non-diabetic and diabetic rats infused with i.v. saline or SIN-1

#: significant difference between saline and SIN-1-treated animals of the same sub-strain

Table 8

LCBF ($ml.100g^{-1}.min^{-1}$) data in conventionally treated diabetic rats (with a single daily subcutaneous injection of insulin) and in rats with implanted insulin rods, providing continuous subcutaneous insulin delivery

	Conventional insulin treatment	*CSII*
Basal Ganglia		
Striatum	87 ± 3	102 ± 9
Globus Pallidus	54 ± 3	59 ± 3
Thalamus		
Lateral geniculate	109 ± 6	122 ± 8
Hippocampus		
CA 2,3	74 ± 7	72 ± 4
Dentate Hilus	72 ± 4	70 ± 3

Data are presented as mean ± s.e.mean, n = 4 in the conventionally treated animals and n = 7 in the CSII.

Table 9

Physiological variables of WKY and SHR rats treated acutely with saline (0.5ml i.v.), L-NAME ($30mg.kg^{-1}$, i.v.) or SB209670 ($10mg.kg^{-1}$, i.v.), just before the LCBF measurement (20min after the injection)

	WKY		***SHR***		
	Saline	*L-NAME*	*Saline*	*L-NAME*	*SB209670*
pCO_2 (mmHg)	41.6 ± 1.8	4 0.3 ± 1.3	39.3 ± 1.8	37.7 ± 1.5	41.0 ± 0.4
pO_2 (mmHg)	89.3 ± 3.4	90.6 ± 4.2	91.4 ± 2.1	86.8 ± 1.0	88.1 ± 1.6
pH	7.487 ±0.010	7.465 ± 0.001	7.475 ± 0.006	7.463 ± 0.006	7.444 ± 0.007
MABP (mmHg)	120 ± 3	152 ± 3 #	175 ± 6	* 207 ± 1	#* 171 ± 8
Heart Rate (bpm)	289 ± 8	210 ± 12 #	382 ± 19	* 323 ± 23	* 355 ± 17
Plasma glucose ($mg.dl^{-1}$)	142 ± 10	135 ± 9	140 ± 13	109 ± 4	126 ± 6
Temperature (°C)	36.4 ± 0.2	36.6 ± 0.4	36.7 ± 0.2	36.7 ± 0.0	37.4 ± 0.1
Haematocrit (%)	49.4 ± 0.6	50.9 ± 1.5	51.6 ± 0.4	* 51.5 ± 0.9	52.5 ± 0.8
Plasma bicarbonate	30.5 ± 0.7	28.3 ± 0.7	28.5 ± 0.9	26.8 ± 0.6	27.3 ± 0.5
Base excess	6.6 ± 0.7	4.2 ± 0.8	4.4 ± 1.0	2.7 ± 0.6	3.2 ± 0.5

Data are presented as mean ± s.e.mean (n = 6 in WKY and n = 5 in SHR saline groups, n = 4 in each other group)

*: significantly different between WKY and SHR with the same treatment

#: significant difference between saline and L-NAME or SB209670 treatment in the same sub-strain

Table 10

Physiological variables in WKY and SHR rats injected acutely with sesame oil (0.6ml, i.p.) or 7-NI ($25mg.kg^{-1}$ i.p.), just before the LCBF experiment (40min after the injection)

	WKY		***SHR***	
	Sesame oil	*7-NI*	*Sesame oil*	*7-NI*
pCO_2 (mmHg)	41.5 ± 2.3	40.0 ± 0.7	38.6 ± 2.1	36.7 ± 0.4
pO_2 (mmHg)	91.5 ± 3.2	97.8 ± 3.8	91.1 ± 2.6	89.2 ± 0.7
pH	7.490 ± 0.011	7.479 ± 0.007	7.481 ± 0.001	7.462 ± 0.009
MABP (mmHg)	119 ± 3	123 ± 3	173 ± 8 *	180 ± 6 *
Heart Rate (bpm)	287 ± 10	280 ± 7	377 ± 24 *	300 ± 11
Plasma glucose ($mg.dl^{-1}$)	142 ± 12	170 ± 12	130 ± 10	146 ± 13
Temperature (°C)	36.5 ± 0.1	37.1 ± 0.2	36.9 ± 0.2	36.5 ± 0.1
Haematocrit (%)	49.3 ± 0.7	50.9 ± 1.5	51.4 ± 0.3	52.2 ± 2.2
Plasma bicarbonate	30.8 ± 0.8	29.2 ± 0.7	28.6 ± 1.2	26.3 ± 0.6
Base excess	6.9 ± 0.8	5.2 ± 0.7	4.5 ± 1.3	2.1 ± 0.7

Data are presented as mean ± s.e.mean (n = 5 in the WKY rats treated with sesame oil, n = 4 in each other group)

*: significant difference between WKY and SHR with the same treatment

Table 11

Physiological variables in WKY and SHR rats treated acutely with saline ($40\mu l.min^{-1}$, i.v. infusion) low SIN-1 ($0.54mg.kg^{-1}.h^{-1}$, i.v. infusion) or high SIN-1 ($1.8mg.kg^{-1}.h^{-1}$, i.v. infusion) dose, just before the measurement of LCBF (20min after the onset of the infusion)

	WKY			*SHR*		
	Saline	*low SIN-1*	*high SIN-1*	*Saline*	*low SIN-1*	*high SIN-1*
pCO_2 (mmHg)	41.1 ± 2.2	38.4 ± 2.0	37.9 ± 1.1	41.0 ± 0.6	37.9 ± 1.0	40.4 ± 1.3
pO_2 (mmHg)	90.1 ± 4.1	85.5 ± 1.0	94.6 ± 2.7	90.1 ± 2.1	90.2 ± 3.0	97.4 ± 1.0
pH	7.488 ± 0.012	7.476 ± 0.007	7.478 ± 0.005	7.474 ± 0.008	7.461 ± 0.007	7.443 ± 0.004
MABP (mmHg)	120 ± 4	96 ± 8	95 ± 6 #	179 ± 6 *	147 ± 2 #*	104 ± 5 #
Heart Rate (bpm)	291 ± 9	287 ± 20	303 ± 3	370 ± 20*	410 ± 24 *	338 ± 16
Plasma glucose ($mg.dl^{-1}$)	146 ± 11	219 ± 46	219 ± 26	144 ± 16	146 ± 7	175 ± 17
Temperature (°C)	36.3 ± 0.2	35.8 ± 0.2	37.0 ± 0.2	36.7 ± 0.2	37.4 ± 0.1	37.3 ± 0.3
Haematocrit (%)	49.3 ± 0.7	49.7 ± 0.3	49.9 ± 1.1	51.6 ± 0.4	50.9 ± 0.5	52.3 ± 1.5
Plasma bicarbonate	30.3 ± 0.9	27.9 ± 0.6	28.0 ± 0.9	29.3 ± 0.4	26.9 ± 0.5	27.0 ± 0.8
Base excess	6.5 ± 0.9	3.8 ± 0.7	3.9 ± 1.0	5.4 ± 0.5	2.7 ± 0.6	2.8 ± 0.8

Data are presented as mean ± s.e.mean (n = 5 in saline-treated WKY, n = 4 in all other groups)

*: significant difference between WKY and SHR following the same treatment

#: significant difference between saline and SIN-1 treatment in animals of the same sub-strain

Table 12

LCBF ($ml.100g^{-1}.min^{-1}$) data in WKY and SHR rats treated acutely with saline (0.5ml, i.v.), L-NAME ($30mg.kg^{-1}$, i.v.) or SB209670 ($10mg.kg^{-1}$, i.v.)

	WKY		***SHR***		
	Saline	*L-NAME*	*Saline*	*L-NAME*	*SB209670*
Neocortex					
Parietal	141 ± 6	124 ± 7	132 ± 8	105 ± 6	158 ± 5
Cingulate	98 ± 4	99 ± 5	117 ± 6	91 ± 6 #	127 ± 23
Corpus Callosum	37 ± 2	20 ± 1 #	32 ± 2	29 ± 2 *	34 ± 6
Basal Ganglia					
Striatum	99 ± 4	87 ± 4	94 ± 5	83 ± 5	110 ± 14
Globus Pallidus	64 ± 4	53 ± 3	68 ± 8	54 ± 3	78 ± 12
Accumbens	98 ± 4	78 ± 5 #	116 ± 3 *	82 ± 5 #	117 ± 13
Thalamus					
Hypothalamus	75 ± 4	49 ± 3 #	83 ± 5	45 ± 3 #	65 ± 3
Lateral geniculate	106 ± 5	89 ± 6	116 ± 9	93 ± 5	126 ± 9
Hippocampus					
CA 3	76 ± 4	60 ± 1 #	85 ± 3	63 ± 4 #	84 ± 2
CA 2	71 ± 3	56 ± 2 #	83 ± 4	59 ± 5 #	82 ± 8
CA 1	68 ± 5	56 ± 1	74 ± 3	61 ± 3	90 ± 11
Molecular layer	73 ± 4	57 ± 1 #	85 ± 2	61 ± 5 #	87 ± 5
Dentate Hilus	76 ± 4	61 ± 1 #	93 ± 6	62 ± 5 #	85 ± 9

Data are presented as mean ± s.e.mean (n = 6 in WKY and n = 5 in SHR saline-treated groups, n = 4 in L-NAME and SB209670-treated groups)

*: significant difference between WKY and SHR

#: significant difference between saline and L-NAME or SB209670

Table 13

LCBF ($ml.100g^{-1}.min^{-1}$) data in WKY and SHR rats injected acutely with sesame oil (0.6ml, i.p.) or 7-NI ($25mg.kg^{-1}$ i.p.)

	WKY		***SHR***	
	Sesame oil	*7-NI*	*Sesame oil*	*7-NI*
Neocortex				
Parietal	143 ± 7	121 ± 5	138 ± 7	78 ± 4 #*
Cingulate	98 ± 5	82 ± 1	120 ± 7	64 ± 6 #
Corpus Callosum	38 ± 2	21 ± 1 #	33 ± 3	21 ± 1 #
Basal Ganglia				
Striatum	100 ± 4	78 ± 3 #	97 ± 5	59 ± 5 #*
Globus Pallidus	66 ± 3	49 ± 2 #	72 ± 9	38 ± 1 #*
Accumbens	99 ± 4	77 ± 5 #	119 ± 2 *	56 ± 1 #*
Thalamus				
Hypothalamus	77 ± 4	50 ±3 #	87 ± 4	36 ± 3 #*
Lateral geniculate	110 ± 4	87 ± 3 #	123 ± 8	69 ± 2 #*
Hippocampus				
CA 3	78 ± 4	57 ± 2 #	88 ± 2	49 ± 3 #
CA 2	72 ± 4	51 ± 1 #	85 ± 4	45 ± 2 #
CA 1	71 ± 4	49 ± 2 #	76 ± 2	44 ± 2 #
Molecular layer	75 ± 4	55 ± 3 #	87 ± 2	45 ± 2 #
Dentate Hilus	78 ± 5	58 ± 3 #	96 ± 7	46 ± 1 #*

Data are presented as mean ± s.e.mean. (n = 5 in WKY treated with sesame oil, n = 4 in all other groups)

*: significantly different between WKY and SHR with the same treatment

#: significant difference between sesame oil and 7-NI - treated rats of the same sub-strain

Table 14

LCBF ($ml.100g^{-1}.min^{-1}$) data in WKY and SHR rats treated acutely with saline ($40\mu l.min^{-1}$, i.v. infusion) low SIN-1 ($0.54mg.kg^{-1}.h^{-1}$, i.v. infusion) or high SIN-1 ($1.8mg.kg^{-1}.h^{-1}$, i.v. infusion) dose, 20min after the onset of the infusion

	WKY			***SHR***		
	Saline	*low SIN-1*	*high SIN-1*	*Saline*	*low SIN-1*	*high SIN-1*
Neocortex						
Parietal	138 ± 6	139 ± 12	206 ± 9 #	127 ± 9	142 ± 10	148 ± 11*
Cingulate	95 ± 3	152 ± 7 #	171 ± 6 #	112 ± 5	143 ± 5 #	138 ± 5 *
Corpus Callosum	35 ± 2	29 ± 3	36 ± 3	32 ± 3	29 ± 1	41 ± 2
Basal Ganglia						
Striatum	96 ± 3	117 ± 5 #	129 ± 6 #	90 ± 4	109 ± 8	107 ± 9
Globus Pallidus	62 ± 4	90 ± 2 #	80 ± 4	61 ± 4	82 ± 4 #	66 ± 4
Accumbens	96 ± 4	136 ± 7 #	153 ± 4 #	114 ± 4	138 ± 11	130 ± 8
Thalamus						
Hypothalamus	71 ± 3	79 ± 5	89 ± 4	80 ± 4	75 ± 7	70 ± 5
Lateral Geniculate	103 ± 5	138 ± 6 #	164 ± 7 #	111 ± 10	139 ± 14	118 ± 8 *
Hippocampus						
CA 3	74 ± 4	98 ± 4 #	104 ± 3#	83 ± 3	100 ± 8	81 ± 9
CA 2	68 ± 3	82 ± 7	92 ± 5 #	80 ± 3	81 ± 9	82 ± 5
CA 1	65 ± 4	80 ± 6	100 ± 5 #	73 ± 3	81 ± 6	76 ± 5 *
Molecular Layer	71 ± 4	95 ± 4 #	103 ± 3 #	84 ± 2	88 ± 8	82 ± 4 *
Dentate Hilus	73 ± 5	94 ± 5 #	112 ± 6 #	90 ± 5	89 ± 9	87 ± 4 *

Data are presented as mean ± s.e.mean (n = 5, in WKY rats treated with saline, n = 4 in all other groups)

*, significant difference between WKY and SHR animals given the same treatment

#, significantly different from saline-treated rats of the same sub-strain

Table 15

Physiological variables during induction of experimental ICH in animals anaesthetized with pentobarbitone (30-45mg.kg^{-1} i.p.), placed on a thermal blanket and receiving supplementary oxygen (4lt.min^{-1}) through a face mask (see also section 2. 16.)

	Non-diabetic		*Diabetic*	
	15 min pre-induction	*15 min post-induction*	*15 min pre-induction*	*15 min post-induction*
pCO_2 (mmHg)	44.5 ± 1.3	44.1 ± 1.3	44.4 ± 2.6	44.7 ± 3.0
pO_2 (mmHg)	80.3 ± 2.8	80.7 ± 2.6	80.2 ± 1.6	80.4 ± 2.5
pH	7.354 ± 0.021	7.353 ± 0.030	7.419 ± 0.016	7.419 ± 0.015
MABP (mmHg)	102 ± 4	112 ± 7	100 ± 4	110 ± 4
Heart Rate (bpm)	n.d.	n.d.	n.d.	n.d.
Plasma glucose (mg.dl^{-1})	142 ± 10	141 ± 6	230 ± 49	225 ± 74
Temperature (°C)	36.1 ± 0.1	36.2 ± 0.4	36.0 ± 0.2	36.1 ± 0.2
Haematocrit (%)	n.d.	n.d.	n.d.	n.d.
Plasma bicarbonate	25.8 ± 1.4	25.4 ± 1.3	28.8 ± 0.5	29.5 ± 1.0
Base excess	-1.2 ± 1.4	-1.5 ± 1.7	3.5 ± 1.5	3.5 ± 1.0

Data are presented as mean ± s.e.mean (n = 6 in non-diabetic and n = 4 in diabetic group)

n.d.: not determined

Table 16

Physiological variables in non-diabetic and diabetic rats subjected to intrastriatal injection of blood or silicon oil (50µl), at the time of the LCBF experiment (24h after the blood or silicon oil injection)

	Non diabetic		***Diabetic***		
	Haematoma	*Silicon oil*	*Haematoma*	*Silicon oil*	
pCO_2 (mmHg)	39.6 ± 1.0	40.5 ± 1.2	39.9 ± 1.9	43.3 ± 0.4	
pO_2 (mmHg)	89.4 ± 2.0	85.1 ± 1.7	89.5 ± 1.7	92.1 ± 3.0	
pH	7.378 ± 0.017	7.422 ± 0.008	7.366 ± 0.038	7.304 ± 0.015	*
MABP (mmHg)	120 ± 3	120 ± 4	118 ± 7	110 ± 5	
Heart Rate (bpm)	428 ± 11	426 ± 22	288 ± 11 *	321 ± 21	*
Plasma glucose ($mg.dl^{-1}$)	176 ± 16	170 ± 10	144 ± 45	251 ± 77	
Temperature (°C)	37.2 ± 0.2	37.4 ± 0.4	37.0 ± 0.2	36.2 ± 0.1	*#
Haematocrit (%)	48.2 ± 1.2	47.3 ± 1.7	48.6 ± 0.9	48.8 ± 0.5	
Plasma bicarbonate	23.4 ± 1.2	26.2 ± 0.9	23.0 ± 1.9	21.7 ± 0.8	*
Base excess	-1.0 ± 1.3	2.5 ± 0.9	-1.9 ± 2.2	-4.7 ± 1.0	*

Data are presented as mean ± s.e.mean (n = 6 in each haematoma and n = 5 in each silicon oil group)

*: significant difference between non-diabetic and diabetic groups receiving the same treatment

#: significant difference between haematoma and silicon oil groups in animals of the same sub-strain.

Table 17

Volumes of striatal oligaemia (in mm^3) in non-diabetic and diabetic animals injected with blood or silicon oil (50µl), measured 24h after the injection

	Non diabetic		***Diabetic***		
LCBF ($ml.100g^{-1}.min^{-1}$)	*Haematoma*	*Silicon oil*	*Haematoma*	*Silicon oil*	
less than 15	0.11 ± 0.05	0.57 ± 0.37	1.91 ± 0.90	0.12 ± 0.04	
between 15 and 25	0.24 ± 0.07	0.07 ± 0.02	1.44 ± 0.24	* 0.05 ± 0.02	#
less than 25	0.35 ± 0.12	0.64 ± 0.38	3.35 ± 1.05	* 0.17 ± 0.05	
between 25 and 35	0.40 ± 0.03	0.11 ± 0.03	# 1.20 ± 0.25	* 0.14 ± 0.06	#
between 15 and 35	0.64 ± 0.10	0.18 ± 0.06	# 2.64 ± 0.44	* 0.19 ± 0.06	#
less than 35	0.75 ± 0.15	0.75 ± 0.41	4.55 ± 1.30	* 0.31 ± 0.08	#

Data are presented as mean ± s.e.mean (n = 6 in each haematoma and n = 5 for each silicon oil group)

*: significant difference between non diabetic and diabetic groups with the same injection (blood or silicon oil)

#: significant difference between haematoma and silicon oil groups in animals of the same sub-strain.

Table 18

Physiological variables in non-diabetic and diabetic rats subjected to intrastriatal injection of blood (50µl), at the time of the AIB experiment (24h after the blood injection)

	Non diabetic	***Diabetic***
	Haematoma	*Haematoma*
pCO_2 (mmHg)	38.7 ± 1.4	39.9 ± 1.7
pO_2 (mmHg)	86.3 ± 2.9	85.1 ± 4.0
pH	7.353 ±0.021	7.362 ± 0.018
MABP (mmHg)	113 ± 3	112 ± 2
Heart Rate (bpm)	414 ± 30	334 ± 32
Plasma glucose ($mg.dl^{-1}$)	138 ± 9	207 ± 64
Temperature (°C)	37.0 ± 0.2	36.2 ± 0.2 *
Haematocrit (%)	47.7 ± 1.8	49.5 ± 1.2
Plasma bicarbonate	21.6 ± 1.4	22.9 ± 0.6
Base excess	-3.1 ± 1.6	-2.3 ± 0.7

Data are presented as mean ± s.e.mean (n = 5 in each group)

*: significant difference between non diabetic and diabetic groups

Table 19

Physiological variables in non-diabetic and diabetic rats subjected to intrastriatal injection of blood (50µl), and treated with either saline (i.p.) or SB209670 (10mg.kg^{-1} i.p. every 6h, starting 30min prior to injection of blood and continuing for 24h) at the time of the LCBF experiment (24h after the blood injection)

	Non diabetic haematoma		***Diabetic haematoma***	
	Saline	*SB209670*	*Saline*	*SB209670*
pCO_2 (mmHg)	39.2 ± 1.1	41.3 ± 0.9	40.1 ± 2.3	44.6 ± 1.4
pO_2 (mmHg)	89.7 ± 2.3	91.3 ± 1.9	90.2 ± 1.8	88.1 ± 2.5
pH	7.376 ±0.011	7.453 ± 0.011 #	7.363 ± 0.047	7.469 ± 0.007
MABP (mmHg)	117 ± 2	115 ± 4	116 ± 9	98 ± 6
Heart Rate (bpm)	425 ± 13	425 ± 9	291 ± 12	285 ± 19
Plasma glucose (mg.dl^{-1})	161 ± 6	169 ± 18	158 ± 10	72 ± 10
Temperature (°C)	37.3 ± 0.3	37.0 ± 0.2	37.0 ± 0.2	36.6 ± 0.4
Haematocrit (%)	47.8 ± 1.4	45.4 ± 1.3	48.4 ± 1.0	46.5 ± 1.4
Plasma bicarbonate	23.0 ± 1.3	28.0 ± 0.9 #	23.0 ± 2.3	32.5 ± 0.8 #
Base excess	-1.3 ± 1.6	4.0 ± 1.0 #	-2.0 ± 2.7	8.4 ± 0.5 #

Data are presented as mean ± s.e.mean (n = 6 in non diabetics treated with SB209670, n = 5 in each other group)

#: significant difference between saline and SB209670-treated animals of the same sub-strain

Table 20

Volumes of striatal oligaemia (in mm^3) in non-diabetic and diabetic animals injected with blood (50µl) and treated with saline or SB209670 (10mg.kg^{-1} i.p. every 6h, starting 30min prior to the injection of blood and continuing for 24h)

	Non-diabetic haematoma		***Diabetic haematoma***	
LCBF (ml.100g^{-1}.min^{-1})	*Saline*	*SB209670*	*Saline*	*SB209670*
less than 15	0.13 ± 0.06	0.0015 ± 0.0001	2.27 ± 1.03	1.30 ± 0.53
between 15 and 25	0.24 ± 0.08	0.013 ± 0.007 #	1.50 ± 0.26	2.10 ± 0.66
less than 25	0.37 ± 0.15	0.01 ± 0.006 #	3.77 ± 1.20	3.40 ± 1.17
between 25 and 35	0.43 ± 0.02	0.08 ± 0.05 #	1.34 ± 0.26	1.74 ± 0.48
between 15 and 35	0.67 ± 0.11	0.10 ± 0.06 #	2.85 ± 0.47	3.85 ± 1.10
less than 35	0.80 ± 0.17	0.10 ± 0.05 #	5.11 ± 1.44	5.14 ± 1.58

Data are presented as mean ± s.e.mean (n = 6 in non-diabetics treated with SB209670, n = 5 in each other group)

#: significant difference between saline and SB209670-treated animals of the same sub-strain

Table 21

Physiological variables during induction of experimental ICH in animals anaesthetized with pentobarbitone (30-45mg.kg^{-1} i.p.), placed on a thermal blanket and receiving supplementary oxygen (4lt/min) through a face mask (see also section 2. 16.)

	WKY		***SHR***	
	15min	*15 min*	*15min*	*15 min*
	pre-induction	*post-induction*	*pre-induction*	*post-induction*
pCO_2 (mmHg)	45.5 ± 0.5	44.7 ± 2.1	44.9 ± 1.9	44.7 ± 2.0
pO_2 (mmHg)	79.5 ± 1.6	81.5 ± 1.2	80.1 ± 1.2	80.9 ± 1.9
pH	7.455 ±0.020	7.457 ± 0.030	7.447 ± 0.016	7.462 ± 0.015
MABP (mmHg)	105 ± 5	115 ± 6	143 ± 7	159 ± 6
Heart Rate (bpm)	n.d.	n.d.	n.d.	n.d.
Plasma glucose (mg.dl^{-1})	159 ± 11	170 ± 12	168 ± 9	175 ± 6
Temperature (°C)	35.9 ± 0.3	36.1 ± 0.3	36.0 ± 0.3	36.2 ± 0.4
Haematocrit (%)	n.d.	n.d.	n.d.	n.d.
Plasma bicarbonate	29.3 ± 0.6	29.2 ± 0.7	26.6 ± 1.5	26.3 ± 1.2
Base excess	5.4 ± 0.4	5.2 ± 0.7	2.6 ± 1.1	2.3 ± 1.0

Data are presented as mean ± s.e.mean (n = 4 in each group)

n.d.: not determined

Table 22

Physiological variables in WKY and SHR rats subjected to intrastriatal injection of blood or silicon oil (50µl), at the time of the LCBF experiment (24h after the blood or silicon oil injection)

	WKY		***SHR***			
	Haematoma	*Silicon Oil*	*Haematoma*		*Silicon Oil*	
pCO_2 (mmHg)	38.2 ± 1.2	42.3 ± 0.6	42.3 ± 0.9		41.2 ± 0.9	
pO_2 (mmHg)	84.7 ± 2.9	80.8 ± 2.0	85.4 ± 1.0		89.1 ± 2.8	
pH	7.456 ± 0.011	7.461 ± 0.008	7.325 ± 0.012		7.372 ± 0.021	
MABP (mmHg)	122 ± 2	122 ± 5	171 ± 5	*	176 ± 9	*
Heart Rate (bpm)	315 ± 3	334 ± 23	422 ± 14	*	351 ± 19	#
Plasma glucose ($mg.dl^{-1}$)	149 ± 19	138 ± 16	158 ± 14		119 ± 5	
Temperature (°C)	36.4 ± 0.1	36.4 ± 0.2	36.7 ± 0.2		37.6 ± 0.4	
Haematocrit (%)	48.9 ± 1.6	48.9 ± 2.0	52.6 ± 0.6		51.3 ± 1.8	
Plasma bicarbonate	26.8 ± 0.8	29.0 ± 0.8	22.1 ± 0.8		23.7 ± 0.7	
Base excess	2.6 ± 0.9	5.0 ± 0.8	-3.7 ± 0.9		-0.8 ± 1.2	

Data are presented as mean ± s.e.mean (n = 6 in the haematoma groups, n = 4 in the silicon oil groups)

#: significant difference between haematoma and silicon oil in animals of the same sub-strain.

*: significant difference between WKY and SHR following the same treatment.

Table 23

Volumes of striatal oligaemia (in mm^3) in WKY and SHR animals injected with blood or silicon oil (50µl), measured 24h after the injection

	WKY		***SHR***	
LCBF ($ml.100g^{-1}.min^{-1}$)	*Haematoma*	*Silicon oil*	*Haematoma*	*Silicon oil*
less than 15	1.48 ± 1.32	1.57 ± 1.14	1.25 ± 0.63	0.51 ± 0.24
between 15 and 25	1.15 ± 0.59	0.77 ± 0.64	1.07 ± 0.33	0.19 ± 0.02
less than 25	2.63 ± 1.88	2.34 ± 1.77	2.32 ± 0.94	0.71 ± 0.25
between 25 and 35	0.82 ± 0.28	0.65 ± 0.48	0.82 ± 0.18	0.19 ± 0.004
between 15 and 35	1.96 ± 0.78	1.42 ± 1.11	1.89 ± 0.50	0.40 ± 0.02
less than 35	3.45 ± 1.97	2.99 ± 2.24	3.14 ± 1.09	0.90 ± 0.24

Data are presented as mean ± s.e.mean (n = 6 in the haematoma groups, n = 4 in the silicon oil groups)

No significant differences found between groups

Table 24

Physiological variables in WKY and SHR rats subjected to intrastriatal injection of blood (50µl), at the time of the AIB experiment (24h after the blood injection)

	WKY	***SHR***	
	Haematoma	*Haematoma*	
pCO_2 (mmHg)	33.1 ± 3.6	38.6 ± 1.4	
pO_2 (mmHg)	84.7 ± 3.3	86.9 ± 2.3	
pH	7.503 ±0.008	7.373 ± 0.013	*
MABP (mmHg)	120 ± 8	172 ± 7	*
Heart Rate (bpm)	313 ± 38	390 ± 21	
Plasma glucose ($mg.dl^{-1}$)	188 ± 51	139 ± 5	
Temperature (°C)	36.3 ± 0.3	37.3 ± 0.4	
Haematocrit (%)	n.d.	51.0 ± 0.7	
Plasma bicarbonate	28.2 ± 1.9	22.3 ± 0.4	*
Base excess	4.1 ± 2.1	-2.0 ± 0.4	*

Data presented as mean ± s.e.mean (n = 8 in SHR and n = 4 in WKY group)

*: significant difference between WKY and SHR groups

n.d.: not determined

Table 25

Physiological variables in WKY and SHR rats subjected to intrastriatal injection of blood (50µl), and treated with either sesame oil (i.p.) or 7-NI (25mg.kg^{-1} i.p. 30min after the injection of blood) at the time of the LCBF experiment (24h after the blood injection)

	WKY		***SHR***		
	Sesame oil	*7-NI(25mg.kg^{-1})*	*Sesame oil*	*7-NI(25mg.kg^{-1})*	
pCO_2 (mmHg)	37.9 ± 1.5	38.7 ± 0.8	42.1 ± 1.1	36.6 ± 1.8	
pO_2 (mmHg)	84.8 ± 3.6	85.5 ± 1.6	86.2 ± 0.5	88.5 ± 2.6	
pH	7.459 ± 0.013	7.455 ± 0.007	7.318 ± 0.012	7.492 ± 0.011	
MABP (mmHg)	122 ± 3	120 ± 5	168 ± 5	192 ± 5	#
Heart Rate (bpm)	318 ± 2	337 ± 22	418 ± 16	346 ± 10	#
Plasma glucose (mg.dl^{-1})	157 ± 21	139 ± 5	156 ± 17	158 ± 3	
Temperature (°C)	36.5 ± 0.1	36.5 ± 0.1	36.8 ± 0.3	37.1 ± 0.2	
Haematocrit (%)	48.5 ± 1.9	50.9 ± 0.9	52.4 ± 0.6	55.0 ± 1.5	
Plasma bicarbonate	26.6 ± 1.0	26.8 ± 0.6	21.6 ± 0.7	28.2 ± 0.7	
Base excess	2.4 ± 1.1	2.6 ± 0.7	-4.2 ± 0.9	4.2 ± 0.7	

Data presented as mean ± s.e.mean (n = 7 in the WKY animals treated with 7-NI, n = 4 in SHR treated with 7-NI and n = 5 in SHR and WKY groups treated with sesame oil)

#: significant difference between sesame oil and 7-NI treatment.

Table 26

Volumes of striatal oligaemia (in mm^3) in WKY and SHR animals injected with blood (50µl) and treated with sesame oil or 7-NI ($25mg.kg^{-1}$ i.p., 30min after the injection of blood), measured 24h after the injection of blood

	WKY Haematoma			***SHR Haematoma***	
LCBF ($ml.100g^{-1}.min^{-1}$)	*Sesame oil*	*7-NI*		*Sesame oil*	*7-NI*
less than 15	1.78 ± 1.57	0.10 ± 0.06		1.23 ± 0.75	0.09 ± 0.05
between 15 and 25	1.38 ± 0.67	0.29 ± 0.15		1.20 ± 0.37	0.55 ± 0.28
less than 25	3.16 ± 2.21	0.38 ± 0.21		2.43 ± 1.13	0.65 ± 0.31
between 25 and 35	0.97 ± 0.29	0.33 ± 0.14		0.97 ± 0.20	0.76 ± 0.34
between 15 and 35	2.35 ± 0.83	0.61 ± 0.28	#	2.17 ± 0.55	1.32 ± 0.62
less than 35	4.13 ± 2.26	0.71 ± 0.33		3.40 ± 1.30	1.41 ± 0.66

Data presented as mean ± s.e.mean (n = 7 in the WKY animals treated with 7-NI, n = 4 in SHR treated with 7-NI and n = 5 in SHR and WKY groups treated with sesame oil)

#: significant difference between sesame oil and 7-NI treatment.

Methods for Fibrinogen Immunohistochemistry

The brain sections were immersed in xylene (100%) for 5min to remove the paraffin wax, then dehydrated in alcohol (70%) for 2min and afterwards in alcohol (100%) for 2min, placed in picric acid for 15min and cleared with water for 20min.

Sections were afterwards treated with trypsin (trypsin 0.1% with 0.1% $CaCl_2$ in Tris-Buffered Saline TBS - pH 7.6) for 20min at 37°C and washed in water for 5min. They were treated with 3% hydrogen peroxide for 5min and washed again in water. They were afterwards washed in TBS for 5min and treated with normal swine serum (NSS) for 20min (Dilution 1:5 NSS/TBS). Excess serum was drained from the sections.

The sections were then incubated in primary antibody for 30min (Primary antibody diluted in 1:5 NSS/TBS). Rabbit anti-human fibrinogen (DAKO) was the primary antibody used. It was dissolved in 0.1M NaCl and 15mM NaN_3 during storage. The antibody titre was 6000mg.l^{-1}. It reacts with native human fibrinogen as well as with the fibrinogen fragments D, E, X and Y and was used in dilution of 1:5000.

The sections were afterwards washed in TBS for 5min twice.

They were then incubated in polyclonal swine anti-rabbit antibody (secondary antibody) for 30min (Dilution 1:200 in 1:5 NSS/TBS). They were washed in TBS for 5min twice and incubated in ABC Elite reagent (DAKO) for 30min. They were washed again in TBS for 5min twice and treated with DAB solution for 10min.

They were washed in water for 5min, counterstained in Mayer's haematoxylin for 20sec, washed again in water for 5min, dehydrated, cleared and mounted.

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Cerebrovascular effects of nitric oxide manipulation in spontaneously hypertensive rats

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1 Evidence that nitric oxide (NO) bioactivity is altered in chronic hypertension is conflicting, possibly as a result of heterogeneity in both the nature of the dysfunction and in the disease process itself. The brain is particularly vulnerable to the vascular complications of chronic hypertension, and the aim of this study was to assess whether differences in the cerebrovascular responsiveness to the NO synthase (NOS) inhibitors, N^G-nitro-L-arginine methyl ester (L-NAME) and 7-nitroindazole (7-NI), and to the NO donor 3-morpholinosydnonimine (SIN-1) might indicate one possible source of these complications.

2 Conscious spontaneously hypertensive (SHR) and WKY rats, were treated with L-NAME (30 mg kg^{-1}, i.v.), 7-NI (25 mg kg^{-1}, i.p.), SIN-1 (0.54 or 1.8 mg kg^{-1} h^{-1}, continuous i.v. infusion) or saline (i.v.), 20 min before the measurement of local cerebral blood flow (LCBF) by the fully quantitative [^{14}C]-iodoantipyrine autoradiographic technique.

3 With the exception of mean arterial blood pressure (MABP), there were no significant differences in physiological parameters between SHR and WKY rats within any of the treatment groups, or between treatment groups. L-NAME treatment increased MABP by 27% in WKY and 18% in SHR groups, whilst 7-NI had no significant effect in either group. Following the lower dose of SIN-1 infusion, MABP was decreased to a similar extent in both groups (around −20%). There was no significant difference in MABP between groups following the higher dose of SIN-1, but this represented a decrease of −41% in SHR and −21% in WKY rats.

4 With the exception of one brain region (nucleus accumbens), there were no significant differences in basal LCBF between WKY and SHR. L-NAME produced similar decreases in LCBF in both groups, ranging between −10 and −40%. The effect of 7-NI upon LCBF was more pronounced in the SHR (ranging from −34 to −57%) compared with the WKY (ranging from −14 to −43%), and in seven out of the thirteen brain areas examined there were significant differences in LCBF.

5 Following the lower dose of SIN-1, in the WKY 8 out of the 13 brain areas examined showed significant increases in blood flow compared to the saline treated animals. In contrast, only 2 brain areas showed significant increases in flow in the SHR. In the rest of the brain areas examined the effects of SIN-1 upon LCBF were less marked than in the WKY.

6 Infusion of the higher dose of SIN-1 resulted in further significant increases in LCBF in the WKY group (ranging between +30% and +74% compared to saline-treated animals), but no significant effects upon LCBF were found in the SHR. As a result, there were significant differences in LCBF between SIN-1-treated WKY and SHR in six brain areas. In most brain areas examined, cerebral blood flow in SHR following the higher dose of SIN-1 was less than that measured with the lower dose of SIN-1.

7 Despite comparable reductions in MABP (~20%) in both groups, calculated cerebrovascular resistance (CVR) confirmed that the vasodilator effects of the lower dose of SIN-1 were significantly more pronounced throughout the brain in the WKY (ranging between −3% and −50%; median = −38%) when compared to the SHR (ranging between −10% and −36%; median = −26%). In the animals treated with the higher dose of SIN-1, CVR changes were broadly similar in both groups (median = −45% in WKY and −42% in SHR), but with the reduction in MABP in SHR being twice that found in WKY, this is in keeping with an attenuated blood flow response to SIN-1 in the SHR.

8 The results of this study indicate that NO-dependent vasodilator capacity is reduced in the cerebrovasculature of SHR. In addition, the equal responsiveness to a non-specific NOS inhibitor but an enhanced effectiveness of a specific neuronal NO inhibitor upon LCBF in the SHR could be consistent with an upregulation of the neuronal NO system.

Keywords: Cerebral blood flow; hypertension; SHR; nitric oxide; L-NAME; 7-nitroindazole; 3-morpholinosydnonimine (SIN-1); quantitative autoradiography

Introduction

Although it has been proposed that an impaired release of endothelial vascular relaxing factors might underlie the pathogenesis of hypertension (Lüscher & Vanhoutte, 1986), the involvement of nitric oxide in the aetiology of the disease process remains controversial. Studies in hypertensive human subjects have shown that there is abnormal nitric oxide (NO) activity associated with hypertension (Calver *et al.*, 1992), but divergent results have also emerged (Cockroft *et al.*, 1994). In experimental animal models of chronic hypertension, there is increasing evidence that endothelium-dependent relaxation is heterogeneously affected (Lüscher, 1992), with normal function maintained in the renal and coronary arteries (Tschudi *et al.*, 1991), but impaired function in aorta, mesenteric, carotid and cerebral circulation (Lüscher & Vanhoutte, 1986; Dohi *et*

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al., 1990; Cuevas *et al.*, 1996). These observations could explain not only contradictory experimental observations, but also the selective vulnerability of certain tissues, such as the brain, to hypertensive complications.

The brain is particularly vulnerable to complications associated with hypertension and both the incidence (Whisnant, 1996) and severity of cerebrovascular ischaemia (Coyle, 1984) are increased in hypertension. Studied performed *in vitro* have raised the possibility that cerebrovascular NO systems are altered in hypertensive rats (Miyata *et al.*, 1990; Malinski *et al.*, 1993), and perturbations in endothelium-dependent relaxation have been identified in pial arteries examined *in situ* (Yang *et al.*, 1991a; Mayhan, 1992). In contrast, cerebrovascular responsiveness to NO inhibition is preserved *in vivo* (Izuta *et al.*, 1995), and basal local cerebral blood flow (LCBF) appears to be unaffected by hypertension (Wei *et al.*, 1992).

Whilst it might appear from these observations that there may be no functional basis to the increased susceptibility to ischaemia in chronic hypertensives, this cannot be discounted in favour of structural dysfunction without first examining the integrity of NO-dependent cerebrovascular dilator reserve and the role of perivascular neuronal NO systems in cerebrovascular control (Kelly *et al*,. 1995). The fact that NO donors reduce infarct size in spontaneously hypertensive rats (SHR) subjected to middle cerebral artery (MCA) occlusion (Zhang *et al.*, 1994), is consistent with a reduced NO-dependent dilator capacity of the hypertensive brain. In the present studies, we have measured local cerebral blood flow (LCBF) in SHR and normotensive WKY controls following treatment with an exogenous NO donor 3-morpholinosydnonimine (SIN-1) and 7-nitro indazole, which shows *in vivo* selectivity for neuronal nitric oxide synthase (NOS) inhibition (Moore *et al.*, 1993a,b). We have also re-examined the effects of the non-selective NOS inhibition N^G-nitro-L-arginine methyl ester (L-NAME) to allow comparisons with 7-NI.

Methods

These studies were performed with a total of 25 SHR and 27 WKY adult male rats between 14 and 16 weeks of age (Charles River, U.K.). Animals were held under normal animal house conditions with free access to food and water.

Measurement of local cerebral blood flow

On the day of the experiment the animals were anaesthetized with halothane (1.5% in a gas mixture of 70% nitrous oxide and 30% oxygen) and prepared for the measurement of LCBF as described previously (Kelly *et al.*, 1994). Following surgery, general anaesthesia was withdrawn and 2 h allowed to elapse before any further experimental manipulation. Approximately equal numbers of SHR and WKY rats were treated with either L-NAME (30 mg kg^{-1} in saline, i.v. over 60 s; $n=4$ from each group), 7-NI (25 mg kg^{-1} in sesame oil, i.p. over 5 s; $n=4$ from each group), SIN-1 (0.54 or 1.8 mg kg^{-1} h^{-1} in saline, continuous i.v. infusion; $n=4$ from each group). In previous studies from this laboratory we have found no difference in LCBF between control animals injected with oil (i.p.) or saline (i.v.), or between saline infusion or bolus injection. In this study, control animals were injected with saline. (1.0 ml i.v.; $n=5$ SHR and $n=6$ WKY). The doses of L-NAME and 7-NI were chosen on the basis of previously published work from this and other laboratories (Macrae *et al.*, 1993; Moore *et al.*, 1993a,b; Kelly *et al.*, 1994; 1995). Doses of 7-NI higher than that used in this study, have been shown to produce further reductions in cerebral blood flow in normal rats (Kelly *et al.*, 1995; Wang *et al.*, 1995) but they may also produce anomalous focal hyperaemia (Kelly *et al.*, 1995).

In a series of preliminary studies, two doses of SIN-1 were identified which produced either similar magnitude of reduction in MABP in WKY and SHR (lower dose, 0.54 mg kg^{-1} h^{-1}), or which reduced MABP to comparable absolute values in both groups (higher dose, 1.8 mg kg^{-1} h^{-1}). In these studies, we also found that an i.v. infusion of SIN-1 at 1.8 mg kg^{-1} h^{-1} for 20 to 30 min completely blocked the expected cardiovascular and cerebrovascular effects of subsequent i.v. injection of L-NAME (30 mg kg^{-1}).

The measurement of LCBF was initiated 20 min after the start of L-NAME, 7-NI, SIN-1 or saline treatments by use of the fully quantitative [^{14}C]-iodoantipyrine autoradiographic technique. As far as possible, control (saline) experiments were performed contemporaneously to the various drug treatments. The protocols were in complete accordance with the methodology as originally published (Sakurada *et al.*, 1978) and as described previously from this laboratory (Kelly *et al.*, 1994). Autoradiographic images were analysed by quantitative densitometry relative to ^{14}C-containing standards and LCBF was calculated by use of the appropriate operational equation for the technique (Sakurada *et al.*, 1978). Areas of interest were chosen to represent brain areas in the vascular territories of the anterior, middle and posterior cerebral arteries. Arterial blood pressure and rectal temperature were monitored continuously in each animal throughout the experiments and heart rate was measured intermittently. Samples of arterial blood were withdrawn before and after treatments, for the measurement of pH, P_{CO_2}, and P_{O_2}.

Calculation of cerebrovascular resistance

Cerebrovascular resistances (CVR) were calculated in animals treated with SIN-1 and the relevant saline-treated rats by dividing mean arterial blood pressure (mmHg) by LCBF values (ml 100 g^{-1} min^{-1}) for each brain area in each individual animal.

Drugs

With the exception of SIN-1 which was a gift from Cassella AG, Frankfurt, Germany, all drugs were purchased from the Sigma Chemical Co.

Statistical analysis

Physiological and LCBF data (presented as mean ± s.e.mean) were analysed by Student's *t* test with Bonferroni correction applied to allow multiple pair-wise comparisons between appropriate groups (maximum number of comparisons = 3). Differences in the CVR response to SIN-1 treatment between the two rat strains were analysed by Mann-Whitney U-test. Acceptable levels of significance were set at $P<0.05$ for all statistical tests.

Results

Physiological parameters

With the exception of mean arterial blood pressure, there were no significant differences in physiological parameters between SHR and WKY rats within any of the treatment groups, or between treatment groups (Table 1). As expected, mean arterial blood pressure (MABP) was 46% greater in SHR compared to WKY rats before any treatment, and saline injection had no effect. Not withstanding the initial difference in blood pressure, L-NAME treatment increased MABP to a similar extent in both groups of rats, whilst 7-NI had no significant effect in either group. Following treatment with the lower SIN-1 dose MABP decreased by −21% in the SHR and by −20% in the WKY (Table 2). However, following the higher dose of SIN-1, MABP was decreased by −41% in SHR but by only −21% in WKY rats, so that after treatment there was no

Table 1 Physiological variables in WKY and SHR groups following saline, L-NAME or 7-NI treatment

	WKY			*SHR*		
	Saline	*L-NAME*	*7-NI*	*Saline*	*L-NAME*	*7-NI*
pH	7.49±0.01	7.47±0.01	7.48±0.01	7.48±0.01	7.46±0.01	7.46±0.01
P_{CO_2} (mmHg)	41.6±2.0	40.3±1.0	40.0±0.7	39.3±2.0	37.7±1.0	36.7±0.4
P_{O_2} (mmHg)	88.1±4.0	90.6±4.0	97.8±4.0	91.4±2.0	86.8±1.0	89.2±0.7
MABP (mmHg)	120±3	152±3*	123±3	175±6#	207±1*#	180±6#
Temperature (°C)	36.3±0.2	36.6±0.4	37.1±0.2	36.7±0.2	36.7±0.1	36.5±0.1
Group *n*	6	4	4	5	4	4

Data are presented as mean±s.e.mean. #Significant difference between WKY and SHR given the same treatment ($P<0.05$). *Significantly different from saline treated rats of the same sub-strain ($P<0.05$).

Table 2 Physiological variables in WKY and SHR groups following saline or SIN-1 treatment

	WKY			*SHR*		
	Saline	*Low SIN-1*	*High SIN-1*	*Saline*	*Low SIN-1*	*High SIN-1*
pH	7.49±0.01	7.48±0.01	7.48±0.01	7.48±0.01	7.46±0.01	7.44±0.01
P_{CO_2} (mmHg)	41.6±2.0	38.4±2.0	37.9±1.0	39.3±2.0	7.9±1.0	40.4±1.0
P_{O_2} (mmHg)	88.1±4.0	85.5±1.0	94.6±3.0	91.4±2.0	90.2±3.0	97.4±1.0
MABP (mmHg)	120±4	96±8*	95±6*	178±6#	147±2#*	104±5*
Temperature (°C)	36.3±0.2	35.8±0.2	37.0±0.2	36.7±0.2	37.4±0.1	37.3±0.3
Group *n*	5	4	4	4	4	4

Data are presented as mean±s.e.mean. #Significant difference between WKY and SHR given the same treatment ($P<0.05$). *Significantly different from saline-treated rats of the same sub-strain ($P<0.05$).

Table 3 Local cerebral blood flow in normotensive (WKY) and spontaneously hypertensive (SHR) rats treated with saline, L-NAME or 7-NI

	Saline		*L-NAME*		*7-NI*	
	WKY	*SHR*	*WKY*	*SHR*	*WKY*	*SHR*
Neocortex						
Parietal	141±6	132±8	124±7	105±6	121±5	78±4*#
Cingulate	98±4	117±6	99±5	91±6	82±1	64±6*
Corpus callosum	37±2	32±2	20±1*	29±2#	21±1*	21±1*
Basal ganglia						
Striatum	99±4	94±5	87±4	83±5	78±3*	59±5*#
Globus pallidus	64±4	68±8	53±3	54±3	49±2	38±1#
Accumbens	98±4	116±3#	78±5	82±5*	77±5	56±1*#
Thalamus						
Hypothalamus	75±4	83±5	49±3*	45±3*	50±3*	36±3*#
Lateral geniculate	106±5	116±9	89±6	93±5	87±3	69±2*#
Hippocampus						
CA3	76±4	85±3	60±1	63±4*	57±2*	49±3*
CA2	71±3	83±4	56±2*	59±5*	51±1*	45±2*
CA1	68±5	74±3	56±1	61±3	49±2	44±2*
Molecular layer	73±4	85±2	57±1	61±5*	55±3	45±2*
Dentate hilus	76±4	93±6	61±1	62±5*	58±3	46±1*#
Group *n*	6	5	4	4	4	4

Data are presented as mean local cerebral blood flow (ml $100\,g^{-1}\,min^{-1}$)±s.e.mean. #Significant difference between WKY and SHR animals given the same treatment ($P<0.05$). *Significantly different from saline-treated rats of the same sub-strain ($P<0.05$).

significant difference in MABP between these two groups (Table 2).

Local cerebral blood flow following L-NAME

Significant differences in basal LCBF between WKY and SHR were found in only of one of the thirteen brain regions examined (nucleus accumbens) (Table 3). Following injection of L-NAME, LCBF in the WKY group was significantly reduced (compared to saline treated animals) in the corpus callosum (−46%), the hypothalamus (−35%) and the CA2 layer of the hippocampus (−21%). Elsewhere in the brain there was a tendency towards decreases in LCBF (with the exception of cingulate cortex) ranging between −12 and −22%, but these differences were not significant. Similarly, in the L-NAME-treated SHR group there was a global trend towards reductions in LCBF ranging between −12 and −22%, which reached acceptable levels of significance in the nucleus accumbens (−29%), hypothalamus (−46%), hippocampal fields CA2 and CA3 (−29% and −26%), and in the molecular layer (−28%) and hilus of the dentate gyrus (−33%). There were no significant differences in the LCBF between L-NAME treated WKY and SHR, apart from in the corpus callosum (Table 3).

Local cerebral blood flow following 7-NI

Following the intraperitoneal injection of SHR with 7-NI, significant reductions in LCBF (ranging between −34 and −57%) were found in 12 of the 13 areas examined when compared to the appropriate saline treated group (Table 3). Although the response to 7-NI in the WKY group was qualitatively similar, significant reductions in LCBF were limited to the corpus callosum (−43%), striatum (−21%), hypothalamus (−33%), and CA2 (−28%) and CA3 (−25%) fields of the hippocampus. However, throughout the brain, the re-

Table 4 Local cerebral blood flow in normotensive (WKY) and spontaneously hypertensive (SHR) rats treated with saline or SIN-1

	Saline		*Low SIN-1*		*High SIN-1*	
	WKY	*SHR*	*WKY*	*SHR*	*WKY*	*SHR*
Neocortex						
Parietal	138±6	127±9	139±12	142±10	206±9*	148±11#
Cingulate	95±3	112±5	152±7*	143±5*	171±6*	138±5#
Corpus callosum	35±2	32±3	29±3	29±1	36±3	41±2
Basal ganglia						
Striatum	96±3	90±4	117±5*	109±8	129±6*	107±9
Globus pallidus	62±4	61±4	90±2*	82±4*	80±4	66±4
Accumbens	96±4	114±4	136±7*	138±11	153±4*	130±8
Thalamus						
Hypothalamus	71±3	80±4	79±5	75±7	89±4	70±5
Lateral geniculate	103±5	111±10	138±6*	139±14	164±7*	118±8#
Hippocampus						
CA3	74±4	83±3	98±4*	100±8	104±3*	81±9
CA2	68±3	80±3	82±7	81±9	92±5*	82±5
CA1	65±4	73±3	80±6	81±6	100±5*	76±5#
Molecular layer	71±4	84±2	95±4*	88±8	103±3*	82±4#
Dentate hilus	73±5	90±5	94±5*	89±9	112±6*	87±4#
Group *n*	5	4	4	4	4	4

Data are presented as mean local cerebral blood flow (ml $100\,g^{-1}\,min^{-1}$)±s.e.mean. #Significant difference between WKY and SHR animals given the same treatment ($P<0.05$). *Significantly different from saline-treated rats of the same sub-strain ($P<0.05$).

Table 5 Calculated cerebrovascular resistance in normotensive (WKY) and hypertensive (SHR) animals, treated with saline or SIN-1

	WKY					*SHR*				
	Saline	*Low SIN-1*	*% change*	*High SIN-1*	*% change*	*Saline*	*Low SIN-1*	*% change*	*High SIN-1*	*% change*
Neocortex										
Parietal	0.87±0.02	0.69±0.05	−21	0.46±0.03	−46	1.43±0.11	1.06±0.08	−26	0.71±0.06	−48
Cingulate	1.27±0.05	0.63±0.05	−50	0.56±0.02	−55	1.58±0.08	1.04±0.05	−34	0.76±0.06	−50
Corpus callosum	3.37±0.21	3.28±0.10	−3	2.66±0.23	−19	5.85±0.79	5.17±0.31	−12	2.55±0.22	−54
Basal ganglia										
Striatum	1.24±0.05	0.82±0.05	−34	0.74±0.04	−39	1.99±0.09	1.38±0.11	−31	0.99±0.11	−48
Globus pallidus	1.95±0.12	1.06±0.08	−46	1.19±0.40	−37	2.85±0.24	1.82±0.12	−36	1.59±0.09	−40
Accumbens	1.26±0.03	0.72±0.03	−43	0.62±0.02	−50	1.54±0.11	1.10±0.10	−29	0.81±0.06	−47
Thalamus										
Hypothalamus	1.68±0.10	1.21±0.04	−28	1.08±0.09	−34	2.26±0.16	2.02±0.21	−11	1.52±0.14	−29
Lateral geniculate	1.17±0.08	0.69±0.05	−41	0.58±0.02	−49	1.66±0.20	1.10±0.13	−34	0.89±0.08	−43
Hippocampus										
CA3	1.64±0.08	0.97±0.07	−41	0.92±0.06	−43	2.17±0.13	1.51±0.15	−30	1.33±0.14	−36
CA2	1.76±0.08	1.18±0.09	−33	1.04±0.03	−39	2.13±0.12	1.91±0.27	−10	1.25±0.09	−41
CA1	1.86±0.13	1.19±0.04	−36	0.95±0.02	−47	2.36±0.16	1.87±0.19	−21	1.39±0.12	−42
Molecular layer	1.71±0.08	1.02±0.09	−40	0.92±0.04	−45	2.03±0.09	1.74±0.21	−14	1.27±0.08	−38
Dentate gyrus	1.65±0.09	1.03±0.10	−38	0.85±0.01	−47	1.92±0.10	1.73±0.23	−10	1.21±0.10	−36
Median effect	–	–	−38	–	−45	–	–	−26§	–	−42
Group *n*	5	4		4		4	4		4	

Cerebrovascular resistances were calculated by dividing mean arterial blood pressure (mmHg) by LCBF values (ml $100\,g^{-1}\,min^{-1}$) for each individual animal and are presented as mean±s.e.mean. The effects of SIN-1 treatments (percentage change) were compared between rat strains by Mann-Whitney U-test. §Significantly different from similarly-treated WKY group ($P<0.01$).

sponse to 7-NI was less marked in the WKY group (ranging between −14 and −43%) when compared to SHR, and in seven brain areas (parietal cortex, striatum, globus pallidus, nucleus accumbens, hypothalamus, lateral geniculate and hilus of the hippocampal dentate gyrus) the decreases in LCBF were significantly greater in the SHR group (Table 3).

Local cerebral blood flow following SIN-1

Following treatment with the lower dose of SIN-1 there were significant increases in LCBF in all but five brain areas (parietal cortex, corpus callosum, hypothalamus, CA1 and CA2 layers of the hippocampus) in the WKY group (Table 4). Significant effects ranged from +22% in striatum, to +60% in cingulate cortex. In contrast, in the SHR group, there were significant effects upon LCBF in only two brain regions (cingulate cortex and globus pallidus) (Table 4). In the rest of the brain areas examined, the effects of this lower dose of SIN-1 upon LCBF were less marked than in the WKY.

Following intravenous infusion of the higher dose of SIN-1 there were significant increases in LCBF in all but three brain areas (corpus callosum, globus pallidus and hypothalamus) in the WKY group (Table 4). Significant effects ranged from +30% in hippocampal field CA2 and striatum, to +74% in cingulate cortex. The CBF values in most of the brain areas examined were higher compared to those obtained following treatment with the lower dose of SIN-1. In contrast, in the SHR group, there were no significant effects of this higher dose of SIN-1 upon LCBF in any region of the brain. In this group, LCBF in grey matter areas ranged from −16% in hypothalamus to +18% in cingulate cortex (Table 4). A comparison of LCBF between WKY and SHR groups treated with the higher dose of SIN-1 revealed significant differences in six of the thirteen brain areas examined (parietal cortex, cingulate cortex, lateral geniculate, hippocampal field CA1, and molecular layer and hilus of the dentate gyrus) (Table 4). Although there were no significant differences between the effects upon LCBF of the two doses of SIN-1 in

SHR, the values obtained following treatment with the higher dose of SIN-1 were generally lower in most of the areas examined (Table 4).

Cerebrovascular resistance (CVR)

Despite comparable reductions in MABP (~20%) in both groups, calculated CVR confirmed that the vasodilator effects of the lower dose of SIN-1 were significantly more pronounced ($P<0.01$, Mann-Whitney U-test) throughout the brain in the WKY (ranging between −3% and −50%; median = −38%) when compared to the SHR (ranging between −10% and −36%; median = −26%) (Table 5). In the groups treated with the higher dose of SIN-1, CVR changes were broadly similar in both groups (median = −45% in WKY and −42% in SHR), but with the reduction in MABP in SHR being twice that found in WKY, this is in keeping with an attenuated blood flow response to SIN-1 in the SHR (Table 5).

Discussion

In the present study we found little evidence of any fundamental difference in basal LCBF between WKY and SHR groups, although in one area of the brain, the nucleus accumbens, blood flow in SHR was significantly higher. These observations are largely in keeping with previous data, where either similar quantitative autoradiographic techniques were used to measure LCBF in conscious rats of these two strains (Wei *et al.*, 1992), or where rats of a similar age to those used in this study were examined with different measurement protocols (Grabowski & Johansson, 1985). Moreover, our physiological studies are in keeping with morphological studies of cerebral capillary bed structure (Lin *et al.*, 1990) and precapillary arterioles on the pial surface (Harper & Bohlen, 1984) which show no differences between SHR and WKY. Although structural changes have been described in larger blood vessels of the cerebrovascular bed in SHR (Folkow, 1990), it is not these vessels which regulate LCBF.

Early *in vitro* investigations identified a decrease in NO-mediated activity in cerebral blood vessels taken from SHR (Miyata *et al.*, 1990; Malinski *et al.*, 1993). Subsequent examination of the basilar artery *in situ* showed that L-NAME induced greater constriction in hypertensive rats (Kitazono *et al.*, 1995). These authors suggested that basal release of NO might be somewhat enhanced in SHR over that in WKY, and *in vivo* studies confirmed that the cerebrovascular response to NO inhibition with N^G-monomethyl-L-arginine (L-NMMA) was greater in SHR (Izuta *et al.*, 1995), although no significant difference in LCBF was found in SHR and WKY treated with N^G-nitro-L-arginine (L-NOARG). In our studies, acute treatment with L-NAME had broadly similar effects upon LCBF in the WKY and SHR groups and were in keeping with previously published results (Izuta *et al.*, 1995; Yang, 1996). The dose of L-NAME used in the present study has previously been found in Sprague-Dawley (SD) rats to produce significant reductions in LCBF at 15 min post-injection (Kelly *et al.*, 1994) which are maintained for at least 3 h (Macrae *et al.*, 1993), but in general it appears from our study that L-NAME is not as efficacious in reducing LCBF in WKY and SHR as it is in Sprague-Dawley (SD) rats. Although it was outwith the present experimental design to make such inter-strain comparisons, it is also noteworthy that differences in vascular structure have been found between normotensive WKY and SD in the cerebral capillary bed (Lin *et al.*, 1990).

Whilst there was no evidence of any difference between WKY and SHR in the response to the non-selective NOS inhibitor L-NAME, the response to the intraperitoneal injection of 7-NI, which *in vivo* is a selective inhibitor of the neuronal isoform of NOS (nNOS; Moore *et al.*, 1993a,b), was significantly greater in the majority of brain areas of hypertensive animals. Although previous studies have suggested that there may be an upregulation of cerebrovascular NO systems in hypertension (Kitazono *et al.* 1995), our observations point to there being a more specific upregulation of neuronal NOS. Studies specifically addressing the role of neuronal NOS in the brains of SHRs are lacking, although it does seem that nNOS expression is normal in the cerebellum and brain stem of 4-, 16- and 24-week-old SHR, compared to age-matched WKY (Iwai *et al.*, 1995). It is interesting to note that the activity of nNOS in cerebral ischaemia is potentially detrimental (Huang *et al.*, 1994) and although perhaps speculative at this stage our findings, consistent with an upregulated nNOS system in hypertension, could offer one explanation for the predisposition of hypertensives to ischaemia following stroke (Coyle, 1984). Our findings of comparable cerebrovascular responses to L-NAME but enhanced responses to 7-NI in the SHR could also be compatible with a down-regulation of endothelial NOS in these animals together with an up-regulation of the neuronal NOS. Testing of this hypothesis awaits the development of specific endothelial NOS inhibitors.

There is growing evidence that endothelium-dependent vascular dilatation is heterogeneously affected in hypertension (Nava *et al.*, 1995) with both regional and species differences (Deng *et al.*, 1995). Studies in hypertensive humans and animals have demonstrated preserved dilatator responses to sodium nitroprusside in peripheral vascular beds (Taddei *et al.*, 1993; Küng & Lüscher, 1995). In contrast to the qualitatively similar effects of SIN-1 upon blood pressure in both WKY and SHR, the effects of SIN-1 upon LCBF were attenuated in our SHR group when compared with WKY. These apparent differences in the response to SIN-1 between vascular beds in the SHR group suggest that they may be regional perturbations of NO-specific vasodilator reserve, and would be consistent with an up-regulation of endogenous cerebral NOS activity. In support of our observations, L-arginine was found to have no effect upon LCBF in the contralateral hemisphere of SHR subjected to middle cerebral artery (MCA) occlusion (Prado *et al.*, 1996), and SIN-1 had no significant effect upon cortical blood flow in SHR subjected to sham-occlusion of the MCA (Zhang *et al.*, 1994). Contradictory results have also been obtained with *in situ* methodology to measure responsiveness of pial arteries, or the basilar artery, to superfusion of NO donors in the stroke-prone substrain of the SHR (Mayhan *et al.*, 1988; Yang *et al.*, 1991a,b; 1993; Kitazono *et al.*, 1993) and also in stroke-resistant SHR (Mayhan *et al.*, 1987; Mayhan, 1991). There are several potential explanations for the apparent differences between these results and the data presented in this paper, including the fact that the previous experiments were performed under the influence of anaesthetics, with their potential influence on cerebrovascular responsiveness (Edvinsson & McCulloch, 1981); the possibility that NO bioactivity might differ markedly between the stroke-prone substrain of SHR and the SHR used in our study (Dominiczak & Bohr, 1995); the fact that our animals were considerably younger than those used previously, and NO responsiveness has been shown to change – at least in renovascular hypertension – as the duration of hypertension progresses (Dubey *et al.*, 1996); most importantly, neither the basilar artery nor the pial vessels constitute the principal source of resistance to flow in the cerebrovascular bed, and are therefore not responsible for the control of LCBF. Recent investigations in conscious patients with arterial hypertension have also confirmed impaired responsiveness to NO-donors (Preik *et al.*, 1996), although once again other, earlier studies have reached contradictory conclusions (Creager & Roddy, 1994; Panza *et al.*, 1995).

Cerebrovascular resistance (CVR), calculated from LCBF and MABP values measured from each individual rat, was found to decrease to a greater extent in WKY (median effect = −38%) treated with the lower dose of SIN-1 when compared to SHR (median effect = −26%). Since the reduction in MABP following this treatment was similar in both WKY and SHR, these differences in CVR directly reflect blood flow changes between the two groups. In the rats treated with a higher dose of SIN-1, CVR changes were broadly similar in both groups, but with the reduction in MABP in SHR

(−41%) being twice that found in WKY (−20%), this is in keeping with an attenuated blood flow response to SIN-1 in the SHR. However, the vessels of the cerebrovascular bed are normally endowed with the ability to alter their calibre in response to fluctuations in perfusion pressure. The resulting changes in vascular resistance ensure the maintenance of constant cerebral blood flow over a wide range of arterial blood pressure, a phenomenon known as autoregulation (Paulson *et al.*, 1990). The lower limit of autoregulation below which the relationship between cerebral blood flow and perfusion pressure becomes linear is not a fixed point, and chronic hypertension is known to raise the arterial pressure threshold at the lower limit of the autoregulatory range (Strandgaard, 1978; Paulson *et al.*, 1990). Although the levels of MABP measured in our SHR in response to the higher dose of SIN-1 (104 mmHg) were above the lower limit of autoregulation reported for this strain (90 mmHg) (Barry *et al.*, 1982; Harper & Bohlen, 1984), the observation that LCBF was higher – though not significantly – in most of the areas examined in the SHR treated with the lower SIN-1 dose compared to those treated with the higher dose, could be interpreted as indicating pressure-dependency in flow to some extent at least. It is known that NO inhibition shifts the upper limit of cerebrovascular autoregulation to higher pressure levels in normal animals (Kelly *et al.*, 1994), so it is conceivable that NO could have a role in determining the lower limit of autoregulation. A definitive conclusion regarding this possibility cannot be derived simply by calculating CVR.

SIN-1 generates NO and superoxide, which can potentially react with NO to form peroxynitrite (Moncada & Higgs, 1995; Plane *et al.*, 1997). The dilator actions of SIN-1 in extracranial tissues are also modulated by the basal production of endothelium-derived NO (Plane *et al.*, 1997). Studies performed *in vitro* with endothelial cells from peripheral tissues of 5 week old SHR showed that superoxide may be responsible for the decreased activity of NO (Grunfeld *et al.*, 1995). There is also growing speculation of an enhanced oxidative stress in the pathogenesis of hypertensive complications (Alexander, 1995). It is clearly possible that the effects of SIN-1 upon LCBF could be related to generation of superoxide, complicated further by the potential involvement of free radicals in the evolution of hypertensive complications. The exact mechanism by which SIN-1 effects changes in cerebral blood flow may therefore be quite complex, but whatever the underlying mechanism there are differences in the cerebrovascular response to SIN-1 in WKY and SHR.

Morphological analysis of those cerebral blood vessels which are largely responsible for the control of LCBF found no structural differences between WKY and SHR (Lin *et al.*, 1990). Whilst it is possible that subtle changes might go undetected in these rather small vessels, the method of analysis did prove sufficiently sensitive to detect differences between vessels from both WKY and SHR, when compared to those from SD rats (Lin *et al.*, 1990). It has been argued that structural changes in the vessel wall associated with chronic hypertension could explain altered responses to vasoactive agents (constrictor and dilator) (Calver *et al.*, 1992), but it might be expected that the response to all vasoactive agents would be affected in a non-specific manner (Harper & Bohlen, 1984; Folkow, 1990). It is possible that structural changes might have been present in our relatively young (14 weeks old) animals, particularly as structural changes appear even before frank hypertension has been established (Folkow, 1990), but structural differences cannot readily provide an explanation for the differential response to the two NOS inhibitors (L-NAME and 7-NI) and nor could it explain the attenuated vasodilator response to SIN-1. Thus our results would appear to support the concept of hypertension-induced functional changes (Winquist & Bohr, 1983) in the cerebrovasculature.

Cerebrovascular dysfunction associated with hypertension is most probably multifactorial. Studies with pial arteries from hypertensive rats have identified altered dilator responses which appear to involve vasoconstrictor prostanoids (Yang *et al.*, 1991a; Mayhan, 1992), and *in situ* studies have shown that the prostaglandin-induced pial arterial vasodilatation is related to NO production (Armstead, 1995). Interestingly, enhanced responses of the basilar artery to activation of endothelin$_B$ (ET_B) receptors in hypertensive rats, independent of NO or prostanoid pathways, have also been observed (Kitazono *et al.*, 1995). The same group also found that the mechanisms responsible for the impaired responses of the basilar artery in SHRs (Mayhan, 1990) are not the same as those responsible for the attenuated responses of the pial vessels.

This present study provides further evidence for heterogeneous perturbation of NO systems in the cerebrovasculature of the SHR strain, with a reduced vasodilator capacity and possibly an up-regulation of neuronally derived NO systems. The importance of these findings may become pronounced in situations of cerebral ischaemia, which is one of the commonest complications of hypertension.

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Cerebrovascular responsiveness to N^G-nitro-L-arginine methyl ester in spontaneously diabetic rats

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1 There is evidence that endothelial dysfunction is associated with diabetes mellitus. The purpose of the present study was to assess local cerebral blood flow (LCBF) and cerebrovascular responsiveness to the NOS inhibitor N^G-nitro-L-arginine methyl ester (L-NAME) in spontaneously diabetic insulin-dependent BioBred (BB) rats.

2 Diabetic rats, and non-diabetic controls, were treated with L-NAME (30 mg kg^{-1}, i.v.) or saline, 20 min prior to the measurement of LCBF by the fully quantitative [^{14}C]-iodoantipyrine autoradiographic technique.

3 There were no significant differences in physiological parameters (blood pH, PCO_2, and PO_2, rectal temperature, arterial blood pressure, or plasma glucose) between any of the groups of rats, and no difference in either the extent or the temporal characteristics of the hypertensive response to L-NAME between diabetic and non-diabetic rats.

4 In diabetic rats, a global reduction in basal LCBF was observed, although significant reductions (between −20 and −30%) were found in only 5 (mainly subcortical) out of the 13 brain regions measured. Following L-NAME injection, significant reductions in LCBF (between −20 and −40%) were found in the non-diabetic animals. In diabetic animals treated with L-NAME, a significant reduction in LCBF was measured only in the hypothalamus (−33%).

5 The cerebrovascular response to acute L-NAME is attenuated in spontaneously diabetic insulin-dependent BB rats. This would be consistent with the endothelial dysfunction in cerebral vessels, known to be associated with diabetes mellitus and it is possible that a loss of NO-induced dilator tone, amongst other factors, may underlie the observed reductions of basal LCBF in these animals.

Keywords: Cerebral blood flow; diabetes mellitus; L-NAME; nitric oxide; nitric oxide synthase inhibition; quantitative autoradiography

Introduction

Diabetes mellitus is a metabolic disorder associated with functional and structural abnormalities in a variety of organs and systems of the body. In the cardiovascular system, diabetes is associated with the development of hypertension, accelerated atherogenesis, thrombosis and ischaemia, which are associated with both macro- and microvascular changes (Colwell, 1991). These cardiovascular complications were thought to be largely independent of the degree of diabetic control (Kannel & McGee, 1979), suggesting that it was the disease process itself which was responsible and not exposure to hyperglycaemia or insulin treatment. However, more recent work (Reichard *et al.*, 1993) suggests a more complex causal relationship between the disease process and therapeutic intervention.

Although the cerebral circulation has been found to be subject to vascular pathology similar to that found in the periphery (Aronson, 1973; Grunnet, 1963), the effects upon cerebrovascular physiology were previously thought to be rather subtle, and often went unrecognised. More recently however, it has become apparent that neither the brain nor its vasculature are spared from the effects of diabetic pathology (Mooradian, 1988; McCall, 1992), and altered blood/brain transport, cerebral blood flow, and brain metabolism, as well as effects on neurones and glia, are all associated with the disease process. Pathophysiological effects of the disease upon the cerebral circulation are manifest in an impaired autoregulatory response to alterations in systemic blood pressure (Kastrup *et al.*, 1986), and altered CO_2 reactivity (Griffith *et al.*, 1987).

In the streptozotocin-induced animal model of diabetes there is clear evidence, from both *in vitro* and *in situ* (cranial window) studies, of impaired cerebrovascular responsiveness to a variety of vasoactive compounds including ADP (Mayhan, 1989), 5-hydroxytryptamine (Rosenblum & Levasseur, 1984; Mayhan, 1989), β-adrenoceptor agonists (Mayhan, 1994), and acetylcholine (Mayhan *et al.*, 1991). Studies performed *in vivo* showed a reduced effect of muscarinic agonists upon blood flow (Pelligrino *et al.*, 1992). Interestingly, however, the streptozotocin rat model does not appear to display the same reduced cerebrovascular CO_2 reactivity found in human subjects (Pelligrino & Albrecht, 1991). Further *in vivo* studies of peripheral vascular beds in streptozotocin-treated rats have revealed a complex endothelial dysfunction with the pressor response to L-NAME being attenuated (Kiff *et al.*, 1991a) whilst vasodilator responses to acetylcholine remained intact (Kiff *et al.*, 1991b).

The BioBred (BB) rat strain provides a useful model for the study of insulin-dependent diabetes mellitus (IDDM). The involvement of genetic and immune aetiological factors in the pathogenesis of the disease, together with the dependence on exogenous insulin for prevention of ketoacidosis and the development of diabetic complications in a variety of organs (Marliss *et al.*, 1982), represent a condition more akin to the human disease process than that afforded by models of drug-induced diabetes (Eizirik *et al.*, 1994). Pathological changes in the retina, kidneys and peripheral nerves have been observed as early as 3 weeks following the onset of diabetes (Baird, 1989).

The purpose of this study was to measure the local cerebral blood flow (LCBF) in insulin-dependent diabetic BB rats to determine whether the decreases in CBF evident in human diabetes were paralleled in this animal model. Given the importance of NO in the regulation of normal cerebral blood

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flow (Faraci & Brian, 1994; Kimura *et al.*, 1994) and the fact that diabetes mellitus is associated with alterations of NO production and release in the extracerebral tissues (Bucala *et al.*, 1991; Corbett *et al.*, 1992; Cohen, 1993), diabetic rats were challenged with the nitric oxide synthase (NOS) inhibitor N^G-nitro-L-arginine methyl ester (L-NAME), to assess the involvement of the NO pathway in any perturbations in basal cerebrovascular control.

Methods

Animals

All rats were supplied from the British Diabetic Association BB (Edinburgh) U.K. Resource Unit. The BB/E colony consists of two lines created by selectively breeding for and against diabetes. In the high-incidence diabetes-prone (DP) main line, the incidence of IDDM is 50–60%, and the age at onset of diabetes is 96 ± 18 days (mean $\pm$ s.d.). In the diabetes-resistant (DR) subline, the incidence of diabetes is $<1\%$.

All rats were maintained at 20°C on a 12 h light/dark cycle and fed rat and mouse Number 1 Expanded Feed (Special Diet Services, Witham, U.K.). Animals were weighed twice weekly from 40 days of age. Failure to gain weight, or loss of weight, was taken as an indication of the possible onset of diabetes, and such rats were tested for glycosuria. If glycosuria was detected, the blood glucose concentration was measured from a sample obtained by tail-tipping. A blood glucose concentration >18 mmol l^{-1} is invariably associated with ketonuria, weight loss and the requirement for daily injection of insulin to survive. These variables constituted our criteria for classifying an animal as having IDDM.

A total of 16 adult male BB rats (weight: 348–495 g) were used in this study from both the DP ($n=8$) and DR ($n=8$) sublines. At the time of the study all rats in the DP group were diabetic, and had been so for between 8 to 20 weeks. These animals had been treated since the onset of diabetes with a single daily subcutaneous injection of medium-acting insulin (2.4–4.0 iu) given at 09 h 00 min each day. Experiments were performed 4 h after the last insulin injection in the diabetic animals. Animals in the DR group were age-matched to those in DP group and served as controls for the effects of the disease processes.

Measurement of local cerebral blood flow

On the day of the experiment the animals were anaesthetized with halothane (1.5% in a gas mixture of 70% nitrous oxide and 30% oxygen) and prepared for the measurement of LCBF as described previously (Kelly *et al.*, 1994). Following surgery, general anaesthesia was withdrawn and 2 h allowed to elapse before any further experimental manipulation.

Equal numbers of non-diabetic and diabetic rats were injected (i.v.) with either L-NAME (30 mg kg^{-1}; $n=4$ from each group) or an equal volume of saline (1.0 ml; $n=4$ from each group) over 60 s via a femoral venous cannula. At this dose, L-NAME reduces LCBF significantly by 15 min post-injection and the effect is maintained stable for at least 3 h (Macrae *et al.*, 1993). The measurement of LCBF was started 20 min after the injection of L-NAME or saline by the fully quantitative [^{14}C]-iodoantipyrine autoradiographic technique. The protocols were in complete accordance with the methodology as originally published (Sakurada *et al.*, 1978) and as described previously from this laboratory (Kelly *et al.*, 1994). Autoradiographic images were analyzed by quantitative densitometry relative to ^{14}C-containing standards, and LCBF was calculated by the appropriate operational equation for the technique (Sakurada *et al.*, 1978). Areas of interest were chosen to represent brain areas in the vascular territories of the anterior, middle and posterior cerebral arteries. Arterial blood pressure and rectal temperature were monitored continuously in every animal throughout the experiments and heart rate was measured intermittently. Samples of arterial blood were withdrawn before and after L-NAME or saline injection, for the measurement of pH, P_{CO_2}, P_{O_2}, plasma glucose and haematocrit.

Data (presented as mean $\pm$ s.e.mean) were analyzed by Student's t test with Bonferroni correction applied to allow multiple pair-wise comparisons between appropriate groups. Acceptable levels of significance were set at $P<0.05$.

Results

Physiological variables

Prior to the injection of either L-NAME or saline there were no significant differences in blood gas tensions, pH, rectal temperature or mean arterial blood pressure (MABP) between non-diabetic and diabetic rats, although heart rate was significantly lower (−22%) in the diabetic group (Table 1). Abnormally high base excess in the diabetic animals confirmed the chronic metabolic disturbances of diabetes, but prior to any drug treatment there were no significant differences in either plasma glucose or body weight between untreated non-diabetic and insulin-treated diabetic rats. There was, however, considerable variation in plasma glucose in both non-diabetic and diabetic rats (coefficient of variation = 58 and 71% respectively). Whilst this possibly indicates a more variable glucose metabolism in the non-diabetic BB subline than would normally be expected, it must be stressed that plasma glucose in all animals was within the normal physiological range (Table 1). Although there was a trend towards increased haematocrit in diabetic rats, this was not significant.

Following the injection of L-NAME, MABP increased to a similar extent in both non-diabetic (+21%) and diabetic rats

Table 1 The effects of L-NAME upon physiological variables in non-diabetic and diabetic rats

	Non-diabetic		*Diabetic*	
	Pre L-NAME	*Post L-NAME*	*Pre L-NAME*	*Post L-NAME*
pH	7.38±0.02	7.38±0.02	7.40±0.01	7.39±0.01
P_{CO_2} (mmHg)	42.5±1.5	35.3±1.8	45.2±1.8	40.8±0.9
P_{CO_2} (mmHg)	89.6±2.7	100.1±1.9	85.9±0.9	92.1±4.4
Base excess	−0.13±0.7	−3.1±1	2.9±0.7	0.3±0.8
Haematocrit (%)	47.8±0.5	50.5±1.0	52.5±1.0	54.9±1.0
Plasma glucose (mmol l^{-1})	15±4.5	14±5	9.5±3.5	7.5±3.5
Heart rate (beats min^{-1})	405±15	293±8#	315±15*	255±15
MABP (mmHg)	117±3	142±2#	121±3	149±4#
Temperature (°C)	36.0±0.1	36.5±0.1	36.4±0.3	36.4±0.2

Data are presented as mean $\pm$ s.e.mean (n = 4 in each group). There were no differences in the values obtained from the saline-treated animals (diabetic and non-diabetic) and those measured prior to injection of L-NAME. *Significant difference between diabetic and non-diabetic animals; #significant difference between pre- and post L-NAME.

(+23%), but the heart rate was reduced significantly only in the non-diabetic group (−28%) (Table 1). In diabetic rats, where heart rate was already significantly lower prior to treatment (−22% compared to non-diabetics), the effect of L-NAME (−19%) was not significant. As a result, heart rates were similar in the two L-NAME-treated groups (diabetic and non-diabetic) following L-NAME (see Table 1).

Local cerebral blood flow

In saline-treated, diabetic rats, mean LCBF was reduced in all 13 brain areas when compared to non-diabetic controls (Table 2). However, the extent of these reductions in LCBF were regionally heterogenous, ranging from −8% in parietal cortex (not significant) to −32% in piriform cortex ($P<0.05$). Using the conservative statistics required for multiple comparisons, the reductions were statistically significant in only five of the areas examined, and these were predominantly sub-cortical (Table 2). Taking each diabetic animal individually, there was no correlation between the extent of LCBF reduction and either duration of diabetes or plasma glucose status at the time of the experiment.

In keeping with previous observations, L-NAME treatment produced reductions in LCBF throughout the brain in non-diabetic animals (Table 2). Only in parietal (−10%) and cingulate areas of cortex (−19%) and in nucleus accumbens (−21%) did the effects of L-NAME fail to reach statistical significance. Elsewhere, significant ($P<0.05$) reductions in LCBF were measured, ranging from −21% in the molecular layer of the hippocampus to −44% in piriform cortex (Table 2). In contrast, L-NAME treatment had no significant effect upon LCBF in diabetic rats, when compared to the appropriate saline-treated (diabetic) group. The one exception to this was the hypothalamus, where a significant (−33%) decrease in LCBF was observed (Table 2). In contrast to the significant differences in flow between the saline-treated non-diabetic and diabetic groups, there were no significant differences in LCBF between the groups treated with L-NAME (Table 2).

The cerebrovascular response to L-NAME will be influenced not only by the direct inhibition of NOS in the blood vessels of the brain, but also indirectly via autoregulatory responses to peripheral hypertension. Following L-NAME

Table 2 Local cerebral blood flow in diabetic and non-diabetic animals, treated with saline or L-NAME

	Saline		*L-NAME*	
	Non-diabetic	*Diabetic*	*Non-diabetic*	*Diabetic*
Neocortex				
Parietal	146±5	134±12	131±7	115±6
Cingulate	148±9	127±16	120±8	113±13
Occipital	133±2	99±11	83±9#	84±13
Piriform	111±6	76±6*	62±6#	66±6
Corpus callosum	39±2	32±1	27±1#	25+4
Basal ganglia				
Striatum	125±5	87±3*	93±2#	84±9
Globus pallidus	73±1	54±3*	50±2#	47±5
Accumbens	124±10	107±6	98±4	89±10
Thalamus				
Hypothalamus	95±3	80±6	59±5#	54±5#
Lateral geniculate	142±7	109±6*	84±2#	86±10
Hippocampus				
CA 2,3	94±5	74±7	60±3#	66±9
Molecular layer	90±2	68±8	70±11#	61±6
Dentate gyrus	92±4	72±4*	59±2#	67±10

Data are presented as mean local cerebral blood flow (ml $100g^{-1}min^{-1}$)±s.e.mean (n = 4 in each group). *Significant difference between diabetic and non-diabetic animals; #significant difference between saline and L-NAME-treated animals.

Table 3 Cerebrovascular resistance in diabetic and non-diabetic animals, treated with saline or L-NAME

	Non-diabetic			*Diabetic*		
	Saline	*L-NAME*	*% change*	*Saline*	*L-NAME*	*% change*
Neocortex						
Parietal	0.76±0.05	1.10±0.08#	45	0.84±0.08	1.32±0.10#	57
Cingulate	0.76±0.07	1.20±1.10#	58	0.90±0.12	1.37±0.16	52
Occipital	0.82±0.04	1.79±0.23#	118	1.15±0.15	1.93±0.37	67
Piriform	1.00±0.10	2.35±0.27#	135	1.46±0.15	2.33±0.30	60
Corpus callosum	2.86±0.13	5.34±0.26#	87	3.40±0.17	6.28±0.93	85
Basal ganglia						
Striatum	0.88±0.05	1.53±0.05#	74	1.26±0.07*	1.84±0.22	46
Globus pallidus	1.51±0.07	2.84±0.12#	88	2.03±0.12*	3.33±0.30#	64
Accumbens	0.91±0.10	1.47±0.07#	62	1.03±0.06	1.76±0.24	71
Thalamus						
Hypothalamus	1.16±0.07	2.49±0.24#	115	1.39±0.13	2.85±0.37#	105
Lateral geniculate	0.77±0.04	1.70±0.05#	121	1.05±0.05*	1.83±0.25	74
Hippocampus						
CA 2,3	1.16±0.01	2.40±0.08#	107	1.52±0.15	2.42±0.39	59
Molecular layer	1.22±0.05	2.37±0.24#	94	1.68±0.23	2.36±0.52	40
Dentate gyrus	1.20±0.05	2.43±0.10#	103	1.52±0.10*	2.43±0.40	60

Cerebrovascular resistances were calculated by dividing mean arterial blood pressure (mmHg) by LCBF values (ml $100g^{-1}min^{-1}$) for each individual animal and are presented as mean±s.e.mean (n = 4 in each group). *Significant difference between diabetic and non-diabetic animals; #significant difference between saline and L-NAME-treated animals.

treatment, calculated mean vascular resistance values were increased in all brain regions in both non-diabetic and diabetic rats (Table 3). In non-diabetic rats, these increases in resistance paralleled a decrease in LCBF in the majority of brain regions (Table 2), but in diabetic rats, whilst there was only moderate change in LCBF (with the exception of the hypothalamus), ranging between −3% and −21% (Table 2), cerebrovascular resistance increased by between 40 and 74% (Table 3). This increased resistance, with no change in flow, is likely to be the result of autoregulatory cerebrovascular constriction in response to peripheral hypertension.

Discussion

This is, to our knowledge, the first study of local cerebral blood flow in sponanteously diabetic insulin-dependent BB rats. The global tendency towards reduced cerebral blood flow which we have observed in these animals parallels to some extent that found originally in human diabetic patients (Kety *et al.*, 1948), but with the greater spatial resolution afforded by the use of quantitative autoradiography in our studies, we have been able to identify a degree of regional heterogeneity in the effects of diabetes upon cerebral blood flow. Whether this apparent differential susceptibility to the disease processes in different parts of the cerebrovascular bed reflects regional variations in vascular pathology, remains to be determined. Regional differences in LCBF have also been described recently in human diabetics when compared to healthy control subjects (Grill *et al.*, 1990; Macleod *et al.*, 1994). In these human studies a relative sparing of flow in fronto-parietal cortex and large decreases in the caudate nucleus show remarkable similarities to the results described here. However, even the most sophisticated imaging techniques currently availabe in man do not have the spatial resolution of animal brain autoradiography, nor can the experimental conditions be as rigorously controlled. It may, therefore, be impossible to find exact parallels between the effects of diabetes upon LCBF described in this study and those in diabetic humans.

A number of studies have examined cerebral blood flow in untreated streptozotocin-induced diabetic rats, with varying results (Duckrow *et al.*, 1987; Harik & LaManna, 1988; Jakobsen *et al.*, 1990; Pelligrino & Albrecht, 1991). Although in general terms reductions in LCBF were found when streptozotocin diabetic rats were compared to controls, the results were often too variable to reach statistical significance, and no clear consensus emerges on the susceptibility of particular regions of the brain to the condition. Interestingly however, if the rats were treated with insulin to normalize glycaemia at the time of the measurement, any differences in LCBF between diabetics and controls were eliminated (Pelligrino & Albrecht, 1991). A similar effect has also been described in peripheral nerve blood flow (Kihara & Low, 1995). In contrast, in the present study of spontaneously diabetic, insulin-dependent rats, significant decreases in LCBF were evident despite the fact that there was no difference in plasma glucose levels between diabetic and non-diabetic animals at the time of the study. This is not to say, however, that the BB rats have not experienced periods of hyperglycaemia. The measurement of LCBF in the diabetic animals was conducted around 4 h after the injection (s.c.) of medium-acting insulin, and the evidence from the physiological data suggests that the hormone was acting to normalize plasma glucose. Over a longer time scale, plasma glucose concentrations in BB/E rats are quite unstable and fluctuate in the course of any 24 h cycle between 3 and 22 mmol l^{-1}. In a parallel study using groups of rats from the same colony, we have found (unpublished observations) that glycosylated haemoglobin (HbA_1) values were elevated ($7.46 \pm 0.87\%$) compared to non-diabetic controls ($3.13 \pm 0.24\%$). There is no doubt, therefore, that our diabetic animals are hyperglycaemic for a large part of the time, although maximum plasma glucose levels are unlikely to reach those found in streptozotocin-treated rats.

In the present study there appeared to be an attenuation of the effects of L-NAME upon LCBF in diabetic rats. It is tempting to speculate that the decreases in LCBF apparent in saline-treated diabetics may be the result of reduced dilatator influence of endogenous NO in determining basal cerebral blood flow, possibly mediated via disinhibition of endothelin release (Gardiner *et al.*, 1995; Kelly *et al.*, 1995; Richard *et al.*, 1995). There is certainly evidence from the peripheral circulation that endothelial NO systems are disrupted in diabetes (Cohen, 1993; Poston & Taylor, 1995), although there is clear evidence of variation in the defect between different vascular beds (Kiff *et al.*, 1991a). In the cerebral circulation the data are equally complex. Indirect evidence for reduced NO activity comes from studies of streptozotocin-induced diabetic rats where a significant, but regionally variable, impairment of endothelium-dependent vascular relaxation was observed following injection of a muscarinic receptor agonist. More direct evidence of an impairment of cerebrovascular NO systems in this diabetic model are however lacking, in that the effects of L-NAME upon cerebral blood flow (Pelligrino *et al.*, 1992) and pial vessel diameter (Mayhan *et al.*, 1991) were reported to be similar in both non-diabetic and diabetic rats. Whilst we similarly found no difference in LCBF values between diabetic and non-diabetic rats following L-NAME, with the exception of the hypothalamus, this did not represent a significant decrease in flow from saline-treated diabetic rats in which blood flow was already depressed. This we interpret as an attenuation of the cerebrovascular response to L-NAME.

It is interesting that whilst we found reduced basal LCBF and an attenuated cerebrovascular response to L-NAME in our diabetic rats, the saline-treated diabetic animals were not hypertensive and L-NAME-treated diabetics displayed the normal blood pressure response, i.e. hypertension. This might suggest that the disease process is more pronounced in the cerebral circulation than it is in other vascular beds. However, the aortae of diabetic BB rats do develop morphological defects in endothelial cells and abnormal endothelium-dependent responses to acetylcholine (Meraji *et al.*, 1987). Moreover, the hypertensive response to chronic L-NAME administration is attenuated in diabetic BB/E rats (Lindsay *et al.*, 1995). Thus it is possible that the mechanisms of NO dysfunction associated with diabetes develop differentially in different vascular beds.

Although there is evidence that the effectiveness of endogenous NO in influencing basal vascular tone may be altered by diabetes (Bucala & Cerami, 1992; Wascher *et al.*, 1994), it is not clear whether this represents a change in synthesis and release, or in activity. There is evidence for reduced levels of L-arginine in the plasma of diabetic rats (Mans *et al.*, 1987) which might reduce NO synthesis, although there is also *in vitro* evidence that hyperglycaemia may actually increase NO production (Wascher *et al.*, 1994). However, *in vivo*, elevated intracellular glucose is converted to sorbitol via the polyol pathway in the endothelium with resultant depletion of cellular NADPH and reduced NOS activity (Cohen, 1993). The same pathway increases the formation of free radicals that inactivate endogenous NO (Cameron & Cotter, 1995). Finally, increased formation of subendothelial advanced glycosylation end products by elevated glucose may quench and inactivate NO (Bucala *et al.*, 1991; Cohen, 1993).

Although hyperglycaemia is believed to be an important factor contributing to vascular dysfunction associated with diabetes, and would certainly account for dysfunction in endothelial NO systems (Cohen, 1993; Poston & Taylor, 1995), other mechanisms may also be involved. Rheological problems such as an increase in plasma viscosity (Barnes *et al.*, 1977) and increased adhesion of platelets to endothelial cells (MacMillan *et al.*, 1978; Wautier *et al.*, 1981) may contribute to cerebrovascular dysfunction in diabetes, and some aspects of diabetic vascular pathology, notably arteriosclerosis (Grunnet, 1963), may be related to the hypertension often associated with diabetes. Hypertension develops only at a later stage in BB rats and is not therefore an issue in these studies, but in a parallel study using groups of rats from the same colony, we have

indeed found increased blood viscosity in BB diabetic animals. However not only is the significance of blood viscosity in determining LCBF contested (Brown & Marshall, 1985; Waschke *et al.*, 1994), it is also unlikely that increased viscosity can explain the heterogeneity in the reduction of blood flow which we observed.

There is increasing evidence that diabetes adversely affects the outcome in experimental models of cerebral ischaemia (Nedergaard & Diemer, 1987; Sutherland *et al.*, 1992) and in human occlusive stroke (Jørgensen *et al.*, 1994). Although the crucial role of elevated plasma glucose in situations of cerebral ischaemia cannot be over-emphasized (Smith *et al.*, 1986), evidence from the present study that there may also be an already perturbed basal blood flow and reduced endothelial NO activity, could represent an important additional factor contributing to the morbidity of stroke in diabetic patients.

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