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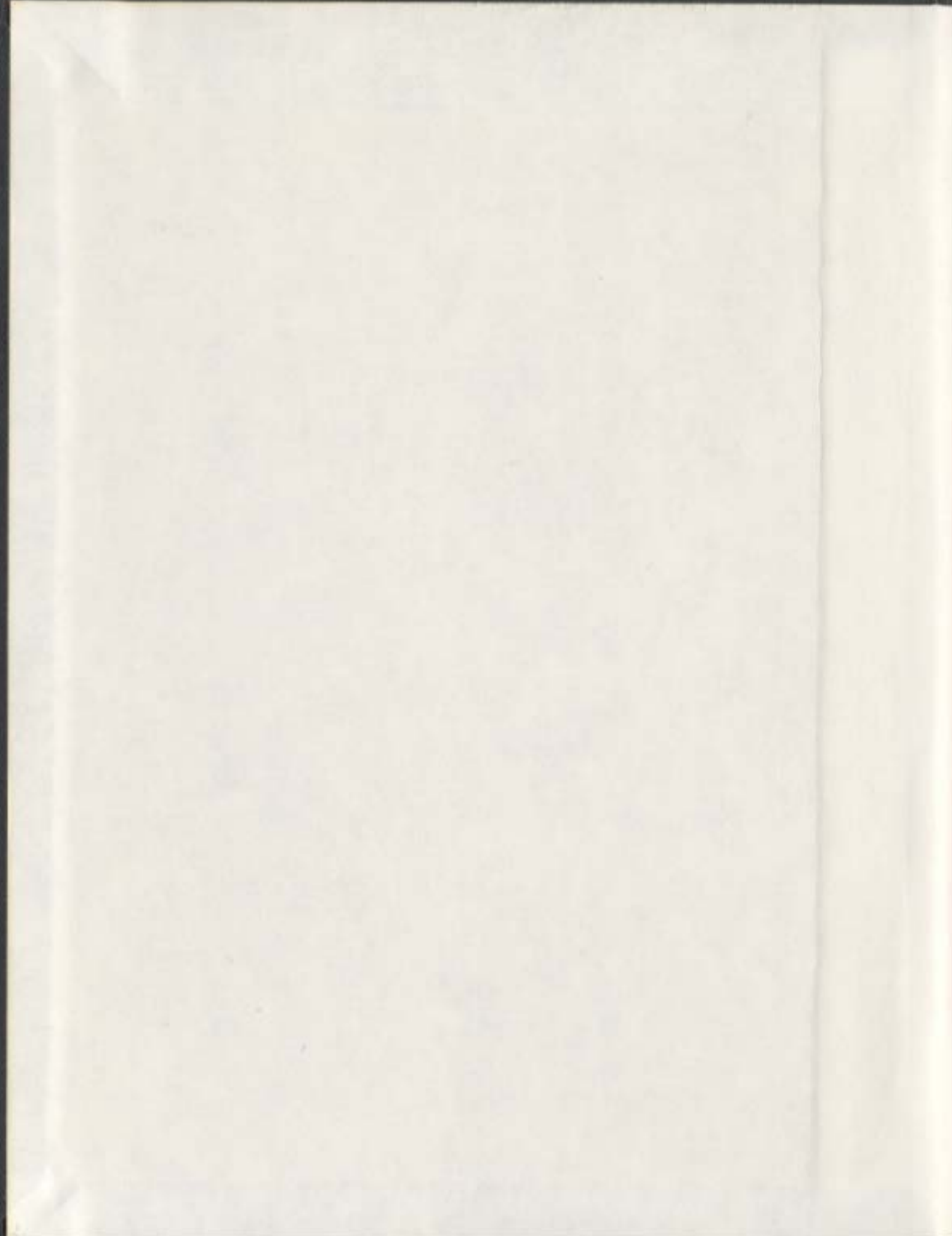
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THE CHARACTERIZATION OF CEREBROVASCULAR DYSFUNCTION ASSOCIATED WITH HYPERTENSIVE ENCEPHALOPATHY IN DAHL SALT-SENSITIVE RATS

by

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ABSTRACT

We initially assessed the characteristics of stroke development in Dahl salt-sensitive rats (Dahl-SS). Dahl-SS developed high blood pressure (BP) when fed a high salt diet (8.7% NaCl) from weaning and a 50% mortality after being fed the diet for 4 to 5 weeks. Prior to death, Dahl-SS exhibited behavioural symptoms (convulsions, seizures, paralysis and stupor) consistent with the possible development of stroke. However, unlike true stroke, the behavioural dysfunctions observed were not associated with cerebral ischemia and occurred in the virtual absence of cerebral hemorrhage. An investigation of the cerebrovascular pathology indicated a breakdown in the integrity of the blood brain barrier and fluid movement into the extravascular space (edema). It was concluded that Dahl-SS best represents a model of hypertensive encephalopathy (HE). In humans HE is produced by brain edema as a result of hypertension in the absence of cerebral ischemia or hemorrhage. It produces convulsions, confusion, and stupor and can result in death. The latter symptoms are consistent with those observed in Dahl-SS fed high salt.

Antihypertensive intervention (captopril) was ineffective in lowering blood pressure or reducing the incidence of mortality. Non-cerebral organ failure was also evident prior to death as demonstrated by kidney dysfunction associated with increased plasma creatinine, urea, urinary protein excretion and decreased plasma albumin levels.

In subsequent experiments we tested the hypothesis that a breakdown in the ability to autoregulate cerebral blood flow (CBF) may contribute to the development of HE in Dahl-SS fed high salt. Such a defect could promote cerebrovascular overperfusion and elevate microvascular blood pressure, alterations that would facilitate blood brain barrier disruption and HE development. Laser Doppler techniques were used to assess the changes in relative CBF with varying BP in the perfusion domain of the middle cerebral arteries (MCA's). Dahl-SS fed 8.7% NaCl for 1 week exhibited an ability to autoregulate near constant CBF up to an upper mean BP of 168 mmHg. Two thirds of the rats lost the ability to autoregulate CBF after they were fed a high salt diet for 3 weeks at a time prior to the development of HE. These rats exhibited a linear increase in CBF with elevations in arterial pressure. The characteristics of the CBF autoregulatory curves suggested that CBF autoregulation was lost under conditions of cerebrovascular constriction.

In other experiments we assessed the hypothesis that the loss of CBF autoregulation in the MCA perfusion domain of Dahl-SS was associated with an inability of the MCA's to elicit pressure dependent constriction (PDC). PDC is an important mechanism involved in promoting CBF autoregulation. Elevations in BP promote cerebrovascular constriction, which raises vascular resistance to blood flow. This counteracts the potential elevation in CBF enabling CBF to remain constant under conditions of elevated BP. Isolated MCA's from asymptomatic Dahl-SS exhibited constriction in response to elevated pressure

and protein kinase (PKC) activation (a signaling intermediate for PDC in MCA's). In addition the MCA's vasodilated in an endothelium dependent manner in response to bradykinin. These functions were lost in the MCA's of Dahl-SS with HE. MCA's from post-HE Dahl-SS that were unable to constrict to pressure lacked the ability to constrict in response to PKC activation via phorbol esters. They exhibited high levels of basal tone and no response to the endothelial specific vasodilator, bradykinin. The loss of PDC in MCA's of Dahl-SS preceded the development of HE and occurred in asymptomatic rats fed high salt for 3 weeks at a time when CBF autoregulation was lost.

It was concluded that defects in the ability of the cerebrovasculature to autoregulate CBF in conjunction with the development of renal dysfunction could contribute to the development of HE in Dahl-SS fed high salt. Cerebrovascular PDC is thought to play an important role in facilitating CBF autoregulation. The loss of this function could contribute to a loss of CBF autoregulation under hypertensive conditions. This could increase cerebrovascular pressures and promote overperfusion in the brain, leading to the development of cerebral edema and HE. The development of HE could be further augmented by a decrease in plasma oncotic pressure promoted by the loss of plasma proteins due to the occurrence of proteinuria. The presence of a dysfunctional PKC system in the vascular smooth muscle of MCA's sampled from Dahl-SS with HE could contribute to the loss of PDC in the arteries.

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ABBREVIATIONS

20-HETE	-	20-hydroxyeicosatetraenoic acid
ACEI	-	angiotensin converting enzyme inhibitor
ANG II	-	angiotensin II
ANOVA	-	one-way analysis of variance
BBB	-	blood brain barrier
BP	-	blood pressure
BUN	-	blood urea nitrogen
Ca^{2+}	-	calcium
CBF	-	cerebral blood flow
cGMP	-	3',5'-guanosine monophosphate
CT	-	computerized tomography
DAG	-	diacylglycerol
Dahl-SR	-	Dahl salt resistant
Dahl-SS	-	Dahl salt sensitive
GFAP	-	glial fibrillary acidic protein
GLM	-	general liner model
HE	-	hypertensive encephalopathy
IP_3	-	inositol triphosphate
MANOVA	-	multiple analysis of variance
MCA	-	middle cerebral artery

N (n)	-	number
NaCl	-	sodium chloride
NO	-	nitric oxide
NOS	-	nitric oxide synthase
PdB	-	phorbol dibutyrate
PDC	-	pressure dependent constriction
PKC	-	protein kinase C
PLC	-	phospholipase C
RAS	-	renin angiotensin system
SHRsp	-	stroke prone spontaneously hypertensive rat
TALH	-	thick ascending loop of henle
TRPc	-	transient receptor potential channel
TTC	-	2,3,5-triphenaltetrazolium chloride
VGCC	-	voltage gated calcium channels
WKY	-	Kyoto wistar

CHAPTER 1

LITERATURE REVIEW

1.1 ETIOLOGY OF STROKE

Stroke is defined as a neurological dysfunction (behavioural, motor and cognitive) produced by ischemia and/or hemorrhage within the brain. It is one of the most prevalent diseases promoting mortality and morbidity in western society.

Ischemic stroke is promoted by an interruption of blood flow through the cerebrovasculature. Blood flow interruption can be precipitated by blockages (sclerotic plaques or emboli) within the cerebral vessels or carotid arteries or as a result of an interruption in blood flow to the brain promoted by decreases in cardiac output (Caplan, 2000). The extent of brain damage that occurs is dependent on the length of time that blood flow is compromised. Neurological deficits will vary depending on location of ischemia.

Hemorrhagic stroke can be subdivided into intracerebral (bleeding directly into the brain) or subarachnoid (bleeding along the surface of the brain within the subarachnoid space). The time course is different for each subtype of hemorrhage. Subarachnoid hemorrhage develops quickly. Blood released from aneurysms of surface arteries fills the subarachnoid space around the brain. This raises intracranial pressure and promotes regional ischemia due to vascular compression and cerebrovascular constriction (Davis and Robertson, 1991). The compression of cranial nerves can also result in a disruption of normal neural

pathway activity (Caplan, 2000). Typically, intracerebral hemorrhage develops more gradually in comparison to subarachnoid hemorrhage. Extravasation of blood from intracerebral arterioles and capillaries creates a situation where blood accumulates within the extravascular space of the brain (Caplan, 2000). In addition to blood, other substances (i.e. glutamate, serotonin, fatty acids, lysosomal enzymes and free radicals) that leak into the parenchyma from the lesion alter vascular integrity and reactivity (Davis and Robertson, 1991). The resultant effects may include changes in endothelial integrity, cell morphology and cell energy metabolism (Baethmann et al., 1980) as well as the production of cerebral vasospasm (Sobey & Faraci, 1998; Sobey, 2001). There are certain situations, such as during cocaine use, where intracerebral hemorrhage can occur rapidly (Conway & Tamargo, 2001).

In addition to the occurrence of ischemic and hemorrhagic stroke, hypertension is associated with other forms of cerebral pathology such as hypertensive encephalopathy (HE). HE is often produced by a sudden rise in blood pressure that promotes widespread edema formation and the loss of cerebral blood flow autoregulation (CBF) (Dinsdale, 1982). The loss of CBF autoregulation promotes overperfusion of the vasculature straining the integrity of the blood brain barrier and resulting in increased movement of fluid (edema) and possibly blood into the extravascular space (Skinhoj & Strandgaard, 1973; Strandgaard et al., 1974). Brain edema produces a rise in intracranial pressure causing neurological abnormalities to develop (Byrom, 1969).

1.2 ROLE OF HYPERTENSION IN STROKE DEVELOPMENT

There is a strong correlation between the rise in blood pressure and the incidence of ischemic and hemorrhagic stroke. Hypertension has been suggested to be the single most prevalent modifiable risk factor associated with stroke development (Strandgaard, 1996). Consistent with this, antihypertensive treatments significantly attenuate the onset of stroke development (Collins *et al.*, 1990; MacMahon, 1990).

1.2.1 Hemorrhagic Stroke and Hypertension

Hemorrhagic stroke represents 10-15% of all stroke cases (Thrift *et al.*, 1995). Intracerebral hemorrhage is the most common form of hemorrhagic stroke. The occurrence of hypertension is more closely related to hemorrhagic than ischemic stroke and antihypertensive treatments reduce the risk of this type of stroke development. (Gebel & Broderick, 2000). The likelihood of intracerebral hemorrhagic stroke development is 2 to 6 times greater in hypertensive individuals versus normotensive individuals (Okada *et al.*, 1976; Lin *et al.*, 1984; Kagan *et al.*, 1985; Brott *et al.*, 1986).

Although human epidemiological studies have confirmed that hypertension is a key risk factor promoting the development of hemorrhagic stroke, eight percent of stroke due to intracerebral hemorrhage occurs without elevated blood pressure. Eight percent of intracerebral hemorrhage occurs in individuals with normal pressure (del Zoppo & Mori, 1992). Patients show an increase in the

vulnerability to develop hemorrhagic stroke during anticoagulant treatment. There are also congenital and acquired factor deficiencies (thrombocytopenia and thrombocytopathic) that increase the likelihood of developing cerebral hemorrhage (Hart et al., 1995). Arteriovenous malformations (enlarged vessels), cavernous angiomas (fibrous deposits in vessel walls) and cerebral tumors increase the risk cerebral hemorrhage (Gebel & Broderick, 2000).

1.3 ANIMAL MODELS OF STROKE

Although the link between hypertension and stroke development is apparent in humans there are few animal models that develop stroke spontaneously during hypertension. Early studies evaluating stroke development tried to mimic stroke, either by occluding blood vessels projecting into the brain (Bederson et al., 1986; Grabowski et al., 1988) or by injecting blood into the brain ventricles (Batton & Nardis, 1987). The emphasis of these studies was on the observation of the secondary changes in cerebral pathology associated with these interventions rather than the underlying mechanisms promoting stroke development. Stroke has been inconsistently noted in Goldblatt renal forms of hypertension (Byrom, 1969). Predictable and consistent stroke development has been described in Kyoto Wistar stroke prone spontaneously hypertensive rats (SHRsp) (Smeda, 1989, 1992) and Dahl salt sensitive rats (Dahl-SS) (von Lutterotti et al., 1992; Lin et al., 1999; Zhang et al., 1999).

1.4 DEVELOPMENT OF THE KYOTO WISTAR STROKE PRONE SPONTANEOUSLY HYPERTENSIVE RAT

Predicable, spontaneous stroke development occurs in SHRsp (Yamori *et al.*, 1984). Okamoto and Aoki (1963) selectively inbred Kyoto Wistar normotensive (WKY) rats that exhibited above average (> 150 mmHg) blood pressures. The selective inbreeding of these rats produced offspring in which high systolic BP (>180 mmHg) was observed by 13 weeks of age (normal systolic BP in a rat is <150 mmHg). This strain of rat was named the Kyoto Wistar spontaneously hypertensive rat (SHR). SHR developed a low incidence of stroke however by selective inbreeding of the offspring of the few SHR that exhibited stroke another substrain of SHR defined as the SHRsp was developed (Okamoto, 1974). Unlike SHR, which had systolic blood pressures of about 180 mmHg, SHRsp developed more robust hypertension (240 mmHg) and when they were fed an appropriate diet, a 100% incidence of stroke occurred prior to death (Yamori *et al.*, 1984).

1.4.1 Role of Diet in the Stroke Development of SHRsp

Diet was shown to play a crucial role in promoting stroke development in SHRsp. SHRsp fed the regular rat chow (Nihon Clea or Purina rat chow), lived between 31 and 41 weeks and developed a low incidence of stroke (30%)(Okamoto *et al.*, 1974; Yamori *et al.*, 1984). However, when the regular diet was substituted with a Japanese, Funahashi-sp diet, the incidence of stroke

increased to 80% (Yamori *et al.*, 1984). The only observable difference between the two diets was a lower protein content (18% compared to 22% in Purina Rat Chow). Wexler (1983) suggested that the origin of the protein within the diet (fish-Funahasi versus plant-Purina) had an important impact on stroke development in SHRsp. Subsequently, a North American–Japanese style diet (a reproduction of the Funahasi-sp diet; produced by Ziegler Brothers) was developed and supplemented with 4% NaCl (Smeda, 1989). The latter diet produced a 100% incidence of hemorrhagic stroke in SHRsp by 16 weeks of age (Smeda, 1989).

1.4.2 Behavioural Symptoms of Stroke Development in SHRsp

The characteristics of stroke development in SHRsp fed a Japanese style diet containing 4% NaCl have been previously outlined in detail (Smeda, 1989, 1992). The onset of stroke development consisted of repetitive convulsive movements of the upper extremities (noticeably forelimbs and head). Subsequently, the animals underwent periods of immobility and marked lethargy coupled with cessation of grooming. The animals adopted a posture, described as "kangaroo like", in which they remained motionless with their legs extended under their bodies and died on average 1.5 weeks after the first behavioural signs of stroke.

1.4.3 Cerebral Pathology and Hemorrhagic Stroke in SHRsp

Okamoto *et al.* (1974) demonstrated the presence of fibrinoid necrosis and hyaline deposits in the cerebral vessel walls in SHRsp with stroke and observed brain hemorrhages and edema. An analysis of the pathological changes in the brains of SHRsp with stroke was conducted by Ogata *et al.* (1981). In this study the brains of 5 SHRsp were serially sectioned for histological analysis. The affected vessels in the parenchyma were thickened due to fibrinoid deposits and most vessels showed stenosis or thrombotic occlusion. Vessels of the subarachnoid layer displayed proliferation of the arterial layer. Tissue injury included cyst formation in the white matter and rarefaction of the neuropil (Ogata *et al.*, 1980). In a more complete microscopic examination 38 brains of old SHRsp (>30 weeks of age displaying neurological symptoms consistent with stroke development) showed that 31 exhibited cerebral lesions. Rarefaction of the neuropil and preservation of the neocortical neurons and adjacent white matter were observed in 29 brains. There was massive intracerebral hemorrhage of 3 brains and observations of old hemorrhages filled with macrophages in 13 brains (Ogata *et al.*, 1982). In more recent studies, intracerebral hemorrhages have been detected on one or both of the hemispheres of the cerebrum in SHRsp fed a Japanese-style diet supplemented with 4% NaCl in which behavioural symptoms of stroke were observed (Smeda, 1989). Hemorrhages did not develop in the cerebellum, or the pons and medulla

regions of the brain. In addition, microhemorrhages were observed around the eyes of rats (Smeda, 1989).

1.4.4 Alterations in the Renin-Angiotensin System and Stroke Development in SHRsp

Recent studies evaluating stroke development in SHRsp have speculated that hyperactivity of the renin angiotensin system (RAS) may play a role in promoting the onset of stroke (Stier *et al.*, 1989; Stier *et al.*, 1991; Stier *et al.*, 1993; MacLeod *et al.*, 1997; Smeda *et al.*, 1999b).

1.4.4.1 Overview of the Renin Angiotensin System

The renin angiotensin system (RAS) regulates sodium and water balance, blood volume and arterial pressure within the body (Guyton, 1992). The main promoter in this cascade is renin, which is released into the circulation by the kidney and catalyzes the conversion of the poly-peptide angiotensinogen to angiotensin I (ANG I). AI is then converted to angiotensin II (ANG II) via the angiotensin converting enzyme (ACE) (Guyton, 1992).

Renin is released from preglomerular arterioles, in response to changes in NaCl concentration and urine flow through the distal renal tubules and is controlled by the juxtaglomerular apparatus (comprised of the section of distal tubule that passes between the efferent and afferent arterioles of the glomerulus). Reductions in the urine concentration of NaCl and/or low urine flow

are detected by the macula densa, which stimulates the release of renin from the afferent arterioles. In addition, the stimulation of β-adrenergic receptors located on renal arterioles can also increase the release of renin (Reid *et al.*, 1978). There are also non-juxtaglomerular mechanisms stimulating the release of renin. In hydronephrotic kidneys (lack functional macula densa), lowering renal perfusion pressure still produces renin secretion (Scholz *et al.*, 1993) suggesting that barostatic control independent of the macula densa exists.

1.4.4.2 The Renin Angiotensin System, Angiotensin II and Hypertension Development

There are two main ANG II receptor subtypes (AT_1 and AT_2), which are stimulated by ANG II (Stroth & Unger, 1999). Most of the classic effects of blood pressure and hypertension are mediated through the AT_1 receptor and can be blocked by AT_1 receptor antagonists such as losartan (Timmermans, 1999). The function of AT_2 receptor activation is less clear but studies have indicated that AT_2 receptors may be involved in modifying proliferation in rat carotid arteries (Nakajima *et al.*, 1995) and apoptosis in cultured cells (Yamada *et al.*, 1996).

ANG II has a profound impact on blood pressure. The stimulation of AT_1 receptors located on presynaptic sympathetic nerve terminals promotes the release of norepinephrine (NE) (Hughes & Roth, 1971). The release of NE acts on smooth muscle α_1 and α_2 receptors to produce vasoconstriction, thus increasing vascular resistance to blood flow and blood pressure. Stimulation of

AT_1 receptors in the adrenal gland by ANG II stimulates the release of aldosterone (Reid *et al.*, 1978; Weir & Dzau, 1999). Aldosterone is an antidiuretic hormone that facilitates the uptake of sodium and water from the urine into the blood. Sodium and water retention could lead to an increase in blood volume and cardiac output, which has the potential to raise blood pressure (Weir & Dzau, 1999).

ANG II can also promote the maintenance of hypertension development by inducing structural alterations in the arteriolar vasculature (Hajdu *et al.*, 1991b; Chillon & Baumbach, 1999). ANG II has been shown to increase smooth muscle cell division (Baumbach & Heistad, 1989). This could lead to the development of vascular hypertrophy (through increased vascular smooth cell multiplication and individual cell growth). An increase in wall thickness facilitates the maintenance of hypertension by increasing vascular contractile reactivity and vascular resistance to flow. Evidence supporting this was demonstrated in SHR. Treatment with angiotensin converting enzyme inhibitors or the AT_1 receptor antagonist losartan normalized blood pressure and promoted a thinning of the cerebral vascular wall (Hajdu *et al.*, 1991b; Chillon & Baumbach, 1999). Other antihypertensive agents such as hydralazine can also normalize blood pressure in SHR but such treatment has no effect on vascular wall thickness (Hajdu *et al.*, 1991a).

1.4.4.3 Alterations in Renin Angiotensin System in SHRsp in Relation to Stroke Development in SHRsp

Plasma renin levels are increased with age in SHRsp (Volpe *et al.*, 1990; Camargo *et al.*, 1991; Gahnem *et al.*, 1994) and are higher in comparison to Kyoto Wistar normotensive control rats (Stier *et al.*, 1991; Kim *et al.*, 1992; Hubner *et al.*, 1995). Normally an increase in dietary salt would reduce the secretion of renin (Stier *et al.*, 1993). However, in SHRsp this produces a paradoxical situation where plasma renin levels are increased (Stier *et al.*, 1991).

Substantial research has been conducted on SHRsp demonstrating that angiotensin converting enzyme inhibitors (ACEIs which inhibit the conversion of ANG I to ANG II) retard the onset of stroke development. This often occurs in the absence of an antihypertensive effect (Stier *et al.*, 1989; Stier *et al.*, 1991; Stier *et al.*, 1993; MacLeod *et al.*, 1997).

Steir *et al.* (1989) studied SHRsp fed a stroke-prone diet supplemented with 1% NaCl in the drinking water. Enalapril treatment (15 mg/kg/day, in the drinking water) at 8 to 9 weeks of age produced a small hypotensive effect and retarded the onset of stroke-associated mortality from about 14 to 36 weeks of age. Captopril treatment (50mg/kg/day) produced a similar effect (Stier *et al.*, 1991). A small reduction in blood pressure was observed between 9 and 11 weeks of age and no stroke-associated mortality was observed up to 26 weeks of age. In other studies, the AT_1 receptor antagonist losartan (10 mg/kg/day) produced similar effects to those observed with ACEI treatment (Stier *et al.*,

1993). No observable signs of stroke were observed up to 28 weeks of age whereas a 100% mortality was observed in untreated SHRsp by 14 weeks of age.

The effects of ACEIs or losartan can not be explained by the modest antihypertensive effects of the drugs. Stier *et al.* (1989) found that the blockade of thromboxane A2 synthesis by dazmegrel in SHRsp fed high salt produced similar antihypertensive effects to those observed during enalapril and captopril treatment. However, the onset of stroke was not altered. This suggested that blockade of RAS with ACEIs or losartan in SHRsp retarded the onset of stroke development in a manner independent of any antihypertensive effect. In this regard, it was suggested that ANG II increased vascular damage in the cerebrovasculature by increasing neutrophil chemoattraction to the vascular endothelium and enhancing cerebrovascular fibrinoid necrosis, thus altering vascular permeability (Stier *et al.*, 1989; Stier *et al.*, 1991). These alterations were suggested to facilitate the development of stroke in SHRsp fed high salt.

Recent studies evaluated the anti-stroke effects of aldosterone suppression on stroke development in SHRsp fed a high salt diet (Japanese-style diet supplemented with 4% NaCl). ACE inhibition with captopril (50mg/kg/day) reduced plasma aldosterone levels and delayed the onset of stroke (MacLeod *et al.*, 1997). It was observed that the administration of aldosterone (via osmotic mini pumps) into captopril treated rats negated the anti-stroke effects of captopril allowing stroke development to occur (MacLeod *et al.*,

1997). Administration of the mineralocorticoid deoxycorticosterone (instead of aldosterone) in captopril treated rat's mimiced the effects of aldosterone whereas treatment with the glucocorticoid dexamethasone did not. This suggested the anti-stroke effects produced by captopril may have occurred in the absence of any direct effect of ANG II and that the suppression of plasma aldosterone during captopril treatment was important in delaying the onset of stroke development in these rats.

1.5 DEVELOPMENT OF THE DAHL SALT SENSITIVE RAT (Dahl-SS) MODEL OF HYPERTENSION

Dahl-SS were developed by Lewis Dahl through the selective inbreeding of Sprague-Dawley rats (Dahl *et al.*, 1962). Two substrains of rats were generated, a salt sensitive strain (Dahl-SS) that developed hypertension in response to a high salt-diet (8% NaCl) and a salt resistant strain (Dahl-SR) that remained normotensive when fed high salt. Blood pressure rapidly rises in the Dahl-SS fed high salt (8% NaCl from weaning) and hypertension (systolic BP of 170mmHg) was observed within 2 weeks (Simchon *et al.*, 1991). After 4 weeks Dahl-SS fed 8% NaCl initially develop hypertension due to an expanded blood volume, which increased cardiac output with no change in peripheral resistance (Simchon *et al.*, 1989). However, by 8 weeks cardiac output normalized and peripheral resistance increased (Simchon *et al.*, 1991). The renal vasculature of high salt fed Dahl-SS also exhibited impaired vasodilation in response to atrial

natriuretic peptide and sodium nitroprusside (Simchon *et al.*, 1996). It was suggested that hypertension development in Dahl-SS (fed high salt) initially involved an elevation in cardiac output secondary to an increase in blood volume associated with an increase in renal vascular resistance to blood flow (perhaps as a result of an inability of the renal vasculature to vasodilate). This was followed by an increase in total peripheral resistance to flow leading to the maintenance of hypertension. (Simchon *et al.*, 1989; Simchon *et al.*, 1991).

1.5.1 The Role of Altered Renal Function in the Development of Hypertension in Dahl-SS

The kidney plays a key role in hypertension development within Dahl-SS. Renal transplant studies have demonstrated that transplanting kidneys from Dahl-SS into Dahl-SR allows the latter rats to develop hypertension when they are fed high salt whereas a reverse renal transplant attenuates hypertension development in Dahl-SS (Dahl & Heine, 1975).

1.5.2 Renal Pathology Associated with Hypertension Development in Dahl-SS

Hypertension development in Dahl-SS is associated with profound morphological alterations in the kidney. Characteristically, the kidneys of Dahl-SS exhibit a progressive thickening of the intrarenal vessels and arteriolar fibrinoid necrosis (Karlsen *et al.*, 1997). Although some glomeruli appear normal, focal changes in glomerular morphology (crescent shape) produced by glomerular

sclerosis and necrosis and widespread renal degeneration were observed. There was marked tubular atrophy and interstitial inflammation (Karlsen *et al.*, 1997) as well as a thickening of the glomerular basement membrane, and a broadening of the podocyte pedicles (Sterzel *et al.*, 1988). Dahl-SS fed high salt also exhibited an accumulation of fibrinoid material in the intima of renal arteries, proteinaceous tubular casts and atrophy of the cortical tubules and glomeruli (Rapp & Dene, 1985). The above alterations were first observed in Dahl-SS 2 weeks after high salt feeding (Rapp & Dene, 1985).

1.5.3 Status of the Renin Angiotensin System in Dahl-SS

Dahl-SS fed 8% NaCl for 4 weeks exhibited suppressed plasma renin levels. Subsequently, renin levels increased to above baseline levels after 8 weeks of high salt feeding (von Lutterotti *et al.*, 1992). The rise in renin activity was correlated with the appearance of renovascular lesions. Renin secretion increased as renal function decreased.

Other studies have shown that the infusion of ANG II (10 or 50 ng/kg/min) into Dahl-SS fed high salt (4% NaCl) promotes proteinuria, glomerular lesions and a reduction in the glomerular filtration rate (Hirawa *et al.*, 1995). These effects were attenuated by ANG II receptor blockade (Hirawa *et al.*, 1995). It was suggested that the renal vasculature of Dahl-SS was more sensitive to ANG II than that of Dahl-SR and this difference in sensitivity predisposed the renal vasculature to develop lesions in response to a high salt diet (Hirawa *et al.*,

1997). Losartan (an AT_1 receptor antagonist), has been shown to reduce the incidence of stroke development in Dahl-SS fed high salt (von Lutterotti *et al.*, 1992).

1.6 STROKE DEVELOMENT in DAHL-SS RATS

Studies have described "stroke" associated mortality in Dahl-SS fed high salt (Tobian *et al.*, 1984; Tobian *et al.*, 1985; Werber *et al.*, 1985; von Lutterotti *et al.*, 1992; Lin *et al.*, 1999; Zhang *et al.*, 1999).

Tobian *et al* (1985) studied Dahl-SS fed a Japanese-style diet supplemented with 4% NaCl between the ages of 3 to 5 weeks and 8% NaCl thereafter. After 9 weeks of salt feeding Dahl-SS exhibited a 55% mortality associated with presence of cerebral hemorrhage or infarcts.

Werber et al. (1985) observed the presence of both hemorrhagic and ischemic lesions in Dahl-SS fed a high salt Japanese or American (Purina) style diet. Hemorrhagic lesions were described as consisting of blood filled intracerebral spaces. Ischemic lesions were classified on the basis on neuronal degeneration, the presence of neutrophils, macrophages, and tissue loss is the absence of hemorrhage (Werber *et al.*, 1985). Kidney damage (nephrosclerosis), lung edema and cardiac hypertrophy were also present (Werber *et al.*, 1985). The incidence of stroke development was higher in Dahl-SS fed a Japanese (83%) versus an American (57%) style diet despite the fact that the latter diet

contained a slightly higher Na^+ content (1.23 vs 1.18 mEq/g) (Werber *et al.*, 1985).

Von Lutterotti *et al* (1992) found that when Dahl-SS were fed 8% NaCl from 5 to 6 weeks of age they developed behavioural abnormalities consistent with 'stroke development. Ischemic and hemorrhagic cerebrovascular lesions were detected 6 weeks after the initiation of a high salt diet and an 82% incidence of lesions was observed in rats fed high salt for 10 weeks. Hemorrhages occurred in 21% of the brains and ischemic infarcts occurred in all brains (von Lutterotti *et al.*, 1992). Animals often exhibited multiple cerebral lesions. Ninety four % of the rats had lesions in the occipital cortex, 68% had lesions in the corpus callosum, 18% in the hippocampus and 25% developed lesions in the brainstem (von Lutterotti *et al.*, 1992).

Other studies observed that Dahl-SS fed 4% NaCl from 4 weeks of age develop behavioral abnormalities consistent with stroke (such as, convulsive repetitive forearm movements, marked lethargy and semiplegia) after 5.5 weeks of high salt feeding (Lin *et al.*, 1999; Zhang *et al.*, 1999). There was no fixed time frame of death after the onset of stroke (Lin *et al.*, 1999; Zhang *et al.*, 1999). Both hemorrhagic (confirmed by the gross evaluation of thick coronal sections) and ischemic (confirmed by the histological use of 2,3,5 triphenyltetrazolium chloride) stroke was verified in these rats (Lin *et al.*, 1999; Zhang *et al.*, 1999).

The age at which a high salt diet is initiated strongly influences the onset of mortality in Dahl-SS (Pfeffer *et al.*, 1984). In this regard, it is not unusual to

observe no mortality in animals until after 16 weeks of age when the rats are fed an 8% NaCl diet from six weeks (Qu *et al.*, 2000) and a 55% mortality associated with stroke by 12 weeks of age when the rats are fed high salt from 3 to 5 weeks of age (Tobian *et al.*, 1985).

1.7 A SUMMARY OF THE RATIONALE AND THE INITIAL OBJECTIVES OF THE CURRENT STUDY

The focus of research within our laboratory is to gain an understanding of the mechanisms involved in producing stroke during hypertension and to develop treatment interventions that can either delay the onset of stroke or prevent death and disability after stroke has developed. The limited availability of animal models that develop stroke without chemical or surgical intervention has been a key challenge in achieving the above goals.

As noted in the preceding review of the literature, very few animal models develop stroke in a spontaneous manner during hypertension. Stroke development has been noted in Goldblatt (one and two kidney) forms of renal hypertension in rats (Byrom, 1969). However, in these models the occurrence of stroke is not predictable. It does not occur in all renal hypertensive rats and when it does occur, the onset of stroke does not follow a consistent chronological pattern after hypertension development (Byrom, 1969). It is therefore difficult to assess the physiological alterations preceding and potentially promoting stroke development using Goldblatt renal hypertensive rats.

SHRsp were the first animal model of hypertension that developed stroke in a predictable manner (Yamori *et al.*, 1984). When SHRsp were fed a Japanese style diet containing 4% NaCl, hemorrhagic stroke developed at about 12 weeks of age and a 100% mortality associated with stroke occurred by 16 weeks of age (Smeda, 1989). Studies of these animals produced unique insights as to the mechanisms that might be involved in promoting hemorrhagic stroke. Initially, researchers believed that hemorrhagic stroke development in SHRsp was a simple consequence resulting from the presence of a very high blood pressure. This view was challenged by studies that indicated that the treatment of SHRsp with ACEIs at doses that did not alter blood pressure delayed stroke development in SHRsp to an extent where the rats survived to a near normal life span (Stier *et al.*, 1989; Stier *et al.*, 1991; MacLeod *et al.*, 1997). It was demonstrated that ACEIs such as captopril suppressed the elevated plasma aldosterone levels observed in these animals and that the beneficial effects of captopril could be negated if during treatment, aldosterone levels were allowed to increase. These studies indicated that hemorrhagic stroke development in SHRsp could be retarded and re-established by modifying the aldosterone arm of the renin-angiotensin-aldosterone system under conditions where the level of hypertension remained unaltered in SHRsp (Stier *et al.*, 1989; Stier *et al.*, 1991; MacLeod *et al.*, 1997). Other studies observed that CBF autoregulation became defective in SHRsp prior to stroke development and that this alteration was associated with defects in the ability of cerebral arteries to constrict in response

to pressure (a mechanism thought to promote CBF autoregulation) (Smeda, 1992). It was hypothesized that hemorrhagic stroke may be produced in SHRsp by a breakdown in the ability of cerebral blood vessels to regulate CBF under hypertensive conditions (Smeda et al., 1999b). Under the latter conditions an elevation in blood pressure could lead to cerebral overperfusion which might promote vessel rupture and cerebral hemorrhage. It was further suggested that the development of renal dysfunction in SHRsp during hypertension (producing a reduction in glomerular filtration) could potentiate stroke development by causing the activation of the renin-angiotensin-aldosterone system and by the induction of bleeding tendencies secondary to uremia (Smeda, 1992, 1997). The observation that hemorrhagic stroke and defects in cerebrovascular pressure dependent constriction could be induced to occur in stroke resistant SHR by renal ·manipulations that produced uremia (Smeda, 1992) and the further observation that ACEI treatment promoted a protective effect against renal dysfunction led to the speculation that alterations in renal function may be responsible for the loss of CBF autoregulation in SHRsp (MacLeod et al., 1997).

A key question raised in relation to the studies involving SHRsp was whether the mechanisms hypothesized to be involved in promoting hemorrhagic stroke development in SHRsp were widely applicable to hemorrhagic stroke development in humans and other animal models. In this regard, an argument could be presented that SHRsp represent a highly inbred, genetically distinct model of hypertension and that the hormonal and cerebrovascular changes

associated with hemorrhagic stroke are unique to this model and not applicable to hemorrhagic stroke in other animals or in humans. Therefore the initial incentive in carrying out the current study was to assess the mechanisms involved in promoting spontaneous hemorrhagic stroke in another animal with the general objective to assess whether some of these mechanisms were common to those observed in SHRsp.

Aside from SHRsp only Dahl-SS have been noted to develop a high incidence of spontaneous stroke during hypertension (Tobian *et al.*, 1984; Tobian *et al.*, 1985; Werber *et al.*, 1985; von Lutterotti *et al.*, 1992; Lin *et al.*, 1999; Zhang *et al.*, 1999).

The majority of studies involving Dahl-SS have assessed the mechanisms involved in promoting hypertension development in this model. The latter studies provide little insight on the specific mechanisms leading to stroke development. The incidence and onset of stroke development reported in Dahl-SS also varies between studies (Tobian *et al.*, 1984; Tobian *et al.*, 1985; Werber *et al.*, 1985; von Lutterotti *et al.*, 1992; Lin *et al.*, 1999; Zhang *et al.*, 1999).

In our view, such variations could be due to the type of diet being fed to the rats (a Japanese style diet versus Purina rat chow), the level of salt in the diet (i.e. 4% versus 8% NaCl) and the time after weaning when a high salt diet is fed to the rats (i.e. 3, 4, 5 or 6+ weeks of age). In addition, the type of stroke that develops (ischemic versus hemorrhagic) and the brain areas where stroke develops are poorly defined. Studies have reported evidence of stroke based on

the presence of neurological dysfunction thought to be consistent with stroke (i.e. seizures and convulsions) without a demonstration of the presence and types of cerebrovascular lesions present (Zhang *et al.*, 1999). In other studies the presence of cerebral infarcts and hemorrhages has been noted without a clear description of the location of these lesions (Lin *et al.*, 1999; Zhang *et al.*, 1999). Based on the current literature it is unclear as to whether Dahl-SS develop ischemic brain lesions in the absence of cerebral hemorrhage. Although cerebral hemorrhage is the predominant lesion observed in Dahl-SS with stroke, cerebral infarcts lacking the presence of hemorrhage have been noted (Tobian *et al.*, 1984; Tobian *et al.*, 1985). Such observations have lead to the speculation that occlusive forms of cerebrovascular disease that produce ischemia might also be present in Dahl-SS.

A clear description of the type of stroke development (ischemic versus hemorrhagic) in the brain is important in assessing the mechanisms of stroke development in Dahl-SS. Distinctly different mechanisms are involved in promoting brain ischemia (occlusive arterial disease/thromboembolism) versus cerebral hemorrhage (over perfusion and vascular rupture). Hence the design of the subsequent experiments would be dictated by the type of stroke development and the cerebral location of stroke would focus the area of study to specific cerebrovascular arterial beds.

Initial objectives of the experiments in the current study were to determine if Dahl-SS fed a high salt diet developed stroke spontaneously in a predictable

manner when fed high salt and to characterize the type of stroke development and the location of cerebrovascular lesions. In an attempt to attain the highest degree of consistency and predictability in stroke development we bred our own Dahl-SS rats and used only male animals in the study. In addition we created our own diet by reconstituting a readily available Purina rat chow formula with accurate concentrations of NaCl. By breeding our own Dahl-SS we could assure genetic uniformity in our rats as well as accurate aging and high salt delivery at exactly 5 weeks of age. We believed that by using this experimental design we could overcome the variations in the incidence and perhaps even the nature of stroke observed in previous studies involving Dahl-SS rats. The working hypothesis at the initiation of the study was that when Dahl- SS rats were fed an appropriate level of salt they would develop hemorrhagic stroke in a predictable manner. We further believed that hemorrhagic stroke would occur at a time when renal damage secondary to hypertension would create a high renin-high aldosterone milieu in the rats and that onset stroke development in the rats would be retarded by ACEI treatment in a manner not dependent on the suppression of blood pressure. At the initiation of the study we felt that the probability of ACEI treatment achieving the latter goals was high due to previous observations that indicated that hemorrhagic stroke development was retarded in Dahl-SS by losartan (AT-1 receptor antagonist) treatment (von Lutterotti *et al.*, 1992).

Subsequent experiments were planned to assess the alterations in CBF autoregulation in the regions where stroke developed and to further assess

cerebrovascular pressure dependent constriction in the vascular beds perfusing these regions. Studies were designed to test the hypothesis that a loss of CBF autoregulation and cerebrovascular pressure dependent constriction preceded blood brain barrier disruptions in cerebral regions involved in stroke development.

The activation of protein kinase C has been shown to be involved in the signal transduction mechanisms promoting cerebrovascular PDC (Osol *et al.*, 1991; Karibe *et al.*, 1997; Kirton & Loutzenhiser, 1998; Smeda *et al.*, 1999a). If defects in cerebrovascular pressure dependent constriction were observed in Dahl-SS, further experiments were planned to assess the hypothesis that a loss in cerebrovascular PDC was associated with defects in the ability of PKC to elicit cerebrovascular constriction.

Many studies involving Dahl-SS have shown that both the basal and antgonist induced vasodilation mediated by nitric oxide released from the endothelium is altered in vascular beds (Chen & Sanders, 1993). Currently there are no studies assessing this function in the cerebrovasculature of Dahl-SS. Studies have shown that the basal release of nitric oxide from the endothelium and non endothelial sources of nitric oxide synthase modulates cerebrovascular PDC. Specifically, the basal release of NO within cerebral vessels governs basal tone and modifies the operating range of cerebrovascular PDC. Endothelial removal or the inhibition of NO synthase causes PDC to occur at more constricted lumen diameters (Smeda, 1993). In view of this, studies were

undertaken to assess endothelial and NO vasodilatory function in the cerebrovasculature of Dahl-SS. The initial aim of these studies was to test the hypothesis that altered NO function in relation to stroke development in Dahl-SS potentially altered basal tone and cerebrovascular PDC in a manner that might be expected to modify CBF autoregulation.

The results and conclusions of the above studies have been organized in sequential chapters titled "The characterization of stroke development in Dahl-SS", "Alterations in cerebrovascular autoregulation and myogenic function in Dahl-SS", "Cerebrovascular alterations in pressure and protein kinase C mediated constriction in Dahl-SS" and "Alterations in cerebrovascular endothelial function in Dahl-SS.

CHAPTER 2

THE CHACTERIZATION OF "STROKE" DEVELOPMENT IN DAHL-SS

2.1 INTRODUCTION

There is a strong correlation between hypertension and stroke development in humans (Collins *et al.*, 1990; MacMahon, 1990; Strandgaard, 1996). No studies have evaluated the cerebrovascular alterations in Dahl-SS that precede the onset of stroke development. Previous studies involving SHRsp have demonstrated the first observations of behavioural signs of stroke at 12 weeks of age (Smeda, 1989, 1992). The type of stroke in this model was hemorrhagic in origin. Although the mechanisms underlying stroke development in SHRsp remain unclear, alterations in renal function and the RAS likely play a role in initiating cerebral hemorrhage. Extensive research has demonstrated that following the administration of a high salt diet, both ACEIs and the ANG II receptor antagonist losartan, delay or prevent the onset of stroke development in SHRsp (Stier *et al.*, 1989; Camargo *et al.*, 1991; Kim *et al.*, 1992; Camargo *et al.*, 1993; Lee & Severson, 1994; MacLeod *et al.*, 1997). This occurs in the absence of an antihypertensive effect during treatments (Stier *et al.*, 1989; Camargo *et al.*, 1991; Camargo *et al.*, 1993; Lee *et al.*, 1994; MacLeod *et al.*, 1997). Plasma renin activity is elevated in SHRsp fed high salt (Volpe *et al.*, 1990; Camargo *et al.*, 1991; Gahnem *et al.*, 1994; MacLeod *et al.*, 1997). This is unique since a high salt diet typically lowers plasma renin activity in SHR (Shibota *et al.*, 1979). The mechanisms underlying the anti-stroke effect produced by ACEIs or losartan

remain unclear but experiments by MacLeod *et al.* (1997) have suggested a potential role for aldosterone in the cascade of events leading to stroke. Elevations in plasma renin activity promote an increase in plasma ANG II levels, which stimulates the release of aldosterone from the adrenal gland (Gupta *et al.*, 1995). Plasma aldosterone levels are elevated in SHRsp after the establishment of hypertension and are further increased in poststroke versus prestroke SHRsp (Kim *et al.*, 1992; MacLeod *et al.*, 1997). ACEI inhibitor treatment with captopril suppresses plasma aldosterone levels and re-elevation of plasma aldosterone (via osmotic pumps) during treatment negates the antistroke effects produced by captopril (MacLeod *et al.*, 1997).

Some studies have shown that Dahl-SS also exhibit a delay in the onset of stroke and protection against the vascular degeneration when they are treated with losartan (von Lutterotti *et al.*, 1992). The beneficial effect of losartan also occurs under conditions where only a modest reduction in blood pressure is observed (von Lutterotti *et al.*, 1992).

2.2 OBJECTIVES AND HYPOTHESES OF THE STUDY

The focus of these experiments was to characterize stroke development in Dahl-SS, comparing its etiology to stroke development in SHRsp. Our hypothesis was that Dahl-SS rats will develop hemorrhagic stroke following the initiation of a high salt diet and that stroke development will be prevented by ACEI treatment

with captopril, through a mechanism involving the reduction of plasma aldosterone.

2.3 METHODS AND MATERIALS

2.3.1 Animals

The Dahl Salt-Sensitive (Dahl-SS) and Salt-Resistant (Dahl-SR) animals were obtained from a maintained colony housed within the Animal Care Facilities (Memorial University of Newfoundland, Health Sciences Center, St. John's). The experiments were performed in accordance with guidelines outlined by the Canadian Council on Animal Care and the Memorial University of Newfoundland Animal Care Committee. Animals were housed in rooms on a 12-hour light/dark cycle. All experiments were conducted on male animals only. The colony was maintained by breeding brother-sister siblings at 8 weeks of age. The litters were weaned at 5 weeks and the separated males were placed on the appropriate diet (see Diet section for more detail).

2.3.2 Diet

Dahl-SS and Dahl-SR rats were separated into groups and fed diets containing differing percentages of NaCl. The diet was made from a standard rat chow (Prolab RMH 3000 formula, PMI Feeds Inc., St Louis MO, USA) containing a basal level of 0.7% NaCl. This diet was then supplemented with NaCl. The diet was ground to a coarse grain using an electric grinder (Dayton gear motor,

Dayton Electric Co., Chicago, IL, USA). NaCl (Sigma-Aldrich Canada Ltd. Oakville, Ontario, Canada) was added supplementing the NaCl levels to 6.7% or 8.7% (by weight). The required level of NaCl was dissolved in a volume of water equaling 70% of the weight of the powder (that was to be reconstituted). The water containing the NaCl was mixed with the powdered chow to form a thick paste that was dried with fans at 23°C for 12 hours. The dried diet was broken into small biscuits and fed to the rats. Fresh diet was made every few days.

2.3.3 Protocols

Dahl rats were separated into 6 groups at 5 weeks of age. These included: Dahl-SS fed normal salt (0.7% NaCl), Dahl-SS fed 8.7% NaCl, Dahl-SS fed moderate salt (6.7% NaCl) as well as Dahl-SS fed 8.7% NaCl that were treated with captopril (50 mg/kg/day). Control groups consisted of Dahl-SR fed normal salt (0.7% NaCl) and Dahl-SR fed 8.7% NaCl were also included in the study. Captopril was administered via the drinking water (the concentration was achieved by modifying drug dosage and drinking rates of animals in order to achieve a 50mg/Kg/day dosage). Each experimental group consisted of 5 animals.

2.3.4 Blood Pressure Measurement

The systolic blood pressure was measured weekly via a tail cuff compression method (IITC Model 29, Pulse Pressure Amplifier, Woodlands Hills,

CA). The rats were placed in a room at a controlled temperature of 35°C for 15 minutes prior to BP measurement. The averages from three recordings were taken once the animal had become acclimated to the environment. Blood pressures were measured on a weekly basis.

2.3.5 Monitoring of Stroke Development

The rats were monitored daily for any signs of stroke development. Previous studies with SHRsp have characterized the symptoms associated with stroke development (Smeda, 1989, 1992). These symptoms include convulsive repetitive forearm movements, which are followed by an altered posture in which the rat is hunched over with its legs hyper-extended (kangaroo-type posture). The animals exhibit poor grooming and lethargy. Dahl-SS rats were monitored closely for the development of any of these symptoms and others that are not congruent with normal behavior. When rats exhibited signs of stroke development, or when death was likely to occur, the rats were anesthetized and a blood sample was taken via cardiac puncture. Subsequently, the brains and kidneys were removed and fixed in 10% formalin for histological examination. All brains were examined closely prior to fixing in order to observe any surface morphological abnormalities consistent with hemorrhagic stroke or other pathological changes.

2.3.6 Assessment of Brain Ischemia, Hemmorhage and Blood Brain Barrier Integrity

Ischemic brain damage was assessed in the following groups: Dahl-SS fed normal (0.7% NaCl) salt, asymptomatic Dahl-SS fed high salt (8.7% NaCl), Dahl-SS fed high salt (post-stroke) and Dahl-SR fed high salt. A 2,3,5 triphenoltetrazolium chloride assay (TTC, Sigma-Aldrich, Oakville, Ontario, Canada) was used to assess the presence of brain ischemia. Six animals were examined from each group. (Lundy *et al.*, 1986) describes the method used. Brains (including brain stem) were removed and placed in oxygenated ice-cooled (4°C) Krebs physiological salt solution (95% O_2, 5% CO_2, pH 7.4). Unfixed brains were then sliced using a jig (FBM-1000c, ASI instruments, USA). Serial, coronal sections of brain were immersed in normal (0.9%) saline containing 4% TTC for 30 minutes at 37°C. Tetrazolium was taken up by the brain and dehydrogenases converted this compound to an impermeable red dye that remained within the cells. Ischemic damage inactivated cellular dehydrogenase leading a failure of TTC to be converted to a red dye. Areas of the brain that had been subjected to ischemic damage failed to react and remained unstained.

The brains of 7 rats from each of the above groups were fixed in buffered 10% formalin, imbedded in paraffin and sectioned (15 μm thick slices). The sections were subsequently stained either with cressyl violet blue or hematoxylin and eosin. Other sections were stained for the presence of glial fibrillary acidic protein (GFAP) which accumulates in astrocytes after Ischemic damage (Burtrum

& Silverstein, 1994). The slides were examined for changes consistent with the occurrence of brain ischemia and hemorrhage. The brains of 26 additional Dahl-SS rats exhibiting signs of stroke were fixed in 10% buffered formalin and sectioned in 1 mm thick coronal sections in an anterior to rostral direction from the olfactory bulbs to the brainstem just behind the cerebellum using a jig (ASI instruments). These sections were studied through a back-light dissecting microscope for the presence of intracerebral hemorrhage. This technique has been previously used to study brains of SHRsp that have developed stroke (Smeda, 1989) and is capable of detecting even the smallest intracerebral hemorrhages.

The integrity of the blood brain barrier was evaluated in 6 Dahl-SS exhibiting behavioural signs of stroke. Rats were anaesthetized with sodium pentobarbital (65mg/Kg, ip) and placed on a heating pad. The right femoral vein was catheterized with PE-10 tubing and Evan's blue dye (30 mg into 1 ml of sterile 0.9% saline) was infused (over a 15 second period) into the animal at a level of 30 mg Evan's blue dye per kg body weight. The dye was allowed to circulate for 12 minutes. After 12 minutes, the abdominal cavity was opened and two hemostats (5 cm tips) were inserted through the diaphragm in a manner where the intercostal arteries and veins located in the thoracic cavity to the right and left of the sternum were clamped. The chest cavity was opened to expose the heart and a PE-50 catheter was inserted into the aorta and tied at the base of the heart. The right and left ventricles were cut allowing free outflow. Clean

0.9% saline was perfused in to the aorta at constant pressure of 200 mmHg for one minute. This latter infusion cleared the Evan's blue containing intravascular blood from the cerebral vasculature leaving only the dye that leaked into the extravascular space. The skull was subsequently opened and the brain (from the olfactory bulbs to the point at the start of the spinal cord where the vertebral arteries join to from the basilar artery) was removed and frozen in liquid nitrogen. To prevent the brain from cracking, the brain was placed on a plastic lid that was floated on the liquid nitrogen. The brains were then stored at -80°C for subsequent analysis.

When this procedure was used, sites of Evan's blue were clearly visible against the opaque matrix of the brain (Evan's blue works by conjugating to plasma albumin). The extravasation of Evan's blue indicated the existence of breaks in the BBB of a size sufficiently large enough to permit the movement of albumin plus conjugated dye (Udaka *et al.*, 1970).

Measurements of the brain wet to dry weight ratio were used to assess brain edema. Animals (n=15) were anesthetized and the brains were removed and weighed to determine the wet weight. Brains were then placed in pre-weighed vials and dried in an oven at 70°C for 24 hours. Subsequently, the dry weight of the brain was determined. Brain water content was determined using the following formula ((wet weight minus dry weight/wet weight) X 100)).

2.3.7 Renal Function Analysis

Dahl-SS fed normal salt (0.7% NaCl) and Dahl-SS and Dahl-SR fed high salt (8.7% NaCl) diets were housed individually in metabolic cages for 24 hours to collect urine samples. Five animals from each group were examined. Food and water was provided. Kidney function was evaluated on the basis of creatinine clearance and total protein excretion in the urine over a 24-hour period calculated from the collected urine samples. Following 24 hours the animals were weighed, total urine volume was determined and blood pressure was measured. The rats were anesthetized and a blood sample was taken via cardiac puncture. The sample of blood (3 ml) was centrifuged (14000 revolutions/sec) and the plasma was retained for analysis. The urine was then analyzed for total protein and creatinine levels and the plasma was assessed for creatinine, albumin and urea content by the hospital (Biochemistry/Hematology Laboratory, Memorial University, Health Science Center Hospital, St. John's, Newfoundland, Canada). Qualified individuals who conduct these tests on a routine basis performed the analyses. The personnel were blind as to the identity of the samples. Creatinine clearance (CC) was determined using the following formula: CC (mL/hr) = [(urine creatinine, μM)(24-hr urine volume, mL) serum creatinine, μM)]/24. The urinary protein excretion (PE) rate was calculated using the following formula: PE (mg/hr) = (urinary protein, mg/L)(24-hr urine volume, L)/24. The urinary excretion of protein was expressed in relation to

creatinine clearance (PE/CC x10^3/Kg), which estimates the amount of protein lost in relation to glomerular filtration.

Due to the rapid expiration of the Dahl-SS (fed 8.7% NaCl) after the observation of behavioural symptoms suggesting stroke (1 day, discussed later), post-stroke Dahl-SS could not be evaluated. Therefore, Dahl-SS fed a high salt diet for 2 weeks (and exhibiting no behavioural signs of stroke), Dahl-SR fed the same diet for 5 weeks and Dahl-SS fed a normal salt were compared and assessed for proteinuria.

2.3.8 Immunoassays for Aldosterone

Dahl-SS (n=24) and Dahl-SR (n=15) were anesthetized and blood samples were taken via cardiac puncture. The sample of blood (3 ml) was centrifuged (14000 revolutions/sec) and the plasma was frozen (-80 °C) for a later analysis of aldosterone. Serum aldosterone was measured by radioimmunoassay techniques by the Memorial University, Health Science Center Renal Diagnostic Laboratory (St John's, Newfoundland, Canada). The assays were performed using a Coat-A-Count radioimmunoassay kit (Diagnostic Products Corp).

2.3.9 Statistical Analysis

Comparisons involving 2 groups of data were assessed with student's t test. A one-way analysis of variance (ANOVA) followed by Fisher's post hoc test was used to assess comparisons involving one parameter in multiple groups.

Data expressed in the forms of curves, which represented groups of animals, were assessed using a general linear model (GLM) of multivariant analysis of variance (MANOVA). The curves representing rat groups were assessed to determine if they significantly differed from each other and were further assessed to determine if a differential interactive effect existed between the response (y-axis, i.e. BP, CBF, vascular constriction) and a given variable (x-axis, i.e. time, dose, age). A significant interactive effect is usually associated with a situation in which curve crossover occurs. Results were considered significant at $P<0.05$ and were expressed as the mean±SEM. In all cases N values represent the number of rats used in each experiment.

2.4 RESULTS

2.4.1 Blood Pressure, "Stroke" Development and Mortality

Dahl-SS and Dahl-SR were fed diets containing varying levels of NaCl from 5 weeks of age. The blood pressure profile for each group is outlined in Figure 1. All Dahl-SS groups fed a high salt (8.7% NaCl) or a moderate salt (6.7% NaCl) diet developed a rapid onset of hypertension (systolic BP >150 mmHg) one week after being fed high salt. Conversely, Dahl-SR fed a normal (0.7% NaCl) or high salt (8.7% NaCl) did not develop hypertension. Dahl-SS fed normal salt (0.7% NaCl) developed moderate levels of hypertension (about 170 mmHg) over the first 5 weeks of feeding. Captopril (50 mg/kg/day) treatment did
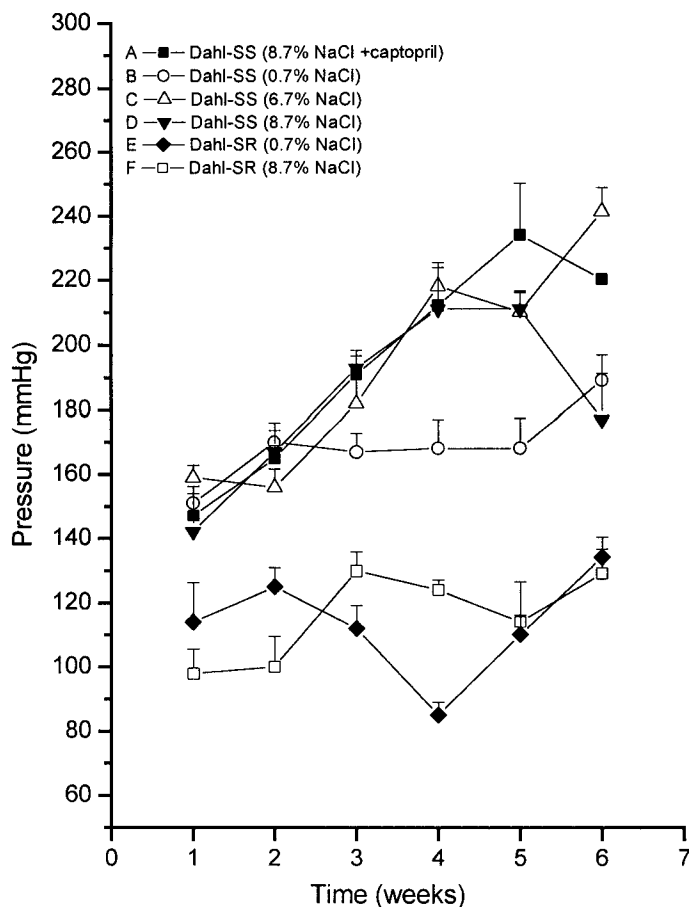
Figure 1: Alterations in systolic blood pressure in relation to feeding duration in Dahl salt-sensitive (Dahl-SS) and Dahl salt-resistant (Dahl-SR) rats fed varying levels of NaCl. Dahl-SS fed a 8.7% NaCl diet and treated with captopril (50 mg/kg/day) exhibited no significant reduction in blood pressure when compared to Dahl-SS fed either 8.7% or an 6.7% NaCl diet without captopril treatment. Blood pressure recording was terminated at six weeks in Dahl-SS following initiation of an 6.7% or 8.7% NaCl diet due to the fact that the rats were either too sick to have their blood pressure recorded or had died prior to six weeks of feeding. All Dahl-SS rats on high salt diets exhibited behavioral symptoms of stroke while Dahl-SR fed the same diet remained asymptomatic. Dahl-SS fed 0.7% NaCl and Dahl-SR fed 0.7% or 8.7% NaCl continued to live up to 17 weeks of age (12 weeks of salt feeding) to the termination of the experiment. These groups exhibited no signs of stroke. Statistics: General Linear Model MANOVA - All high salt groups (except F) were significantly different from normal salt groups (B and E). All Dahl-SS groups (A,B,C,D) were significantly different from Dahl-SR groups (E and F), P<0.05. Values equal the mean ± SEM (5 animals per group).

not effect the onset of hypertension or the maximum level of hypertension (systolic BP >200 mmHg) attained in Dahl-SS fed 8.7% NaCl (Figure 1).

All Dahl-SS fed 8.7%, 6.7% NaCl or an 8.7% NaCl diet combined with captopril treatment exhibited behavioural abnormalities consistent with the development of stroke. This included repetitive head movements, flexion of the forelimbs, which was usually confined to the right side. Postural abnormalities previously defined as a "kangaroo stance" (whereby the animal was seated upright with hindlimbs underneath the body) were observed. The animals often entered a phase of marked lethargy and immobility, confirmed by patches of urine soaked bedding. All the animals died or were sacrificed (when it was evident that death was imminent) within 24-48hrs after the symptoms were detected. The animals were carefully examined prior to death. Common terminal features observed included immobility, dramatic weight loss and cool body temperature possibly due to poor circulation. There was often a sustained erection, possibly indicative of brain pathology that may have led to abnormalities in the autonomic nervous system. Post-mortem analysis revealed marked accumulation of fluid in the extravascular space of the abdominal cavity (ascities). Figure 2 outlines the mortality profiles of the rats in relation to duration of high salt feeding (initiated a 5 weeks of age). Fifty-percent mortality occurred in the Dahl-SS fed 8.7% or 6.7% NaCl after 3 to 4 weeks feeding. Dahl-SR fed the 8.7% salt diet did not develop stroke associated behavioural symptoms and exhibited a 100% survival rate for the duration of the experiment (12 weeks of

100

80

Percent Survival (%)

60

40

20

0

A —■—Dahl-SS(8.7% NaCl +captopril)
B —O—Dahl-SS (8.7% NaCl)
C —▲—Dahl-SS (0.7% NaCl)
D —▽—Dahl-SR (8.7% NaCl)
E —◆—Dahl-SR (0.7% NaCl)
F — —Dahl-SS (6.7% NaCl)

0   2   4   6   8   10   12

Time (weeks)
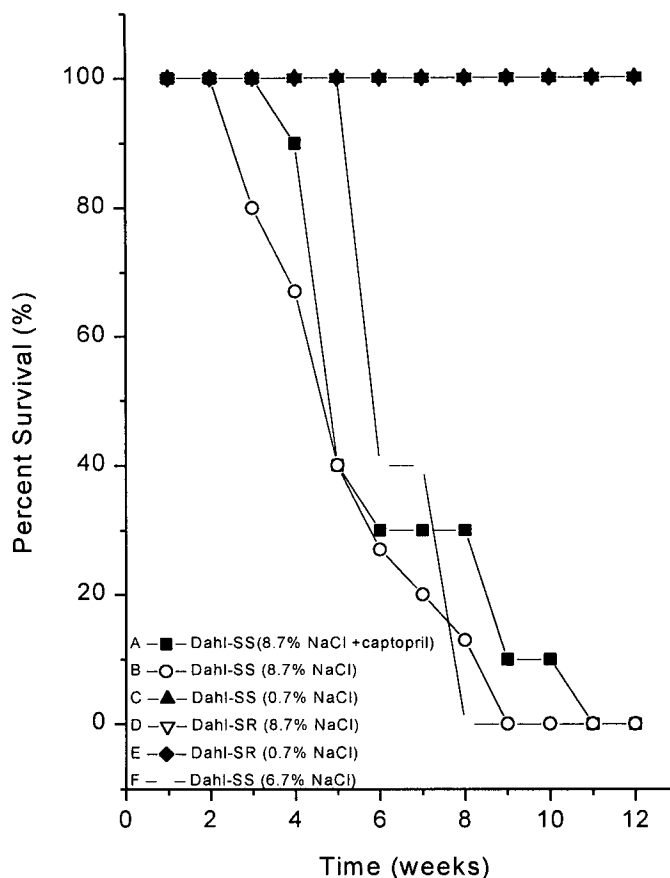
Figure 2: Mortality profile of Dahl-SS and Dahl-SR fed a diet containing various concentrations of NaCl (+/- captopril treatment). All Dahl-SS fed high salt diets (8.7%, 6.7% and 8.7% NaCl+captopril) exhibited comparable mortality profiles in relation to duration to feeding. Death was preceded by the presence of stroke-like behavioural symptoms. A 50% level of mortality was reached around 4.5 weeks. No significant difference in mortality with respect to feeding duration was observed between Dahl-SS fed 6.7%, 8.7% NaCl or NaCl with captopril treatment. No mortality was observed in the Dahl-SR groups fed 0.7% and 8.7% NaCl and in Dahl-SS fed 0.7% NaCl. These latter groups represented by the star like overlay extending from 100% survival over the duration of the experiment. (n=5 animals per group). Statistics: General Linear Model MANOVA- Groups A, B, and F were significantly different from C, D and E, P<0.05. Symbol across from 100% on the y-axis represents groups C, D and E.

salt feeding). Captopril had no effect on the onset of mortality in Dahl-SS fed 8.7% NaCl.

2.4.2 An Assessment of Cerebrovascular Lesions in Dahl-SS Exhibiting Behavioural Symptoms of Stroke

Brains were removed from asymptomatic Dahl-SS, Dahl-SS with behavioural symptoms of stroke and Dahl-SR fed 8.7% NaCl for 2, 3.5 and 5 weeks respectively. The brains were serially sectioned in 1mm thick coronal sections from the olfactory bulb area to the brain stem at a point where vertebral arteries join to form the basilar artery. The sections were examined using TTC assay outlined in the methods. Bright brick red staining occurred within all brains across all sections (n=6 rats/group). The red reaction indicated the robust cellular dehydrogenase conversion of TTC to a red dye, a feature that is inconsistent with the presence of cerebral ischemia.

The brains of 7 Dahl-SS fed 8.7% NaCl were histologically examined. These brains were fixed in formalin and imbedded in paraffin. Each brain was coronally serially sectioned in 15μm thick sections. The sections were stained with one of the following stains: Cressyl violet, hematoxylin and eosin or for the presence of glial fibrillary acidic protein (which accumulates in the astrocytes after ischemic damage). These histological analyses failed to detect any evidence of ischemic damage.

Other brains (n=26 rats) from Dahl-SS exhibiting behavioural symptoms of stroke were sectioned in 1mm thick coronal sections and studied under a dissecting microscope for the presence of intracerebral hemorrhage. All the analyses indicated the presence of intracerebral hemorrhage in 7 out of 39 Dahl-

SS that exhibited symptoms consistent with stroke. Hemorrhagic lesions were present in the cerebrum in all 7 out of 39 rats. One rat had hemorrhagic lesions in both the cerebrum plus the cerebellum and brainstem. Brain sections from the latter rat that were stained with cressyl violet are shown in Figure 3.

Five of 6 rats demonstrated focal areas of extravasation of Evans blue dye in the cerebrum and the remaining rat exhibited a general extravasation of the dye. Figure 4 demonstrates focal areas of Evans blue extravasation surrounded by lighter areas of edema surrounding the extravasated dye.

To further confirm the presence of edema the wet to dry weight ratio of brains was measured in Dahl-SS that exhibited stroke-like behaviour (n=5) and compared to the ratio found in asymptomatic Dahl-SS fed 8.7% NaCl for 2 weeks and Dahl-SR fed 8.7% NaCl for 5 weeks. The results of these experiments are shown in Figure 5. Dahl-SS exhibiting stroke-like behaviour had brains containing a higher percentage of water than either asymptomatic Dahl-SS or Dahl-SR. This latter finding is consistent with the occurrence of brain edema in the Dahl-SS exhibiting stroke-like abnormal behaviour.

At this point we concluded that the results suggest that the stroke-like behavioural symptoms observed best represent the development of *hypertensive encephalopathy rather than true stroke*. By definition, stroke is produced by cerebral lesions secondary to the presence of brain ischemia or

Figure 3. A representative picture of brain (coronal section) from a Dahl-SS fed 8.7% NaCl for 3.5 weeks that exhibited behavioural symptoms consistent with stroke. Brain stained with cressyl violet blue. The sites of hemorrhage within the cerebrum and brainstem are visible as red stained material.
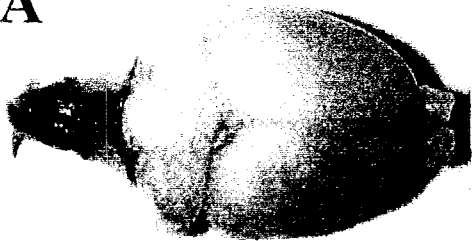
Figure 4: Evans blue extravasation in the brain of a Dahl-SS exhibiting seizures with behavioural abnormalities exhibited blood brain barrier disruption and the extravascular movement of Evans blue dye (arrows). The light colored areas surrounding the extravasated dye correspond to areas of edema. Cerebral ischemia was absent and hemorrhage rarely occurred (7/39) rats. Based on the brain pathology the behavioural dsyfunctions observed best represent the occurrence of hypertensive encephalopathy.
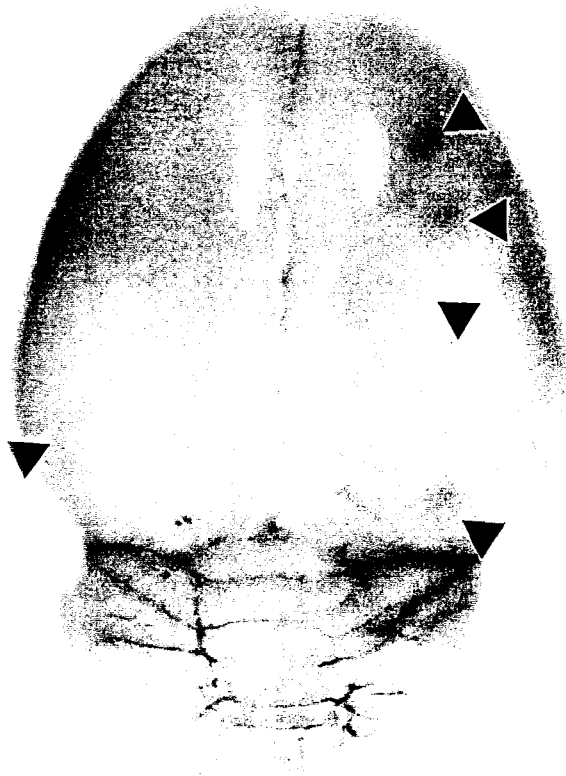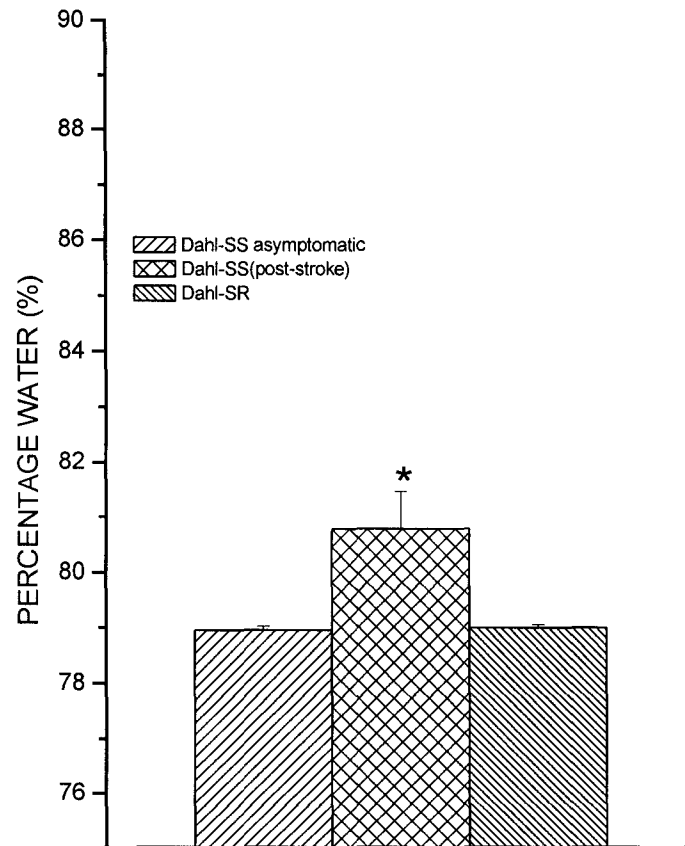
Figure 5: Levels of brain edema assessed by the water content of brains sampled from asymptomatic, post-HE Dahl-SS and Dahl-SR fed high salt (8.7% NaCl). The level of water content in the brains of Dahl-SS exhibiting "stroke-like" behaviour was significantly elevated when compared to asymptomatic Dahl-SS and Dahl-SR. Stroke is defined as the occurrence of neurological based abnormalities that result from a cerebrovascular accident which produces brain ischemia and or hemorrhage. Based on the absence of any evidence of brain ischemia and low incidence of cerebral hemorrhage combined with clear evidence of brain edema and blood brain barrier disruption we concluded that "stroke-like" symptoms observed in our colony of Dahl-SS best represents a condition of hypertensive encephalopathy as apposed to true stroke. Therefore, from this point on in the thesis Dahl-SS exhibiting "stroke-like" behaviour will be referred to as having hypertensive encephalopathy (HE) as apposed to stroke. Statistics: ANOVA with Fisher post-hoc test. Significance was determined at (*) P<0.05. Values represent the mean ± SEM (all groups had 5 animals per group).

hemorrhage. In view of the near absence of the latter forms of lesions it is incorrect to define the current colony of Dahl-SS as having stroke based only on the presence of behavioural symptoms.

Based on the above conclusion from this point to the end of the thesis, Dahl-SS exhibiting hypertensive encephalopathy (HE) will be referred to as post-HE as apposed to rats with stroke or stroke-like behaviour.

2.4.3 An Analysis of Renal Function in Dahl-SS and Dahl-SR Fed High Salt

Alterations in the plasma levels of urea (blood urea nitrogen-BUN), creatinine and albumin are outlined in Figure 6. Plasma BUN and creatinine were significantly elevated in post-HE Dahl-SS fed 8.7% NaCl (for 3.5 weeks) and their plasma compared to the asymptomatic Dahl-SS fed the same diet (for 2 weeks). In Dahl-SR fed 8.7% NaCl (for 5 weeks), which do not develop HE, similar plasma levels of BUN and creatinine are observed in comparison to asymptomatic Dahl-SS fed high salt. Plasma albumin was significantly lower in the post-HE Dahl-SS compared to asymptomatic Dahl-SS rats. Proteinuria was assessed by determining urinary protein loss in relation to the creatinine clearance over a 24-hour period. This parameter represents an estimate of the level of protein loss into urine in relation to glomerular filtration. As shown in Figure 7, the urinary
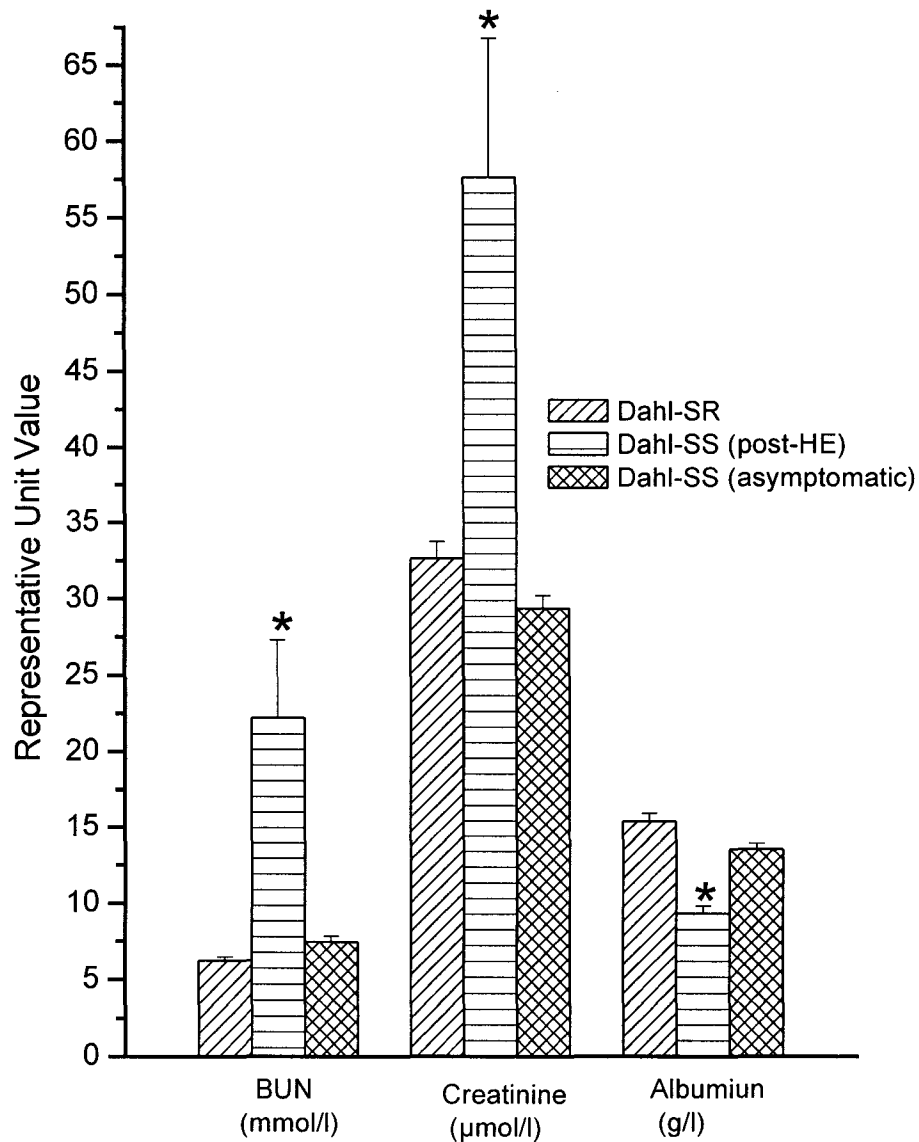
Figure 6: Blood plasma profile of urea (BUN), creatinine and albumin in asymptomatic Dahl-SS, post-HE Dahl-SS and Dahl-SR fed 8.7%NaCl. Post-HE Dahl-SS were fed a 8.7% NaCl diet for 3.5 weeks. Dahl-SR were fed a high salt diet for 5 weeks whereas asymptomatic Dahl-SS received the diet for 2 weeks. Statistics: ANOVA with Fisher post hoc. BUN and creatinine and albumin values were significantly different between post-HE Dahl-SS and asymptomatic Dahl-SS and SR groups, (*) P<0.05. Values represent the mean ± SEM (asymptomatic Dahl-SS=11, post-HE Dahl-SS=16 and Dahl-SR=5.
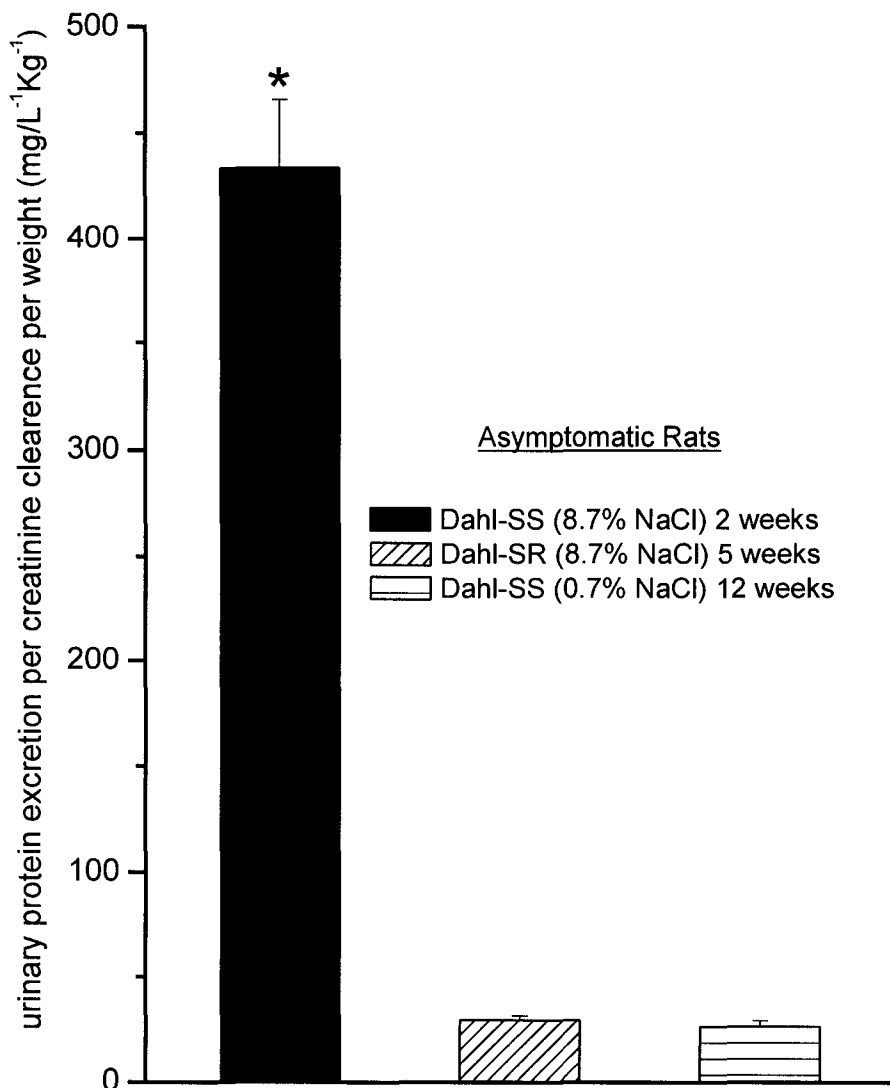
Figure 7: An analysis of proteinuria in asymptomatic Dahl-SS fed 0.7% or 8.7% NaCl diets and Dahl-SR fed an 8.7% NaCl diet. The results show that urinary protein excretion per creatinine clearance was significantly elevated in Dahl-SS fed 8.7% NaCl for 2 weeks when compared to Dahl-SR fed a 8.7% NaCl diet and Dahl-SS fed a 0.7% NaCl diet. Statistics: ANOVA with Fisher post-hoc. (*) $P<0.05$. Values represent the mean $\pm$ SEM (all groups had 5 animals per group).

protein excretion per creatinine clearance was 9-fold higher in the asymptomatic Dahl-SS fed 8.7% NaCl (for 2 weeks) when compared to the Dahl-SS fed 0.7% NaCl (for 12 weeks) or Dahl-SR fed 8.7% NaCl for 5 weeks.

2.4.4 Plasma Aldosterone Levels

Figure 8 outlines the changes in plasma aldosterone levels in relation to diet (8.7% versus 0.7% NaCl), strain (Dahl-SS versus Dahl-SR rats) and HE development in the Dahl-SS. Our results indicate that plasma aldosterone levels were significantly lower in asymptomatic Dahl-SS fed 8.7% NaCl (for 2 weeks) in comparison to Dahl-SS fed 0.7% NaCl for 12 weeks.

Post-HE Dahl-SS fed 8.7% NaCl (for 3.5 weeks), had high plasma aldosterone levels. The level of plasma aldosterone in these rats was 4 times higher than that presented in asymptomatic Dahl-SS and double of that observed in Dahl-SS rats fed a normal diet (Figure 8). Dahl-SS fed 8.7% NaCl (for 3.5 weeks) and subjected to captopril (50 mg/kg/day) treatment that developed HE had half the levels of plasma aldosterone observed in untreated age matched Dahl-SS with HE. The level of plasma aldosterone observed in captopril treated Dahl-SS with HE was equal to that present in asymptomatic Dahl-SS fed normal NaCl.
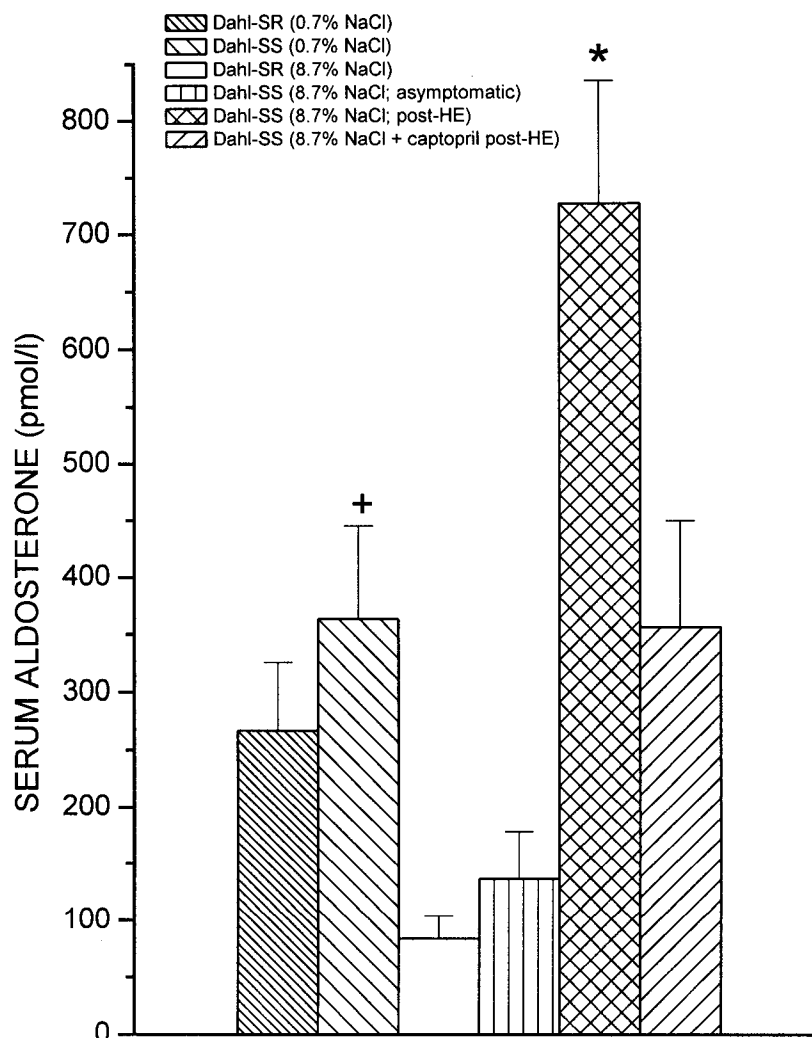
Figure 8: Effect of an 8.7% NaCl and 0.7% NaCl diet on plasma aldosterone levels in asymptomatic and post-HE Dahl-SS rats in comparison to Dahl-SR fed high (8.7%) and normal (0.7%) NaCl and post-HE Dahl-SS fed high salt along with captopril (50mg/kg/day) treatment. Plasma aldosterone levels in post–HE Dahl-SS were significantly elevated when compared to all other groups (*) and the levels in Dahl-SS (normal salt) were significantly higher than both asymptomatic and post-HE Dahl-SS rats (+). Statistics: ANOVA with Fisher post-hoc test. Significance was determined at P<0.05. Values represent the mean ± SEM (asymptomatic Dahl-SS=6, post-HE Dahl-SS=8, Dahl-SS normal salt=7 Dahl-SS +captopril=3, Dahl-SR high salt=5 and Dahl-SR normal salt=10).

2.5 DISCUSSION

Dahl-SS fed 8.7% NaCl rapidly developed high blood pressure levels (> 200 mmHg) that were eventually associated with neurological abnormalities (such as seizures, convulsive repetitive forearm movements and altered posture) and behavioural abnormalities consisting of poor grooming and lethargy. All Dahl-SS that were not sampled at the time when these abnormalities were first observed died within 1 day. The brains of the rats showed no evidence of ischemia and the observation of intracerebral hemorrhage was rare. Both brain edema and BBB disruption was observed in Dahl-SS that developed neurological and behavioural abnormalities. Evidence of these alterations was demonstrated by the extravasation of Evans blue dye and by an increase in brain water content. Alterations in renal function occurred in Dahl-SS fed high salt. Dahl-SS exhibiting neurological and behavioural abnormalities had higher levels of plasma aldosterone compared to Dahl-SR fed high salt. These animals also developed uremia and significant elevations in plasma creatinine. Plasma albumin levels were decreased and proteinuria was observed in the rats.

2.5.1 Development of Hypertensive Encephalopathy

Our working hypothesis was that the neurological and behavioural abnormalities observed in Dahl-SS fed 8.7% NaCl were due to stroke. However, in the present study, Dahl-SS exhibiting stroke-like behaviour were completely devoid of ischemic areas (indicated by bright red staining of the brain slices in

response to TTC). In addition, histological evaluation using GFAP staining revealed a complete absence of GFAP reaction (which accumulates in astrocytes after ischemic damage). These assessments indicate that the stroke-like behaviour was not a result of ischemic brain damage. Intracerebral hemorrhage formation in Dahl-SS exhibiting stroke-like behaviour was rare and occurred in less than 20% of the brains examined. Since by definition stroke is produced by brain ischemia and or hemorrhage (Sacco and Mayer, 1994) behavioural abnormalities observed in Dahl-SS fed 8.7% NaCl were not the result of stroke.

We believe that the stroke-like behavior observed in Dahl-SS was a result of the development of HE. HE is neurological dysfunction brought about by a sudden rise in BP that has been primarily described in humans (Vaughan & Delanty, 2000). It occurs in the absence of cerebral ischemia or hemorrhage and produces stroke-like symptoms such as lethargy, confusion, headache, visual impairments and generalized seizures (Healton et al., 1982; Vaughan & Delanty, 2000). HE is associated with a loss in CBF autoregulation in response to the rapid rise in BP (Dinsdale, 1982; Vaughan & Delanty, 2000). This promotes a breakdown in the BBB (Oztas & Turkel, 2001) that is coupled with fluid movement into the extravascular space and cerebral edema formation (Vaughan & Delanty, 2000). The occurrence of BBB breakdown and HE development is dependent on the level and duration of hypertension present and is potentiated by a sudden abrupt elevation in blood pressure (Sokrab et al., 1988; Johansson, 1999).

All the above alterations (motor dsyfunctions, seizures, BBB breakdown, brain edema in the absence of cerebral ischemia or hemorrhage) associated with HE development in humans are consistent with the changes observed in Dahl-SS from the present study. The development of such alterations at the first point where maximal hypertension was established in our colony of Dahl-SS (4 to 5 weeks salt feeding) just after the phase of rapid hypertension development is consistent with the characteristics of HE development in humans. In view of the similarities in characteristics of HE development in humans and our observations in Dahl-SS we believe that our rats best represent a model of hypertensive encephalopathy. This in itself is important since no animal model that develops HE in a reliable fashion is currently available for study. BBB breakdown in Dahl-SS rats with HE was exclusively observed in the cerebrum. This may give insight into the particular behavioural abnormalities (seizures and involuntary limb movement) that these rats exhibited since the cerebrum is primarily involved in regulating motor control.

Cerebral edema may by virtue of applying intracranial pressure, disrupt the medullary control and/or cortical of heart function creating the potential for cardiac arrhythmias to occur (Hachinski, 1993; Oppenheimer, 1994). This type of pathway connecting hypertensive BBB disruption to heart dysfunction may have caused our Dahl-SS rats to die abruptly after HE development.

In other studies Dahl-SS fed high salt tended to develop both cerebral hemorrhage and ischemia (Tobian *et al.*, 1985; Werber *et al.*, 1985; von Lutterotti

et al., 1992; Lin *et al.*, 1999; Zhang *et al.*, 1999). However in our colony of Dahl-SS observations of hemorrhages were rare and there was an absence of ischemia following high salt feeding. This low propensity for stroke development in our model compared to others may have been due to genetic differences between Dahl-SS colonies. The observation of predictable HE development within our colony of Dahl-SS in response to high salt feeding may be of greater value than a situation where the rats uniformly developed stroke since at the present time no animal model of HE exists.

2.5.2 Renal Function During Hypertensive Encephalopathy

The kidneys of Dahl-SS with hypertension undergo changes in morphology that modify kidney function (Rapp & Dene, 1985; Sterzel *et al.*, 1988; Karlsen *et al.*, 1997). A morphological assessment of renal structural alterations was not the objective of this study. However, we did histologically section and stain (H & E) four kidneys from Dahl-SS with HE. These sections exhibited vascular and glomerular degeneration as well as the appearance of fibrinoid deposits in renal arterioles and capillaries. The presence of these lesions would decrease blood flow resulting in reduced glomerular filtration (Hirawa *et al.*, 1997). A reduction in glomerular filtration would facilitate the production of uremia and exacerbate the development of hypertension due to volume expansion via salt and water retention.

Certain characteristics of renal dysfunction could potentiate HE development in Dahl-SS. The excess loss of protein into the urine (proteinuria) and the associated decreased plasma albumin could decrease colloidal osmotic pressure and promote edema formation throughout the vasculature. Post-HE Dahl-SS from the current study also exhibited massive fluid accumulation within the abdominal cavity (ascities) suggesting that global non-cerebral movement of fluid into extravascular space occurred. The development of proteinuria could be important in promoting HE development since asymptomatic Dahl-SR fed high salt fail to develop proteinuria and maintain plasma albumin levels that are equivalent to those observed in Dahl-SS fed normal salt.

Humans exhibiting end-stage renal disease (i.e. uremia, proteinuria, blood in the urine and abnormal electrolyte levels) also commonly show signs of HE (Agildere et al., 2001). It has been suggested that an inability of the kidney to control fluid homeostasis results in a rapid elevation of blood pressure as glomerular filtration is significantly reduced (Agildere et al., 2001). Such individuals also commonly develop proteinuria, hence it is possible that a drop in plasma oncotic pressure may also aggravate brain edema formation and HE development.

2.5.3 The Effects of ACEIs on the Development of Hypertensive Encephalopathy

The beneficial effect of captopril in both protecting and treating the onset ·of stroke has been demonstrated in the SHRsp (Stier *et al.*, 1991; MacLeod *et al.*, 1997). The mechanisms by which captopril exerted its anti-stroke effect was postulated to be through the reduction in plasma aldosterone levels (MacLeod *et al.*, 1997) possibly by decreasing the development of kidney and cerebral lesions (Stier *et al.*, 1991).

Evaluation of plasma aldosterone in the present study revealed that plasma aldosterone was significantly elevated in post-HE Dahl-SS compared to asymptomatic Dahl-SS fed 8.7% NaCl for 2 weeks. However, the aldosterone levels observed in post-HE Dahl-SS were significantly lower than the levels observed in the post-stroke SHRsp (700 pmol/L versus 4000 pmol/L respectively) (MacLeod *et al.*, 1997).

As discussed within the literature review, renin release and consequently ANG II formation and plasma aldosterone levels are controlled by tubular urine flow and urine NaCl concentration at the macula densa. In high salt fed SHRsp and Dahl-SS, two opposing factors will govern renin release. Renal pathology develops causing a restriction in renal blood flow and glomerular filtration. This reduces primary urine production and flow past the macula densa and facilitates renin release. The latter effect is opposed by high salt ingestion which should increase the urinary Na levels passing the macula densa and in doing so reduce

the release of renin into the blood. It is the balance of these two opposing forces that will ultimately govern the level of renin release into the blood. It is possible since Dahl-SS were fed an 8.7% NaCl diet that the balance favored a lower renin release than that observed in SHRsp fed 4% NaCl diet. Therefore, although plasma levels of aldosterone elevated, the levels observed in SHRsp were not achieved in Dahl-SS fed high salt.

Other factors may also govern the above balance. Dahl-SS exhibit far greater renal dysfunction and kidney ischemia than SHRsp when they are fed a high salt diet. In order for renin to enter the circulatory system it must flow through the glomerular capillaries into the venous circulation. If total renovascular occlusion occurred blocking this circulation, any potential renin released into the preglomerular arteriolar lumen will not make its way into the circulation. This would lead to the production of lower blood ANG II levels and consequently a smaller release of aldosterone from the adrenal gland. Finally, it is possible that the levels of renin release in response to Na^+ concentration and urine flow may be set in a manner where the level of renin released per equal level of stimulation is less in the kidneys of Dahl-SS versus SHRsp. At the present time sufficient experimental evidence supporting or contradicting the above theories is not available.

High plasma aldosterone levels play an important role in the specific development of intracerebral hemorrhage. In this regard the absolute plasma aldosterone levels observed in post-HE Dahl-SS are comparable to levels found

in SHRsp treated with captopril that are protected from hemorrhagic stroke development and are below the levels observed in prestroke SHRsp not treated with captopril (MacLeod *et al.*, 1997). Hence, if one accepts the premise that aldosterone plays a specific role in cerebral hemorrhage formation, the low aldosterone levels observed in asymptomatic and post-HE Dahl-SS may account for the low incidence of this lesion in this strain.

In this study, captopril (50mg/kg/day) treatment produced no antihypertensive effects and did not delay the onset of mortality in Dahl-SS fed high salt. Dahl-SS fed high salt plus captopril treatment exhibited significant levels of hypertension and no change in HE-associated mortality. If elevated levels of plasma aldosterone play an important role in hemorrhagic stroke development and the subsequent death in SHRsp, it is reasonable to assume that since the levels of plasma aldosterone were not elevated to the same degree in post-HE Dahl-SS, captopril treatment and aldosterone suppression would not alter HE development. The ineffectiveness of captopril treatment in altering HE development and mortality in Dahl-SS would also indicate the mechanisms promoting death in this model do not involve the activation of the renin angiotensin system. However, one could speculate that if mortality after HE could be delayed in Dahl-SS the level of aldosterone might continue to rise and the incidence of hemorrhage formation in Dahl-SS would increase.

2.6 CONCLUSIONS

Dahl-SS develop significant rapid renal insufficiency in response to a high salt diet (8.7% NaCl). Established hypertension (> 200 mmHg) occurs by two weeks. This promotes the development of cerebral edema and HE, subsequently leading to death 1 day after initial observations of behavioural abnormalities associated with HE. The cellular mechanisms underlying the development of HE need to be addressed further to better understand the development in the Dahl-SS model. It has been shown previously that HE is associated with the loss of cerebral blood autoregulation (Dinsdale, 1983) promoted by the rapid elevation in blood pressure. This could lead to an overperfusion of the brain and facilitate edema formation. In the next chapter we will evaluate cerebral blood flow autoregulation in Dahl-SS in response to feeding high salt.

CHAPTER 3

ALTERATIONS IN CEREBROVASCULAR AUTOREGULATION AND

MYOGENIC FUNCTION IN DAHL-SS

3.1 Introduction

CBF autoregulation is defined as the maintenance of constant CBF under conditions of varying blood pressures. (Paulson *et al.*, 1990). This mechanism ensures that the brain obtains adequate supplies of oxygen (O_2) and nutrients even when blood pressure falls below normal levels (Harder *et al.*, 2002). In situations where blood pressure is elevated (i.e. hypertension), the autoregulatory control of CBF acts as a protective mechanism. In hypertensive individuals the CBF versus BP autoregulatory curve is shifted to the right (Strandgaard & Paulson, 1995). This ensures that during periods of increased blood pressure, CBF remains constant and overperfusion is prevented (Strandgaard & Paulson, 1995).

The shifting of the upper BP limit of CBF autoregulation to higher limits during periods of increased blood pressure is likely the result of vascular remodeling (i.e. decreased lumen and increased wall thickness) which causes increased vascular contractility (Heagerty *et al.*, 1993; Asmar *et al.*, 1997). A displacement of CBF autoregulation to higher BP limits can also occur due to increases in sympathetic nerve activity, which commonly occur during hypertension (Edvinsson *et al.*, 1978; Paulson *et al.*, 1990). These rightward

displacements in the autoregulatory curve in response to increased BP may protect against overperfusion and subsequent hemorrhage occurrence (Smeda *et al.*, 1999b).

In humans exhibiting hypertension, CBF autoregulation is maintained during therapeutic treatments with Ca^{2+} channel antagonists (Gaab *et al.*, 1990). However, in animals, the administration of high doses of nimodipine (Ca^{2+} antagonist) abolishes CBF autoregulation (Gaab *et al.*, 1990) and promotes the extravasation of fluid and blood into the extravascular space when BP is raised. This suggests that loss of CBF autoregulation under hypertensive conditions has the potential to facilitate the development of edema and hemorrhages.

It has been suggested that the development of hypertensive encephalopathy (HE) in humans, is promoted by the loss of CBF autoregulation, which could facilitate the formation of cerebral edema and increase the risk of hemorrhage formation (Dinsdale, 1983).

3.2 OBJECTIVES AND HYPOTHESES OF STUDY

The observations outlined in Chapter 2 demonstrated that Dahl-SS fed a high salt diet develop marked elevations in BP and behavioural signs consistent with HE. The development of HE occurred in a predictable manner. The purpose of the following study was to test the hypothesis that HE development in our colony of Dahl-SS was preceded by an inability of the cerebrovasculature to autoregulate constant CBF. An important mechanism thought to produce CBF

autoregulation is pressure dependent constriction (PDC) (Johnson, 1986). Elevations in blood pressure which potentially increase flow are counteracted by cerebrovascular constriction, which increases vascular resistance to flow and helps maintain CBF constant (Johnson, 1986). In view of the above, we tested if potential loss of CBF autoregulation in the middle cerebral artery (MCA) perfusion domain was associated with an inability of the MCAs to constrict in response to elevations in pressure.

To test the above hypotheses we evaluated Dahl-SS fed 8.7% NaCl. Laser Doppler techniques were used to measure CBF autoregulation in the perfusion domain of the MCA prior to HE development. In separate experiments, distal segments of the MCAs were isolated from the brain and the ability of these arteries to constrict to elevations in transmural pressure was tested and related to both HE development and CBF autoregulatory function.

3.3 MATERIALS AND METHODS

3.3.1 Experimental Animals, Diet and Systolic Blood Pressure

These experimental protocols are outlined in detail in chapter 2. Specifically a description of the Dahl Salt-Sensitive Colony and the breeding protocol is outlined in section 2.3.1. The development of the appropriate high salt diet used is outlined in section 2.3.2. The techniques used to measure blood pressure in the particular experimental groups are outlined in detail in section

2.3.4. The behavioural changes associated with the development of hypertensive encephalopathy are outlined in section 2.3.5.

3.3.2 The Measurement of CBF Autoregulation

Laser Doppler techniques were used to measure CBF. The rats were anesthetized with sodium pentobarbitol (65mg/kg, i.p.) and a tracheal tube (PE-250) was inserted into the airway and connected to a ventilator. Pure O_2 was mixed with inspired air to produce a PaO_2 of about 250 mmHg (>98% hemoglobin saturation). The left femoral artery and vein were catheterized with PE-50 ad PE-10 tubing respectively that were filled with lactate Ringer's solution. The animal's head was then immobilized in a stereotaxic device. The animal's temperature was measured with a digital rectal thermometer and maintained at 37°C through the use of a heating pad. The skull cap was exposed and a 2 mm hole was drilled into the cranium without breaking the dura. The hole was positioned in the MCA perfusion domain of the right cerebral hemisphere, 1 mm right of lateral bregma in the oculus sinister parietale bone at a point where the outside edge of the 2 mm hole was just medial to a bony ridge. The latter ridge (unnamed) runs from the orbital socket in a posterior direction and separates the oculus sinister temporale and pars squamosa bones that form the lateral skull surface. A laser probe (PF 403, Perimed, Jarfalla, Sweden) was lowered into the hole by a micromanipulator until it touched the dura without penetrating this layer. The CBF flux was monitored using a Perimed 4001 laser Doppler flow meter

(Perimed, Jarfalla, Sweden). Prior to the start of the experiments the laser probe was calibrated between fluxes of 0 to 250 using a PF100 Perimed external calibration standards.

CBF was measured based on the fact that when laser light hits a moving object the wavelength of the light is (Doppler) shifted. In the brain, the laser beam penetrates the tissue. *In vivo,* only plasma is moving within the vasculature of the brain. The proportion of Doppler shifted light (reflected from the moving blood cells in the blood) in relation to the light hitting immobile matter was measured. A flux value was calculated which was proportional to the amount of blood flowing (velocity x blood cell concentration) below the probe.

The BP of the animal was measured through a femoral arterial catheter, in which the catheter was connected to a Statham P23 ID pressure transducer (Gould Electronique, Ballainvillers, France) and amplified through a Gould Model 81888 recorder containing a Universal amplifier. The BP and CBF flux were synchronized by an input into a computer and the analogue signals were converted to digital data (C10-AD 16 JR-AT, Acquire Program, Computer Boards Inc., Mansfield, MA, USA). The raw CBF flux and corresponding mean arterial blood pressure (MAP) data was stored in the computer as ASCII files.

Following a equilibration period, blood $PaCO_2$ pH, HCO_3 and hemoglobin O_2 saturation (Ciba Corning 278 Blood Gas System Analyzer, Medfield, MA, USA) were analyzed from an arterial blood sample collected from the femoral catheter (normal ranges; $PaCO_2$ >38 mmHg; pH 7.35-7.45; HCO_3 24-27mM and

hemoglobin O_2 saturation >99%). The animal was then given an injection of hexamethonium (5 mg/kg, ip) to inhibit the sympathetic nervous system and prevent baroreflex action. This also typically lowered the animal's BP. The BP was then raised by infusing 4.6 x 10^{-2} mg norepinephrine/ml lactate ringers solution into the femoral vein via a syringe pump (Model 355, Sage Instruments, Cambridge, MA, USA) to produce a slow rise in BP. Norepinephrine was used to increase the BP since it can contract virtually all systemic blood vessels but has no effect on the vessels of the cerebrovasculature (Paulson *et al.*, 1990). Hence systemic BP can be raised without producing cerebrovascular constriction.

In each experiment, BP was raised to the highest possible level. The CBF present at a given blood pressure was normalized to the CBF present at a MAP of 100 mmHg to give a relative CBF value (i.e. relative CBF at a given MAP = (Flux at the MAP/Flux at a MAP of 100 mmHg)). The relative CBF was then plotted against MAP.

3.3.3 Pressure Myograph Studies

The PDC was determined in MCAs of Dahl-SS fed 8.7% NaCl prior to and following the development of behavioural symptoms consistent with the development of HE. In addition, we also sampled MCAs from Dahl-SR fed both a high (8.7%) and normal (0.7%) NaCl diet. There were 5 animals in each group. Isolated MCAs segments were examined for the presence of a functional PDC response using a pressure myograph apparatus described by Osol and Halpren

(1985). Briefly, rats were anaesthetized with sodium pentobarbital (65mg/Kg/i.p.). The thoracic cavity was opened to allow access to the heart. The left ventricle was cut to exsanguinate the animal. Following this, the skull cavity was opened and the brain was removed and placed in ice cooled Krebs saline solution bubbled with 95%O_2/5%CO_2. An isolated arteriolar segment of the MCA was removed and canulated on a hollow glass micropipette (~20 μm tip) of the pressure myograph apparatus. The distal end was tied with 10-0 suture creating a closed sac. Pressure was increased within the lumen of the artery through the connection of the proximal end of the micropipette connected to a Krebs saline filled reservoir. The reservoir was then connected to a gas cylinder (95%O_2/5%CO_2). The preparation bath, containing Krebs saline solution was oxygenated (95%O_2/5%CO_2). All drugs used in the study were applied to the exterior of the artery within the Krebs saline suffusing the artery. The outer diameter of the artery was viewed through a microscope system (Wild Leitz M3 microscope, Wild Heerbrugg, Switzerland) and the dimensional changes were ·recorded on videotape and measured at 322x magnification.

Following an initial equilibration period of 30 minutes at 100 mmHg, the pressure was reduced to 0 mmHg for an additional six minutes. Solution within the vessel chamber was held constant at 37°C. The PDC response was recorded as the amplitude of vessel contraction following a rapid pressure increase to 100mmHg over a four minute increment. Following an initial dilation (1sec after pressure was applied) the vessel contracted over the four minutes at

100 mmHg. Figure 9 is a representative example of PDC in a MCA from an asymptomatic Dahl-SS rat. Maximal diameter of the vessel is recorded as the amplitude of vessel vasodilation following nifedipine (3 μM) application.

3.3.4 Statistical Analysis

We used either a one-way analysis of variance (ANOVA) followed by Fisher post-hoc test or GLM multivariant analysis of variance (MANOVA) to determine if significant differences existed between groups of data. Results were considered significant at $P<0.05$. The mean ± the standard error measurement is shown in the data. N values in the PDC experiments always equal one MCA from one rat used. A more detailed description of the statistical analysis is outlined in section 2.3.9.

3.4 RESULTS

3.4.1 Blood Pressure and the Behavioural Symptoms of HE in Dahl-SS

Dahl-SS fed 8.7% NaCl for > 3 weeks exhibited behaviour consistent with HE (described in chapter 2). Dahl-SS fed a normal 0.7% NaCl diet for up to ten weeks were asymptomatic and appeared healthy throughout the duration of the experiments.

The blood pressure profiles of Dahl-SS fed either 8.7% NaCl or 0.7% NaCl are outlined in Figure 10. Dahl-SS fed high salt exhibited a rapid onset of hypertension (defined as systolic >150 mmHg) whereas Dahl-SS fed normal salt
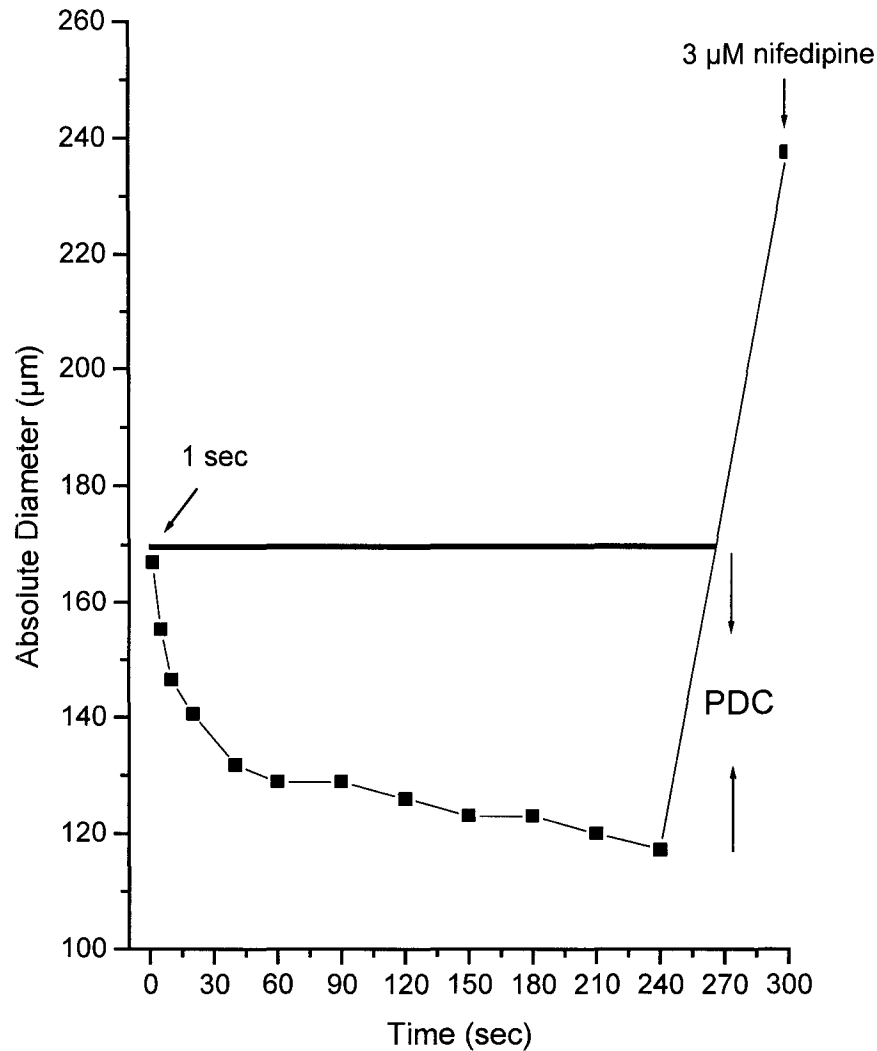
Figure 9. An example of the lumen diameter alterations occurring in a middle cerebral artery in response to a 100 mmHg pressure step. The artery was sampled from a asymptomatic Dahl salt-sensitive rat and equilibrated to 0 mmHg pressure for six minutes. This eliminates pressure dependent tone from the artery. Then the pressure was increased abruptly to 100 mmHg. Initially, one second after pressurization, prior to a significant engagement of pressure dependent constriction (PDC), the arterial lumen expands. Subsequently PDC reduces the lumen size to a smaller diameter (which is dependent on the applied pressure). PDC was measured as the decrease in lumen diameter between 1 and 240 seconds after the application of a 100 mmHg pressure step. The application of nifedipine to the artery produced maximal vasodilation.
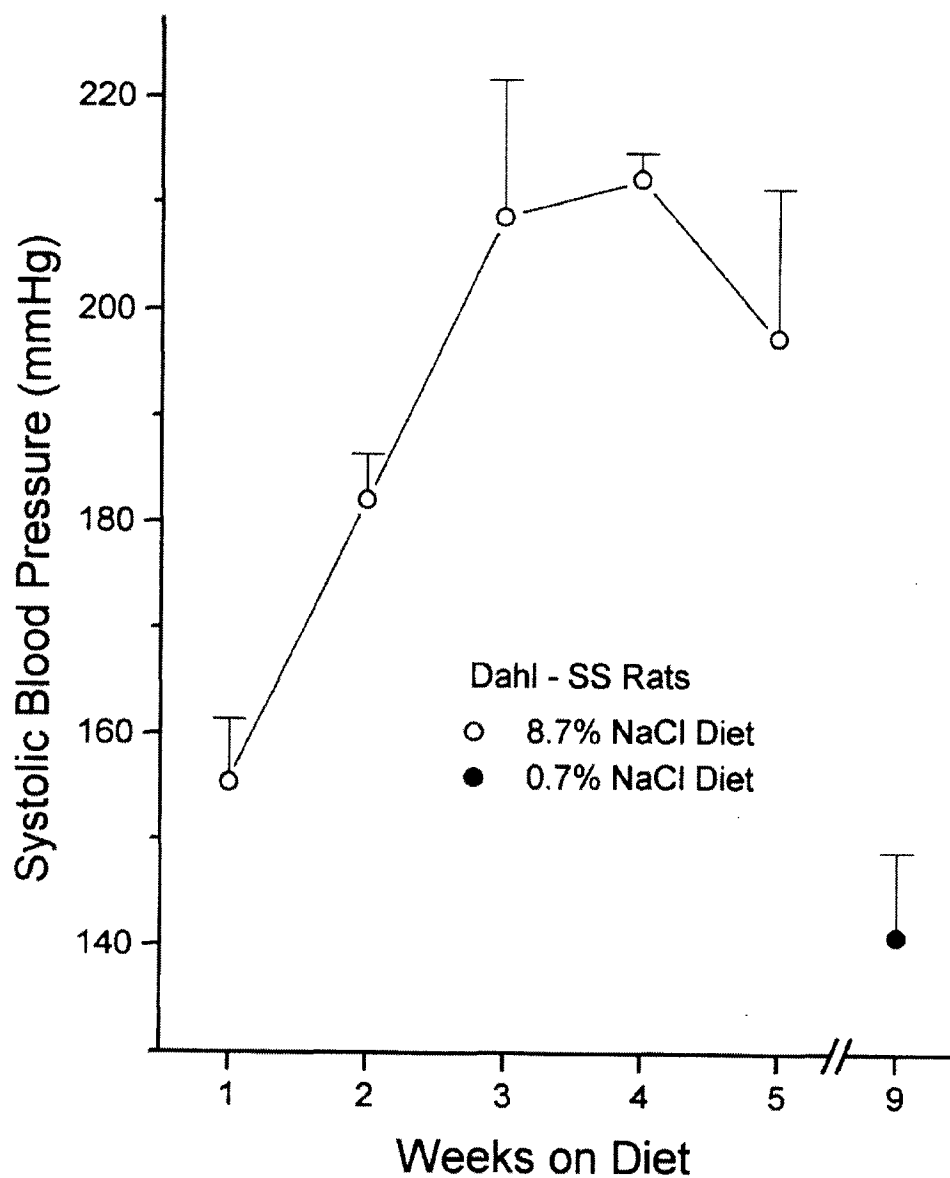
Figure 10. The systolic blood pressure profile of Dahl-SS rats fed 8.7% NaCl (n=6) and 0.7% NaCl (n=7). Dahl-SS fed high salt after weaning (5 weeks of age) exhibited a rapidly developing onset of hypertension after only one week. Dahl-SS fed 0.7% NaCl diet for 9 weeks exhibited normotensive blood pressures. Values represent mean ± SEM.

diet remained normotensive. Maximal systolic BP's were >200 mmHg after a three week period. Subsequently, some of the Dahl-SS fed a high salt diet began to exhibit behavioural signs of HE. Dahl-SS fed a normal diet for nine weeks typically exhibited normal to borderline hypertensive (150-165 mmHg) levels up to a maximal systolic blood pressure of 160 mmHg. This level of hypertension was not associated with any of the behavioural symptoms of HE observed in Dahl-SS rats fed a high salt diet.

3.4.2 Alterations in CBF Autoregulation in Dahl-SS

Table 1 outlines the blood gas parameters for the groups of rats used in these autoregulatory studies. Through the control of respiratory rates and the administration of oxygen, arterial pCO_2 was maintained at normal levels and blood hemoglobin oxygen saturation was always greater than 99.6%. Blood pH and HCO_3 levels were normal and none of the parameters differed significantly between the groups studied.

Table 2 summarizes the characteristics of CBF autoregulation observed in the rats. All of the Dahl-SS rats fed 8.7% NaCl for one week or 0.7% NaCl for nine weeks exhibited an ability to autoregulate blood flow up to an upper mean arterial pressure limit of respectively 168±6 mmHg and 204±12 mmHg. One of the six rats fed 8.7% NaCl for two weeks and four of the six rats fed 8.7% for three weeks lacked the ability to autoregulate blood flow with varying blood

Table 1: The arterial blood gas characteristics of anesthetized

asymptomatic Dahl-SS used in cerebral blood flow experiments.

Dietary NaCl (%)	Weeks On Diet	n	Blood pH	$PaCO_2$ (mmHg)	Blood HCO_3 (mM)	HbO_2 Saturation (%)
8.7	1	6	7.42 ± 0.01	40 ± 2	26.5 ± 0.8	99.9 ±0.02
8.7	2	6	7.38 ± 0.01	40 ± 2	23.9 ± 0.9	99.7 ± 0.05
8.7	3	6	7.40 ± 0.01	39 ± 2	24.7 ± 1.0	99.7 ± 0.05
8.7	9	7	7.42 ± 0.01	40 ± 2	26.1 ± 0.5	99.7 ± 0.05

Values = mean ± S.E.M.

$PaCO_2$ = arterial CO_2 tension

HbO_2 saturation = saturation of arterial blood hemoglobin with oxygen.

Statistics (ANOVA) – arterial blood pH, $PaCO_2$, HCO_3 and HbO_2 saturation did not significantly

differ between groups.

Table 2: Characteristics of cerebral blood flow (CBF) autoregulation (mmHg) in anesthetized Dahl-SS.

Dietary NaCl (%)	Weeks on Diet	n	Proportion of rats exhibiting autoregulation	Upper BP Limit of Autoregulation	ΔrCBF/ ΔmmHg
8.7	1	6	6/6	168 ± 6^a	4.73 ± 1.17
8.7	2	6	5/6	181 ± 9	9.2 ± 1.13^b
8.7	3	6	2/6	206 **	7.12 ± 0.94
0.7	9	7	7/7	204 ± 12	5.41 ± 0.85

Values = Mean ± S.E.M.

BP = mean arterial pressure

ΔrCBF/ΔmmHg = change in relative cerebral blood flow between BP's of 90 to 120 mmHg ($\times 10^{-3}$).

** the 2 of the 6 rats exhibiting CBF autoregulation had a upper BP limits of 227 and 185 mmHg

Statistics – ANOVA (Fisher post hoc)

a – significantly different from rats on a 0.7% NaCl diet.

b – significantly different from rats on a 8.7% NaCl diet for 1 week and rats on a 0.7% NaCl diet.

pressures. These defects in CBF autoregulation occurred in the rats prior to the development of any behavioural abnormalities suggesting HE.

Representative examples of the CBF autoregulation (presence and absence) are outlined in Figure 11. This data was obtained from two Dahl-SS fed 8.7% NaCl. The rat demonstrating an ability to autoregulate CBF maintained a relatively constant CBF as mean BP was increased from about 63 mmHg to an upper BP limit of CBF autoregulation (188 mmHg). The elevation of the mean arterial pressure above this point resulted in an abrupt increase in CBF. The second animal exhibited a linear increase in CBF with mean arterial pressure and an absence of an upper pressure limit to autoregulation, suggestive of an inability to autoregulate CBF in response to increases in BP (Figure 11).

The changes in relative CBF with varying arterial pressure in Dahl-SS fed a 8.7% NaCl diet for one, two and three weeks are outlined in Figure 12. The rats in these groups were healthy and demonstrated no observable behavioural signs of HE. The changes in CBF demonstrate that a majority of asymptomatic Dahl-SS fed a 8.7% NaCl diet for three weeks lose their ability to autoregulate CBF. Dahl-SS fed a 0.7% NaCl diet for nine weeks all maintained the ability to autoregulate CBF (Figure 13). The CBF alterations in rats fed a normal salt diet were comparable to those observed in Dahl-SS fed 8.7% NaCl diet for two weeks.

Although a majority (4 of 6) of Dahl-SS fed a 8.7% NaCl diet for three weeks lacked the ability to autoregulate CBF, the relative changes in CBF
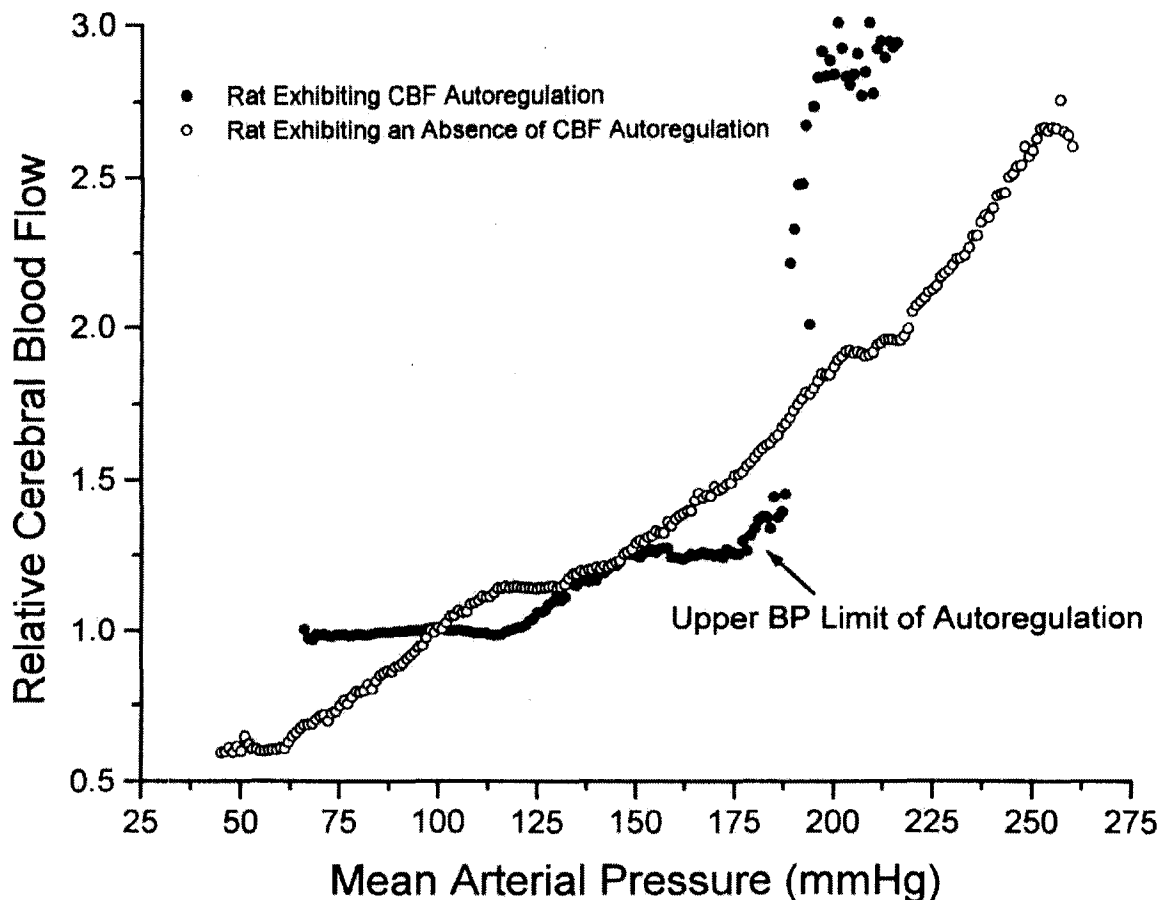
Figure 11: An example of the changes in relative cerebral blood flow (CBF) with mean blood pressure in anesthetized asymptomatic Dahl-SS exhibiting the presence and absence of CBF autoregulation. The curves represent experiments on two rats fed 8.7% NaCl for 3 weeks. Rats exhibiting CBF autoregulation exhibit very moderate changes in CBF up to the upper blood pressure limit termed breakthrough. In the absence of CBF regulation there is a linear increase in CBF with a blood pressure with no apparent breakthrough. In the absence of CBF autoregulation, the slope of the changes in relative CBF with respect to blood pressure will depend on the degree of vasoconstriction present in the cerebrovasculature.
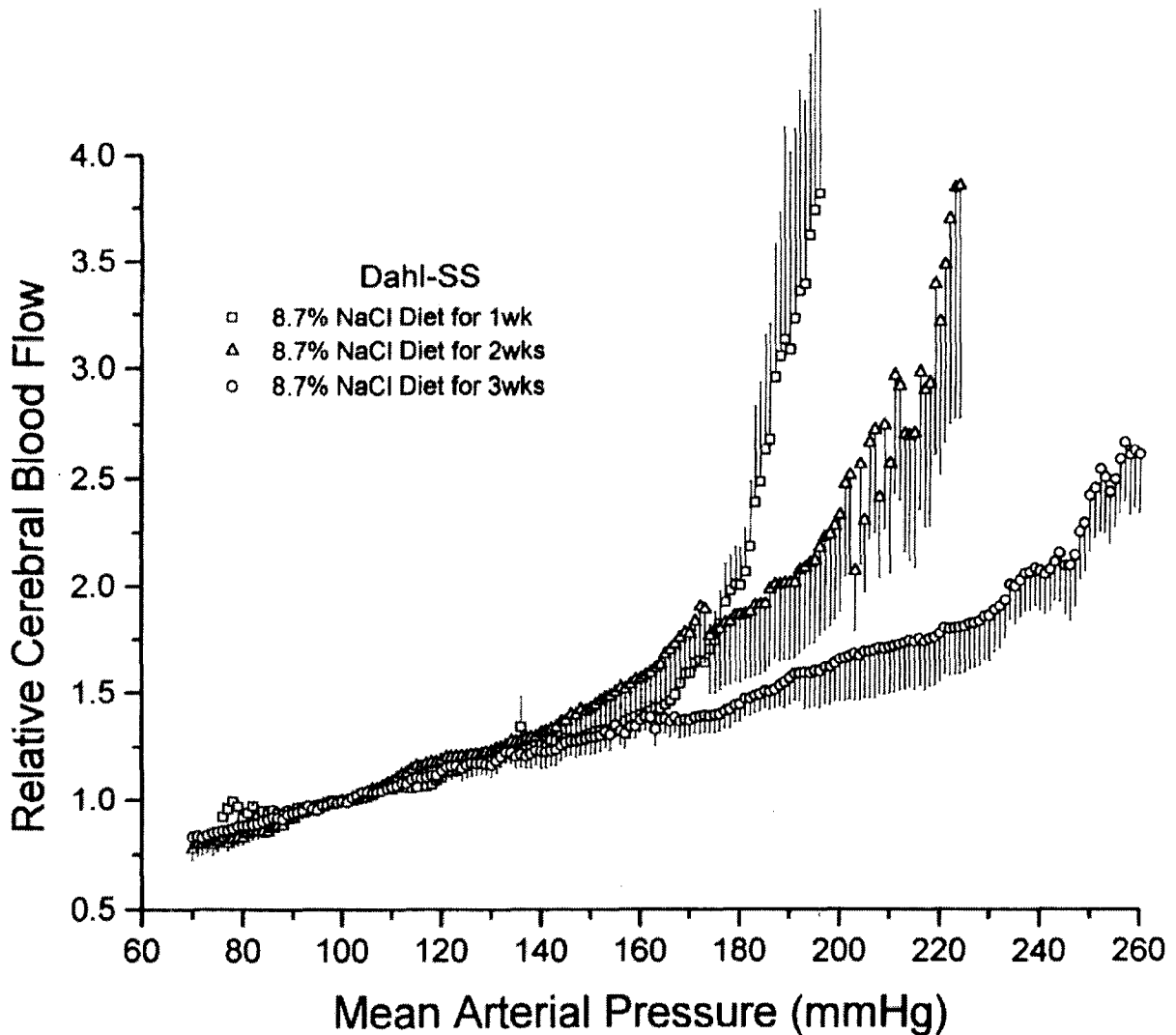
Figure 12: Alterations in cerebral blood flow (CBF) autoregulation in anaesthetized asymptomatic Dahl-SS fed 8.7% NaCl for varying duration. Dahl-SS fed 8.7% NaCl for 1 week from weaning (5 weeks of age) exhibited ability to autoregulate CBF. The majority of these rats (5/6) fed this diet for 2 weeks also exhibited an ability to autoregulate CBF and the upper limit of CBF autoregulation was shifted to higher blood pressure limits (see Table 2). The majority of asymptomatic rats (4/6) fed 8.7% NaCl for 3 weeks lacked an ability to autoregulate CBF and exhibited a linear relationship between CBF and blood pressure. Statistics: General Linear Model MANOVA on curves. Each curve is significantly (p<0.05) different from all other curves in absolute levels over common blood pressures and in terms of an interactive effect of relative CBF with blood pressure (p<0.05 in all cases). (n=6 rats in each group).
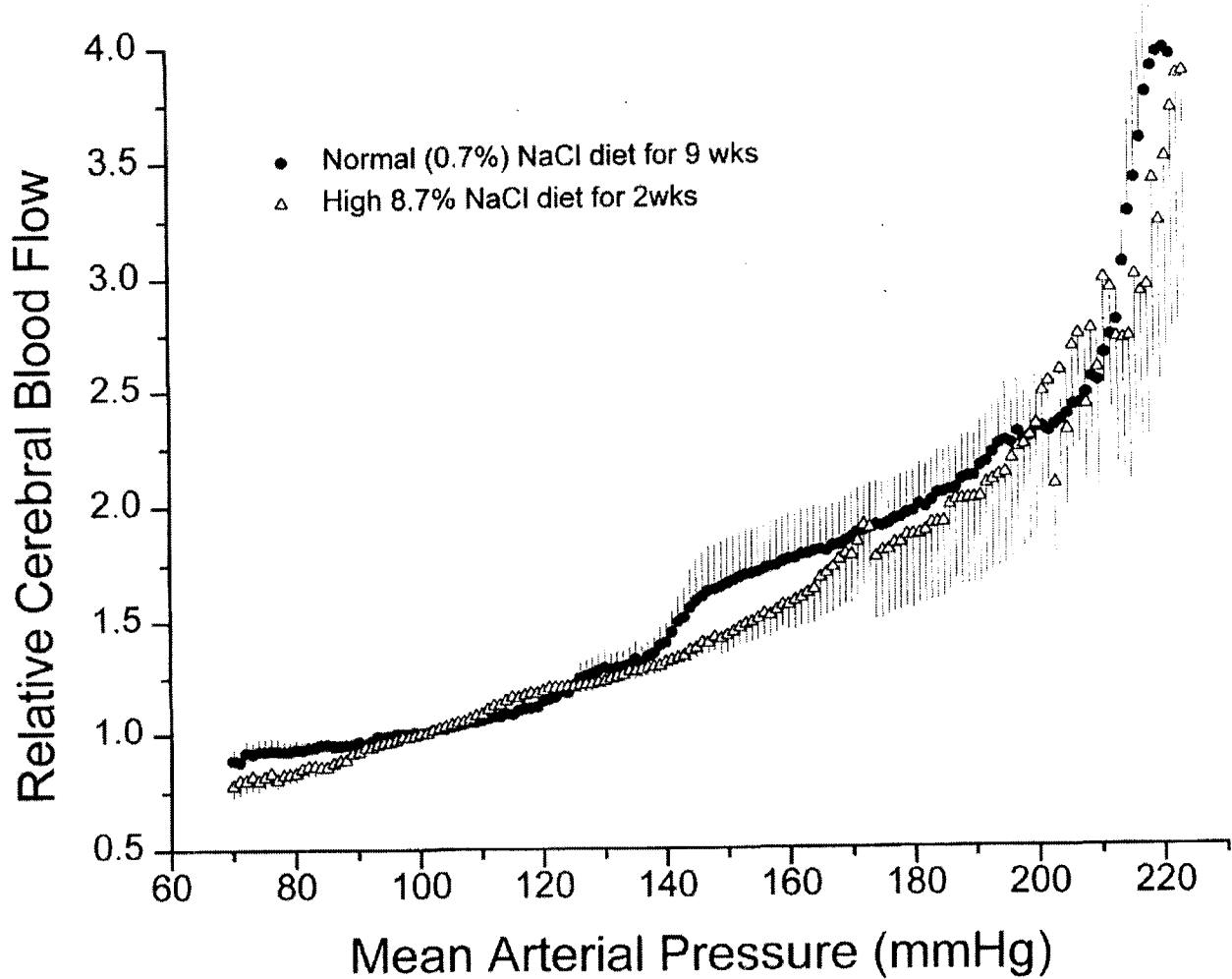
Figure 13. CBF autoregulation in anaesthetized Dahl-SS fed 0.7% NaCl. Rats (n=7) fed 0.7% NaCl from weaning (5 weeks of age) for 9 weeks maintained an ability to autoregulate CBF in a manner virtually identical to rats fed 8.7% NaCl diet for 2 weeks (n=6). Statistics: General Linear Model MANOVA on curves: no significant differences were observed between groups.

between BP of 90 to 120 mmHg were comparable between the groups (Table 2). The Dahl-SS fed a 8.7% NaCl diet that exhibited autoregulation had upper limits of CBF regulation which tended to be displaced to higher pressures in relation to duration of time that the rats on high salt diet. The upper limit of CBF regulation was high (204±12 mmHg) in Dahl-SS fed a normal salt diet despite the fact that these rats had near normal BP's. This observation suggests that factors other than elevations in BP also can lead to an elevation in the upper limit of CBF autoregulation in Dahl-SS.

CBF autoregulation experiments were attempted on Dahl-SS with HE. Unfortunately after developing HE the rats became quite sick and were extremely vulnerable to death once they were anesthetized with sodium pentobarbital. All the animals studied in this condition died within the first 30 minutes of anesthesia. This time period was insufficient to conduct the autoregulatory protocol outlined. However, the observation that a majority of asymptomatic Dahl-SS lost the ability to autoregulate blood flow after being fed a high salt diet would suggest that defects in CBF autoregulation precede HE development and it is reasonable to believe that this dysfunction is maintained after HE development.

3.4.3 Alterations in Cerebrovascular Pressure Dependent Constriction In Dahl-SS

The percent decrease in MCA lumen diameter in response to a 100 mmHg pressure step in the lumen diameter of MCAs sampled from Dahl-SS fed a 8.7% or 0.7% NaCl diet is shown in Figure 14. The ability to constrict in response to an increase in pressure declined in relation to the duration of high salt (8.7% NaCl) feeding in MCAs of Dahl-SS. This response was severely attenuated in rats subjected to the diet for three weeks. Post-HE Dahl-SS (which were fed a 8.7% NaCl diet for three or four weeks) had MCAs that did not constrict to pressure. The pressure dependent constrictor response observed in the MCAs of Dahl-SS fed a 0.7% NaCl diet for nine weeks was comparable to that observed in rats fed a 8.7% NaCl diet for two weeks.

The nature of the loss of PDC in the MCAs of post-HE Dahl-SS is further characterized in Figure 15. This figure demonstrates that asymptomatic Dahl-SS fed a 8.7% NaCl diet for three weeks and post-HE Dahl-SS (fed the same diet for three or four weeks) have MCAs with reduced lumen diameters under maximally dilated conditions (3 μM nifedipine) when compared to Dahl-SS maintained on the diet for one or two weeks. The absence or attenuation of PDC in the MCAs of post-HE Dahl-SS fed 8.7% NaCl for three weeks was associated with the maintenance of a very large degree of basal tone (calculated as % constriction in relation to the maximal dilated lumen diameter in response to nifedipine 3 μM) and a reduced diameter at the start of pressurization (which would limit the
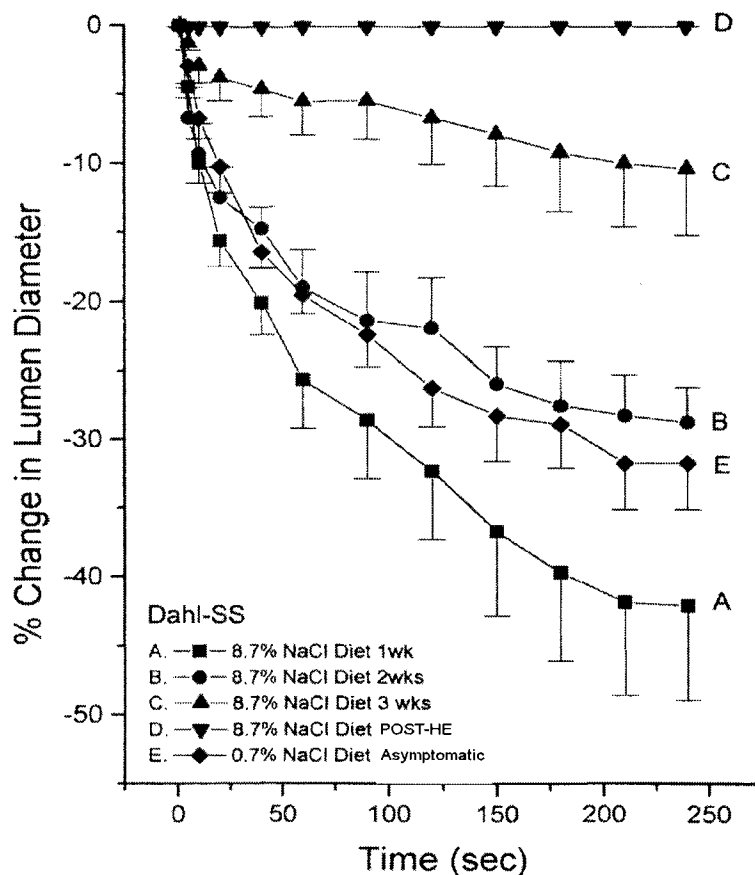
Figure 14: Pressure dependent constriction in the MCAs of Dahl-SS prior to and after HE development. The percent decrease in the lumen diameter with respect to the time after the application of a 100 mmHg pressure step is shown. The MCAs of the Dahl-SS fed 8.7% NaCl for a period of 1, 2 or 3 weeks after weaning (5 weeks of age) progressively developed a decreased ability to constrict in response to pressure. The ability to constrict to pressure was absent in MCAs sampled from post-HE Dahl-SS and was quite robust in Dahl-SS fed 0.7% NaCl for 9 weeks. Statistics: General Linear Model MANOVA on curves: All curves were significantly different ($p < 0.05$) from each other in terms of amplitude of the response with the exception of curve B vs E. All curves also significantly ($p < 0.05$) differ from each other in terms of a differential interactive effect of response with respect to time, except for the following comparisons A vs B, A vs E, B vs E, C vs D. ($n = 5$ rats within each group). Values represent mean ± S.E.M.
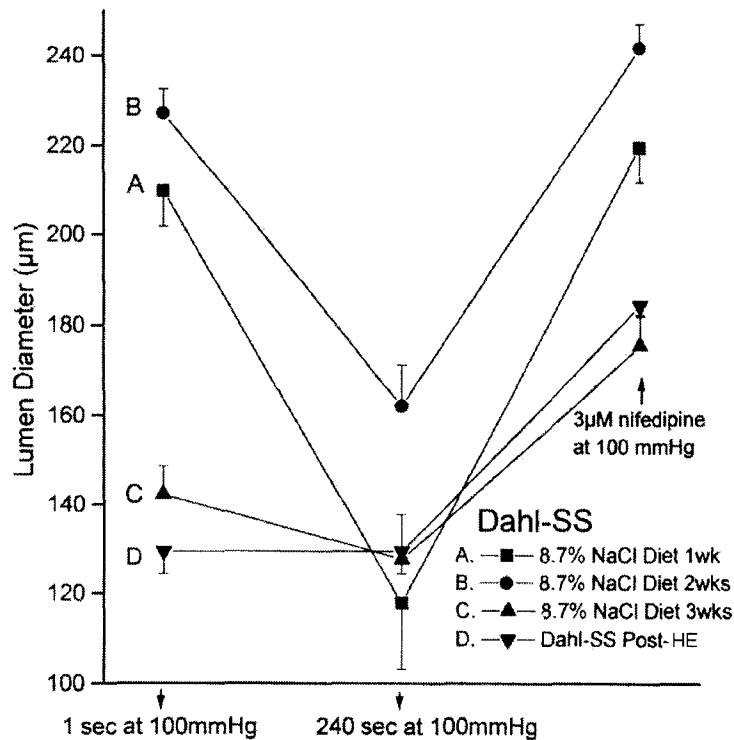
Figure 15: The absolute changes in lumen diameter in response to pressure and nifedipine in MCA are sampled from Dahl-SS. The figure shows the lumen diameters present in MCAs 1 second after the application of 100 mmHg pressure prior to significant constriction in response to pressure and the lumen diameter present after 240 seconds at a time when pressure dependent constriction is complete. Subsequently nifedipine was added to the bath producing maximal dilation of the arteries. Under maximally vasodilated conditions (in the presence of nifedipine) there is a reduction in lumen diameter of MCAs from asymptomatic Dahl-SS fed 8.7% NaCl for 3 weeks and post-HE Dahl-SS (3 or 4 weeks on the diet) when compared to Dahl-SS fed 8.7% NaCl for 1 or 2 weeks post weaning. Statistics: ANOVA plus Fisher post hoc test. Significant ($p<0.05$) differences: At 1 second at 100 mmHg- A vs C, B vs C and D. At 240 seconds at 100 mmHg- B vs C and D. At 100 mmHg with nifedipine- A vs B, C and D, B vs C and D. (n=5 rats within each group). Values represent mean ± S.E.M.

further ability of the MCAs to constrict to pressure). As shown in Figure 16, the degree of basal tone present in the MCAs increased with the duration of time the rats were maintained on high salt (8.7% NaCl). We also evaluated Dahl-SS fed 0.7% NaCl for 9 weeks. Data for these rats is not included in Figure 14 to increase the clarity of the figure. The MCA lumen diameters 1 second and 4 minutes after pressurization to 100 mmHg and after maximal vasodilation to 3 μM nifedipine were respectively 190±14, 127±9 and 213±9μm, mean± SEM (n=5 per group). These values were not significantly different from the same parameters in Dahl-SS fed a 8.7% NaCl diet for one week. The degree of basal tone present in Dahl-SS fed a 0.7% NaCl diet for 9 weeks was less than that observed in Dahl-SS fed a 8.7% NaCl diet for 3 weeks and not significantly different from rats fed high salt for 1 or 2 weeks (Figure 16).

3.5 DISCUSSION

The studies of this chapter were carried out to test the hypothesis that HE development was preceded by a defect in the ability of the cerebrovasculature to autoregulate CBF and to further assess the possibility that the latter defects coincided with the inability of the vascular segments to elicit constriction to pressure. Dahl-SS fed 8.7% NaCl developed behavioural symptoms consistent with HE. The origin of the symptoms likely resulted from the breakdown or loss in integrity of the BBB as evidenced by significant edema formation and extravasation of Evans blue dye, previously outlined in chapter 2 (Figure 7). A
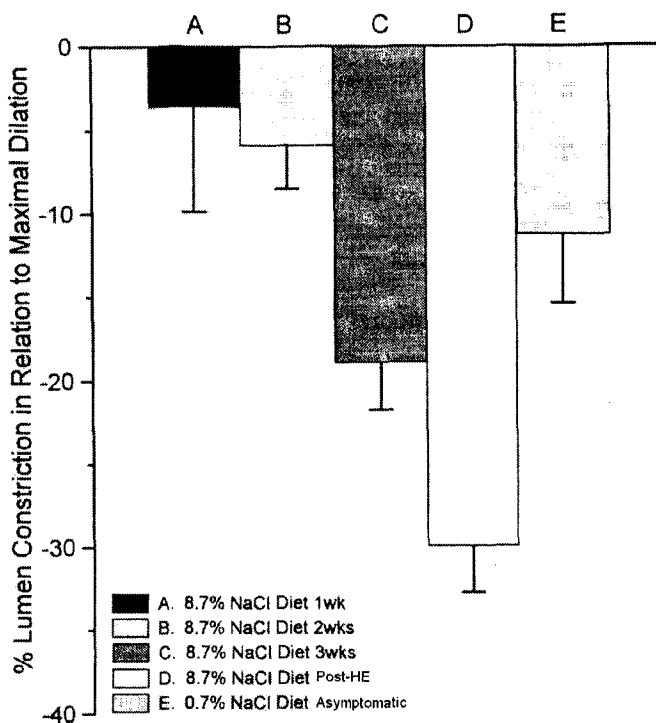
Figure 16: The basal tone in MCAs of Dahl-SS at 100 mmHg prior to the initiation of pressure dependent constriction. The basal tone present in the MCAs prior to pressure dependent constriction was calculated as the percent constriction present from the maximally dilated state (i.e.[(lumen diameter 1 sec after pressurization to 100 mmHg/lumen diameter in presence of nifedipine at 100 mmHg)-1] x 100. Statistics: ANOVA plus Fisher post hoc test. Significant ($p < 0.05$) difference A vs C and D, B vs C and D, D vs E (n=5 rats within each group). Values represent the mean ± S.E.M.

key finding in the present study was that the ability of the MCAs to constrict to increases in pressure (i.e. the PDC response) decreased in relation to the duration that the rats were fed high salt. This response was absent in MCAs sampled from rats exhibiting HE. Conversely, the MCAs of Dahl-SS fed a 0.7% NaCl diet for 9 weeks continually exhibited robust constriction to pressure. Chronologically, the virtual loss of PDC in the MCAs of asymptomatic Dahl-SS fed a 8.7% NaCl diet coincided with the loss of CBF autoregulation in matched rats. This raises the possibility that a loss of cerebrovascular PDC may be an important mechanism contributing to the loss of CBF autoregulation.

3.5.1 Characteristics of Cerebral Blood Flow Autoregulation

The hypothesis that CBF autoregulation was lost prior to HE development was supported by the observation that asymptomatic Dahl-SS fed a 8.7% NaCl diet for 3 weeks showed an inability to regulate CBF (the relationship between blood pressure and CBF was linear). Interestingly, the change in relative CBF in response to BP (Table 2) was similar between all groups despite the absence or the presence of autoregulation. The latter phenomenon could be explained by a situation where the loss of CBF autoregulation occurred in the presence of either a reduction in lumen diameter and/or vasoconstriction of the vessels. This potential mechanism is explained in detail in Figure 17.

In addition, after 3 weeks of high salt feeding the lumen diameters were reduced in the MCAs of asymptomatic Dahl-SS fed high salt (see figure 15, C., 3
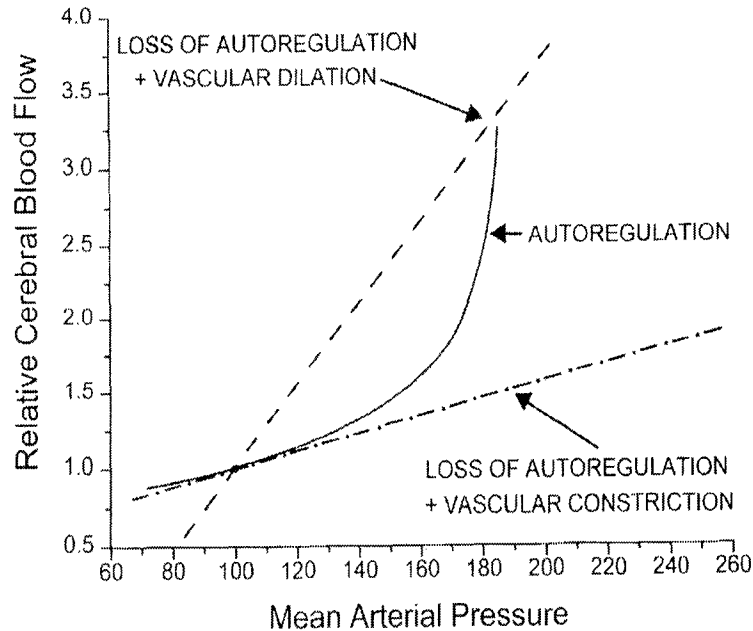
Figure 17: A hypothesis of the type of change occurring in the cerebrovasculature of asymptomatic Dahl-SS fed 8.7% NaCl for 3 weeks. The solid line curve labeled AUTOREGULATION represents the approximation of the average change in relative cerebral blood flow (CBF) with blood pressure in asymptomatic Dahl-SS fed 8.7% an NaCl diet for one week. Evidence of CBF autoregulation with the presence of a distinct upper limit is present. The loss of CBF autoregulation under conditions of cerebrovascular vasodilation would produce hyper-perfusion at higher blood pressures, a straight line relationship without the presence of a distinct upper limit to autoregulation such as that shown in the figure (see LOSS OF AUTOREGULATION + VASCULAR DILATION). The occurrence of a loss of autoregulation and the presence of cerebrovascular vasoconstriction would decrease the slope of the relationship of relative CBF to blood pressure from that present under dilated conditions as shown in the diagram (see LOSS OF AUTOREGULATION + VASCULAR CONSTRICTION). This is representative of the type of regulation present in the asymptomatic Dahl-SS fed 8.7% an NaCl diet for 3 weeks (see Figure 12). If the types of alterations observed in the isolated MCAs of Dahl-SS an fed 8.7% diet are representative of the typical types of alterations in the cerebrovasculature. The loss of CBF autoregulation in rats fed the diet for 3 weeks could be due an ability to constrict to pressure, (see Figure 14) coupled with the development of a structurally reduced lumen diameter (see Figure 15) plus the presence of a large degree of basal tone (see Figure 16). This could exert a protective effect, preventing maximal cerebrovascular over perfusion under conditions where cerebral blood flow is lost.

µM nifedipine). This suggests that in addition to the presence of increased basal tone a structural reduction in the lumen diameter existed in asymptomatic Dahl-SS fed high salt for 3 weeks. We believe that as hypertension continued to develop in Dahl-SS at approximately 3 weeks of high salt feeding, cerebrovascular PDC was attenuated causing the loss of CBF autoregulation. Autoregulation could have been lost under conditions of massive vasodilation. If this was the case it would result in a situation of overperfusion at higher blood pressures and a straight line relationship with no distinct upper limit as described by the curve (LOSS OF AUTOREGULATION + VASCULAR DILATION) in Figure 17. In this instance the change in CBF in relation to the change in BP would increase. In contrast, the loss of autoregulation in the presence of extreme vasoconstriction and/or a structural reduction in lumen diameter would create a linear relationship with a decreased slope in the relationship of relative CBF versus BP. This is best described by the relationship labeled "LOSS OF AUTOREGULATION + VASCULAR CONSTRICTION" in Figure 17.

Overall, the latter situation best represents autoregulatory characteristics observed in Dahl-SS fed 8.7% NaCl for 3 weeks (see Figure 12). This feature is also consistent with the presence of large degrees of basal tone (Figure 16), a structurally reduced lumen diameter (Figure 15) and an attenuation of PDC (Figure 14) in the MCAs of asymptomatic HE Dahl-SS fed high salt for 3 weeks and for post-HE rats.

3.5.2 Pressure Dependent Constriction and CBF Autoregulation

In the microvasculature of the brain, pressure dependent constriction (PDC) is thought to play an important role in the autoregulation of blood flow (Johnson, 1986). Previous studies have shown that elevations in blood or transmural pressure under both *in vivo* conditions and in isolated cerebral vessels promote constriction in small cerebral arteries (Harder, 1984; Tamaki *et al.*, 1984; Faraci *et al.*, 1989). The constriction of cerebral vessels in response to elevations in blood pressure elevates vascular resistance to flow. This counteracts the subsequent potential increase in blood flow that might be expected to occur. Thus CBF remains constant despite the change in blood pressure.

In the present study, MCAs from Dahl-SS fed high salt lost their ability to respond to increases in pressure (i.e. PDC) prior to the development of hypertensive encephalopathy. This loss in PDC was dependent on the duration that that animal was fed high salt. In Dahl-SS, there was a significant decrease (> 80%) in the ability of the MCAs to constrict in response to a 100 mmHg pressure step when Dahl-SS were fed high salt from 1 week to 3 weeks. This suggests that changes are occurring within the vasculature that are altering the PDC response. The significance of this is that an inability to decrease lumen diameter in response to elevated pressure during hypertension could promote cerebrovascular overperfusion. The increased downstream pressure and elevated endothelial shear resulting from the loss of PDC could compromise the

blood-brain barrier and contribute to the initiation of HE. The 80% attenuation of the PDC in the MCAs of asymptomatic HE Dahl-SS fed high salt (Figure 14) is consistent with the observation that CBF autoregulation is lost *in vivo* in the MCA perfusion domain of comparable Dahl-SS fed high salt for the same duration (Figure 12).

A potential limitation in studies evaluating PDC and CBF autoregulation in the MCAs perfusion domain is that it only provides a snapshot as to how the entire cerebrovasculature is responding to increases in pressure. In addition since the MCA is a relatively large vessel we do not know if the types of alterations that occur in the MCA mimic those present at the level of the microcirculation. That said, there is some consistency between the types of changes observed in the MCA and the nature of CBF autoregulatory loss observed, which suggests that a similar alteration exists in the microvessels fed by the MCA.

3.5.3 Autoregulation: Dahl-SS versus SHRsp

The development of HE in Dahl-SS fed a high salt diet shows changes within the cerebrovasculature that exhibit similarities and contrasts to the types of changes present in the SHRsp following stroke development (Smeda, 1992; Smeda *et al.*, 1999b). Both Dahl-SS and SHRsp lost their ability to autoregulate CBF and developed defects in PDC within the MCAs prior to the development of stroke and HE. However, unlike Dahl-SS, the loss of autoregulation observed in

SHRsp was associated with an increase in $_\Delta$ CBF/ $_\Delta$mmHg BP (Smeda *et al.*, 1999b). The resultant effect of this relationship of CBF to BP was that overperfusion was enhanced to a greater degree than if regulation were lost under conditions of cerebrovascular vasoconstriction, (as is predicted in Dahl-SS). This could explain the higher incidence of hemorrhage formation in SHRsp versus Dahl-SS.

3.5.4 The Loss of Renal Blood Flow Autoregulation in the Kidneys of Dahl-SS

Besides the present study, no other cerebral circulatory studies have been performed on Dahl-SS rats. However, it is of interest to note that similar alterations in autoregulatory dysfunction and defects in PDC have been noted in the renal vasculature of Dahl-SS (Karlsen *et al.*, 1997). As previously described, the kidney vasculature has the ability to regulate constant blood flow over a range of varying pressure under *in vivo* conditions. Studies involving isolated kidneys sampled from Dahl-SS have shown that elevations in renal perfusion produce constriction in the renal interlobular arteries and afferent arterioles (Takenaka *et al.*, 1992; Hayashi *et al.*, 1996). The response time to pressure elevations are faster in the renal versus the cerebral vasculatures but Ca^{2+} channel antagonists inhibit PDC in both vascular beds (Takenaka *et al.*, 1992; Hayashi *et al.*, 1996). Studies involving Dahl-SS rats fed an 8% NaCl diet for five weeks post weaning have indicated a complete loss in the ability of the renal

vasculature to autoregulate blood flow (Karlsen *et al.*, 1997). Likewise the loss of autoregulation was associated with the presence of a large increase in the renal vascular resistance to flow (Karlsen *et al.*, 1997), suggesting that the loss in renal blood flow autoregulation was associated with vasoconstriction and/or a structural reduction in the lumen diameter. Studies involving perfused kidneys have also indicated that Dahl-SS rats fed an 8% NaCl diet have interlobular arteries and afferent arterioles that lose their ability to constrict to pressure (Takenaka *et al.*, 1992; Hayashi *et al.*, 1996). It is possible that defects in the ability to autoregulate blood flow may occur in multiple vascular beds in Dahl-SS fed high salt diet.

3.5.5 CONCLUSIONS

Overall, we can conclude that the ability to constrict to pressure and regulate CBF is lost prior to the development of hypertensive encephalopathy in MCAs from post-HE Dahl-SS. The increase in basal tone observed in MCAs from post-HE Dahl-SS fed a 8.7% NaCl diet suggests that they lose their ability to autoregulate CBF under conditions of vasoconstriction. The loss of CBF autoregulation under conditions of cerebrovascular constriction would still result in a situation where *in vivo* alterations in BP would evoke changes in CBF, however, the presence of cerebrovascular constriction would provide some protection despite the loss of autoregulation by dampening the level of hyperperfusion during hypertension. This could account for the lower incidence of

cerebral hemorrhage in this model in comparison to SHRsp, which lose CBF autoregulation under conditions of cerebrovascular vasodilation. The chronological, quantitative and qualitative changes in CBF autoregulation in the MCA perfusion domain coincide with the loss of PDC in isolated MCAs perfusing this area suggesting that PDC is an important mechanism supporting autoregulation in these rats.

CHAPTER 4

CEREBROVASCULAR ALTERATIONS IN PRESSURE AND PROTEIN KINASE C MEDIATED CONSTRICTION IN DAHL-SS

4.1 INTRODUCTION

The intrinsic ability of arterioles to rapidly constrict or dilate in response to an increase or decrease in intraluminal pressure is defined as the myogenic response or pressure dependent constriction (Meininger & Davis, 1992; Davis & Hill, 1999; Hill *et al.*, 2001). This mechanism provides the fundamental basis for regulation of blood flow within the cerebrovasculature that insures that the necessary nutrient and oxygen demand required for normal function is achieved (Harder *et al.*, 2002). In addition, it provides a protective mechanism whereby cerebrovascular overperfusion is prevented during hypertension (Strandgaard & Paulson, 1995).

The signal transduction mechanisms linking elevations in pressure to arterial constriction have not been fully characterized. It is unlikely that one signaling pathway is responsible for mediating the PDC response. Both pharmacomechanical (constriction without a change in smooth muscle membrane potential) and electromechanical (constriction associated with smooth muscle depolarization) coupled pathways are involved in producing PDC (Hill *et al.*, 2001)

Protein kinase C (PKC), modulates PDC during low levels of intracellular Ca^{2+} (Gokina *et al.*, 1999). PKC has also been shown to open voltage gated calcium channels (VGCC's) independent of membrane depolarization (Fish *et al.*, 1988). In more recent studies, PKC activation in response to a cascade initiated by elevations in pressure have been hypothesized to open the transient receptor potential cation channels (TRPc). It was hypothesized that an influx of cations through TRPc's promoted vascular smooth muscle depolarization in the cerebrovasculature leading to the opening of VGCC's that lead to the initiation of constriction (Welsh *et al.*, 2002). All of the above evidence suggests that PKC is an important signal transduction agent and mediator of PDC in many arterial systems.

The studies outlined in chapter 3 have demonstrated that PDC in the MCAs of Dahl-SS fed a high salt diet becomes attenuated prior to HE development and is lost in the MCAs of rats with HE. The loss of the PDC response in the MCAs of SHRsp with stroke was coupled to alterations in PKC function (Smeda *et al.*, 1999a). This suggested that the PKC system in the cerebrovascular smooth muscle plays an important role in governing PDC and that this system can become defective. In view of this, studies were carried out to determine if the loss of PDC observed in the MCAs of Dahl-SS exhibiting HE was associated with the presence of altered PKC function in the smooth muscle of the arteries.

4.2 OBJECTIVES AND HYPOTHESES

It is our belief that PKC is an important signaling agent involved in promoting or permitting the development of PDC in the MCAs of Dahl-SS. We tested the hypothesis that the loss of PDC in the MCAs of Dahl-SS with HE was associated with the occurrence of defects in the ability of PKC to mediate MCA constriction. To test for this possibility, MCAs from Dahl-SS were sampled prior to and following the observation of behavioural signs associated with HE. The ability of MCAs from these rats to constrict to a 100 mmHg pressure step or to the PKC activator, phorbol dibutyrate was assessed. The ability of the artery to contract in response to pressure was related to the constriction produced by PKC activation.

4.3 MATERIALS AND METHODS

4.3.1 Experimental Animals and the Monitoring of HE Development

These aspects of the experimental protocol are outlined in detail in chapter 2. Specifically, a description of the Dahl Salt-Sensitive Colony and the breeding protocol is outlined in section (2.3.1). The rats were examined for signs of HE which are defined in section 2.3.5.

4.3.2 Pressure Myograph Experiments

The evaluation of PDC was tested in the MCAs of Dahl-SS fed an 8.7%

NaCl diet prior to (n=17) and following (n=10) the observations of symptoms

consistent with the development HE. In addition, we also sampled MCAs from

Dahl-SR fed 8.7% (n=10) and 0.7% (n=8) NaCl. Dahl-SR remained healthy and

asymptomatic for the duration of the experiments when fed high or normal salt

diet. PDC was measured in the MCAs using the techniques previously outlined in

Chapter 3 (section 3.3.3)

Following the evaluation of PDC to a 100 mmHg pressure step (methods

described earlier in detail in section 3.3.3), the MCAs were maintained at 100

mmHg pressure and then maximally vasodilated with nifedipine (3 μM). The

PKC activator, phorbol dibutyrate (0.1μm) was applied to the bath (n=6) and the

degree of constriction was measured. Validation experiments (outlined in the

results section) were carried out which demonstrated that under the latter

conditions constriction in response to phorbol dibutyrate was mediated by PKC

activation, which was inhibited by the PKC inhibitors chelerythrine (12 μM) or

bisindoylmaleimide (5 μM). MCAs were further evaluated in their ability to

constrict to a 100 mmHg pressure step in the presence of the PKC inhibitors

outlined above (n=4 per inhibitor). Control experiments evaluated the

reproducibility of PDC in the presence of vehicle (50% dimethyl sulfoxide; n=4).

In additional experiments (n=5), the ability of vasopressin (0.17 μM) to constrict

MCAs in the presence of 3 μM nifedipine was tested. Vasopressin can constrict

MCAs by the release of an intracellular source of Ca^{2+} in a manner independent of PKC activation. The presence of constriction in response to vasopressin indicated that the contractile apparatus remained functional.

4.3.3 Statistical Analysis

We used either a one-way analysis of variance (ANOVA) followed by a Fisher post-hoc test or GLM multivariant analysis of variance (MANOVA) was used to determine if significant differences existed between groups of data. Results were considered significant at $P<0.05$. The mean ± the standard error is shown in the data. N values always equal the number of rats used in the experiment. A more detailed description of the statistical analysis used is outlined in section 2.3.9.

4.4 RESULTS

4.4.1 Pressure Dependent Constriction

MCAs sampled from Dahl-SS with HE were unable to constrict in response to a 100 mmHg pressure step (Figure 18). Asymptomatic Dahl-SS fed 0.7% NaCl or 8.7% NaCl for 2 weeks as well as Dahl-SR fed 0.7% NaCl or 8.7% NaCl had MCAs that exhibited comparable and robust constriction in response to increases in pressure.
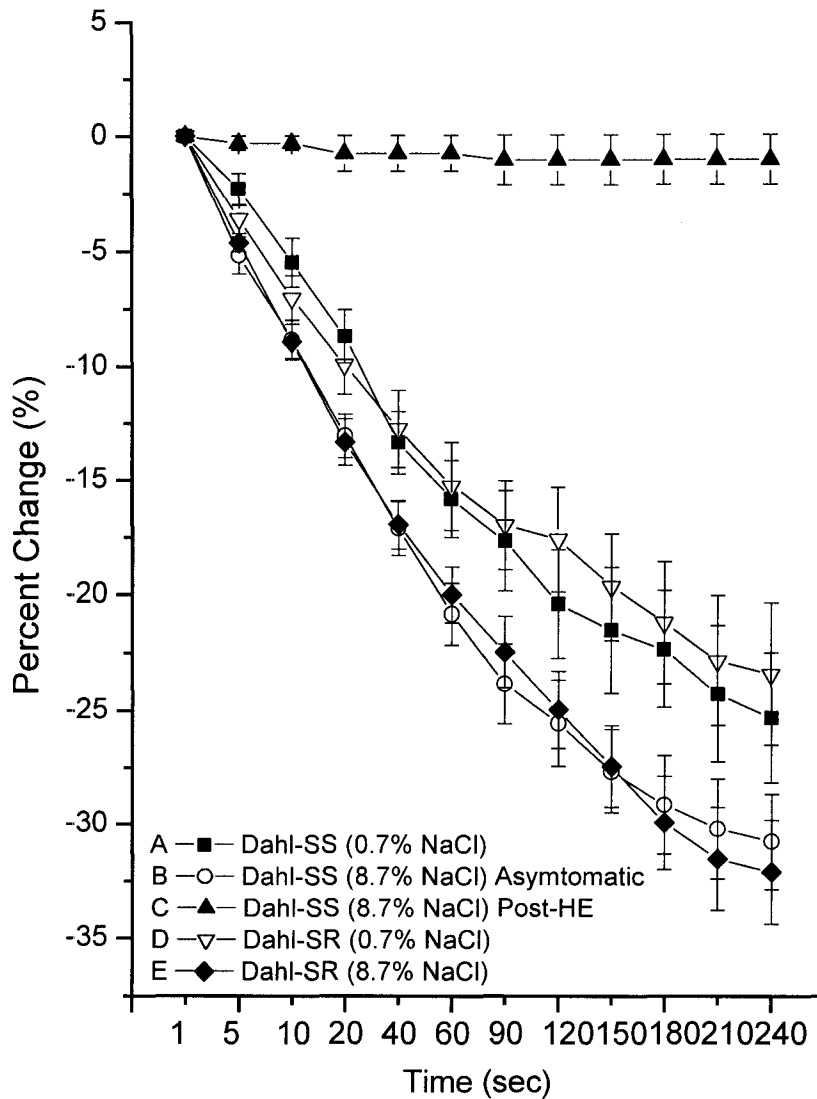
Figure 18: Pressure dependent constriction response in isolated middle cerebral arteries (MCAs) in response to a 100 mmHg pressure step. Rats were fed 8.7% or 0.7% NaCl from weaning (five weeks of age). Dahl-SS exhibiting behavioral signs of HE (n=10), had MCAs that did not constrict to pressure. Dahl-SR fed 8.7% NaCl for 5 weeks (n=11) and asymptomatic Dahl-SS fed 8.7% NaCl for 2.0 weeks (n=17) had MCAs that constricted robustly to the applied pressure. Dahl-SS (n=10) and Dahl-SR (n=8) fed 0.7% NaCl also constricted to pressure. Statistics: General Linear Model MANOVA: A vs B, C, D or E response over time p<0.05. Values represent the mean ± S.E.M.

4.4.2 Pressure Dependent Constriction and PKC Activation

The effects of the PKC inhibitors chelerythrine and bisindoylmaleimide on PDC and phorbol dibutyrate induced constriction in MCAs are outlined in Table 3. MCAs used in these experiments were sampled from asymptomatic Dahl-SS rats. As shown in Table 3 (1st PDC) arteries from these rats exhibited constriction in response to a 100 mmHg pressure step. Pre-incubation with bisindoylmaleimide or chelerythrine prevented the development of PDC in the MCAs (2nd PDC, Table 3) in response to a subsequent equal pressure step, and (in the presence of nifedipine (3 µM) prevented constriction in response to phorbol dibutyrate (0.1 µM). The effect of bisindoylmaleimide on the time course of PDC is shown in Figure 19. These data demonstrated that in MCAs, PDC can not occur under conditions where PKC activity is inhibited and that in the presence of nifedipine, phorbol dibutyrate mediates constriction by the activation of PKC.

4.4.3 Alterations in the PKC System in Relation to the Development of Hypertensive Encephalopathy

In separate MCAs, we assessed the function of the PKC system in asymptomatic Dahl-SS and Dahl-SR fed an 8.7% or a 0.7% NaCl diet as well as in Dahl-SS exhibiting HE (fed 8.7% NaCl). MCAs of Dahl-SS with HE which were unable to constrict to a 100 mmHg pressure step (see Figure 18) also did not constrict to phorbol dibutyrate in the presence of nifedipine (i.e. via PKC

TABLE 3. Effect of PKC Inhibition on the PDC Response in MCAs of Asymptomatic Dahl-SS.

PKC Inhibitor or Vehicle	1st PDC (no inhibitor or vehicle)	2nd PDC (+ inhibitor or vehicle)	PDB-mediated Constriction
Control (n=5)	-28.5 ± 2.4	-26.2 ± 1.7	-37.3 ± 6.4
Vehicle (n=4)	-24.8 ± 6.2	-31.0 ± 4.7	-25.0 ± 8.7
Chelerythrine (n=4)	-21.9 ± 2.9	+13.0 ± 5.5[a]	0 ± 0[b]
Bisindolylmalemide (n=4)	-31.2 ± 4.2	-7.9 ± 5.6[a]	-2.3 ± 1.3[b]

Values represent the mean ± S.E.M.

[a,b] $P < 0.05$ compared with all 1st PDC responses and all control and vehicle responses (ANOVA with Fisher's post hoc test).

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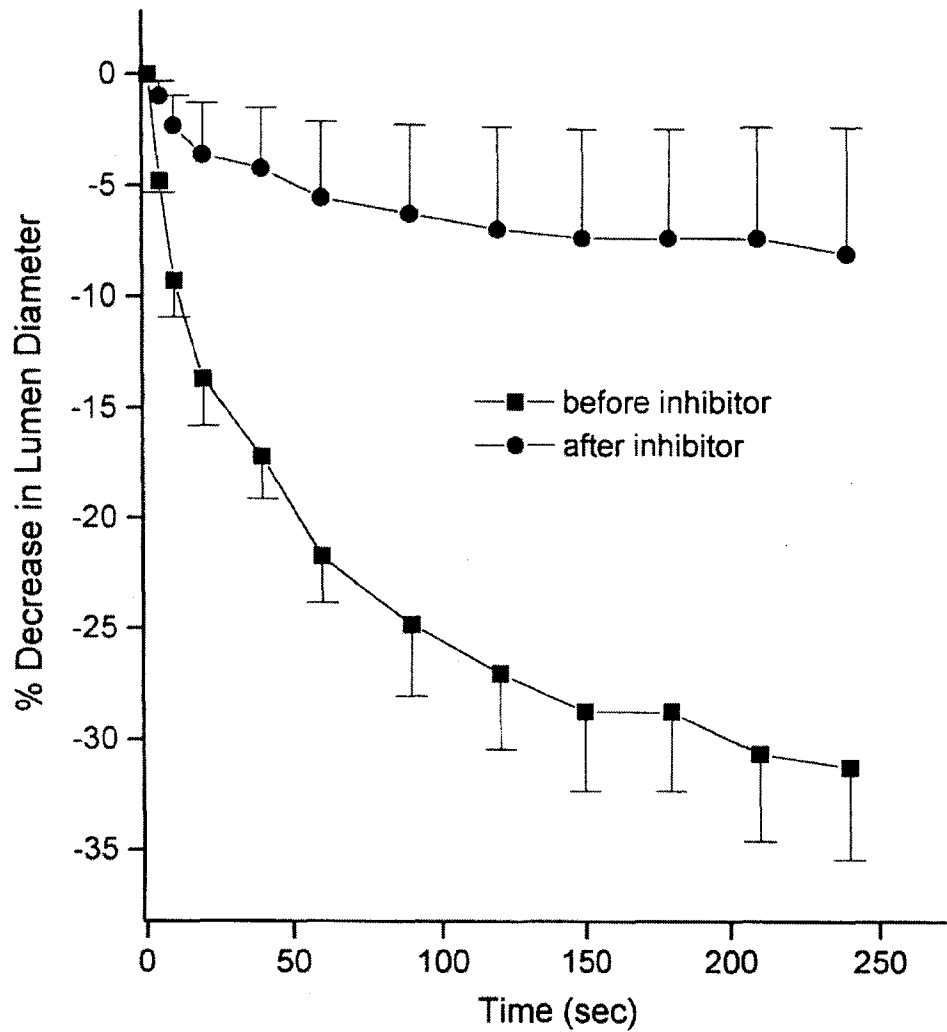
Figure 19: The effect of PKC inhibition on PDC in isolated middle cerebral arteries MCA(s). PKC inhibitor bisindolylmaleimide (5 μM) and chelerythrine (12 μM) (see Table 1) significantly inhibited the ability of the MCAs to constrict to a 100 mmHg pressure step. (n=4 asymptomatic Dahl-SS). Values represent the mean ± S.E.M.

activation). On the other hand, the MCAs of asymptomatic Dahl-SS and Dahl-SR fed a high or normal salt diet exhibited constriction both to pressure (Figure 18) and to phorbol dibutyrate (Figure 20).

Comparisons of the left versus the right MCA of the same rats indicated that Dahl-SS with HE (which had MCAs that lacked the ability to constrict to phorbol dibutyrate) exhibited phasic constriction to vasopressin in the presence of nifedipine (see C versus F in Figure 20). Asymptomatic Dahl-SS and Dahl-SR had MCAs that constricted to both phorbol dibutyrate and vasopressin in the presence of nifedipine (Figure 20).

4.5 DISCUSSION

Dahl-SS fed an 8.7% NaCl diet exhibited behavioural symptoms consistent with HE after three weeks of high salt feeding. Isolated MCA segments from these rats lacked a functional PDC response and could not contract to the PKC activator, phorbol dibutyrate in the presence of nifedipine. Pre-treatment with PKC inhibitors (chelerythrine and bisindoylmaleimide) abolished the PDC response. The contractile apparatus of MCAs from post-HE Dahl-SS was functional since MCAs that were unable to elicit PDC could still constrict to vasopressin in the presence of nifedipine.
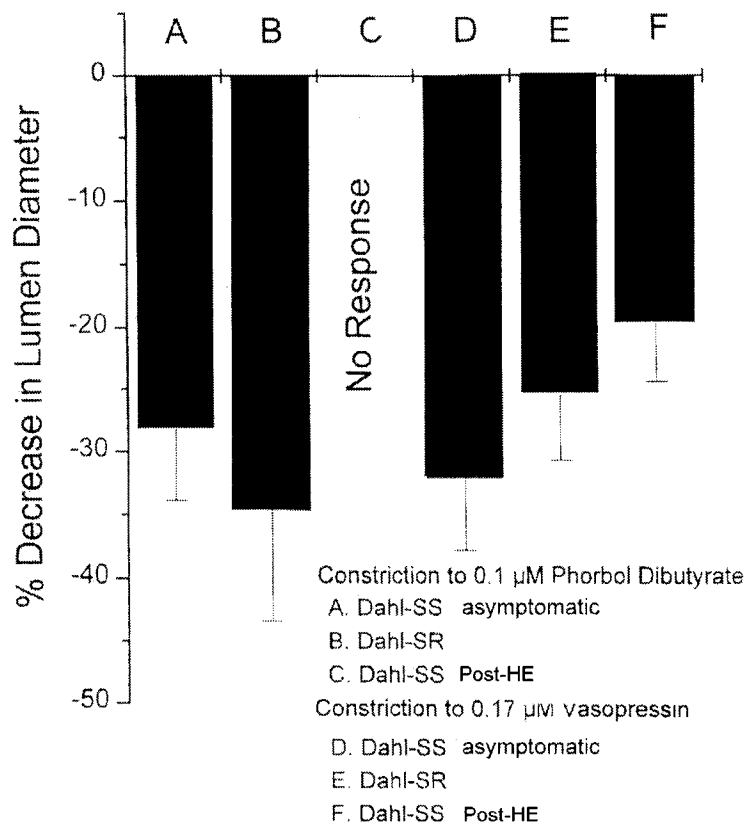
Figure 20. Constriction of isolated MCAs in response to phorbol dibutyrate or vasopressin in the presence of nifedipine. The experiment demonstrates that the MCAs of post-HE Dahl-SS that have an inability to constrict to pressure (See Figure 18) have a defective PKC system (see C). The inability to constrict in response to pressure or PKC activation is not due to a dysfunctional contractile apparatus since the isolated MCAs of post-HE Dahl-SS constrict in response to vasopressin (see F). (n-values, A-6 rats, B to F- 5 rats per group; different arteries were used for the phorbol dibutyrate and vasopressin constriction experiments. Statistics: ANOVA + Fisher post hoc – A vs C, B vs C, significant, $p<0.01$, A VS B –NS, D E and F are not significantly different ($p<0.05$) from each other. Values represent the mean ± S.E.M.

4.5.1 PKC Signaling Pathway and Pressure Dependent Constriction

The importance of PKC in the promoting vascular PDC has been demonstrated in a number of vascular beds (Osol *et al.*, 1991; Karibe *et al.*, 1997; Kirton & Loutzenhiser, 1998; Smeda *et al.*, 1999a). Previous studies have evaluated the role of PKC in the signaling cascade that promotes PDC. In pressurized human coronary arterioles the maintenance of tone was dose dependently reduced when the PKC inhibitor, calphostin C, was administered (Miller *et al.*, 1997). Furthermore, in vascular arterioles that lack the ability to constrict to pressure, the addition of the PKC activator phorbol 12-myristate 13-acetate resulted in the establishment of a PDC response. (Miller *et al.*, 1997).

Other experiments demonstrated that the PKC activator, indolactam enhanced the level of tone in rat posterior cerebral arteries when arteries were pressurized to 125 mmHg whereas the PKC inhibitor, staurosporin, also produced a dose dependent vasodilation of (Osol *et al.*, 1991).

Upstream signaling promoters of PKC may also be involved in initiation of PDC. Phospholipase C (PLC) activity within the smooth muscle membrane can lead to the formation of inositol triphosphate (IP_3) and diacylglycerol (DAG) or DAG in the absence of IP_3 (Narayanan *et al.*, 1994). In pressurized rat posterior cerebral arteries the inhibition of PLC with U-73122 produced vasodilation (Osol *et al.*, 1993). In the latter study, the selectivity of U-73122 as a PLC inhibitor was validated by the attenuation of PLC mediated constriction in response to 5-HT. This suggested that vascular pressure induced constriction was mediated

through the activation of PLC. The latter hypothesis is consistent with the observation that the smooth muscle levels of DAG are elevated in dog canine renal arteries in response to elevations in pressure (Narayanan *et al.*, 1994). More recently, in human subcutaneous vessels, the inhibition of DAG kinase by the inhibitor, RHC-80267 abolished PDC (Coats *et al.*, 2001). These studies all suggest that the inhibition of PLC or the inhibition of downstream cellular components generated by PLC activation (i.e. DAG), inhibits the PDC response. These findings suggest that PLC activation may be involved in promoting PDC.

4.5.2 PKC Activation and the Downstream Signaling Promoting PDC

The potential signal transduction pathway promoting PDC in response to PKC activation may involve the opening of the transient receptor potential channel (TRPc). These channels have been characterized in the smooth muscle of various vascular beds (Clapham *et al.*, 2001; Inoue *et al.*, 2001; Welsh *et al.*, 2002). Recently, mRNA coding for the TRPc6 channel was identified in smooth muscle cells of cerebral arteries. The inhibition of the TRPc6 channel with oligodeoxynucleotide antisense treatment inhibited pressure dependent but not high $[K^+]_o$ depolarization induced constriction in posterior cerebral arteries (Welsh *et al.*, 2002). It was hypothesized that elevations in pressure activated PLC, which (through a cascade of reactions) led to the formation of DAG. Increases in DAG activated PKC, which opened the TRP6 channels. The opening of these nonspecific cation channels produced vascular smooth muscle membrane

depolarization, which promoted the opening of VGCC leading to an influx of Ca^{2+} and myogenic contraction (Welsh *et al.*, 2002). The loss of PDC in the MCAs of post-HE Dahl-SS may be attributable to the fact that TRP6 channels are unable to open due to the presence of a defective PKC system. Since the MCAs of post-HE Dahl-SS were capable of constricting to vasopressin (a response that is mediated by PLC activation), it is likely that the PLC component of the PDC signaling pathway that precedes PKC activation (i.e. is involved in generating DAG) remained functional. Although the present study has demonstrated that the MCAs of post-HE Dahl-SS exhibit a defect in the ability to constrict to PKC activation, this does not preclude the possibility that other components in the pathway are also defective. Recent studies (Smeda, unpublished results) indicate that the MCAs of post-HE Dahl-SS exhibit an attenuated ability to constrict in response to high $[K+]_o$ induced depolarization, indicating that the mechanisms promoting the voltage dependent opening of VGCC's may also be defective in these arteries.

Alternative signaling pathways have also been proposed to explain PDC in the cerebrovasculature of rats. An increase in pressure in rat cerebral arteries has been shown to increase the production of 20-hydroxyeicosatetraenoic acid (20-HETE), a cytochrome p450 metabolite of arachidonic acid (Gebremedhin *et al.*, 2000). The inhibition of 20-HETE formation or its vasoconstrictor action inhibits PDC in isolated rat cerebral arteries (Gebremedhin *et al.*, 2000). Constriction in response to 20-HETE is also thought to be promoted by PKC

activation and smooth muscle depolarization (via a decrease in K+ conductance, that promotes Ca^{2+} influx through VGCC) (Lange *et al.*, 1997; Gebremedhin *et al.*, 2000). If this unique signaling pathway is involved in promoting PDC in the cerebrovasculature, the presence of a defective PKC system would still lead to the inhibition of PDC.

4.6 CONCLUSIONS

There is overwhelming evidence that a functional vascular smooth muscle PKC system is necessary to allow PDC to occur in the cerebrovasculature. Our studies demonstrated that the inhibition of the PKC system in the vascular smooth muscle of MCAs inhibits PDC in the arteries. In addition we demonstrated that in the MCAs of Dahl-SS with HE, the loss of PDC coincides with the presence of a PKC system that is dysfunctional in terms of its ability to elicit constriction. Recent studies (Smeda & Payne, 2003) have indicated that there is a progressive decline in the ability of the isolated MCAs to elicit PDC in asymptomatic Dahl-SS fed an 8.7% NaCl diet for progressively longer durations. The ability of the MCAs of these rats to elicit PDC was directly related in a quantitative manner to the ability of the same arteries to constrict to PKC activation by phorbol dibutyrate (Smeda, unpublished results). Finally, virtually every pathway proposed to explain the signal transduction mechanisms promoting constriction in response to elevations in pressure have included PKC activation as an important, if not a critical, component. In view of the above

evidence there is a strong possibility that the presence of a defective PKC system may contribute to the loss of PDC in MCAs of Dahl-SS with HE. In view of the importance of cerebrovascular PDC in the maintenance of CBF autoregulation (discussed in Chapter 3), a defect in the vascular smooth muscle PKC system may contribute to the loss of CBF autoregulation observed in Dahl-SS fed a high salt.

CHAPTER 5

ALTERATIONS IN CEREBROVASCULAR ENDOTHELIAL

FUNCTION IN DAHL-SS

5.1 INTRODUCTION

Nitric oxide (NO) is a potent vasodilator that is intrinsically produced in the vasculature. The basal release can occur from adventitial and endothelial sources in many vascular beds (Moncada *et al.*, 1991). The levels of NO released from the endothelium can be elevated by the actions of agonists that act on the endothelium to increase the activity of endothelial nitric oxide synthase (NOS). In addition, the endothelium very likely can release a non-NO dilatory factor such as endothelial-derived hyperpolarizing factor (EDHF) (Busse *et al.*, 2002). Vasodilation can be evoked via the endothelial-mediated release of both NO and EDHF in response to stimulation by prostacyclins (Armstead, 1995), bradykinin and acetylcholine (ACh) (Faraci & Heistad, 1998).

Endothelial functional impairment contributes to the development of hypertension (Boulanger, 1999). Endothelium dependent vasodilation is impaired in many models of both experimental and human hypertension (Luscher & Vanhoutte, 1986; Panza *et al.*, 1990). A reduction in vasodilation to ACh, methacholine, bradykinin and ADP has been observed in the cerebrovasculatures of SHRsp and SHR (Faraci & Heistad, 1998), whereas non-

endothelial mediated vasodilation in response to nitroprusside (Baumbach *et al.*, 1994) and adenosine (Mayhan *et al.*, 1987) remain un-impaired.

Nitric oxide synthase (NOS) activity is altered in hypertensive Dahl-SS fed high salt (Chen & Sanders, 1993). The administration of L-arginine (a substrate that is broken down to produce NO by NOS) to Dahl-SS fed high salt reverses hypertension and improves renal hemodynamics (Mattson *et al.*, 1997), whereas NOS inhibition increases blood pressure (Chen & Sanders, 1993). In view of this it has been suggested that hypertension development in Dahl-SS may be partially maintained by the reduced production or decreased availability of NO (Hayakawa & Raij, 1998).

5.2 OBJECTIVES AND HYPOTHESIS

There is a paucity of information regarding the development of hypertension and associated changes in endothelial function within the cerebrovasculature of Dahl-SS. Currently, it is unclear whether the basal release of endothelial and non-endothelial sources is altered in the cerebrovasculature of Dahl-SS with hypertension and the potential alterations in these functions in relation to HE or stroke development remain unexplored. In addition, no information is available as to whether NO released from the endothelium or the adventitia can modulate PDC in the cerebrovasculature of Dahl-SS. If such modulation exists, it could have an impact on CBF autoregulation.

It is clear that NOS related-function is attenuated within the renal vasculature of hypertensive Dahl-SS (Chen & Sanders, 1993; Hayakawa & Raij, 1998). Therefore, it is plausible that hypertension and or HE related alterations in endothelial function may impact the ability of cerebral arteries to elicit PDC. Dahl-SS developed large degrees of basal tone in the MCAs following the development of HE (Chapter 3). This was not due to the presence of a circulating factor since these arteries were studies under *in vitro* conditions where they were suffused with a physiological saline solution. The lack of a circulating factor would suggest that the development of basal tone was intrinsic in origin. It is possible that elevations in basal tone could have occurred due to the attenuation of the basal release of NO from an adventitial and/or endothelial source or via the attenuation of the release of a non-NO endothelial derived vasodilator.

The first objective of this study was to determine if the occurrence of HE in Dahl-SS was associated with changes in NOS-related function. The second objective was to assess if such changes altered the modulation of PDC in the cerebrovasculature?

5.3 MATERIALS AND METHODS

5.3.1 Animals

A description of the Dahl Salt-Sensitive Colony and the breeding protocol is outlined in section (2.3.1). The techniques used to measure blood pressure in the particular experimental groups are outlined in detail in section (2.3.4).

5.3.2 Pressure Myograph Studies

The evaluation PDC was tested in Dahl-SS (n=10 per group) fed an 8.7% NaCl diet prior to and following the development of behavioural symptoms consistent with the development of HE. The methodology for the evaluation of PDC was described in detail in chapter 3 (see section 3.3.3). All drugs used in the study were added to the external surface of the bath.

5.3.3 Assessment of the Effects of the Endothelium on Pressure Dependent Constriction

MCAs were removed from the brains of Dahl-SS fed 0.7% NaCl (n=5) and mounted on to a pipette of the pressure myograph used to measure PDC within arteries. Prior to tying the arteries the arterial lumen of the proximal half of the artery was rubbed (15X) against the pipette tip to remove the endothelium. An intact endothelium was maintained on the distal end of the artery. Subsequently, the artery was tied on to the pipette and pressurized to 100 mmHg. Bradykinin (1.6 μM) was used to evaluate completeness of endothelial removal in the proximal segments of the vessel. Using this technique the distal endothelial intact segment of the artery acted as a control for the proximal endothelial denuded segment. Vasodilation to bradykinin was also evaluated in MCAs from Dahl-SS fed 8.7% NaCl for 1 week (n=4) and Dahl-SS with HE (n=5) feed 8.7% NaCl for > 3 weeks. The effects of NOS inhibition (100 μM L-NAME) on bradykinin

vasodilation was tested in MCAs sampled from asymptomatic Dahl-SS fed 8.7% NaCl for 1 week (n=4).

5.3.4 Role of Nitric Oxide Synthase (NOS) in Modulating PDC

PDC response to a 100 mmHg pressure step was measured in the areas of the MCA containing an intact and absent endothelium in the presence or absence of 100 μM L-NAME (i.e. NOS inhibition). The latter experiments were performed in MCAs sampled from Dahl-SS 0.7% NaCl for 9 weeks (n=5). The effects of L-NAME were also assessed in MCAs from Dahl-SS with HE (n=5) that were fed 8.7% NaCl. The MCAs were incubated with L-NAME for 10 minutes prior to the evaluation of its effects on PDC or vessel diameter. The specificity of NOS inhibition by L-NAME was evaluated in MCAs from Dahl-SS fed 0.7% NaCl (n=4) by determining if the competitive inhibition produced by the NOS inhibitor could be reversed by L-arginine (8 mM, the normal substrate for NOS). The specificity of the reversal of L-NAME inhibition by L-arginine was further tested by the inability of D-arginine (an incompatible substrate for NOS) to duplicate the effects of L-arginine.

5.3.5 Statistical Analysis

We used either a one-way analysis of variance (ANOVA) followed by Fisher post-hoc test, GLM multivariant form of analysis of variance (MANOVA) or student's t-test to determine if significant differences existed between groups of

data. Results were considered significant at P<0.05. The mean ± the standard error measurement is shown in the data. N values always equal the number of rats used in the experiments. A more detailed description of the statistical analysis is outlined in section 2.3.9.

5.4 RESULTS

5.4.1 Pressure Dependent Constriction

MCAs sampled from of Dahl-SS with HE (fed 8.7% NaCl) exhibited an attenuation of the PDC response to a 100 mmHg pressure step (Figure 21). Asymptomatic Dahl-SS fed 8.7% NaCl for 1 week displayed robust (>25%) constriction in response to pressure.

Equal levels of PDC were observed in response to a 100 mmHg pressure step in the endothelial intact versus the denuded segments of MCAs that were sampled from Dahl-SS fed a 0.7% NaCl for 9 weeks (Figure 22). The denuded areas exhibited a greater vasodilation in response to nifedipine (3 μM) when compared to the endothelial intact segments of the MCAs. Endothelial removal abolished the ability of bradykinin (1.6 μm) to elicit vasodilation (Insert: Figure 22).
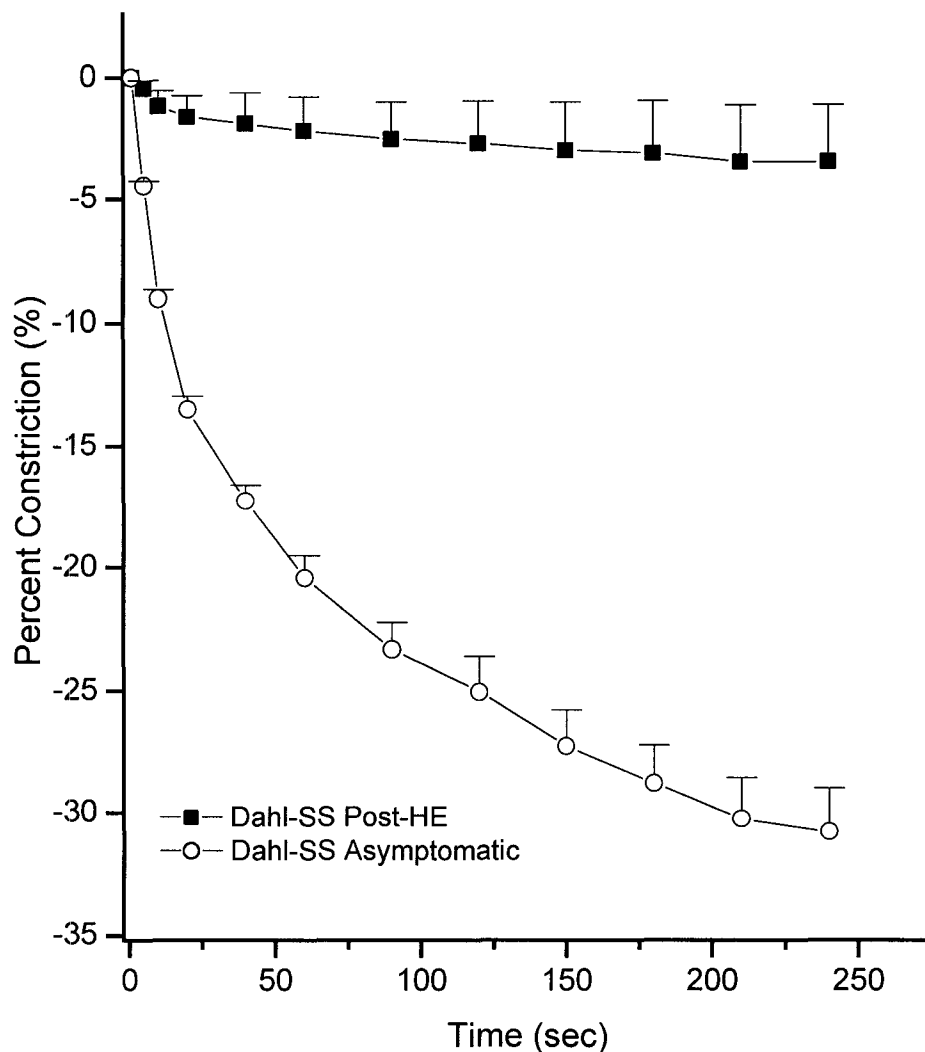
Figure 21: MCA pressure dependent constriction in response to a 100 mmHg pressure step. MCAs were sampled from asymptomatic Dahl-SS fed 8.7% NaCl for 1 week and post-HE Dahl-SS fed 8.7% NaCl. Statistics: General Linear Model MANOVA post-HE Dahl-SS group significantly different from the asymptomatic group, p<0.01, n=10 per group. Values represent the mean ± S.E.M.
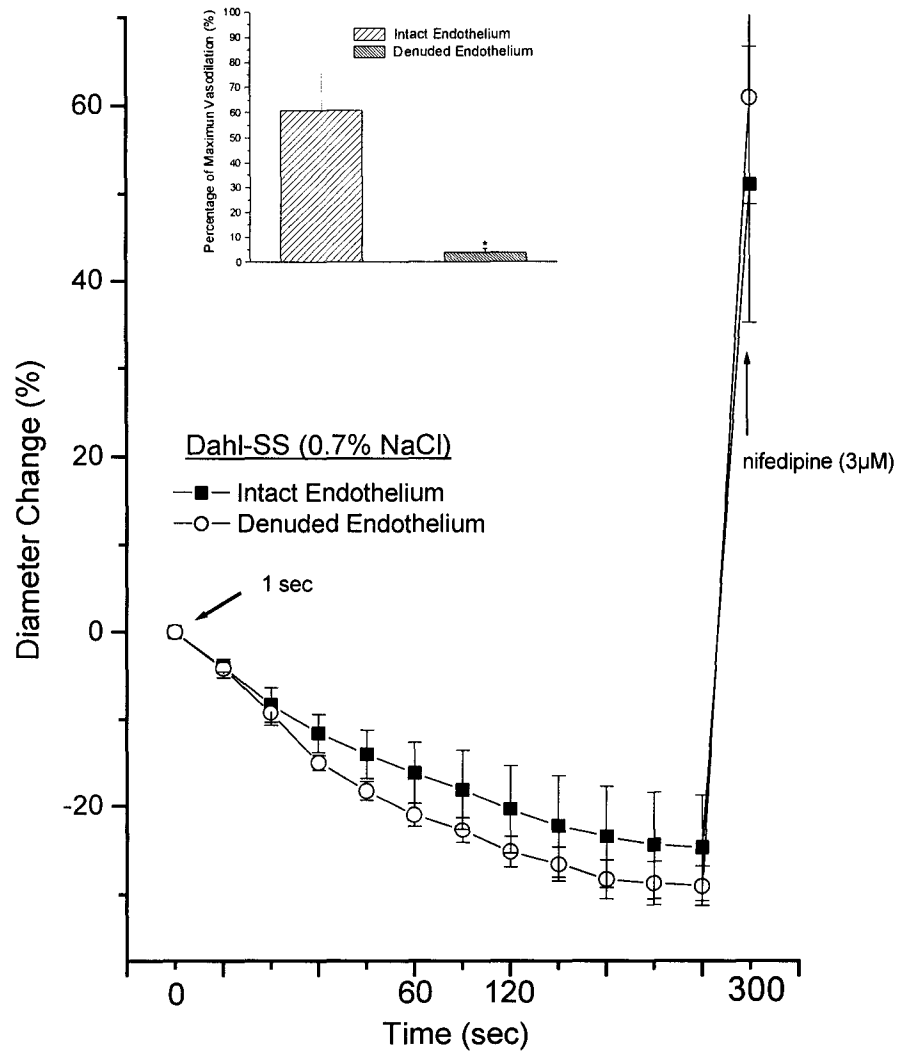
Figure 22: Pressure dependent constriction in response to a 100 mmHg pressure step in MCAs from Dahl-SS (low salt diet) in which one area of the vessel was subjected to de-endothelialization. Insert: Endothelial removal was confirmed by the absence of vasodilation in response to bradykinin (1.6 μM). Statistics: General Linear Model MANOVA: Endothelium intact group not significantly different from endothelial denuded group, p>0.05, n=5 per group. (*) Student t-test –paired. Bradykinin relaxation was significantly different (p <0.05) between the endothelial intact and denuded segments, n=5 per group. Values represent the mean ± S.E.M.

5.4.2 Endothelium-Dependent Vasodilation and Arteriolar Diameter

Bradykinin mediated vasodilation was attenuated in (endothelial intact) MCAs sampled from Dahl-SS with HE (fed 8.7% NaCl). The response was approximately 25% of that observed in the MCAs of asymptomatic Dahl-SS fed 8.7% NaCl for 1 week (Figure 23). Vasodilation to bradykinin was still observed after NOS inhibition (100 µM L-NAME) in MCAs sampled from Dahl-SS fed 8.7% NaCl for 1 week indicating that significant a degree of endothelial dependent bradykinin vasodilation must be produced by a non-NO vasodilator (Figure 23).

5.4.3 Modulation of PDC by the Endothelium and Nitric Oxide Synthase

The endothelium intact MCAs of asymptomatic Dahl-SS fed 8.7% NaCl for 1 week constricted to L-NAME (100 µM) indicating the presence of the release of basal NO. MCAs sampled from post-HE Dahl-SS exhibited an attenuated ability to constrict in response to L-NAME (Figure 24) suggesting the possibility that the basal release or action of NO may have been reduced. The level of vasoconstriction to L-NAME observed in asymptomatic Dahl-SS fed 8.7% NaCl for 1 week was significantly lower than that observed in intact endothelial .segment's of MCAs from Dahl-SS fed a normal diet (Figure 24). In MCAs sampled from Dahl-SS fed 0.7% NaCl, endothelial removal attenuated but did not abolish the ability of L-NAME to elicit vasoconstriction (Figure 24). This indicated that a non-endothelial source of basal NO release must be involved in promoting vasodilation in the MCAs (Figure 24).
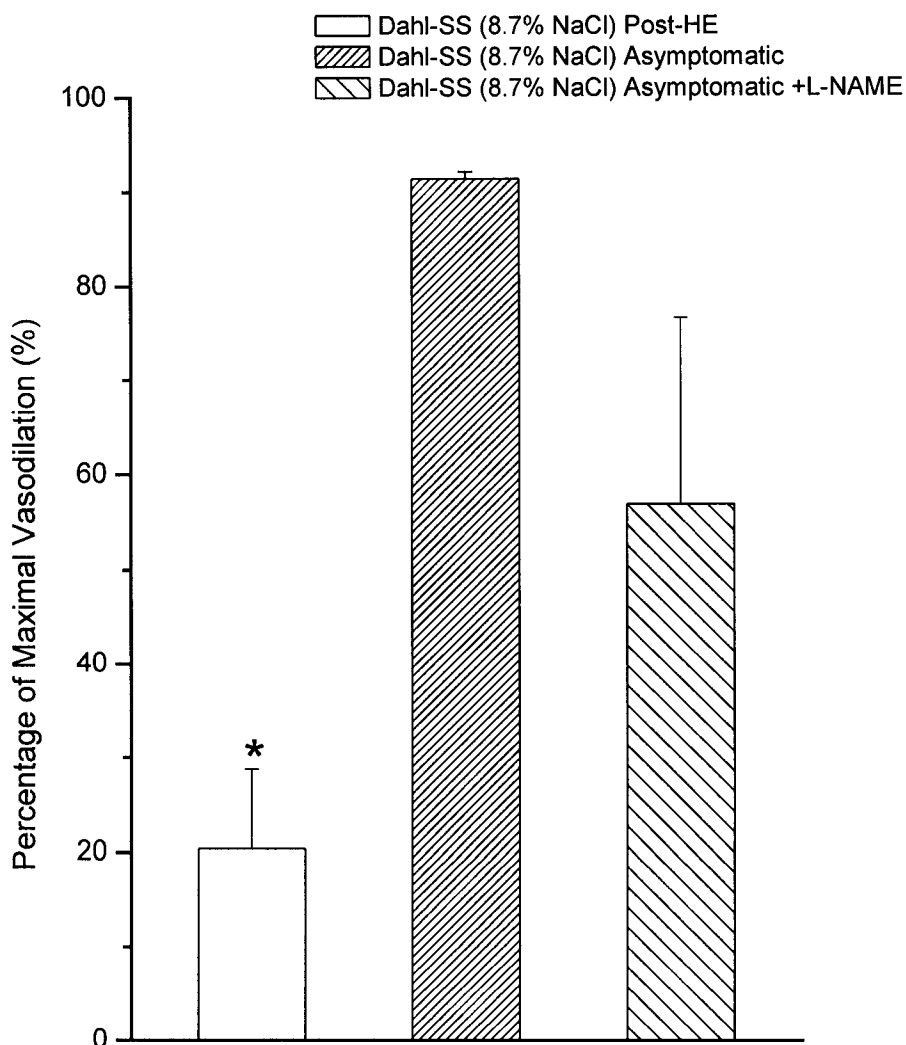
Figure 23: Endothelial mediated bradykinin vasodilation of MCAs sampled from Dahl-SS. The MCAs of asymptomatic Dahl-SS fed 8.7% NaCl for 1 week (n=4) and post-HE Dahl-SS fed 8.7% NaCl (n=9) were compared. The ability of NOS inhibition to inhibit bradykinin mediated vasodilation was assessed in the same MCAs that were sampled from Dahl-SS fed 8.7% NaCl for 1 week (n=4). Responses to 1.6 μM bradykinin are shown. Statistics: ANOVA with post hoc Fisher test. Significant *, post-HE group vs asymptomatic and asymptomatic + L-NAME, $p < 0.05$. Values represent the mean ± S.E.M.
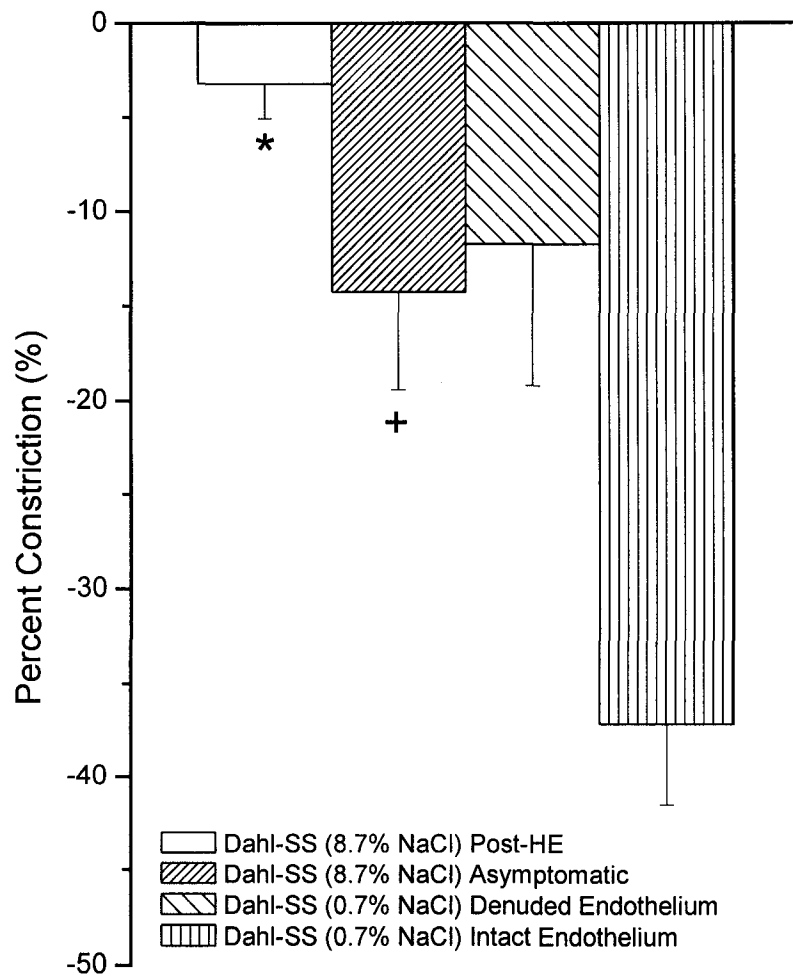
Figure 24: Effect of nitric oxide synthase (NOS) inhibition (100 µM L-NAME) on the MCA diameter in arteries sampled from asymptomatic (n=4), post-HE Dahl-SS (n=5) fed 8.7% NaCl and Dahl-SS fed 0.7% NaCl. The effect of endothelial removal on L-NAME induced vasoconstriction was assessed in MCAs sampled from Dahl-SS fed 0.7% NaCl (n=4). Statistics: ANOVA with fisher post-hoc test. Significant *, post-HE group vs asymptomatic Dahl-SS group vs Dahl-SS endothelial intact group, +, asymptomatic Dahl-SS group vs Dahl-SS endothelial intact group p<0.05. Values represent the mean ± S.E.M.

Table 4 outlines the effects of endothelium removal and NOS inhibition
with L-NAME on PDC. MCAs used in the experiments were sampled from Dahl-
SS fed 0.7% NaCl (n=4). Endothelial removal did not alter the amplitude of the
PDC response to 100 mmHg pressure step. However, the dimensional range
over which constriction to pressure occurred was shifted to more constricted
lumen diameters in the absence of endothelium (i.e. + endothelium from 195 ± 7
µm at the start of PDC to 154 ± 7 µm at the end of PDC versus − endothelium
140 ± 15 µm at the start of PDC to 108 ± 13 µm at the end of PDC). This
indicated that although the endothelium is not involved in promoting PDC, the
presence of an intact endothelium exerts a dilatory influence on the MCAs
shifting the operating range of PDC to larger lumen diameters. If the dilatory
influence of the endothelium was only mediated by the basal release of NO from
the endothelium then it would be expected that after endothelium removal the
subsequent addition of the NOS inhibitor, L-NAME would not alter arterial lumen
dimensions. As shown in Table 4, this proved not be the case. After endothelial
removal, L-NAME produced greater degrees of constriction. This indicated that a
non-endothelial source of NO must have also been influencing basal tone in the
MCAs. Finally, we assessed the possibility that the endothelium released a non
NO vasodilator that was capable of influencing PDC and basal tone. IF
endothelial and non-endothelial NO were the only source of vasodilatory
influence in the MCAs and the endothelium produced no other vasodilator
capable of influencing basal tone then under conditions where NOS was inhibited

TABLE 4: Effect of L-NAME (100µM) on PDC in Intact and Denuded segments of MCAs of Dahl-SS

Group	1st PDC Start (µm)	1st PDC Finish (µm)	1st PDC (%)	2nd PDC Start (µm) + L-NAME	2nd PDC Finish (µm) +L-NAME	2nd PDC (%) +L-NAME
Intact Endothelium (n=4)	195 ± 7^a	154 ± 7	-21.0^b	146 ± 30	130 ± 22	-10.9
Denuded Endothelium (n=4)	140 ± 15	108 ± 13	-22.8^b	106 ± 17	103 ± 16	-2.8

Values represent the mean $\pm$ S.E.M.

[a] $P<0.05$ Lumen size at start of 1st PDC between intact and denuded segments.

[b] $P<0.05$ Amplitude of 1st PDC versus 2nd PDC

, the subsequent removal of the endothelium would be inconsequential. As shown in Table 4 this was not the case. In the presence of L-NAME, endothelial removal further constricted the MCAs indicating that basal tone was influenced by a non-NO vasodilator that originated from the endothelium.

A shown in Table 4, the amplitude of the PDC in the MCAs was attenuated in the presence of L-NAME and further reduced when endothelium was removed during NOS inhibition. In out view, this observation could be misinterpreted to indicate that basal NO release (± endothelial non-NO vasodilatory influence) may be involved in promoting PDC. A more likely explanation of this phenomena is that the sequential abolishment of all basal NO influence (via L-NAME) plus endothelial dependent non-NO effects (via endothelial removal in the presence of L-NAME) increases basal tone in the MCAs to a point where further constriction by other influences is limited. Hence the ability of pressure to induce further constriction is attenuated and the amplitude of PDC to a 100 mmHg pressure step is reduced (Table 4).

Table 5 demonstrates that in MCAs sampled from Dahl-SS fed 0.7% NaCl (for 9 weeks), excess levels of L-arginine (8mM) or D-arginine (8mM) did not effect the ability of the MCAs to elicit PDC in response to a 100 mmHg pressure step. Pre-treatment with L-arginine but not D-arginine inhibited the ability of L-NAME to induce vasoconstriction. Since L-NAME is a competitive inhibitor of NOS, the ability of high levels of L-arginine (the proper substrate for NOS) to

TABLE 5: Role of Arginine Isomers (L/D) on PDC and L-NAME (100 µM)

Induced Constriction in MCAs of Dahl-SS

Group	PDC (%)	Lumen (µm) Pre-arginine Application	Lumen (µm) Post-arginine Application	Δ Lumen Diameter (µm)	% lumen constriction in response to L-NAME (100 µm) in the presence of L or D-arginine
L-Arginine (8µM) (n=4)	27.7±3.2	129.7±11.1	131.1 ±3[a]	1.4±10.5	0[b]
D-Arginine (8µM) (n=4)	33.1±4.5	127.7±10.4	113.7±5.4	-14.1±9.4	-22.6±3.8

Values represent the mean ± S.E.M

a P<0.05 vs Lumen Diameter after Arginine Incubation

b P<0.05 vs L-Name Response in D-Arginine Group

prevent L-NAME action would suggest that the vasoconstriction produced by L-NAME was in fact being mediated by the inhibition of NOS.

5.5 DISCUSSION

MCAs of post-HE Dahl-SS fed 8.7% NaCl displayed an inability to respond to a 100 mmHg pressure step whereas the MCAs from asymptomatic Dahl-SS fed 8.7% NaCl for 1 week exhibited robust PDC. The influence of NO and the endothelium on basal tone and PDC were assessed in MCAs sampled from healthy Dahl-SS fed a normal salt (0.7% NaCl). We observed that endothelial removal did not alter the amplitude of PDC in the MCAs but shifted the operating range of the lumen diameter changes in response to pressure to a more constricted state. This indicated that the endothelium exerted a basal vasodilatory influence that modulated PDC. The observation that endothelial removal under conditions of NOS inhibition further enhanced vasoconstriction suggested that a proportion of the vasodilation produced by the endothelium was mediated by a non-NO vasodilator. The ability of the endothelium to release a non-NO vasodilator was further supported by the finding that a substantial proportion of vasodilation produced by bradykinin in the MCAs was endothelial dependent but not NO mediated. In the absence of endothelium, NOS inhibition enhanced MCA constriction indicating that a non-endothelial source of NO influenced basal tone in the MCAs the sequential removal of the endothelium and the total inhibition of NOS and presumably all NO production progressively

increased basal MCA constriction. In doing so, it limited the further ability of the MCAs to constrict in response to pressure and thus reduced the amplitude of PDC observed in response to a 100 mmHg pressure step.

MCAs sampled from Dahl-SS with HE that were fed 8.7% NaCl exhibited an attenuated ability to constrict in response to NOS inhibition and to dilate in response to bradykinin when compared to arteries sampled from asymptomatic Dahl-SS fed 8.7% NaCl or Dahl-SS fed normal salt. This suggested that the basal release of NO (from endothelial and non-endothelial sources) is reduced and that endothelial dependent vasodilation initiated by bradykinin and mediated by NO and non-NO vasodilators are compromised in the MCAs of Dahl-SS with HE.

5.5.1 Nitric Oxide and Hypertension Development

The endothelium plays an important role in regulating cerebrovasculature tone through the release of constrictor and vasodilatory factors (Faraci, 1993; Faraci & Heistad, 1998) and the impairment of endothelial function plays role in hypertension development (Boulanger, 1999). In the present study, the cerebrovascular tone in MCAs from Dahl-SS is likely regulated by the release of vasodilatory factors from the endothelium. The observation that endothelial removal produced a significant decrease in lumen diameter in comparison to intact endothelial segments suggests that the endothelium decreases basal tone. Despite this increase in basal tone after endothelial removal, the dynamics of the

PDC response were not significantly different in endothelial intact segments versus denuded segments (see Table 4). This is consistent with previous studies in which the removal of the endothelium in a number of different vascular beds did not attenuate the PDC response (McCarron et al., 1989; Kuo et al., 1990; Falcone et al., 1991).

Prior to this study, altered NO activity has not been shown in the cerebrovascular of Dahl-SS. However, the kidney has shown impaired NO/NOS activity (Chen & Sanders, 1993; Chen et al., 1993). Oral administration of L-arginine (the biological substrate for NO) reverses salt induced hypertension in Dahl-SS rats and NO production is reduced when Dahl-SS (but not Dahl-SR) are fed high salt (8% NaCl) (Chen et al., 1993). Furthermore, Dahl-SS fed high salt (8% NaCl) also have lower levels of NOS activity (Hayakawa & Raij, 1998). Therefore, it is plausible to suggest that defects in NO generation may play a role in hypertension development in Dahl-SS and that such alterations extend to multiple vascular beds, which include the cerebrovasculature.

5.5.2 Endothelium Dependent Vasodilation in MCAs of Dahl-SS

Endothelium-dependent vasodilation is impaired in many models of both experimental and human hypertension (Luscher & Vanhoutte, 1986; Panza et al., 1990). This is consistent with the present study in which MCAs of post-HE Dahl-SS fed high salt exhibited a significant attenuation of vasodilation to bradykinin (endothelial-dependent vasodilator) in comparison to MCAs of asymptomatic

Dahl-SS fed a high salt diet. In the presence of L-NAME, vasodilation evoked by bradykinin was still substantial in MCAs from Dahl-SS fed a high salt diet for 1 week. Vasodilation to bradykinin observed in MCAs from asymptomatic Dahl-SS in the presence of NOS inhibition with L-NAME suggests that another factor other than NO is responsible for vasodilation.

Vasodilation in response to a number of vasodilators such as acetylcholine, have been suggested to be evoked by EDHF (Feletou & Vanhoutte, 1988; Dong et al., 2000; Golding et al., 2002). There has been much debate over the past decade as to the mechanisms underlying EDHF evoked vasodilation but a general consensus is that it is mediated by the opening of K^+ channels (Triggle et al., 1999; Dong et al., 2000; Busse et al., 2002). The mechanisms underlying EDHF mediated vasodilation is complex because it is dependent on the particular vascular bed of interest (Edwards et al., 1998; Dong et al., 2000). The additional non-NO vasodilatory factor observed in the present study may be EDHF however at the present time sufficient experimental evidence supporting or contradicting the above theories is not available.

5.6 CONCLUSIONS

As Dahl-SS develop hypertension in response to high salt feeding there is a significant increase in basal tone in the cerebrovasculature of these animals. The increased level of basal tone observed in the MCAs of Dahl-SS fed high salt could be precipitated by the decreased influence or release of endothelial

sources of NO and or non-endothelial vasodilators. The loss of PDC and CBF autoregulation observed in Dahl-SS with HE was not produced by an endothelial defect since PDC was robust in MCAs of Dahl-SS lacking a functional endothelium. The importance of the increased basal tone observed in Dahl-SS with HE may be that it acts as a protective mechanism to counteract increases in CBF, due to increased BP. This is consistent with the observation that hemorrhage formation is rare in these animals following development of HE and the characteristics of CBF autoregulation observed in Dahl-SS fed high salt ·(discussed in Figure 17).

5.7 OVERALL SUMMARY OF STUDY

The overall focus of the present study was to evaluate the Dahl-SS as a model for stroke development. Dahl-SS rapidly developed high blood pressure when fed a high salt diet (8.7% NaCl). This was subsequently followed by death 2.5 weeks after the initiation of the diet. Prior to death, Dahl-SS exhibited behavioural symptoms, which were thought to be consistent with the development of stroke.

We characterized the cerebrovascular pathology of rats exhibiting behavioural signs thought to be consistent with stroke. These rats displayed significant levels of edema and fluid extravasation indicative of the breakdown in the integrity of the blood brain barrier and fluid movement into the extravascular space. Brain ischemia was absent and intracerebral hemorrhage was rare. We

concluded that the behavioural abnormalities observed in Dahl-SS prior to death were indicative of hypertensive encephalopathy. This condition occurs during hypertension and is associated with the development of seizures, coma, stupor and can produce death in humans. It is differentiated from true stroke by the fact that it can occur in the absence of cerebral ischemia and hemorrhage.

Plasma aldosterone was elevated in Dahl-SS with stroke but the levels of this hormone were far less than those observed in a high renin-angiotensin model of stroke development such as SHRsp. The antihypertensive agent (captopril) was ineffective in lowering blood pressure or preventing death. Multiple end organ failure was evident prior to death as demonstrated by kidney dysfunction characterized by an increase in plasma creatinine, urinary protein excretion, and plasma urea nitrogen as well as decreased plasma albumin.

The ability of Dahl-SS to autoregulate CBF was altered in relation to the duration that the rats were fed 8.7% NaCl. After 3 weeks of high salt feeding, even asymptomatic Dahl-SS lost the ability to autoregulate CBF. This indicated that CBF autoregulation was lost prior to HE development. We subsequently assessed PDC in isolated MCAs. Pressure dependent constriction of the cerebrovasculature is thought to be an important mechanism involved in maintaining CBF autoregulation. Elevations in blood pressure promote cerebrovascular vasoconstriction. The latter response increases cerebrovascular resistance to flow and counteracts the potential increase in CBF that might be

produced by the elevated BP. The net result is that CBF remains constant despite the elevation in BP.

PDC in the MCAs was attenuated in asymptomatic Dahl-SS fed high salt for 3 weeks and was totally abolished in rats exhibiting HE. Chronologically, the defects in PDC coincided with the loss of CBF in the MCAs. The characteristics of CBF regulation with varying BP and the nature of the changes observed in isolated MCAs suggested that CBF autoregulation was lost under conditions consistent with the presence of cerebrovasculature vasoconstriction. This alteration could have blunted the potential overperfusion of the cerebrovasculature that might have been expected under conditions where CBF autoregulation was lost in the presence of hypertension. This may have exerted a protective effect, which prevented the progression of HE to the development of intracerebral hemorrhage thus accounting for the low incidence of cerebral hemorrhage observed in our model.

The loss of PDC appeared to be coupled with a dysfunctional PKC system. Dahl-SS with HE were unable to constrict to PKC activation via phorbol esters. Since PKC antagonist's inhibit PDC, it is possible that the absence of PDC in the MCAs of Dahl-SS with HE may have been produced by a dysfunctional PKC system. There was also an abnormal response to endothelial-derived vasodilators. MCAs from post-HE Dahl-SS exhibited significantly higher levels of basal tone and no response to the endothelial dependent vasodilator, bradykinin. The basal production of NO and non-NO endothelial dependent

vasodilators and or the actions of these dilators were diminished in Dahl-SS with HE. All the latter alterations could contribute to the production of increased basal tone in MCAs of Dahl-SS. This abnormal function may have been beneficial. The decreased vasodilatory capacity of the cerebral vasculature could have increased cerebrovasculature under *in vivo* conditions. In doing so, it might have reduced the degree of overperfusion that might have occurred under hypertensive conditions, thus retarding the progression of HE to cerebral hemorrhage in Dahl-SS.

A schematic diagram outlining the possible mechanisms promoting the development of hypertensive encephalopathy in Dahl-SS is outlined in Figure 25. Hypertension development in Dahl-SS is promoted by volume loading (i.e. Na^+ and water retention) in response to a high salt diet. As the kidney becomes damaged, proteinuria and uremia develop. The development of proteinuria decreases plasma protein and oncotic pressure. The latter alterations, in combination with high hydrostatic pressure, facilitate the movement of fluid into the extravascular space, causing HE development (i.e. edema formation). As BP increases, PDC (via alterations in PKC activity) and CBF autoregulation becomes dysfunctional. This ultimately leads to an overperfusion of the cerebrovasculature further enhancing edema formation causing hypertensive encephalopathy development.

The Mechanisms Promoting the Development of Hypertensive Encephalopathy in Dahl-SS

Increase Dietary Salt

Dahl-SS

Dahl-SR

No Effect
Normotensive

Kidney

Brain

▲ Na^+/H_2O retention

Normal **PDC**

Normal **CBF AUTOREGULATION**

↑ BP

↓ **PDC**

↑ Basal Tone (decrease NO activity)

Kidney Dysfunction
(proteinuria, ↓ plasma protein
↓ decrease oncotic pressure)
Uremia (high serum BUN)

Organ Failure and Development
of ascities due to movement
fluid in extravascular space via
decrease oncotic pressure

Loss of **PDC** (loss of PKC System)
Loss of **CBF AUTOREGULATION**

Overperfusion of cerebrovasculature
Extravasation of fluid

HYPERTENSIVE   ENCEPHALOPATHY

5.8 Future Experimental Directions

Future studies using the Dahl-SS model of HE should focus on a number of key areas that have immerged from the present study. These include further analyses of the PKC signal transduction pathway evaluating specific isoform(s) of PKC as well as its downstream targets involved in promoting PDC. In addition, addressing these questions in light of the development of HE to determine the specific nature of PKC dysfunction that is involved in HE development. Changes in NO activity, including the contribution of other forms of endothelium dependent vasodilators (i.e. EDHF) that may play a role in modulating activity prior to and following HE development also need investigation. Further, do alternative pools of NO (i.e. smooth muscle) exert any effects on modulating myogenic activity? Finally, with the present study being the first to develop a animal model for HE, a further investigation how the animal model correlates with the onset of HE in humans in terms of pathology and possible treatment of the symptoms observed in the animal model.

The present study clearly demonstrates that PKC activation is involved in the pressure dependent constriction response and further; PKC signaling is altered following the development of HE. These observations lead us to postulate how PKC activation is altered. Using specific antibodies against the various isoform(s) of PKC we will be able to determine what isoform is present prior to development of HE and further if there is a downregulation of a particular isoform following the development of HE. Secondly, with the recent advancement in

selective antagonists of the specific isoforms of PKC we can determine if inhibition of particular isoforms of PKC mimic the effects observed with global PKC inhibition effects outlined in chapter 4. Of the current 11 isoforms of PKC, two candidates have recently been suggested to be involved in promoting myogenic activity, PKC-α and PKC-ϵ. Both PKC-α and PKC-ϵ have been implicated in myogenic contractions of the coronary microcirculation (Dessy *et al.*, 1998; Dessy *et al.*, 2000).

In addition to the possible specific isoforms of PKC involved in promoting the PDC response in this model evaluation of downstream targets like the TRPc6 channel described in chapter 4 are worth further investigation. Using oligodeoxynucleotide antisense treatment we can determine if inhibition of this channel disrupts PDC in MCA's from Dahl-SS to mimic the loss of PDC observed in post-HE Dahl-SS. Also, if this is an important target of PKC in generating PDC, does the mRNA for the TRPc6 decrease as PDC becomes attenuated during the development of HE.

Although the mechanisms involved in generating PDC are mediated in the smooth muscle the endothelium also plays an important role in modulating myogenic activity. In the present study two interesting observations were noted. These were that there was an endothelium dependent vasodilation present in the presence of NOS inhibition and also that constriction to NOS was present in vessels in which the endothelium was removed.

The first observation suggests the presence of a non-NO dilatory substance. As outlined in Chapter 5 the likely candidate is EDHF. EDHF is interesting as the specific mechanism underlying its activation remains to be elucidated. According to a recent review article evidence exists for three possible mechanisms, (i) the activation of the cytochrome p450 pathway, (ii) endothelial cell hyperpolarization that is transmitted to the smooth muscle via gap junctions and (iii) endothelial released K^+ that acts on smooth muscle potassium channels or activates Na^+-K^+-ATPase inducing smooth muscle hyperpolarization (Busse *et al.*, 2002). The initiation of these mechanisms all requires endothelial K^+ channels activation prior to EDHF release. Located on endothelial cells are three main K^+ channels that are classified on their conductance states. These include small conductance channels (sK), intermediate conductance channels (iK) or large conductance channels (BK) Busse *et al.*, 2002).

To determine if the observed dilation to bradykinin in the presence of NOS inhibition was mediated via EDHF the classical experiment is the selective inhibition of both the iK (apamin) and sK (charybdotoxin) channels. If EDHF is the residual dilatory component released in the MCAs of Dahl-SS, the selective inhibition of these K^+ channels will inhibit the remainder of the dilatory component to bradykinin that is insensitive to NOS inhibition. If there is an EDHF component, the selective inhibition of cyotochrome p450 with 17-ODYA or gap junction inhibitors (GAP 27) will further our knowledge as to the specific mechanisms that

EDHF may be mediated through in promoting vasodilation in MCAs from Dahl-SS.

The observation that NOS inhibition with L-NAME still evoked a vasoconstriction in MCAs in which the endothelial had been removed suggests the involvement of a non-endothelial source of NO. In order to evaluate this in our model, cross sections of MCA's could be stained nitric oxide. This will determine where pools of NO are located within these vessels. Also, if this particular pool of NO is important, does this pool become reduced in response to HE development.

Finally, this is the first animal model of HE and further investigation into what precipitates its onset it needed. Based on the observations in present study Dahl-SS undergo a rapid elevation in BP following the feeding of a high salt diet whereas Dahl-SR does not. In humans, patients who exhibit signs of HE treated by reducing the hypertension which restores normal cerebral blood autoregulation and stops on the developing edema formation. In order to substantiate this model and a viable model for HE we have to demonstrate that a reduction in BP abrogates the onset of HE in these animals. However conventional antihypertensive treatments using ACEIs and AT_1 receptor antagonists (losartan) failed to reduce the BP or the onset of HE in this model. Therefore, in future experiments, new anthypertensive treatments are required to try and reduce the BP in Dahl-SS fed high salt and retard the onset of HE. One such treatment might be through the use of diuretics like hydralazine. Since, the

hypertension observed in this model is via volume overload maybe the use of diuretics could lower BP reverse the effects of HE development. A corollary experiment would be to induce high BP in Dahl-SR to determine if the raising of BP in these animals mimics the HE symptoms observed in Dahl-SS or if the raising of BP not the precipitating factor but is through a yet unknown mechanism that is unique to Dahl-SS that predisposes them for the development of HE.

Overall, by further investigating the underlying mechanisms at the level of the smooth muscle and endothelium that become dysfunctional in response to HE development along with the global precipitating mechanisms (i.e. increases in BP) our understanding of the first animal model of HE will be furthered. Untimely will help understand the onset of HE in humans leading to better prevention and treatment paradigms.

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