THE EFFECTS OF CAPTOPRIL TREATMENT ON HEMORRHAGIC STROKE DEVELOPMENT IN STROKE-PRONE SPONTANEOUSLY HYPERTENSIVE RATS

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THE EFFECTS OF CAPTOPRIL TREATMENT ON HEMORRHAGIC STROKE DEVELOPMENT IN STROKE-PRONE SPONTANEOUSLY HYPERTENSIVE RATS

by

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ABSTRACT

We tested the hypothesis that inhibiting stroke development in Kyoto Wistar stroke-prone rats (SHRsp) with captopril treatment (50 mg/kg) was, in part, mediated by reduced plasma aldosterone levels, independent of an antihypertensive effect.

Plasma aldosterone levels were measured before and after stroke development in untreated and captopril treated SHRsp. Plasma aldosterone levels suppressed by captopril were re-elevated by aldosterone infusion (16 µg/day) into captopril treated SHRsp. The resulting blood pressure (BP) and stroke development was evaluated. We also examined the BP and antistroke effects of a vasodilating agent, hydralazine (40-100 mg/L), and aldosterone antagonism by spironolactone (20 mg/kg).

Untreated SHRsp developed hypertension and 100% mortality associated with intracerebral hemorrhage by 14 weeks of age. Captopril treatment from 6 weeks of age did not lower BP but increased survival past 35 weeks of age. Hydralazine lowered BP but only mildly retarded stroke development compared with captopril treated rats. Plasma aldosterone levels increased with age in prestroke SHRsp (0.25 to 3.9 nmol/l) and rose further following stroke (11.4 nmol/l). Captopril treated SHRsp showed suppressed aldosterone values (0.5 nmol/l). Restoring hyperaldosteronemia in captopril treated SHRsp negated captopril's ability to retard stroke development. Spironolactone treatment reduced BP with little effect on

stroke development.

Additional studies assessed the hypothesis that captopril treatment helped preserve contractile function (related to blood flow autoregulation) of isolated middle cerebral arteries (MCA) from SHRsp. This was accomplished by examining lumen diameter changes in response to pressure, protein kinase C activation and potassium induced depolarization in the MCA from untreated and captopril treated, pre and post stroke SHRsp.

Stroke development was associated with a defect in the ability of the MCA to constrict to elevated transmural pressure (pressure dependent constriction) and phorbol dibutyrate induced protein kinase C activation. Captopril treatment preserved these functions.

We concluded that elevated plasma aldosterone levels promoted stroke development within captopril treated SHRsp through mechanisms not involving mineralocorticoid receptor stimulation or the exacerbation of hypertension. The antistroke effect of captopril may be partially mediated through plasma aldosterone suppression. We further suggest that the ability of captopril treatment to preserve MCA pressure dependant constriction may also contribute to the antistroke effects of captopril treatment in SHRsp

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TABLE OF CONTENTS

LIST OF FIGURES

ABBREVIATIONS

ACE	-	angiotensin converting enzyme
AI	-	angiotensin I
AII	-	angiotensin II
ANOVA	-	analysis of variance
11βHSDH	-	11β - hydroxysteroid dehydrogenase
BF	-	blood flow
BP	-	systolic blood pressure
$[Ca^{2+}]_i$	-	intracellular calcium concentration
CBF	-	cerebral blood flow
cGMP	-	cyclic guanosine monophosphate
CT	-	computerized tomography
DAG	-	diacylglycerol
DDMS	-	N-methylsolfonyl-12, 12-dibromododec-11-enamide
DMSO	-	dimethyl sulfoxide
DNA	-	deoxyribonucleic acid
EIAP	-	ethylisopropylamiloride
Em	-	resting membrane potential
GR	-	glucocorticoid receptor
20-HEDE	-	20-hydroxyeicosa-6(Z), 15(Z)-dienoic acid

VSMC	-	vascular smooth muscle cells
WKY	-	Wistar Kyoto normotensive rat

THE EFFECTS OF CAPTOPRIL TREATMENT ON HEMORRHAGIC STROKE DEVELOPMENT IN SHRsp

CHAPTER 1: THE INVOLVEMENT OF HYPERTENSION AND THE RENIN ANGIOTENSIN SYSTEM IN HEMORRHAGIC STROKE DEVELOPMENT

LITERATURE REVIEW

1.1 THE RELATIONSHIP OF HEMORRHAGIC STROKE-DEVELOPMENT TO HYPERTENSION IN HUMANS

Stroke is defined as an abrupt onset of focal or global neurological symptoms caused by ischemia or hemorrhage in the brain resulting from diseases of the cerebral blood vessels (Sacco and Mayer,1994). Consequently, stroke is generally described as being either ischemic or hemorrhagic in origin.

Ischemic stroke is produced by the regional blockage of blood flow to the brain resulting in tissue death. The latter occurrence of stroke typically involves the development of arteriosclerotic or atherosclerotic lesions in the cerebral arteries emanating from the circle of Willis, the vertebral-basilar arterial system and or within the carotid arteries. These lesions result in the production of arterial lumen occlusion. The areas containing these lesions are predisposed to the entrapment of circulating clots as well as the local development of clot formation. When the process evolves to the point where blood flow is interrupted focal or regional brain ischemia occur, subsequently resulting in neural death. The definition of ischemic

stroke has also encompassed the development of brain ischemia secondary to the entrapment of large clots into cerebral vascular areas without arterial disease. This occurs when large clots formed in the left ventricle (particularly in the valve region) dislodge, and make there way to the brain.

Hemorrhagic stroke results from bleeding directly into the brain (intracerebral hemorrhage) or along the surface of the brain within the subarachnoid space (subarachnoid hemorrhage). Cerebral bleeding can produce injury via an increase in intracranial pressure leading to a reduction in cerebral perfusion pressure with subsequent regional ischemia (Davis and Robertson, 1991) or it can cause damage by cutting off connecting neural pathways and compressing cranial nerves (Caplan, 1993). Biochemical substances released during and after hemorrhage may adversely affect nearby vascular or brain tissue (Caplan 1993). Fluid entering the parenchyma can include glutamate, serotonin, fatty acids, products of the kallikrein-kininogen system, free radicals and lysosomal enzymes (Davis and Robertson, 1991). The possible damage mediated by these agents includes changes in blood brain barrier permeability, cell swelling and damage to cell energy metabolism (Baethmann *et al.*, 1980).

Intracerebral hemorrhage generally occurs less frequently than ischemic stroke. Results from a multicentre American study of 1805 patients admitted for acute stroke revealed intracerebral hemorrhage accounted for 13% of all acute hospitalizations. The diagnosis was based on computerized tomography (CT) scan,

clinical symptoms, angiogram or surgical and/or autopsy results (Foulkes *et al.*, 1988). According to Sacco and Mayer (1994) this translates into approximately 65000 new cases of intracerebral hemorrhage per year in the U.S. This rate however can be considerably higher depending on the population investigated. CT scans of 2168 Japanese stroke patients revealed a 30% incidence of intracerebral hemorrhage (Suzuki *et al.*, 1987).

There is evidence that the prognosis for patients suffering from hemorrhagic stroke is worse than those suffering from ischemic stroke. The Framingham study followed 5184 subjects for 26 years with initial strokes occurring in 394 patients. The 30 day case fatality rate for intracerebral hemorrhage was 82% (14/17) compared to 15% (33/222) for ischemic stroke (Sacco *et al.*, 1982). Given the incidence of hemorrhagic stroke and the poor prognosis associated with this condition, scientific investigation into the mechanisms promoting this disorder is justified.

Hypertension has been reported to be the most common modifiable risk factor associated with intracerebral hemorrhage (Caplan, 1993; Wilterdink, 1994). Exposure to high pressure can result in vessel wall thickening and reduction in lumen diameter which may protect capillary beds from the elevated pressure. However, with continued exposure to hypertension, lipid and hyaline material can be deposited in the walls of arterioles compromising their structural integrity (lipohyalinosis). The resulting weakening of the vessel wall leads to the formation

of Chacot-Bouchard Aneurysms. These are small fusiform aneurysms typically located at the trunk of a vessel and are predisposed to rupture and hemorrhage (Rubin and Farber, 1995).

The importance of hypertension in the development of hemorrhagic stroke is supported by human epidemiological studies which suggest that a reduction in the incidence of intracerebral hemorrhage seen in the USA between 1970-1980 may be the result of improved antihypertensive treatment. A study conducted by Furlan *et al.* (1979) in Rochester, Minnesota, observed that a decline in the incidence of intracerebral hemorrhage was associated with increased use of antihypertensive agents and a decline in the incidence of hypertension from 15.7/100 000 between 1945 and 1952 to 7.3/100 000 between 1969 to 1976. This study found that between 1950 and 1954, hypertension was present in nearly all patients with intracerebral hemorrhage. More than half the patients had mean arterial blood pressure (BP) of greater than 150mmHg. Twenty-five years later, only 48% of patients with intracerebral hemorrhage were hypertensive and none had mean arterial blood pressure greater than 130mmHg (Drury *et al.*, 1984). This suggests that as hypertension became better managed, the non-hypertensive risk factors for hemorrhagic stroke became more prominent. In Finland, a decrease in the annual rate of intracerebral hemorrhage from 26.2/100 000 to 15.4/100 000 was reported between 1970 and 1979. This study concluded that although the improved care of hypertension produced a decline in the incidence of cerebral hemorrhage,

cerebral hemorrhage did occur in the absence of hypertension (Kotila, 1984). There are other reports of intracerebral hemorrhages occurring in the absence of chronic hypertension. Brott *et al.* (1986) found that 56% (87/154) of patients in Cincinnati hospitals who had sustained an intracerebral hemorrhage had no evidence of hypertension by history and an absence of electrocardiographic (ECG) changes (left ventricular hypertrophy) consistent with the past presence of hypertension. These authors proposed that hypertension be viewed as one of several important risk factors for the occurrence of spontaneous intracerebral hemorrhage. Consistent with this possibility, there are established non-hypertensive causes of intracerebral hemorrhage including hematologic abnormalities and vascular malformations. According to del Zopo and Mori (1992), hematologic causes of hemorrhagic stroke include primary causes such as deficiencies in coagulation factors and secondary causes such as thrombocytopenia (abnormally low platelet count, often resulting from spleen disorders). Various vascular malformations and congenital abnormalities are also associated with the development of cerebral hemorrhage. Arteriovenous malformations are characterized by many enlarged, engorged vessels of varied diameter. The thickness of the wall is irregular, composed mostly of fibrous tissue with occasional smooth muscle fibers and can be associated with saccular aneurysms. Cavernous hemangiomas are another type of vascular malformation which is associated with hemorrhagic stroke which appear as closely clustered, thick fibrous walled vessels.

Based on the above evidence one can conclude that although there is an association between intracerebral hemorrhagic stoke development and the occurrence of hypertension other as yet undiscovered factors independent of the presence of hypertension likely also contribute to the initiation of hemorrhagic stroke.

1.2 ANIMAL MODELS OF HEMORRHAGIC STROKE

1.2.1 Development of Kyoto Wistar Stroke Prone Hypertensive Rats

In 1963 Okamoto and Aoki developed the Kyoto Wistar spontaneously hypertensive rat (SHR) by the selective inbreeding of Wistar rats with above average blood pressures. These animals had a genetic form of hypertension that was transmitted to their offspring and was considered to be analogous to essential hypertension in humans. By 13 weeks of age, the male SHR developed systolic blood pressures (BP) of 180mmHg. In comparison, the normotensive Wistar rats used to develop the SHR strain (subsequently named Kyoto Wistar rats (WKY)) had BP's of approximately 135mmHg. Despite the presence of hypertension, SHR only rarely developed stroke. In 1974, Okamoto *et al.* developed a substrain of the SHR by selectively breeding SHR progeny from the few parents who subsequently developed stroke. These animals were termed Kyoto Wistar spontaneously hypertensive stroke-prone rats (SHRsp) to differentiate them from their stroke resistant parent breed. SHRsp attained BP's greater than 240mmHg during established hypertension. Stroke development in SHRsp was strongly affected by

diet. While maintained on regular rat chow (Nihon Clea), the lifespan of SHRsp was between 33 and 41 weeks of age (Okamoto *et al.*, 1974). Yamori *et al.* (1984) found that by 36 weeks of age, 88% of SHRsp fed a Japanese made Funahashi-SP diet developed cerebrovascular lesions compared to less than 30% of SHRsp fed a Ralston Purina National Institute of Health open formula diet. The authors reported that the only remarkable difference between the 2 diets was that the American diet had a 22% protein content compared with 15% present in the Japanese diet. The development of stroke in SHRsp increased to 83% at 24 weeks of age by adding a 4% NaCl supplement to a North American Japanese-style diet produced by Zeigler Bros in the USA (Tobian, 1986). More recently, selective colonies of SHRsp were developed where the incidence of stroke associated with intracerebral hemorrhage was 100% by 16 weeks of age when the animals were fed the same version of the 4% NaCl Japanese style diet (Smeda, 1989). This resulted in a rapid and well defined period of stroke onset beginning at 12 weeks of age. BP values in these animals increase with age with typical values ranging from 141 to 233 mmHg between 6 and 16 weeks of age. A predictable time course of stroke development in SHRsp was a prerequisite necessary for studying events which preceded the development of stroke in this animal model.

1.2.2 SHRsp as a Model of Human Hemorrhagic Stroke

The extent to which hemorrhagic stroke in SHRsp is an accurate model of human hemorrhagic stroke was addressed by Yamori *et al.* (1976). These

investigators reported that the most common site for hemorrhagic stroke in SHRsp was in the cortical region (69.8%). In contrast, human cortical hemorrhages represent just 13.1% of intracerebral hemorrhages. However, a common site for stroke in SHRsp and in humans is the basal ganglia which represents 24.5% of hemorrhagic strokes in rats and 66.5 % in humans. The anterior and posterior cerebral arteries which seemed to be responsible for the majority of cortical lesions in the rats possessed patterns of recurrent branching. The lenticulostriate arteries from the middle cerebral artery which are responsible for stroke in the basal ganglia in both SHRsp and in humans also branch recurrently. Recurrent arterial branching occurs when the secondary branches leaving a parent arterial branch are angled in a manner where the direction of flow in the secondary branch is angled greater than 90° to the parent branch. Arteries not responsible for cortical lesions were found to have few recurrent branchings.

1.2.3 Cerebral Pathological Alterations Associated with Hemorrhagic Stroke in SHRsp

The pathological changes in various organs of SHRsp were described by Ogata *et al.* (1982). Using microscopic examination of the brains of 38 SHRsp showing signs of stroke such as roughened fur and emaciation (of which 31 were subsequently observed to have cerebral hemorrhage) arteriolar fibrinoid necrosis was identified in the neocortex of 29/38 animals. Arterial fibrinoid necrosis in the brain and kidneys is associated with vascular injury secondary to malignant

hypertension (Benditt and Schwartz, 1994). The disorder is characterized by smooth muscle cell necrosis, loss of endothelial cell integrity and increased vascular permeability. This leads to the deposition of fibrin and the entry of other plasma proteins into the vessel wall. There is subsequent smooth muscle cell proliferation and an increase in the number of concentric layers of smooth muscle within the arterial media resulting in an onion skin appearance. Using serial sections of brains from five SHRsp with neurological deficits, Ogata *et al.* (1981) reported that arterioles with insudation of fibrinoid material showed stenosis and thrombotic occlusion. The occurrence of microaneurysms was rare. Massive intracerebral hemorrhages were identified in 3 animals whereas multiple petechial hemorrhages were seen in 22/38 brains. Multiple old hemorrhages, consisting of small collections of hemosiderin laden macrophages were seen in 13/38 animals. Micro infarcts were seen in 3 animals while large infarcts were not encountered. Regions of rarefaction of the neuropil were also identified in 31/38 animals. Wakita *et al.* (1995), explained that chronic hypoperfusion (possibly due to the disruption of downstream blood flow following cerebral hemorrhage) activated phagocytic microglia in the white matter resulting in rarefaction in the region.

1.2.4 Behavioral Symptoms of Hemorrhagic Stroke in SHRsp

Smeda (1989) described the behavioral signs of stroke in SHRsp. An initial common sign was convulsive rhythmic movements of the head and one forelimb which persisted no longer than 2-3 days and were periodically sufficiently violent

as to knock the rat over on its side. Next, mild lethargy occurred and the rats began to appear poorly groomed. The lethargy worsened within a few days and the ability of the animals to right itself from a side lying position became compromised. The rats also adopted an unusual posture characterized by having the rear legs extended and maintained underneath the body such that the toes extended past the animals head. Subsequently, the animals became quite immobile and 50% died within 1.5 weeks of exhibiting the first behavioral signs of stroke.

1.3 THE RENIN-ANGIOTENSIN SYSTEM

1.3.1 General Overview of the Renin Angiotensin System

The renin angiotensin system (RAS) is a mixed enzymatic-hormonal system controlling Na+ and water balance, blood volume and arterial blood pressure (Guyton, 1991). The biochemical pathway which comprises the RAS is characterized by the secretion of the enzyme renin from the preglomerular arterioles of the kidney. Renin then catalyzes the rate limiting conversion of the plasma protein angiotensinogen to angiotensin I (AI). AI is then converted to angiotensin II (AII) via angiotensin converting enzyme (ACE). ACE is located primarily in the endothelial lining of capillaries in many vascular beds. In addition to catalyzing the formation of AII, ACE is also responsible for the degradation of the vasodilator bradykinin (Vollaten, 1987).

The release of renin is modulated primarily by the juxtaglomerular apparatus

(JGA) whose function it is to translate changes in tubular salt loads (which result from changes in salt intake) into changes in glomerular filtration rate and renin secretion. The JGA is the structure formed by the distal tubule passing between the afferent and the efferent arterioles of the nephron. The epithelial cells of the tubule which contact the arterioles are known as the macula densa while the renin containing juxtaglomerular or granular cells are modified smooth muscle cells of the afferent arteriole (Hackenthal and Nobiling, 1994). Low Na^+ and Cl load (concentration times flow within the tubule) past the macula densa mediates the release of renin from the afferent preglomerular arterioles.

Renin release from the afferent arterioles is regulated by several other factors including beta receptor adrenergic stimulation via sympathetic innervation of the arterioles (Reid *et al.*, 1978; Vallotten 1987). There is evidence suggesting that dopaminergic innervation may also stimulate renin secretion (Sowers, 1984) and that non-JGA mechanisms may play a role in controlling renin release in response to salt loads. This latter hypothesis is based on the observation that animals with hydronephrotic kidneys (no functional tubular structure and therefore no macula densa) are able to adapt to chronic changes in salt ingestion. For example, renal renin release and renin mRNA transcription are stimulated to a similar extent in the hydronephrotic and contralateral normal kidney of Na^+ depleted mice (Munter and Hackenthal, 1989). There is also evidence for an intrarenal baroreceptor as a regulator of renin release and an inverse relationship exists

between renal perfusion pressure and renin release (Skinner *et al.*, 1964; Eide *et al.*, 1973). Negative feedback inhibition from AII is also thought to influence the release of renin (Griendling and Alexander, 1994).

1.3.2 The Systemic Actions of Angiotensin II

The classic AII effects described above are mediated via the AT_1 receptor which is blocked by the AII receptor antagonist losartan (Timmermans, 1999). This receptor has been further subdivided into the AT_{1A} and AT_{1B} subtypes. However, the functional significance of each is not understood (Gunning et al., 1996). An AT_4 receptor has also been reported (Zhuo et al., 1998). It has shown high specificity for the AII degredation fragment AIV (i.e., AII - [3-8]) and low specificity for AII and losartan. Radiolabelled AIV binding studies in the brain revealed an association with cholinergic neurons, as well as motor and sensory nuclei. The authors suggested a possible role in central motor and sensory activities and memory (Zhuo et al., 1998). An AT_3 in mouse neuroblastoma cells with high affinity for AII, low affinity for AIII and no affinity for losartan has also been reported (Dinh et al., 2001).

Stimulation of the AT_1 receptor in the adrenal cortex stimulates the release of aldosterone into the blood (Reid, 1985). Aldosterone is a steroid hormone that facilitates the uptake of Na^+ and water from the urine in the distal tubules of the kidney. The physiology, pharmacology and mechanisms of aldosterone action will be discussed in detail in the literature review section of Chapter 2 in this thesis. At this point in the discussion it is sufficient to say that aldosterone promotes fluid and

Na^+ retention which has a tendency to expand blood and extravascular fluid volumes, an alteration that can potentially elevate BP (Reid 1985). The AT_2 receptor has been noted to activate cellular protein phosphatases, nitric oxide-cGMP and phospholipase A2 signaling pathways in cell systems (Nouet and Nahmias, 2000), however its overall physiological effects are uncertain. The abundance of the AT_2 in fetal and neonatal rat tissue suggests a possible developmental role for the receptor (Griendling and Alexander, 1994).

Circulating plasma levels of AII are typically in the pico to nanomolar range (Morishita *et al.,* 1995). At these levels of AII, the stimulation of AT_1 receptors on vascular smooth muscle cells can facilitate constriction mediated (via stimulation of alpha 1 and 2 receptors) by norepinephrine (the predominant neurotransmitter present in the sympathetic nervous system) (Kawasaki *et al.*, 1988). Stimulation of AT_1 receptors on the presynaptic nerve terminals of the sympathetic nervous system further enhances the release of the vasoconstrictor norepinephrine from the nerves (Kawasaki *et al.*, 1982). The central administration of AII into the cerebral spinal fluid results in a massive activation of the sympathetic nervous system, an effect that is also mediated by AT_1 receptors in the central nervous system (Ma *et al.* 1999). It has been suggested that blood circulating AII may gain access to the central nervous system through normal or pathologically produced discontinuities in the blood brain barrier and thus potentially promote sympathetic nerve activation. At high levels greater than 10 nM, AII stimulation of vascular smooth muscle AT_1

receptors promotes vascular constriction (Smeda *et al.*, 1988a). Although such high levels of AII are not typically seen in the circulation, arguments have been presented that the local tissue production of AII via nonrenal sites of renin production (discussed later) may lead to the accumulation of AII higher than that seen in the circulation and possibly high enough to promote direct vasoconstriction.

An important vascular effect of AII is its ability to markedly constrict the efferent arteriole (Mene and Munn, 1992). This has the effect of decreasing urine output due to a reduction of blood flow through the glomerulous. The reduced blood flow in the paritubular capillaries augment the tubular reabsorption of fluid. AII can also act directly on the proximal tubule by decreasing Na^+ and water reabsorption via an enhanced Na^+, H^+ exchanger (Guyton 2000; Mene and Dunn, 1992).

In summary, elevations in circulating AII via the activation of the sympathetic nervous system, the presynaptic enhancement of norepinephrine release from the sympathetic nerves and the facilitation of norepinephrine constriction would enhance vascular constriction and raise vascular resistance to blood flow while the AII mediated release of aldosterone would facilitate blood volume expansion, an alteration that can increase cardiac blood flow output. Since BP is a product of cardiac output times vascular resistance to blood flow (discussed in detail in Chapter 3), elevations in AII production can produce hypertension. Hence, many antihypertensive agents (ACE inhibitors or AT_1 receptor antagonists) exert there

action by either preventing the formation of AII or inhibiting its action on tissues.

Although AII has a profound effect on blood pressure, the primary role of AII in the body is to control salt and water balance (Mulrow, 1999). This goal is often achieved by modifying the systemic blood pressure. For example, severe blood loss can produce a condition termed shock in which blood pressure can decrease to a point, below which normal glomerular filtration can occur. This promotes a decrease in primary urine production and a decrease in salt load being presented at the macula densa of the kidney. Renin secretion will increase, AII formation will be enhanced which in turn will facilitate the elevation in blood pressure which will attempt to restore normal glomerular filtration and hence salt and water balance (Reid, 1985). In this instance AII induced aldosterone secretion will occur. However, if very little primary urine is being produced the ability of aldosterone to extract Na^+ or water at the distal tubules will be limited. Hence, in this instance, its contribution to the maintenance of homeostasis would also be limited. In other situations, pathological vascular alterations in the kidney can activate RAS. In SHRsp an important pathological feature observed in the kidney is the occurrence of fibronecrosis in the afferent preglomerular arterioles (Ogato *et al.*, 1982). This would produce a decrease in glomerular filtration under normal BP conditions and by the mechanisms described above promote RAS activation. The effect of AII formed under the latter conditions would be to raise the BP above normal levels to promote near normal filtration and the aldosterone formed would facilitate Na^+ and

fluid retention further contributing to the elevation in BP. The net effect would be that the elevated BP would be essential to the maintenance of normal glomerular filtration and salt and water balance.

A second potentially important effect of AII in the maintenance of hypertension is in the ability of AII to remodel the structure of arteries (Hajdu *et al.*, 1991; Chillon and Baumbach, 1999). SHR typically have normal plasma AII levels (Morishita *et al.*, 1995) but do exhibit a hypercontractile reactivity to AII (Smeda *et al.*, 1988a). The treatment of these animals with ACE inhibitor or AT_1 receptor antagonist can normalize BP. Long term treatment of SHR produces a normalization of BP even when treatment is withdrawn (Gillies and Lee, 1996; Gillies *et al.*, 1997). Structural analysis of the vasculature (such as the cerebral and mesenteric vasculature) of these animals indicates that the inhibition of AII action causes the vascular walls to thin and the vascular lumen to enlarge when the arteries are studied under dilated conditions (Hajdu *et al.*, 1991; Gillies and Lee, 1996; Gillies *et al.*, 1997; Chillon and Baumbach, 1999). All these alterations would contribute to promoting a decrease in vascular resistance to blood flow and are thought to contribute to the maintenance of near normal blood pressure even after treatment is withdrawn. These effects are likely not due to the antihypertensive effects of ACE inhibitors or AII antagonists since other equally effective antihypertensive agents that promote their effects through vasodilation can not alter vascular structure in SHR (Smeda, 1988b; Smeda and Lee, 1991). In view of this it has been suggested

that AII can exert a trophic influence on vascular growth which through the promotion of increased vascular smooth muscle cell multiplication of individual cell growth may contribute to the production of vascular smooth muscle cell hyperplasia and hypertrophy commonly seen in the arteries of SHR and other hypertensive models. Wall thickening produced by cellular hyperplasia is thought to facilitate the maintenance of hypertension. This hypothesis is consistent with observation made in cultured vascular smooth muscle cells that indicate that AII can accelerate growth in culture (Stouffer and Owens, 1992; Loukotova *et al.*, 1998).

1.3.3 Nonrenal Pathways of Angiotensin II Generation

AII can be generated via a non-renal pathways. Human heart chymase which has high substrate specificity for AI and cannot degrade bradykinin is the major AII forming enzyme in the human heart (Urata *et al.*, 1990). Components of the above system have been localized in extrarenal tissues including the brain, adrenal gland and the vascular wall (Samani, 1994). These extrarenal or tissue RAS have been implicated in hypertension. For example, Schelling *et al.* (1982) found that during the developing phase of hypertension, renin activity of brain homogenates from bilaterally nephrectomized SHRsp was elevated relative to age matched WKY. Renin activity and AII was also found to be elevated in the adrenal gland of nephrectomized SHRsp relative to WKY at 18 weeks of age (Kim *et al.*, 1992). Further, ACE activity in the aorta of SHR was found to be elevated compared to WKY and this elevation correlated well with the developing phase of hypertension.

Although the significance of tissue RAS's and the non-ACE AII pathway remains unclear, there exists a possibility that this system could contribute to hypertension.

1.3.4 The Renin Angiotensin System In SHRsp

Studies involving SHRsp have shown that both plasma renin levels and activity increase with age (Volpe *et al.*, 1990; Camargo *et al.*, 1991; Gahnem *et al.*, 1994; Glubner *et al.*, 1995) and that plasma renin activity and AII levels are elevated in SHRsp with established hypertension when compared to normotensive Kyoto Wistar control rats. (Stier *et al.*, 1991; Kim *et al.*, 1992; Hubner *et al.*, 1995). The renin angiotensin system responds to dietary NaCl in a unique manner in SHRsp. Whereas elevations in dietary sodium suppress renin activity in normal rats (Stier *et al.*, 1991), renin activity (Stier *et al.*, 1991; Shibota *et al.*, 1979), as well as stroke development, (Nagaoka *et al.*, 1976) is enhanced in SHRsp. The mechanism underlying the inability of a high salt to suppress renin secretion in SHRsp has not been adequately studied, however high dietary salt has been shown to accelerate renal damage in SHRsp and specifically enhance the formation of preglomerular arteriolar fibriniod necrosis, a change that would limit glomerular filtration (Ogata et al., 1982). Hence, it is possible that the effect of this vascular occlusive disease in potentiating renin release by restricting glomerular filtration may outweigh the suppressive effects of a high salt diet on renin release, resulting in a net elevation in plasma renin and AII levels.

These findings are consistent with the results of Enea et al., (2000) using a

Japanese style diet plus 1% NaCl drinking solution in SHRsp. The investigators found that a high salt diet induced a rise in plasma aldosterone in SHRsp whereas SHRsr plasma aldosterone was suppressed. This finding was paralleled by an increase in adrenal aldosterone synthase mRNA is SHRsp whereas the same enzyme mRNA levels were reduced in SHRsr. In contrast however, plasma and adrenal renin activity as well as AII receptor mRNA were comparably reduced in response to the high salt intake in both strains. The authors proposed an early impairment in high salt intake induced aldosterone synthesis as a possible mediator of pathogenesis in SHRsp.

1.4 ANGIOTENSIN CONVERTING ENZYME INHIBITORS

1.4.1 General Overview

ACE inhibitor history begins with the discovery of a bradykinin potentiating factor from the venom of the South American pit viper. This substance was a mixture of peptides which inhibited ACE (Ferreira *et al.*, 1965). Captopril, the first orally active, non-peptide ACE inhibitor was synthesized based on research involving the most potent of these peptides (Ondetti *et al.*, 1977). There are 3 chemical classes of ACE inhibitors based on differences in binding to a zinc ligand on the converting enzyme. These chemical classes differ in several pharmacokinetic variables including elimination half life and time to onset of action. Apart from implications on dosing frequency however, differences in the therapeutic effectiveness of the different classes of ACE inhibitors is likely small (Gums, 1992).

All ACE inhibitors exert their hemodynamic effects primarily via the inhibition of AII formation (Gums, 1992). This results in a reduction in peripheral resistance. Plasma AI and plasma renin activity may increase while plasma aldosterone will decreases. The decrease in aldosterone will promote Na^+ and water loss in the urine (Gums, 1992). There is however evidence that plasma AII and aldosterone levels can, return to or, exceed pre-treatment levels with chronic ACE inhibitor exposure. For example, Mento and Wilkes (1987) treated adults Sprague-Dawley rats with enalapril (30 mg/kg, d.w.) for 1 week and for 2 months. The animals were fed standard rat chow and given tap water. Investigators reported a similar and significant decrease in mean arterial pressure (relative to controls) at 1 week and 2 months. PRA did not change significantly during either time period whereas plasma AII was elevated significantly at 1 week and 2 months relative to controls. Similar investigations were conducted by Lijnen et al., (1982) in hypertensive patients. The onset of captopril treatment was followed by a gradual rise in plasma aldosterone levels. Pre-treatment control values were significantly exceeded at 12 months. A hypotensive effect was reportedly maintained during the course of the study with plasma AII levels remaining suppressed.

ACE inhibitors can also decrease the breakdown of the vasodilator bradykinin. This influence on bradykinin has been implicated in the dry cough sometimes associated with ACE inhibitor therapy (Karlberg, 1993). There is evidence that bradykinin plays a role in the antihypertensive effect of ACE inhibitors

in some forms of hypertension (Bae *et al.*, 1992). ACE inhibitors have also been reported to ameliorate cardiovascular damage via the accumulation of bradykinin and nitric oxide (Kitikaze et al., 2000). In patients with a highly active RAS caused by Na^+ depletion or other factors, ACE inhibition produces an especially marked reduction in BP.

ACE inhibitors have also been demonstrated to interfere with sympathetic transmission, most probably by antagonizing the facilitating action of AII on presynaptic transmitter release (Zimmermann, 1981). A more long term antihypertensive mechanism may reside in the inhibition of the trophic actions of AII (Gohlke and Unger, 1994).

The clinical use of ACE inhibitors includes first line therapy in hypertension and congestive heart failure (The Joint National Committee on Detection, Evaluation and Treatment of High Blood Pressure, 1988). ACE inhibitors have also been suggested as a possible treatment for ischemic heart disease (Gohlke and Unger, 1994), kidney disease such as diabetic nephropathy (Marre *et al.*, 1988) as well as an agent to enhance cognitive function (Costall *et al.*, 1989). Recent studies have indicated that ACE inhibitors such as captopril exhibit an antioxidant effect and can scavenge superoxide radicals (Chopra *et al.*, 1989). It is possible that some of the therapeutic effects of captopril treatment in heart or renal disease could be mediated via this mechanism.

1.4.2 The Effect of Angiotensin Converting Enzyme Inhibitors on SHRsp

There is a very common view that the differences in the incidence of stroke that are observed in SHR verses SHRsp are simply due to the fact that SHRsp have higher blood pressures than SHR and that the development of stroke in SHRsp is simply a consequence of the presence of extreme hypertension. A growing body of evidence suggests that ACE inhibitors retard or prevent mortality associated with hemorrhagic stroke in SHRsp with minimal or no significant reduction in BPs. Stier *et al.* (1989) fed SHRsp a Japanese style stroke-prone rodent diet supplemented with a 1% NaCl drinking solution from 7 to 8 weeks of age. Treatment with the ACE inhibitor enalapril (15mg/kg/day, mixed in drinking water) began at 8-9 weeks of age. Using the tail cuff compression method, weekly BPs measurements revealed a significant decrease in BPs at 10 and 12 weeks of age. Although the hypotensive effect was mild, the mortality rate was dramatically altered with no stroke related deaths in the enalapril treated group at 36 weeks of age when the study was discontinued. In contrast, rapid mortality occurred in the untreated control animals between 12 and 14 weeks of age. The authors did not directly comment on a possible mechanism for this protective effect. However, the results did suggest the possibility of a weak association between BPs reduction and mortality associated with stroke in SHRsp.

Under virtually identical conditions as above, Stier *et al.* (1991), found that using the ACE inhibitor captopril (50mg/kg/day) yielded very similar results. Captopril treatment produced a small but significant reduction in BP between 9 and

11 weeks of age relative to untreated control animals. There were no stroke related deaths in the captopril treated group noted through 26 weeks of age at which time the study was terminated. Untreated control animals died between 12 and 16 weeks of age. In spite of these observations, the paper suggested that the therapeutic benefit of captopril treatment was independent of a hypotensive effect and inferred that RAS inhibition could be an important factor in the protective effects of ACE inhibitor treatment.

Stier *et al.* (1993) treated SHRsp with the AII receptor antagonist losartan (10mg/kg/day, by gavage). Losartan treatment produced similar results to those reported with ACE inhibitors in that the onset of death from stroke was retarded. However, no significant differences in BPs were detected in the losartan treated group compared to untreated SHRsp. In spite of the lack of a significant hypotensive effect, the losartan treated animals were able to survive until the study was discontinued at 28 weeks. Untreated control SHRsp achieved 100% mortality by 14 weeks of age. The authors concluded that AII may play a significant role in the development of stoke in SHRsp. This study indicates that virtually identical results were obtained from either ACE inhibition or AII receptor blockade. This suggests that ACE inhibitor induced bradykinin potentiation may not be an important mediator of the reduced mortality rate in these animals.

Further investigations into ACE inhibitors in SHRsp were conducted by Ogiku *et al.* (1993). SHRsp were fed a Na^+ loaded, low protein, Japanese style diet

and were administered the ACE inhibitor imidapril (50mg/kg/day by gavage) beginning at 11 weeks of age. The imidapril treated SHRsp were not found to have significantly different BP values relative to untreated controls. The control animals died between 13 and 23 weeks of age whereas the first death in the imidapril treated group did not occur until 31 weeks of age (2 weeks after treatment was withdrawn).

Investigations by Wang *et al.* (1995) found that perindopril treatment in SHRsp resulted in significantly lower BPs during 9 and 10 weeks of age. The ACE inhibited animals also lived much longer than controls. Although these results were similar to those of Stier *et al.* (1991), the conclusion differed. The onset of stroke in SHRsp was significantly correlated to the age when BPs exceeded a 240 mmHg. It was suggested that a modest hypotensive effects capable of maintaining an animal's BPs below 240 mmHg could exert a profound antistroke effect. However, close scrutiny of the data indicates that there are inconsistencies with the above hypothesis. Within the various experiments performed by Wang *et al.* (1995) some groups of control SHRsp developed stroke before reaching a systolic BP of 240 mmHg and low doses of perindopril treatment failed to produce any antihypertensive effect but still protected the animals from stroke. Finally even in cases where perindopril suppressed BPs during the developing phase of hypertension and prevented stroke, some SHRsp treated with perindopril developed BPs values greater than 240 mmHg and yet did not develop stroke. These studies

suggest at best a weak association between BPs and stroke related mortality in ACE inhibited SHRsp.

Stier *et al.* (1988) studied the effects of inhibition of thromboxane A_2 synthesis by dazmegrel in salt loaded SHRsp. They found that such treatment resulted in a significant reduction in BPs in SHRsp at 10 and 11 weeks of age. This hypotensive effect was virtually identical to that reported with enalapril treatment (Stier *et al.*, 1989), captopril treatment (Stier *et al.*, 1991) and perindopril treatment (Wang *et al.*, 1995) yet no reduction in mortality rate was achieved. An additional problem with many of the above studies was in the statistical tests chosen for data analysis. In several instances, a series of t-tests were used to analyze BPs values of treated and control animals over several weeks. Given the repeated measures nature of the BPs measurements, an analysis of variance of the effects of treatment with age would have been a more appropriate test to use. This would eliminate the potential source of error introduced by repeated t-tests.

In view of the above controversies we decided to undertake experiments to more adequately assess the possibility that ACE inhibitors exert an antistroke effect independent of their ability to lower blood pressure. If this proved to be the case, our intentions were to attempt to study the mechanisms by which the ACE inhibitor captopril exerted it antistroke effects.

EXPERIMENTAL RESEARCH

1.5 THE EFFECT OF BLOOD PRESSURE SUPPRESSION ON STROKE DEVELOPMENT IN SHRsp DURING TREATMENT WITH THE ANGIOTENSIN CONVERTING ENZYME INHIBITOR CAPTOPRIL

1.5.1 THE OBJECTIVES OF THE STUDY

We attempted to determine if treatment with the ACE inhibitor captopril was capable of preventing hemorrhagic stroke development within our colony of SHRsp with the intention of carrying out further studies to determine whether or not captopril treatment requires a significant reduction in BPs in order to significantly retard mortality in SHRsp. To answer the above question, an attempt was made to elevate BPs during captopril treatment by treating the animals concurrently with the glucocorticoid dexamethasone (an agent known to produce hypertension in normal animals) or the mineralocorticoid deoxycorticosterone. The results of these experiments would then be used to determine if an elevation in BPs or a suppression of any potential antihypertensive effects of captopril treatment affected the ability of captopril to retard stroke development. In other experiments, SHRsp were treated with the antihypertensive vasodilator hydralazine . The objective of these experiments was to determine if antihypertensive treatment of SHRsp via a non ACE inhibiting drug was equally effective in altering stroke development in SHRsp.

1.5.2 HYPOTHESES

We tested the hypothesis that the elevations in plasma AII either directly or via the increase in plasma aldosterone contribute to the initiation of hemorrhagic stroke in SHRsp in a manner that is not totally dependent on the hypertensive effects of AII. Furthermore, although captopril treatment of SHRsp may suppress blood pressure in these animals, the potential antistroke effects of such treatment will not be affected by cotreating the animals with drugs that will prevent the suppression of blood pressure during captopril treatment. We also examined the impact of hydralazine treatment, a non-ACE inhibitor antihypertensive agent, on BP and hemorrhagic stroke development in SHRsp.

1.5.3 MATERIALS AND METHODS

1.5.3.1 Experimental Animals

The SHRsp used in these experiments were taken from a colony housed in Animal Care Services, within the Health Sciences Centre at Memorial University of Newfoundland (St. John's). The experiments were performed in conformity with the guidelines set by Canadian Council on Animal Care and with the approval of the Memorial University Animal Care Committee. Animals were kept in rooms on a 12 hour light/dark cycle at 20-21° C with a humidity of approximately 50%. The characteristics of the strain were preserved by brother and sister matings. To avoid potential complications produced by hormonal fluctuations during the estrous cycle, only male animals were used. Our best breeding results were obtained by placing

1 male with 1-3 females at approximately 9 to 10 weeks of age. Animals were kept together for approximately 1 week. At 4 weeks of age, animals were weaned, placed 1 rat per cage and given standard rat chow (Agway Inc., Syracuse, NY, USA; Prolab Rat, Mouse/Hamster 3000). At 6 weeks of age male SHRsp were placed on a stroke-prone rodent diet containing 4%NaCl and 0.75%K^+ (Zeigler Bros Inc., Gardners, Pennsylvania, USA). All drug treatments were initiated at 6 weeks of age and water and food were provided in unlimited quantities. Animals were weighed to the nearest gram every other day between 6 and 12 weeks of age and once weekly thereafter.

1.5.3.2 Administration of Drugs

All drugs and chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA) unless otherwise stated. Captopril was dissolved in drinking water at a concentration to provide an average dose of 50 mg/kg/day. The concentration of captopril in the drinking water was based on the average 24 hour drinking rate of the captopril treated animals as evaluated once weekly. Drinking rates for these animals averaged approximately 350 ml/kg/day.

Dexamethasone was first dissolved in dimethyl sulfoxide (DMSO), at 3.0 mg\100µl. Olive oil was then added to this mixture to attain a final volume of 10ml. The dose used was 0.1mg/kg/day injected subcutaneously. The latter treatment has been previously shown to enhance hypertension development in SHR (Nagoka *et al.*, 1976). Deoxycorticosterone Acetate was dissolved in DMSO, at 100mg/200µl.

Olive oil was then added to the mixture to attain a final volume of 2.5ml. A dose of 40mg/kg was injected subcutaneously into the rats twice weekly. This treatment with deoxycorticosterone corresponds to a standard dose used to produce deoxycorticosterone hypertension in a normotensive rats. Hydralazine was mixed in the drinking water at a concentration of 20, 40, 60, 80, and 100mg/l. The two highest doses of hydralazine are typically sufficient to lower BP to normal levels in SHR (Smeda *et al.*,1988b; Smeda and Lee, 1991).

1.5.3.3 Systolic Blood Pressure Determinations

Systolic BP's were measured in unanesthetized rats using a tail cuff compression method and equipment purchased from IITC Inc. (Woodland Hills, CA, USA).. Prior to testing, animals were warmed in their cages at 35° C for approximately 1 hour. This exposure to heat was found to be necessary in order to obtain a tail pulse. When the heating period was completed, animals were placed one at a time in a restrainer (IITC Model 81, 82 or 83 depending on the size of the rat). The pulse sensor and tail cuff which were contained in a single unit (IITC Model B60-3/8" or B60-7/16") was placed over the animals tail. The rat was then allowed 5 minutes to settle in the restrainer. The tail cuff/pulse sensor unit was connected to an amplifier unit (IITC Model 29-SSP BP Meter with built in Artifact Filter) and then to a 2 channel chart recorder (Linear instruments Corp., Irvine, CA; Model 285, MM). The chart recorder was calibrated before each session to a range of 0 to 300mmHg. The tail cuff was inflated to a pressure where the

photoelectrically detected tail pulse was blocked. The pressure within the cuff was then allowed to slowly decay until the tail pulse returned. The pressure within the cuff when the tail pulse returned was taken as the systolic BP. Each animal had its BPs taken 3 times per session and the average was recorded as that week's value. The weekly BPs measurements were completed during regular office hours on the same day each week.

1.5.3.4 The Monitoring of Stroke Development and the Sampling of Animals

The rats were monitored daily for the occurrence of stroke. The symptoms associated with stroke development within SHRsp have been previously described (Smeda, 1989, 1992). Initially, SHRsp develop convulsive repetitive forearm movement followed by inappropriate posture where the rat sits with its legs hyper extended in a "kangaroo type" posture. The latter symptom was often associated with lethargy and poor grooming. There is typically a 1.5 week period between the onset of the first behavioral symptoms of stroke and death.

Some SHRsp died abruptly, not allowing us to obtain blood samples or to carry out functional studies on the cerebrovasculature. However, the majority of animals exhibited signs of stroke that progressed to the point where death was imminent. In such instances, the animal was anesthetized with a mixture of Xylazine and Ketamine (10 mg Xylazine (Rompun, Bayer Inc., Ont. , Canada) plus 50 mg Ketamine (Ketalean, MTC Pharmaceuticals, Ont., Canada) per kg body weight, i.m). A thoracotomy was performed and approximately 2 ml of blood was

collected into heparinized syringes via cardiac puncture. Blood was centrifuged at 14000 rpm for approximately 1-2 minutes (Brinkmann Instruments Inc., Westbury, NY, USA: Eppendorf Centrifuge 5415C). Serum was collected and stored at -20° C for aldosterone determinations to be described in chapter 2. The animals were then exsanguinated and the middle cerebral arteries (MCA) were collected and used in functional studies to be described in chapter 3. The brains of all animals were removed and fixed in 84mM PO_4 buffer containing 4% formaldehyde and 1% glutaraldehyde at a pH of 7.4. The fixed brains were later examined for the presence or absence of hemorrhagic stroke by gross visual inspection. In the event that no stroke was identified using this method, brains were cut into .5-1.5mm sections and examined microscopically (Olympus America Inc., Melville NY, USA; Model SZ40 microscope).

1.5.3.5 Statistical Analysis

Alterations of blood pressure with age with respect to individual treatments were analyzed using a general linear model of multivariant analysis of variance (subsequently referred to as ANOVA). The effects of varying treatments on blood pressures were determined with respect to age over common ages between treatment groups. Mortality with age was assessed using a Mann-Whitney rank order test. Results were considered significant at $P<0.05$. All results are expressed as the mean +/- one standard error measurement. All tests were performed using

Minitab Statistical Software (State College, PA, USA)

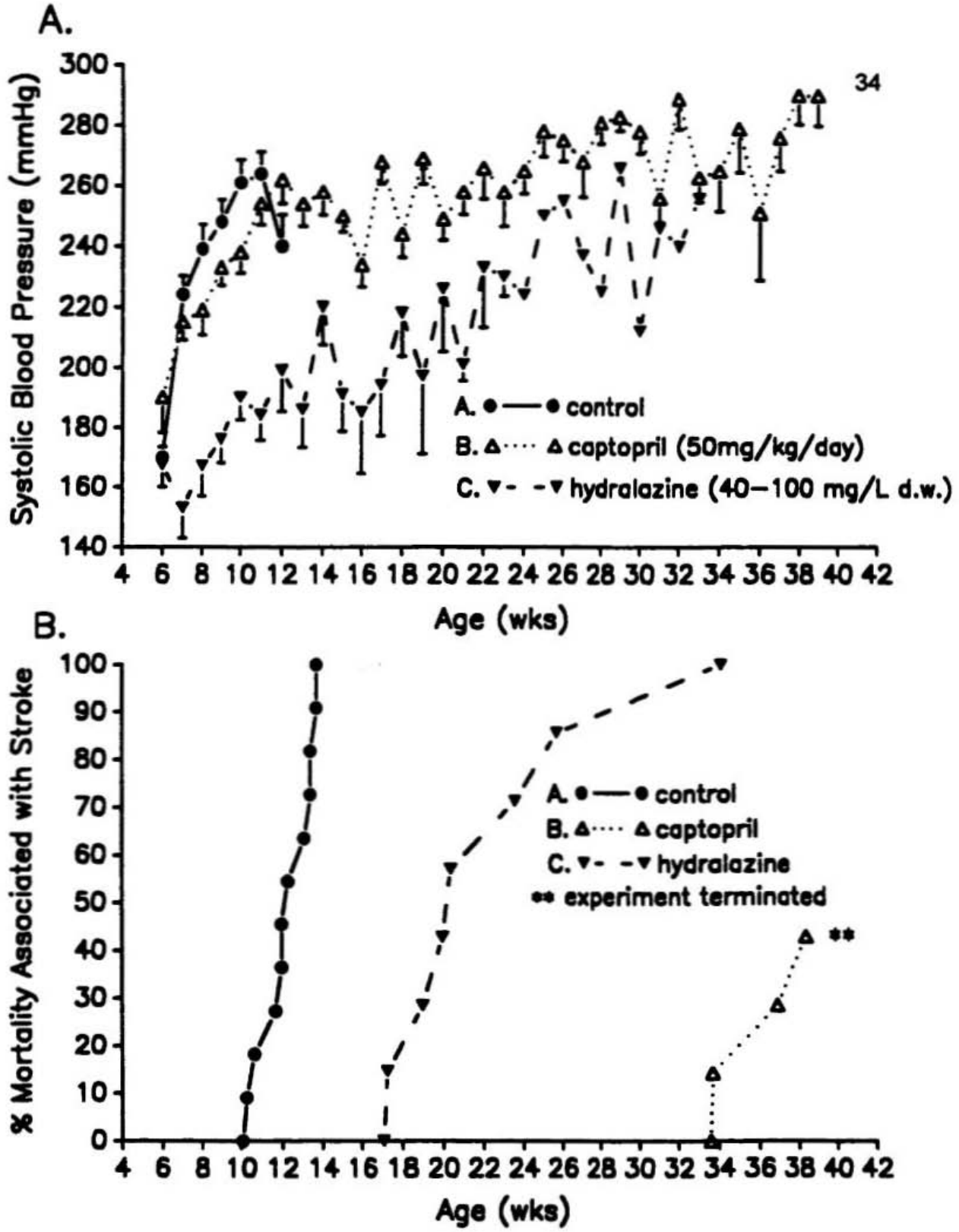
1.5.4 RESULTS

1.5.4.1 The Effects of Captopril and Hydralazine Treatment on Blood Pressure and Mortality Associated With Stroke

The effect of captopril and hydralazine treatment on the systolic blood pressure and mortality of SHRsp is outlined in Figure 1A and 1B. Captopril treatment produced a small reduction in the mean BPs of SHRsp when compared to controls between the ages 8 and 11 weeks. However, when the BP data was analysed over the lifespan of the controls (ANOVA) no significant differences were observed. Captopril treated SHRsp survived to an older age than control rats and at ages greater than 12 weeks of age (the age at which the control rats developed a 100% mortality associated with stroke) captopril treated SHRsp exhibited BPs, which surpassed those recorded earlier in untreated control group. For example, BPs values from control animals peaked at approximately 265mmHg at 11 weeks of age while the BPs of captopril treated SHRsp often attained BP's greater than 275 mmHg after 22 weeks of age.

SHRsp were treated with the antihypertensive agent hydralazine at doses of 20, 40, 60, 80 and 100 mg/L of drinking water. The antihypertensive effects of this drug were variable between animals. It was not uncommon for the animals to initially exhibit a suppression of BPs only to have their BP's re-elevate to levels that were present in control animals. Since the objective of the study was to achieve an

Figure 1. Alterations in systolic blood pressure (A) and mortality (B) associated with hemorrhagic stroke in untreated control SHRsp and SHRsp treated with captopril or hydralazine . Over the lifespan of the control SHRsp, captopril treatment (50 mg/kg/day, orally) did not significantly alter blood pressure in SHRsp and all SHRsp survived up to 34 weeks of age. The experiment was terminated at 38 weeks of age (**) with 4 captopril treated SHRsp surviving. These animals lacked behavioral symptoms of stroke but 2 of 4 SHRsp exhibited small intracerebral hemorrhages. To study the effects of reduced blood pressure on stroke development, SHRsp were treated with hydralazine (40-100 mg/L, d.w.). Mortality from stroke was studied in SHRsp that responded to hydralazine treatment by exhibiting average systolic blood pressures <220 mmHg from 10 weeks of age to death. Despite the presence of lower blood pressures in the hydralazine treated group, the onset of mortality associated with stroke occurred at an earlier age than that observed in captopril treated SHRsp. Statistics: Fig. 1A - ANOVA - over the lifespan of A, AvsB-NS, AvsC-P< 0.001, over the lifespan of C, BvsC - P<0.001; Fig. 1B - Mann-Whitney - AvsB-P<0.001, AvsC - P<0.001, BvsC - P<0.01. (n = values A = 11, B = 7, C = 7 SHRsp).


A.
34
Systolic Blood Pressure (mmHg)
300
280
260
240
220
200
180
160
140
4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42
Age (wks)
A. ● — ● control
B. △ ···· △ captopril (50mg/kg/day)
C. ▼- -▼ hydralazine (40–100 mg/L d.w.)
B.
% Mortality Associated with Stroke
100
90
80
70
60
50
40
30
20
10
0
4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42
Age (wks)
A. ● — ● control
B. △ ···· △ captopril
C. ▼- -▼ hydralazine
** experiment terminated
**

antihypertensive effect and not to study the effects of hydralazine *per se*, mortality was only studied in SHRsp that exhibited average weekly BPs values of less than 220mmHg from 10 weeks of age until the time of death. Three SHRsp treated with 40mg/L, 1 treated with 60mg/L, 2 treated with 80mg/L and 1 treated with 100mg/L of drinking water met these criteria.

The ability of hydralazine to suppress BP was greater during the first half of their lifespan when compared to the second half, frequently not surpassing values of 200mmHg. The hydralazine treated SHRsp were shown to have significantly reduced BPs when compared to the untreated control group when the BP's were compared over the lifespan of controls. The hydralazine treated animals were also shown to have a significantly reduced BPs profiles compared to the captopril treated group when analyzed over the common lifespans of both groups.

Untreated SHRsp controls began to die at approximately 10.5 weeks of age. A 100% mortality associated with stroke was attained before any of the rats achieved 13 weeks of age. None of the SHRsp treated with captopril developed stroke or died up to 19 weeks of age. At this point in time, four captopril treated SHRsp were randomly picked from the group and sampled while the remaining seven captopril treated animals were allowed to live until the termination of the experiment (40 weeks of age). The sampled captopril treated SHRsp were used in other investigations to be described later. This also allowed us to examine the brains of the captopril treated rats for any potential physical evidence of

hemorrhagic stroke. No evidence of infarcts or hemorrhagic stroke was observed in the sampled captopril treated SHRsp.

The mortality profile of the captopril treated SHRsp indicates that the earliest stroke related death in this group did not occur until approximately 34 weeks of age. At 38.5 weeks of age, only three captopril treated animals had died with evidence of hemorrhagic stroke and at 40 weeks of age, the remaining four animals were sampled with no apparent signs of stroke. Upon autopsy however, 2 of 4 of these animals were found to have hemorrhagic lesions. Statistical analysis of the mortality rates of untreated control verses the captopril treated SHRsp indicated that captopril treatment significantly increased the lifespan of SHRsp.

The mortality profile of the hydralazine treated animals showed that this group began to die at approximately 17 weeks of age and six of the seven animals were dead by 26 weeks of age, about 2 months prior to the age at which the first stroke related death occurred in the captopril treated group. Statistical analysis revealed that the mortality in hydralazine treated SHRsp occurred at a younger age than captopril treated animals despite the fact that the same treatment resulted in a more effective reduction in BP. Hydralazine treatment did produce a beneficial effect in that it retarded the onset of mortality associated with stroke when compared to the absence of treatment.

In summary, captopril treatment significantly retarded the onset of mortality associated with stroke in the absence of a statistically significant (i.e., $P>0.05$)

change in BP over that present in untreated rats. The latter observation combined with the observation that hydralazine treatment did significantly lower the BP of SHRsp but could not duplicate the antistroke effects of captopril would suggest that captopril's antistroke effects are likely not mediated by the suppression of hypertension.

1.5.4.2 The Effects of Dexamethasone and Deoxycorticosterone Treatment on Blood Pressure and Mortality Associated With Stroke in SHRsp Treated with Captopril

Due to the expected long duration of the proposed experiments (40 weeks), the experiments studying the effects of dexamethasone and deoxycorticosterone cotreatment on stroke development in captopril treated SHRsp were performed concurrently with the experiments studying the individual effects of captopril treatment on SHRsp. This was done with the anticipation that captopril treatment would suppress the BP of SHRsp and the realization that there may not be enough time during the graduate studies time period to run consecutive multiple 40 week duration experiments. The observation that captopril treatment did not significantly lower the BP over that present in untreated SHRsp diminished the need to re-elevate BP using dexamethasone or deoxycorticosterone treatment. However, since these experiments were in progress we completed the studies as outlined. The effect of dexamethasone and deoxycorticosterone cotreatment on blood pressure and mortality associated with stroke in captopril treated SHRsp is outlined in Figure

Figure 2. Alterations in systolic blood pressure (A) and mortality associated with hemorrhagic stroke (B) within untreated control SHRsp and SHRsp treated with captopril in the presence or absence of co-treatment with dexamethasone or deoxycorticosterone. The co-treatment of captopril (50mg/kg/day, orally) with deoxycorticosterone (40 mg/kg, 2X/week, s.c.) but not with dexamethasone (0.1 mg/kg/day, s.c.) prevented captopril treatment from retarding the onset of hemorrhagic stroke. None of the captopril treated SHRsp co-treated with dexamethasone died or developed symptoms associated with hemorrhagic stroke, however when the latter SHRsp were sampled between 24 to 30 weeks of age (**), 2 of 6 SHRsp showed histological evidence of intracerebral hemorrhage. Statistics - ANOVA - in Fig. 2A - over the lifespan of A, AvsC and AvsD - NS, over lifespan of C, BvsC - $P < 0.001$, over lifespan of D, BvsD $P < 0.001$; In Fig. 2B - Mann Whitney - AvsC - $P < 0.001$, AvsD - NS, BvsC and BvsD - $P < 0.001$ (n values A = 11, B = 7, C = 6, D = 6).

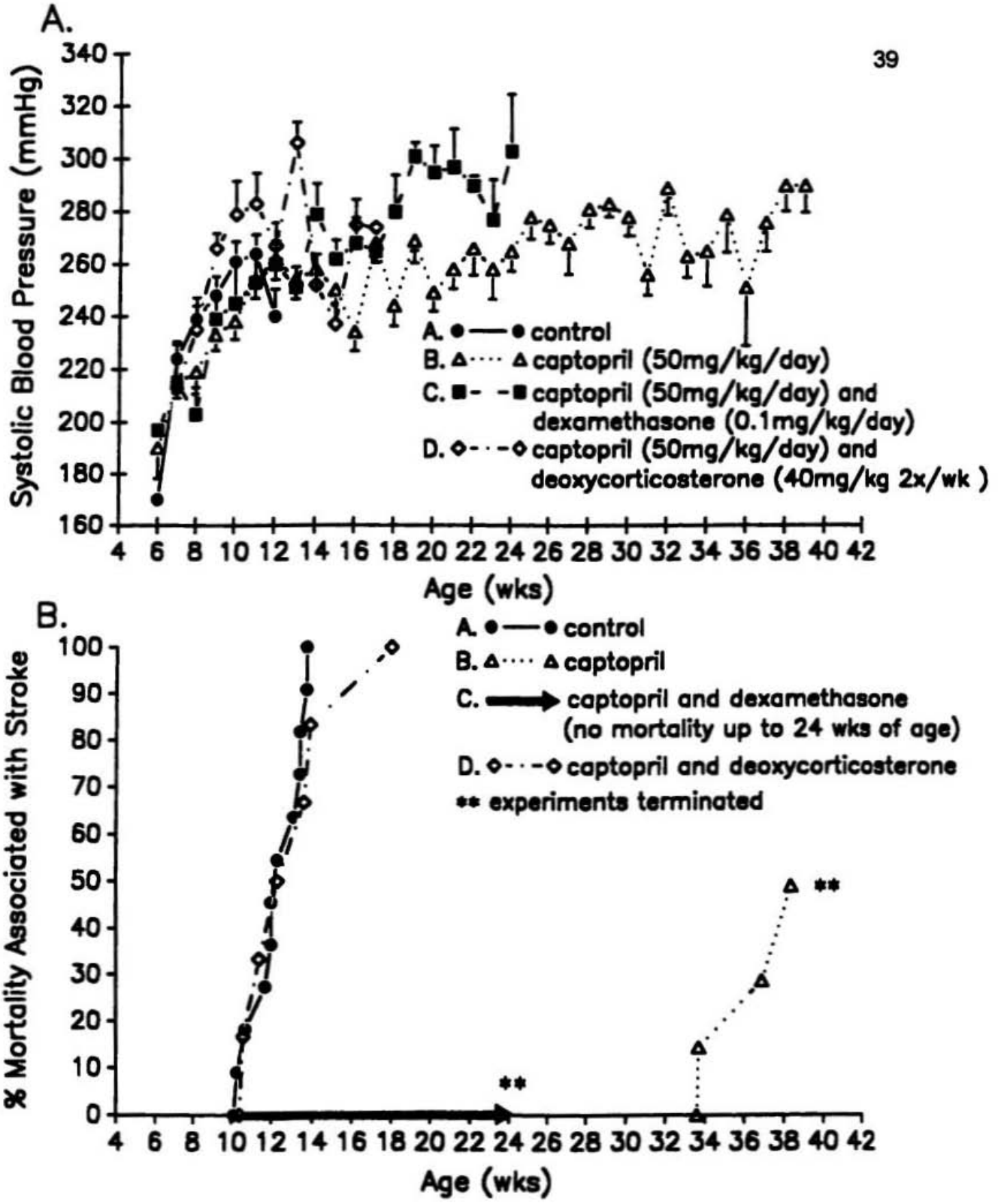
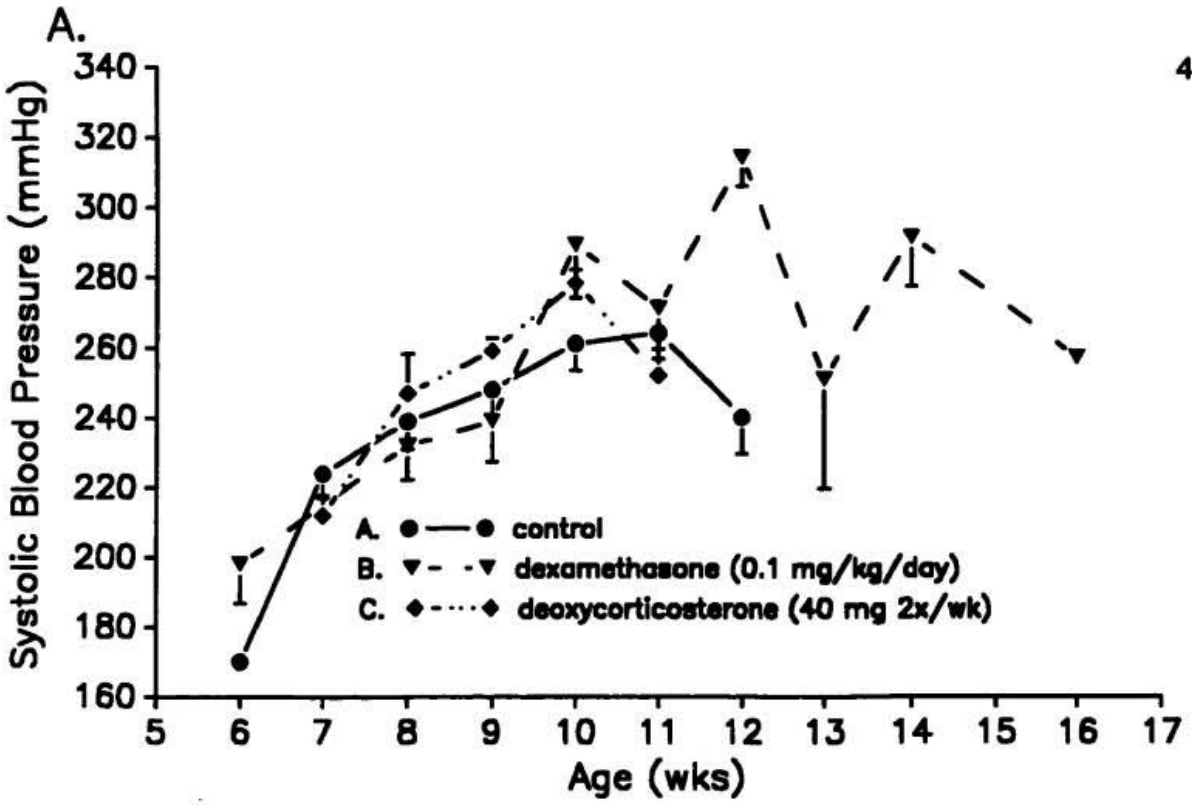
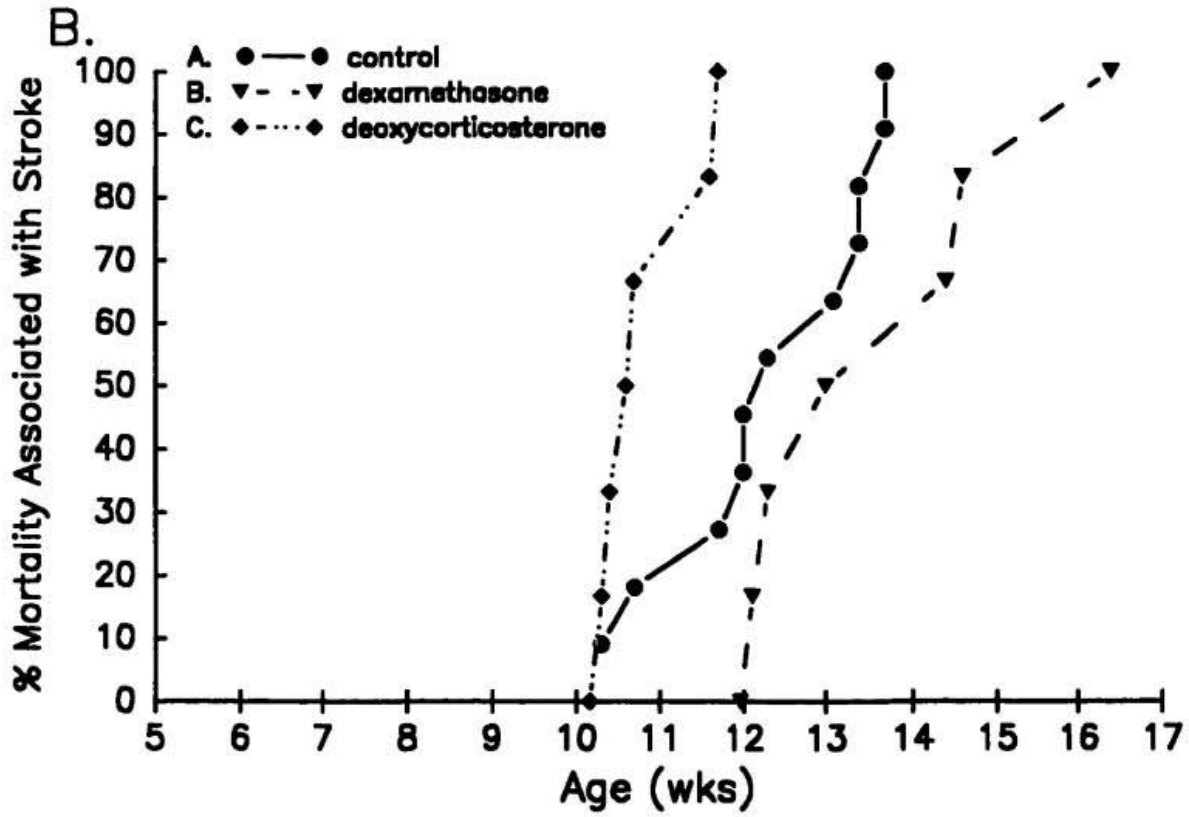

A.
Systolic Blood Pressure (mmHg)
340
320
300
280
260
240
220
200
180
160
4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42
Age (wks)
A. control
B. captopril (50mg/kg/day)
C. captopril (50mg/kg/day) and dexamethasone (0.1mg/kg/day)
D. captopril (50mg/kg/day) and deoxycorticosterone (40mg/kg 2x/wk)
B.
% Mortality Associated with Stroke
100
90
80
70
60
50
40
30
20
10
0
4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42
Age (wks)
A. control
B. captopril
C. captopril and dexamethasone (no mortality up to 24 wks of age)
D. captopril and deoxycorticosterone
** experiments terminated
**
**

Figure 3. Alterations in systolic blood pressure (A) and mortality associated with hemorrhagic stroke (B) in untreated SHRsp and SHRsp treated with dexamethasone or deoxycorticosterone. Treatment of SHRsp with deoxycorticosterone (40 mg/kg, 2X/week, s.c.) accelerated the onset of stroke development in SHRsp while treatment with dexamethasone (0.1mg/kg/day, s.c.) had no effect on the onset of death associated with stroke. Statistics -ANOVA- Fig 3A- over overlapping life spans, A vs B, A vs C - all NS; In Fig 3B - Mann Whitney - A vs B - NS, A vs C - $P < 0.01$ (n values A = 11, B = 6, C = 6).


A.
Systolic Blood Pressure (mmHg)
340
320
300
280
260
240
220
200
180
160
5
6
7
8
9
10
11
12
13
14
15
16
17
Age (wks)
A. control
B. dexamethasone (0.1 mg/kg/day)
C. deoxycorticosterone (40 mg 2x/wk)


B.
% Mortality Associated with Stroke
100
90
80
70
60
50
40
30
20
10
0
5
6
7
8
9
10
11
12
13
14
15
16
17
Age (wks)
A. control
B. dexamethasone
C. deoxycorticosterone

2A and 2B.

SHRsp were treated with captopril from 6 weeks of age and deoxycorticosterone (40 mg/kg 2x/week) or dexamethasone (0.1 mg/kg/day) was injected into the animals. As shown in Figure 2A, when compared over common life spans neither of the latter injections significantly elevated the BP of SHRsp over that present in untreated control animals but, both deoxycorticosterone and dexamethasone injections significantly elevated the BP of captopril treated animals. The injection of deoxycorticosterone into captopril treated SHRsp prevented captopril treatment from retarding mortality associated with stroke development whereas, dexamethasone did not (Figure 2B).

None of the 6 rats treated with captopril plus dexamethasone died or exhibited symptoms related to stroke development (Figure 2B). The captopril plus dexamethasone treated SHRsp were sampled between 24-30 weeks of age. None of the rats exhibited behavioral symptoms of stroke, however, histological examination revealed the presence of intracerebral hemorrhage in 2 of the 6 animals.

The conclusions reached were that despite the ability of dexamethasone to elevate blood pressure in captopril treated SHRsp, the latter glucocorticoid did not greatly reverse the antistroke effects of captopril treatment on mortality. To our surprise deoxycorticosterone cotreatment of captopril treated rats produced comparable blood pressures in SHRsp to those observed in dexamethasone

cotreated animals yet unlike the latter treatment, cotreatment with deoxycorticosterone completely nullified the antistroke effects of captopril treatment in SHRsp. Unlike dexamethasone, deoxycorticosterone binds to the same mineralocorticoid receptors and mimics the action of aldosterone. Since the renin angiotensin system is hyperactive in SHRsp we contemplated the possibility that the potential mineralocorticoid receptor stimulation via aldosterone, secondary to the presence of AII mediated hyper-release of aldosterone may play an important role in promote hemorrhagic stroke development in SHRsp.

We also tested the individual effects of dexamethasone and deoxycorticosterone on blood pressure and mortality associated with stroke within SHRsp not treated with captopril. The results of this experiment are shown in Figure 3A and 3B. The blood pressures of SHRsp subjected to deoxycorticosterone or dexamethasone injections did not significantly differ from untreated control SHRsp when compared over corresponding life spans (Figure 3A). Deoxycorticosterone injections accelerated the onset of mortality associated with stroke on average by 2.2 to 2.5 weeks when compared to untreated SHRsp. The injection of dexamethasone did not significantly alter stroke development in SHRsp (Figure 3B).

1.5.5 DISCUSSION

We have shown that captopril treatment of SHRsp is capable of promoting a dramatic reduction in the onset of mortality associated with hemorrhagic stroke

without significantly altering BP. Hydralazine treatment produced a much greater reduction in BP than captopril treatment but was less effective than captopril treatment in retarding the onset of hemorrhagic stroke development. The antistroke effects of captopril treatment were preserved under conditions where the level of hypertension was enhanced by cotreatment of the rats with dexamethasone. In view of these findings it seems unlikely that the antistroke effect of captopril treatment was the result of the small (statistically insignificant) hypotensive effect seen between 8 and 11 weeks of age in SHRsp. It would appear that ACE inhibition and the suppression of AII independent of the production of hypertension may play a role in promoting the antistroke effects of captopril in SHRsp. It is important to be cognizant of the fact that the aforementioned results do not indicate that elevated BP plays no role in the promotion of hemorrhagic stroke in SHRsp. The observation that hydralazine treatment of SHRsp resulted in significantly reduced BP and a retarded onset of mortality acknowledges this association. It was considered that the hydralazine mediated BP reduction could have increased RAS activity and partially offset a potential antistroke effect of BP reduction. Therefore, a number of studies were reviewed in order to gain an understanding of the influence of hydralazine treatment on the RAS.

Campbell et al. (1975) reported serum renin activity and serum aldosterone values were significantly elevated by a single intraperitoneal dose of hydralazine (1 mg/kg), peaking at 20 and 60 minutes respectively. The results derived from

Wistar rats were not accompanied by BP values. Similarly, treating SHR with hydralazine (3 mg/kg, d.w.) for 2 weeks resulted in a 2 to 3 fold increase in PRA and a significant reduction in mean arterial pressure relative to untreated controls. Neither value changed significantly when re-examined at 3 and 6 months (Antonaccio et al., 1979).

In a clinical study by Abraham et al. (1987), patients diagnosed with essential hypertension and treated with hydralazine (163 mg/day, p.o.) for 8.3 weeks, showed a significant reduction in supine diastolic blood pressure relative to a placebo treatment phase. However, there were no significant changes in supine systolic, upright systolic or upright diastolic blood pressures. Among those patients found to be controlled on hydralazine monotherapy (supine diastolic blood pressure < 91 mmHg and reduced at least 10 mmHg relative to placebo) no significant changes were found in supine and upright PRA or supine and upright plasma aldosterone levels.

Additional animal studies were conducted by Morishita et al. (1995) and found BP and plasma AII levels significantly elevated in 2 kidney, 1 clip (2K1C) Wistar rats relative to a sham operated group. Treating the 2K1C group with hydralazine (20mg/kg, d.w.) for 4 weeks significantly decreased BP but had no significant effect on plasma AII concentration. The investigators further reported that the same dose of hydralazine given to SHR reduced BP to normotensive WKY levels and also had no significant effect on plasma AII concentration. Furthermore,

treating SHRsp with hydralazine (30 mg/kg, d.w.) for 28 days resulted in significantly reduced BP, significantly elevated PRA but no significant change in kidney renin mRNA levels. The control group was untreated SHRsp and all animals had unrestricted access to standard rat chow and distilled water (Keuneke et al., 1995).

Unfortunately, the investigations outlined above differ from those described in the thesis in several potentially important ways. For example, there is little consistency among studies in terms of the particular component(s) of the RAS examined, methods of blood pressure evaluation, the species studied as well as the hydralazine dose, delivery method and duration of treatment. In addition, due to time limitations, we were unable to examination the RAS in our hydralazine treated animals. Consequently, the relevance of these findings to our own is questionable and we are unable directly address the status of the RAS is SHRsp . Based on our results, we suggest that variables in conjunction with the presence of hypertension can determine whether stroke develops in SHRsp.

Although the present preliminary study can not ascertain the mechanisms via which captopril exerts its antistroke effects, the observation that deoxycorticosterone cotreatment of captopril treated rats reversed the antistroke effects of captopril is a significant observation. The level of captopril given to the rats far exceeds the levels typically required to completely inhibit both circulatory and tissue ACE activity in rats (Sweet *et al.*, 1987). Hence, under the conditions of

the experiment, local and circulating AII levels should have been suppressed in SHRsp by captopril treatment. Studies outlined in the introduction of this chapter presented evidence that AII (Mento and Wilkes, 1987) and aldosterone (Lijnen, 1982) may return to, or exceed pre-tretement levels with chronic exposure to ACE inhibition. However, these studies differ from those outlined in the thesis in potentially important factors such as the species studied and the duration of treatment. Consequently, it is difficult to relate these results to our own. The question of plasma aldosterone levels in SHRsp under chronic captopril treatment however will be specifically addressed in chapter 2.

The observation that deoxycorticosterone treatment in the presence of AII suppression permitted stroke to develop would suggest that mineralocorticoid action independent of AII direct effects can facilitate the onset of hemorrhagic stroke development. Since aldosterone is comparable to deoxycorticosterone in its structure and acts to stimulate the same receptors (Agarwal and Mirshahi, 1999; Rogerson and Fuller, 2000) and in view of the fact that activation of the renin angiotensin system occurs in SHRsp, it is possible that elevations in aldosterone and not the direct actions of AII may play a role in promoting stroke development in SHRsp. If this were to be the case, the antistroke effects of captopril could be mediated via the suppression of plasma aldosterone, a hypothesis that will be tested in Chapter 2 of this thesis.

During our investigations, four captopril treated and six captopril plus

dexamethasone treated SHRsp were sacrificed prior to the development of any overt signs of stroke. Of interest, two of the four captopril treated SHRsp and two of the six captopril plus dexamethasone treated SHRsp were found to have suffered a hemorrhagic stroke when the autopsies were performed. As previously discussed, SHRsp only live for 1.5 weeks after the onset of stroke (Smeda, 1989). This observation suggests the possibility that captopril treatment not only delays the onset of stroke but prolongs life after a stroke has occurred. This is consistent with the observations made in recent studies that captopril treatment not only retards the onset of stroke in SHRsp, but also significantly prevents death even when the treatment is started up to 6 days after the onset of hemorrhagic stroke development in SHRsp (Smeda *et al.*, 1999a). The mechanism via which this effect takes place is unclear, however MRI studies of the brains of SHRsp during the initial onset of stroke indicate that an early event preceding the development of cerebral hemorrhage is the occurrence of intra to extravascular plasma movement across the vascular wall causing the subsequent formation of edema (Takashi *et al.*, 1994; Blezer *et al.*, 1998). Treatment with an ACE inhibitor prior to the formation of edema retards this event and treatment after edema has formed, reverses this pathology. It is possible that the extravascular movement of fluid occurs at the first sites of vascular rupture and is followed by the movement of blood cells into the brain tissue. ACE inhibitors could produce a physiological change altering cerebral blood flow to the region in a manner that reduces local hydrostatic pressures. This,

in turn, could preserve the integrity of the endothelium and thereby prevent or limit rupture of the blood brain barrier.

A second possible action of captopril could be as a renal protectant. SHRsp develop renal dysfunction prior to stroke (Smeda, 1997). Morphological examination of the kidneys indicates the presence of ischemic underperfused areas. Glomerular filtration decreases, urea and likely other uremic toxins are not filtered and are retained in the blood (uremia). The nature of filtration changes are such that plasma proteins such as albumin are lost into the urine (Smeda, 1997). These alterations could be promoted as a result of the development of preglomerular arteriolar occlusive disease such as the occurrence of arterial fibrinoid necrosis in the kidneys of SHRsp (Ogata *et al.*, 1982). Such defects could play an important role in the development of hemorrhagic stroke in SHRsp. The development of uremia independent of hypertension is associated with the development of bleeding disorders due to the anticoagulant effects of uremic toxins (Smeda,1992, 1997). Hence a small rupture in the blood brain barrier that might be repaired by clotting could, in the presence of uremia expand to form a large intracerebral hemorrhage. Since albumin contributes to the oncotic pressure of the blood, which tends to draw fluid from the extra into the intravascular space, a decrease in blood albumin level would promote the extravascular movement of fluids, an event that has been shown to precede hemorrhagic stroke development in SHRsp (Smeda, 1997). The potential importance of the above alterations in relation to stroke development is

emphasized by studies involving SHR which typically do not develop stroke. Partial nephrectomy of SHR which produces a decrease in glomerular filtration, uremia, and proteinuria allows this stroke resistant model to develop stroke (Smeda, 1992). Nephrectomized SHR can developed hemorrhagic stroke under conditions where the blood pressure of the animals is not further elevated (Smeda, 1992). ACE inhibitor treatment of SHRsp prior to stroke has been shown to inhibit the development of preglomerular arteriolar fibrinoid necrosis, prevent the decrease in glomerular filtration and inhibit the development of uremia and proteinuria (Stier *et al.*, 1989; Blezer *et al.*, 1998). It is possible that some of the beneficial antistroke effects of ACE inhibition could be the result of the renal protective effects exerted by this class of drugs and the ability of these drugs to inhibit the development of arterial fibrinoid necrosis in the kidney. Recent studies have suggested that elevations in plasma aldosterone levels in SHRsp may play a particularly important role in promoting the type of renal damage described above (Rocha *et al.*, 1999).

An interesting finding in our study is the observation that captopril treatment did not significantly suppress the blood pressure of our SHRsp. The paradox of this situation is that as discussed in the literature review, SHRsp develop high plasma renin and AII levels (Stier *et al.*, 1991; Kim *et al.*, 1992; Hubner *et al.*, 1995), an alteration that should facilitate the enhancement of hypertension. Ironically, ACE inhibitors are effective in lowering the BP of SHR to near normotensive levels despite the fact that plasma renin and AII levels are normal in these rats (Morishita

et al., 1995). There are individual populations of SHRsp that do not develop a high incidence of stroke, ACE inhibitor treatment does suppress the BP in these groups of SHRsp (Hadju *et al.*, 1991; Chillon and Baumbach, 1999). The reason why our SHRsp, and other SHRsp (that develop a high incidence of stroke) do not respond to ACE inhibitor treatment by exhibiting a suppression in BP is unknown. It is possible that the structural alterations in the renal arteriolar vasculature are of such a massive degree in our SHRsp that kidney function is permanently reset. The high BP present could be a consequence of a structurally reset kidney and may be necessary to maintain a viable level of glomerular filtration, salt and water balance (Guyton *et al.*, 1986). If the system was set in a manner where a given range of high BP's were required to maintain a viable salt and water balance, a decrease in BP which potentially could perturb this balance (i.e., via ACE inhibitor treatment) would be resisted by the body since the disruption in the fluid water electrolyte balance would cause death. The presence of the renal dysfunction would also activate RAS and elevate plasma AII levels which could increase the risk of stroke. SHRsp colonies that do not develop stroke could have more normal renal function and normal plasma AII levels as is the case in SHR, hence the risk of stroke may be absent. In these colonies of SHRsp a decrease in BP promoted by ACE inhibitor treatment could be sustained without compromising renal filtration, salt and water balance to a point where death is immanent.

CHAPTER 2: THE MECHANISMS PROMOTING THE ANTISTROKE EFFECTS OF ANGIOTENSIN CONVERTING ENZYME INHIBITORS IN SHRsp

LITERATURE REVIEW

2.1 AN ASSESSMENT OF THE POSSIBLE MEDIATORS INVOLVED IN PROMOTING THE ANTISTROKE EFFECTS OF CAPTOPRIL

2.1.1 Introduction

In chapter 1 we provided evidence to support our conclusion that captopril treatment of Kyoto Wistar stroke prone spontaneously hypertensive rats (SHRsp) retards mortality associated with stroke independent of a significant reduction in blood pressure (BP). Consequently, our focus progressed to investigations aimed at identifying possible mediators of captopril's antistroke effects in these animals. The major pharmacologic effects of angiotensin converting enzyme (ACE) inhibitors are known to be the inhibition of angiotensin II (AII) formation and the reduction of plasma aldosterone levels (Gums, 1992). There are also reports that captopril treatment exerts a sympatholytic effect (Xie *et al.*, 1994). These three effects were therefore considered as possible mediators of the therapeutic actions of captopril treatment in SHRsp

2.1.2 The Involvement of the Sympathetic Nervous System in the Antistroke Effects of Captopril Treatment

The possibility that captopril's therapeutic effects were mediated as a result

of a sympatholytic effect was considered, but seemed unlikely. Mueller and Black (1985), measured the sympathetic nerve activity from the superior cervical trunk at 12 to 25 weeks of age in SHRsp, Kyoto Wistar stroke prone spontaneously hypertensive rats (stroke resistant strain) (SHR) and Wistar Kyoto rats fed a standard rat chow diet. Peak sympathetic nerve activity (achieved via the induction of central ischemia) and resting sympathetic nerve activity were found to be significantly elevated in SHR compared to SHRsp. The authors reported that the reduced sympathetic nerve activity in SHRsp relative to SHR may be a contributing factor in the development of hemorrhagic stroke. Similarly, Sadoshima *et al.* (1983) examined the effects of unilateral cervical ganglionectomy, performed at 7 months of age, in SHRsp fed a Japanese style diet and a 1% NaCl drinking solution. These investigators found that stroke occurred only in the denervated side in 79% of the animals which underwent the procedure. In sham operated controls, the incidence of stroke was similar in the 2 hemispheres. It was also reported that vascular hypertrophy of pial and intraparenchymal brain arteries (external diameter < 80μm) was reduced in the denervated hemisphere. The authors concluded that wall hypertrophy may protect the cerebral microcirculation from hemorrhage. The same sympathectomy procedure performed at 3 months of age had no effect suggesting that sympathectomy altered cerebrovascular development only at a young age. Other studies have shown that the density of cerebrovascular sympathetic innervation is not altered with stroke or between SHR and SHRsp (Smeda,1990).

The above studies would contradict the hypothesis that a decrease in sympathetic nerve activity during captopril treatment promotes an antistroke effect.

2.1.3 The Involvement of the Renin Angiotensin System in the Antistroke Effects of Captopril Treatment

It seemed unlikely that captopril treatment exerted its antistroke effects in SHRsp via sympatholytic mechanisms. Consequently, our search for mediators of captopril's therapeutic actions in SHRsp focused on the renin angiotensin system (RAS). The justification for taking the investigations in this direction was derived from experimental evidence indicating that various components of the RAS in SHRsp seem to be elevated during a high Na^+ diet. It was reasoned that this may be an inappropriate response as normally, the activity of the RAS would be suppressed during a high salt diet (covered in Chapter 1).

Stier *et al.* (1991), reported that following 5 weeks of exposure to the stroke-prone rodent diet and 1% saline drinking solution, 12.5 week old Kyoto Wistar normotensive (WKY) rats revealed plasma renin activity (PRA) values of 0.6 ng AI/ml/hr compared with 3.5 ngAI/ml/hr in WKY on a standard died and water. SHRsp of the same age however, placed on the same high salt diet produced PRA values of 29.1 ngAI/ml/hr compared to 9.2 ngAI/ml/hr in SHRsp on a standard diet and water. These results suggest that while PRA may be suppressed in response to an increased salt intake in WKY, the PRA of SHRsp may rise inappropriately under identical, high salt conditions.

Further anomalies in the RAS of SHRsp were reported by Volpe *et al.* (1993). These authors found that during a 1 week exposure to the stroke-prone rodent diet, the PRA of 6 week old SHRsp, SHR and WKY was suppressed approximately equally relative to baselines established prior to being placed on the special diet. However, plasma aldosterone suppression and urinary Na^+ excretion was less in SHRsp compared with both SHR and WKY over the same time period. BP among the 3 groups of animals was not significantly different at any time during the study. The investigators concluded that young SHRsp show a blunted suppression of aldosterone and defective Na^+ excretion during high salt intake.

In another study of the RAS in SHRsp, Volpe *et al.* (1990) examined the changes in plasma renin activity and plasma aldosterone levels in response to a 12 week exposure to a high salt, stroke-prone diet, beginning at 6 weeks of age. After 4 weeks on the diet, there was no significant difference in plasma renin activity or plasma aldosterone between the salt loaded SHRsp and a group of SHRsp fed a standard diet. At 8 weeks and again at 12 weeks however, the plasma renin activity in the salt loaded SHRsp rose to markedly high levels compared with animals on the regular diet, which maintained constant levels over the 12 week period. Plasma aldosterone levels also rose dramatically at 8 and 12 weeks of age in salt loaded SHRsp compared to baseline values. Similar results were reported by Camargo *et al* (1991) who began feeding SHRsp the stroke-prone diet at 6 weeks of age. Following 4 weeks on the Na^+ loaded diet, plasma renin activity was not suppressed

relative to baseline values determined prior to beginning the high salt diet. However, at 8 and 12 weeks of exposure to the special diet, PRA increased and remained greater than twice baseline values.

In addition to the elevations in plasma renin activity and plasma aldosterone, plasma AII levels have also been shown to be high in SHRsp using radioimmunoassay. For example, Hubner et al. (1995), found that plasma AII levels were significantly elevated in SHRsp compared with WKY both at baseline and after a 12 day exposure to a 1% NaCl drinking solution. In another study, plasma aldosterone levels determined by radioimmunoassay were found to be nearly four times higher in 25 week old hypertensive SHRsp compared with age-matched normotensive WKY (Kim *et al.*, 1992).

The studies described above indicate that various components of the RAS may be inappropriately elevated when SHRsp are maintained on a high salt diet for greater than 4 weeks. The normal suppression of plasma aldosterone and increase in Na^+ excretion in response to a Na^+ loaded diet may also be impaired. In addition, as discussed in chapter 1, deoxycorticosterone treatment was shown to increase the mortality rate of SHRsp relative to untreated control animals. Deoxycorticosterone was also able to eliminate the antistroke effects observed in captopril treated SHRsp. Compared with untreated controls, deoxycorticosterone or dexamethasone treatment produced very similar BPs effects. However, deoxycorticosterone treatment enhanced the onset of mortality rate in SHRsp

whereas dexamethasone did not. Human studies also report an association between aldosteronism and hemorrhagic stroke. For example, Nishimura et al. (1999), reported that stoke and proteinuria were particularly common in a study group of 58 patients diagnosed with primary aldosteronism. Additionally, the incidence of cerebral hemorrhage was significantly greater in patients with primary aldosteronism relative to age and sex matched controls with essential hypertension. Diastolic blood pressure was also significantly higher in the primary aldosteronism group. However, diastolic blood pressure was not significantly different between primary aldosteronism groups with and without vascular complications (Takeda et al., 1995).

Based on these observations it was speculated that the mortality rate in SHRsp may be related to a mineralocorticoid effect. Consequently, we focused our search for a possible mediator of captopril's antistroke action on aldosterone. The following sections will review the pharmacology and physiology of aldosterone.

2.2 PHYSIOLOGICAL AND PHARMACOLOGICAL ACTIONS OF ALDOSTERONE

2.2.1 Introduction

Aldosterone is the main mineralocorticoid in mammals and plays an important role in Na^+, K^+ and H^+ homeostasis regulation. This occurs primarily via its actions on the distal tubules cortical collecting duct of the nephron (Bartlet-Bas and Doucet, 1995). Other aldosterone target tissues include the urinary bladder, colon, salivary and sweat glands (Garty, 1986). Regulation of aldosterone

secretion takes place via changes in the activity of one or more of its biosynthetic enzymes. Conditions that tend to reduce renal perfusion pressure (e.g., sodium deprivation, congestive heart failure) increase aldosterone secretion indirectly via the RAS. Aldosterone secretion is also influenced by plasma potassium levels, possibly via an effect on Na+, K+-ATPase activity in the adrenal glomerulosa. Plasma aldosterone levels are increased and decreased by raised and depressed plasma potassium concentrations, respectively (Sealey and Laragh, 1995).

Additionally, aldosterone release under conditions of acute stress appear to be mediated by an adrenocorticotrophic hormone effect on adrenal cortical biosynthesis. Further control is mediated by atrial natriuretic factor which has been shown to suppress both aldosterone release as well as the aldosterone response to adrenocorticotrophic hormone (Gunning et al., 1996).

Aldosterone exerts its effect by regulating the expression of several proteins which function either as ion transporters or regulatory proteins which modulate the activity of specific pre-existing ion transporters. Supporting evidence was presented by Williamson (1963) who found that aldosterone induced antinatriuresis in adrenalectomized rats was completely blocked by the inhibitor of transcription, actinomycin D. Recently, Bartlet-Bas and Doucet (1995), described 3 distinct molecular targets which could explain the effects of aldosterone on Na^+ transport in the collecting duct. These targets are the apical Na^+ channels, the basolateral Na^+, K^+-ATPase and mitochondrial enzymes involved in the synthesis of ATP (the

fuel required for the Na^+,K^+-ATPase).

2.2.2 The Action of Aldosterone on the Apical Na^+ channel of the Collecting Duct

Aldosterone is known to increase the apical entry of Na^+ into the nephron epithelium through amiloride sensitive channels. The exact mechanism via which aldosterone increases apical Na^+ entry is unclear (Bartlet-Bas and Doucet, 1995). Palmer *et al.* (1982) examined the effects of aldosterone on the isolated urinary bladder from Na^+ loaded toads. These investigators found that aldosterone produced an increase in apical Na^+ channel area density thereby suggesting the synthesis of new channels. In contrast, Garty and Edelman (1983) reported that trypsinization of the apical Na^+ channels of isolated toad bladder before the addition of aldosterone reduced the aldosterone induced effects on Na^+ transport. These results suggested that aldosterone did not induce the synthesis of new proteins but instead activated channels which pre-existed in the membrane. A possible explanation for this apparent discrepancy was proposed by Bartlet-Bas and Doucet (1995) who suggested that the apical Na^+ channel may be composed of both a pore-forming (amiloride binding) subunit and a regulatory subunit which may control the activity of the pore forming subunit. The pore-forming subunit may pre-exist in the membrane with aldosterone inducing the expression of new regulatory subunits.

2.2.3 The actions of Aldosterone on the Basolateral Na^+, K^+- ATPase of the Collecting Duct

Several studies have established a relationship between plasma aldosterone levels and Na^+, K^+-ATPase activity in the collecting duct of mammals (Bartlet-Bas and Doucet, 1995). Evidence in support of an aldosterone induced synthesis of new Na^+, K^+-ATPase pumps includes the results of Mernissi and Doucet (1984). Using isolated collecting ducts from Na^+ loaded, adrenalectomized rabbits these authors found that aldosterone simultaneously restored both Na^+, K^+-ATPase activity (as measured by quantification of radiolabelled phosphate liberated from hydrolyzed ATP) and the number of catalytic subunits of Na^+, K^+-ATPase (as determined by ^{3}H-oubain bound/mm tubule length). Studies conducted by Farman *et al*, (1992) offered additional evidence of an aldosterone induced synthesis of new Na^+, K^+-ATPase proteins using kidney slices from rats. In situ hybridization showed a reduction in mRNA encoding for the α_1 -subunit (catalytic component) in adrenalectomized (aldosterone deplete) versus adrenal intact rats.

2.2.4 The Effect of Aldosterone on the Oxidative Metabolism of the Collecting Duct

The third mechanism generally thought to be responsible for the actions of aldosterone on electrolyte transport in epithelia is the induction of oxidative enzymes important for ATP generation (used as fuel by the Na^+ , K^+-ATPase). According to Bartlet-Bas and Doucet (1995), the antinatriuretic actions of

aldosterone are blunted by anoxic conditions. This issue was addressed by Law and Edelman (1978) who examined citrate synthase activity (Krebs cycle enzyme) in Na^+ loaded adrenalectomized rats. Renal citrate synthase activity was found to be significantly elevated by aldosterone treatment compared with controls. The enzyme activity was determined by the rate of Coenzyme A-SH generation from mitochondrial fractions. Law and Edelman (1978) also found that aldosterone augmented the incorporation of radiolabelled methionine into citrate synthase from mitochondrial fractions compared with controls. This effect was obliterated by actinomycin D. Together these results strongly suggest that aldosterone increases the activity of citrate synthase and that this is due to the induction of new proteins as opposed to modulation of pre-existing ones.

2.2.5 The Effect of Aldosterone on K^+ Transport in the Collecting Duct

Morel and Doucet (1992), explained that K^+ transport begins with K^+ being actively pumped into the tubule epithelial cell via the basolateral Na^+, K^+-ATPase followed by K^+ leaking passively into the lumen of the tubule through Ba^{2+} sensitive apical K^+ channels. The entry of Na^+into the epithelium promotes depolarization of the tubule cell increasing the driving force for K^+ exit from the apical boarder. Bartlet-Bas and Doucet (1995), however point out that confusion persists regarding whether aldosterone exerts a direct effect on K^+transport or if the kaliuretic changes occur indirectly as a result of other aldosterone induced effects.

2.2.6 The Effect of Aldosterone on H^+ Transport in the Collecting Duct

Studies generally agree that the aldosterone induced acidification of urine occurs via an upregulation of the apical, electrogenic H^+-ATPase in the collecting duct. For example, Khadouri *et al.* (1987), examined the H^+-ATPase activity in isolated renal tubules from adrenalectomized rats. The activity of this H^+-ATPase (which is blocked by N-ethylmaleimide and assayed by the quantification of labeled phosphate liberated/mm of tubule length/hr) was found to be significantly decreased in the collecting ducts of adrenalectomized rats compared with controls. Similarly, Mujais (1987) also using isolated tubules from rats found that the activity of N-ethylmaleimide sensitive H^+- ATPase was significantly increased in the collecting duct by prolonged administration of aldosterone.

2.2.7 Rapid, Nongenomic Effects of Aldosterone

Historically, the actions of aldosterone on epithelial transport of electrolytes have been thought to be mediated via an intracellular mineralocorticoid receptor. With the binding of aldosterone to its receptor, the steroid-receptor complex is thought to translocate to the nucleus where binding to specific DNA segments occurs resulting in subsequent production of specific proteins involved in ion transport.

Recently, aldosterone has been described as exerting nonclassical mineralocorticoid effects (to be described below) via a putative membrane receptor. The primary characteristics of these effects which renders them inconsistent with

the classical theory of steroid action is the rapidity with which aldosterone is able to induce the effects and by the fact that these effects are not blocked by inhibitors of transcription, protein synthesis or the mineralocorticoid receptor antagonists spironolactone and canrenone (Wehling, 1995). Furthermore, the specificity at the membrane receptor is such that aldosterone tends to be approximately 1000 times more potent than glucocorticoids (cortisol). Aldosterone's actions on the surface receptor tends to occur within minutes of its administration (versus hours for traditional steroid effects) and are thought not to directly involve actions at the nucleus. These effects are often referred to as rapid or nongenomic effects of aldosterone.

Most evidence for the rapid effects of aldosterone have been derived from human mononuclear leukocytes and vascular smooth muscle cells (VSMC) from rat thoracic aorta (Wehling, 1995).

Christ *et al.* (1995a), found that aldosterone was able to significantly increase $^{22}Na^+$ influx relative to pretreatment baselines in isolated VSMC's from rat thoracic aorta. A near maximal response was achieved within 5 minutes. The response was blocked by the inhibitor of the Na^+/H^+ exchanger ethylisopropylamiloride (EIPA) but not by the inhibitor of transcription, actinomycin D or the inhibitor of protein synthesis, cyclohexamide. Compared with pretreatment control conditions, intracellular levels of inositol 1,4,5-triphosphate (IP_3) were also shown to be significantly elevated within 30 seconds of aldosterone administration.

The aldosterone induced stimulation of the Na^+/H^+ exchanger was inhibited by the phospholipase C (PLC) inhibitor neomycin. The results described above suggest that the administration of aldosterone may activate the Na^+/H^+ exchanger in VSMC's via a nongenomic pathway and may possibly involve PLC (an enzyme that initiates the formation of diacylglycerol (DAG) and inositol triphosphate (IP_3), two important signal messengers in VSMC's).

In an additional study by Christ *et al.* (1995b), using the same in vitro VSMC preparation, a significant increase in intracellular DAG levels was found 30 seconds after administration of aldosterone. The administration of aldosterone was also found to stimulate the translocation of protein kinase C (PKC) from cytosol fractions to membrane fractions within 15 minutes. The rapid effect of aldosterone on DAG levels was blocked by a 30 minute preincubation with the inhibitors of PLC, neomycin and U-73122. Furthermore, the effects of aldosterone on PKC translocation were mimicked by the PKC activator phorbol myristate. Also using VSMC's from rat aorta, Wehling *et al.* (1994) found that free intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) significantly increased with the administration of aldosterone within 30 seconds and peaked within 2 to 4 minutes. Similar results regarding $[Ca^{2+}]_i$ were observed in cultured endothelial cells from the pig aorta.

Christ *et al.* (1995b), speculated on a possible signal transduction pathway involved in the rapid effects of aldosterone in VSMC. They reported that the signaling involved the PLC second messenger cascade. PLC activation via

membrane aldosterone receptors could result in the formation of IP_3 and DAG with IP_3 promoting the sacroplasmic release of Ca^{2+} from intracellular stores and DAG activating PKC. They further reported that it remains unclear if PKC is responsible for the rapid aldosterone effects on the Na^+/H^+ exchanger.

Aldosterone induced rapid increases in Na^+/K^+ exchanger activity have also been reported in human mononuclear leukocytes. Wehling *et al.* (1991), used cell swelling in an isotonic Na^+ solution as an indicator of Na^+/H^+ exchanger activity. With this *in vitro* preparation the authors reported a dose dependant, aldosterone induced significant increase in cell swelling relative to control conditions of up to 30 to 50%. This effect was statistically significant as early as 1 minute after aldosterone application and was blocked by EIPA. Hydrocortisone and dexamethasone were effective agonists only at concentrations much greater than aldosterone. Canrenone, K^+-canrenoate (antagonists of the intracellular mineralocorticiod receptor), actinomycin D and cyclohexamide did not antagonize the response. The investigators proposed the possibility that this rapid effect of aldosterone on the Na^+/H^+ exchanger in human mononuclear leukocytes may be mediated via distinct membrane receptors with high affinity for aldosterone.

The rapid effects of aldosterone have also been studied in epithelial cells. Oberleithner *et al.* (1987), reported nongenomic effects of aldosterone in a renal tubule epithelial cell preparation after the induction of a transient intracellular acidosis. These authors used the time taken for intracellular pH to recover as an

indication of Na^+/H^+ exchanger activity. The application of aldosterone allowed recovery of intracellular pH significantly more quickly than control preparations without aldosterone. This response was noted as early as 20 minutes after the addition of the steroid.

Wehling (1995) proposed a possible two step model to explain the apparent diversity of mineralocorticoid actions described above. Step one involves aldosterone binding to membrane receptors resulting in changes to electrolyte transport systems characterized by their rapid onset (the nongenomic effects of aldosterone). The second step is based on a completely different response which begins to affect ion transport after a latency of 30 to 60 minutes. This is the classical area of steroid action involving the intracellular MR and synthesis of ion transport related proteins (the genomic effects of aldosterone).

2.3 THE INTRACELLULAR ALDOSTERONE MINERALOCORTICOID RECEPTOR

2.3.1 The Pharmacology of the Intracellular Mineralocorticoid Receptor

According to Funder (1995), receptors normally discriminate between potential signals via two basic mechanisms. First, the ligand with the greater affinity for the receptor will be more likely to bind. Secondly, the binding of some ligands will activate the receptor (the agonist) while others will not, regardless of how well they bind (the antagonist). Mineralocorticoid receptors (MR) do not appear to conform to these generalizations. For example, Arriza *et al.* (1987), found that unlabeled aldosterone, corticosterone, deoxycorticosterone and cortisol competed

approximately equally with aldosterone binding in cytosol from a monkey kidney cell line transfected with human MR complementary DNA. This indicates that all the steroids examined had similar affinities for the human MR. Similar results were reported by Funder *et al.* (1973a), who studied the ability of aldosterone, deoxycorticosterone and corticosterone to compete with aldosterone binding in rat kidney slices incubated with the steroids in vitro. They found that deoxycorticosterone was approximately 80% and corticosterone was approximately 10% as effective as aldosterone in competing with aldosterone binding. The authors stated that in light of the much higher plasma levels of corticosterone (compared with aldosterone), without further discriminating factors, the aldosterone binding sites would be inappropriately occupied by corticosterone.

Funder *et al.* (1973a) further reported that when the steroids were infused *in vivo*, the ability of corticosterone to compete for aldosterone binding sites was reduced compared with observations made *in vitro*. This indicated that corticosterone was a weaker competitor for aldosterone *in vivo* than *in vitro*. These investigators reported that the *in vitro* renal aldosterone binding sites fell into two classes; Type I with high affinity for aldosterone and Type II with a lower affinity. Corticosterone was shown to have high affinity for the Type II receptor but less than 2% of aldosterone's affinity for the Type I receptor. Funder (1995) later described these sites as the Type I high affinity for aldosterone receptor (the mineralocorticoid receptor) and the Type II receptor having low affinity for aldosterone and high

affinity for dexamethasone and physiological glucocorticoids (the glucocorticoid receptor). The MR abbreviation will henceforth denote the Type I receptor and GR will denote the type II receptor.

The studies described above suggest the existence of mechanisms which offer the MR selectivity for aldosterone over glucocorticoids in renal tissue *in vivo* which are not functional *in vitro*. However, this does not appear to be the case in nonclassical mineralocorticoid target tissue. For example, Sheppard and Funder (1987) found that *in vitro* cytosol preparations from rat pituitary, colon and parotid tissue bound aldosterone and corticosterone with similar affinity to the MR. In contrast, *in vivo* studies using rats injected with tritiated aldosterone and corticosterone, found that pituitary tissue retained both aldosterone and corticosterone whereas colon and parotid tissue retained only aldosterone. These authors used excess RU 28362, a highly specific synthetic glucocorticoid to prevent binding of the labeled aldosterone and corticosterone to the GR. These results suggest that the mechanism which confers specificity *in vivo* to the MR in epithelial tissue may not exist for nonclassical mineralocorticoid target tissue such as pituitary.

2.3.2 The Specificity Conferring Mechanism for the Mineralocorticoid Receptors

Funder *et al.* (1988), proposed the enzyme 11β-hydroxysteroid dehydrogenase (11βHSDH) as the specificity conferring mechanism for classical

mineralocorticoid target tissue. These investigators examined the rate of conversion of ^{3}H-cortisol to ^{3}H-cortisone (as an assay of 11βHSDH activity) in rat heart, hippocampus, kidney, parotid and colon tissue. No activity was found in the hippocampus or heart tissue whereas the kidney, parotid and colon tissue exhibited 11βHSDH activity. Funder *et al.* (1988), also found that the differential ability of classical and nonclassical mineralocorticoid target tissues to show 11βHSDH activity was virtually abolished by pretreatment with carbenoxolone, a blocker of 11βHSDH. The authors reported that the action of 11βHSDH is essential to explain the selectivity of the MR action in classical mineralocorticoid target tissue. It was reported that the reason that in vitro preparations do not show the selectivity of the MR is that 11βHSDH is a microsomal enzyme not present in cytosolic preparations.

Mineralocorticoid effects from glucocorticoid and mineralocorticoid receptor activation have been reported. There is evidence that GR activation can induce a classical mineralocorticoid response. Funder *et al.* (1990) reported that patients with pseudohypoaldosteronism (undetectable MR levels) who were treated with carbenoxolone exhibited an antinatriuretic response. Similar results were observed with the highly specific glucocorticoid RU 28362 given to adrenalectomized rats. This mineralocorticoid effect became more marked with concurrent carbenoxolone administration and was not affected by the MR antagonist RU 28318. The authors concluded that activation of the GR could be followed by mineralocorticoid effects on urinary electrolytes. They summarized by stating that not only is the MR

inherently nonselective but the mineralocorticoid response elements (the sequence of DNA targeted by the steroid receptor/agonist complex) appear to be similarly nondiscriminating.

In summary, the *in vitro* MR appears to have the capacity to bind both aldosterone and physiological glucocorticoids. However, *in vivo*, the physiological glucocorticoids are prevented from binding to the MR by the enzyme 11βHSDH. This specificity conferring mechanism does not seem to operate in non-epithelial tissue such as pituitary. Furthermore, evidence exists that mineralocorticoid effects can result from GR activation under certain conditions.

2.3.3 Spironolactone

Spironolactone antagonizes the actions of aldosterone and is used in the treatment of hypertension, hyperaldosteronism and edematous states such as congestive heart failure. Spironolactone and/or its metabolites are thought to act as a competitive antagonist at the MR (Los and Colby, 1994). It is not completely understood whether the antimineralocorticoid effects of this drug are the direct result of spironolactone itself or if they are mediated primarily by metabolites of the parent compound. Furthermore, there appears to be uncertainty in the literature regarding which of the spironolactone metabolites play an important role in the aldosterone antagonism. The major pathway leading to the metabolism of spironolactone was outlined by Los and Colby (1994) who reported that spironolactone is metabolized into both sulphur containing and non-sulphur

containing metabolites. The non-sulphur containing metabolites include canrenone and its hydrolytic product canrenoate while the sulphur containing metabolites include 7α-thiospironolactone and 7α-thiomethylspironolactone.

The basic pharmacology of spironolactone was discussed by Sherry (1986) who outlined that spironolactone is quickly metabolized so that very little of the parent compound is found in biological fluids. In the past it has generally been believed that the primary circulating metabolite of spironolactone was canrenone. Also, that because of canrenone's antimineralocorticoid activity, it was thought to be the primary mediator of spironolactone's effects. However, it was also noted that canrenone levels may have been overestimated by the older relatively non-selective fluorimetric assay and that canrenone alone can account for only a small part of the antimineralocorticoid actions of spironolactone. The newer highly selective high performance liquid chromatography (HPLC) analyses later showed that plasma canrenone levels were overestimated by the fluorimetric method.

The spironolactone metabolite 7α-thiomethylspironolactone has received attention as an important mediator of spironolactone's antimineralocorticoid activity. For example, 7α-thiomethylspironolactone was shown to have greater affinity for the MR than canrenone using competitive binding studies with rat kidney slices (Funder *et al.*, 1973b). Overdiek *et al.* (1985) examined the serum concentration of spironolactone, canrenone, 7α-thiomethylspironolactone and 6β-hydroxy-7α-thiomethylspironolactone in healthy human males. The HPLC results of eight serial

blood samples over a 24 hour period following one 200mg oral dose of spironolactone showed that 7α-thiomethylspironolactone attained the highest levels among the compounds examined. They reported that 7α-thiomethylspironolactone is the primary metabolite following one oral dose of spironolactone. However, these authors noted that because canrenone had a much longer elimination half life (11.2 hrs) compared with 7α-thiomethlylspironolactone (1.8 hrs), 6β-hydroxy-7α-thiomethylspironolactone (3.1 hrs) and spironolactone (1.0 hrs), further investigations with multiple dosing of spironolactone were needed. In a later study, Fanestil (1988), noted that the high levels of 7α-thiomethylspironolactone were higher than the other substances investigated only for approximately the first 6 hours after spironolactone administration. It should be noted that the serum metabolite profile of spironolactone derived from the first few hours after one dose may be very different from the profile which develops during chronic administration. It may be that due to canrenone's relatively long elimination half life, this metabolite plays the dominant role in the antimineralocorticoid effects seen with long term spironolactone administration.

In spite of the limitations of the study described above, results favoring an important role for 7α-thiomethylspironolactone were also reported by Los and Colby (1994). These investigators found that following three days of spironolactone treatment, 7α-thiomethylspironolactone was the most abundant metabolite in the steroid receptor fraction of guinea pig kidney cytosol compared to 7α-

thiospironolactone and canrenone. However, the steroid receptor fraction from renal nuclei contained approximately equal amounts of 7α-thiomethylspironolactone, 7α-thiospironolactone and canrenone. The authors concluded that while 7α-thiomethylspironolactone appears to be the major spironolactone metabolite that interacts with the cytosolic MR in kidney, 7α-thiospironolactone and canrenone may play their primary role contributing to nuclear receptor binding.

EXPERIMENTAL RESEARCH

2.4 THE INVOLVEMENT OF PLASMA ALDOSTERONE IN PROMOTING HEMORRHAGIC STROKE IN CAPTOPRIL TREATED SHRsp

2.4.1 THE OBJECTIVES OF THE STUDY

The mechanisms via which ACE inhibition alters mortality and stroke development in SHRsp is not well understood. To date little consideration has been given to the possibility that elevations in plasma aldosterone may play a role in stroke development in SHRsp. Plasma aldosterone increases in a parallel manner to renin activity with increasing age in SHRsp (Okomoto *et al.*, 1991) and during established hypertension, the levels of plasma aldosterone and AII are both 4 to 6 times the levels present in normotensive rats (Kim *et al.*, 1991). ACE inhibitor treatment should lower plasma aldosterone levels in SHRsp.

The general objective of the proposed experiments was to gain an understanding of the role that plasma aldosterone suppression plays in the prevention of hemorrhagic stroke during captopril treatment. In pursuit of this goal, plasma aldosterone levels were assayed in relation to age and stroke development in SHRsp to determine, if in fact plasma aldosterone levels do elevate in relation to stroke development in SHRsp and to further determine if captopril treatment reduces plasma aldosterone levels in these animals. Subsequently, further studies were undertaken where aldosterone was infused into captopril treated SHRsp via the use of osmotic minipumps. The objective of these experiments was to re-elevate

the plasma aldosterone levels during captopril treatment to those levels present in SHRsp not treated with captopril to determine the effect that this manipulation would have on BP and mortality associated with stroke development. In the latter experiments, tissue and plasma AII levels should be suppressed hence the selective involvement of elevated plasma aldosterone could be assessed. Finally, the ability of the mineralocorticoid receptor antagonist spironolactone to alter stroke development was assessed in SHRsp.

2.4.2 HYPOTHESES

It is our hypothesis that plasma aldosterone levels are elevated in SHRsp prior to stroke development and that elevations of plasma aldosterone levels play an important role in promoting stroke in these animals. If this is the case, the antistroke effects of captopril treatment in SHRsp would be expected to be mediated by the ability of captopril treatment to suppress plasma aldosterone. If this proved to be the case then the re-elevation of plasma aldosterone during captopril treatment should inhibit the antistroke effects of the treatment. Finally, if the antistroke effects of captopril treatment are due to the suppression of plasma aldosterone and if the effects of aldosterone are mediated by the stimulation of the aldosterone mineralocorticoid receptor, treatment of SHRsp with spironolactone, an aldosterone mineralocorticoid receptor antagonist should mimic the antistroke effects of captopril treatment.

2.4.3 MATERIALS AND METHODS

2.4.3.1 Experimental Animals, Systolic Blood Pressure Determinations, the Monitoring of Stroke Development and the Sampling of the Animals.

These aspects of the experimental protocol are identical to those used in the experiments outlined in Chapter 1. Specifically a description of the SHRsp colony and the breeding protocol is outlined in section 1.5.3.1. The techniques used to measure blood pressure in the rats is outlined in section 1.5.3.3. The monitoring of stroke development and the sampling of the animals is outlined in section 1.5.3.4.

2.4.3.2 Plasma Aldosterone Determinations

Radioimmunoassay techniques were used to measure plasma aldosterone levels. These were performed by the Memorial University of Newfoundland Health Sciences Renal Diagnostic Laboratory (St. John's, Newfoundland, Canada) by qualified personnel that perform these assays on a routine basis. The assays were performed using a Coat-A-Count radioimmunoassay kit (Diagnostic Products Corporation, Los Angeles, CA, USA). The personnel who performed the assays were blinded of the identity of the samples.

2.4.3.3 Administration of the Drugs

All drugs and chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA) unless otherwise stated. Aldosterone was first dissolved in DMSO, 5mg/0.4ml. This mixture was then added to polyethylene glycol (300MW). The

drug solution was loaded into osmotic minipumps (Alzet Corporation, Palo Alto, CA) and implanted subcutaneously in the flank region under anaesthesia (10 mg Xylazine (Rompun, Bayer Inc., Ont. , Canada) plus 50 mg Ketamine (Ketalean, MTC Pharmaceuticals, Ont., Canada) per kg body weight, i.m). Pumps having a 2 or 4 week pumping capacity were implanted into the SHRsp depending on the size and age of the animal. The aldosterone/polyethylene glycol concentration was adjusted per pump to deliver aldosterone at 0.67 µg/hr or 16 µg/day (Morris and Kenyon, 1982). Vehicle control SHRsp were also studied. These SHRsp contained the implanted pump with polyethylene glycol minus aldosterone. The 2 week pumps delivered vehicle at 0.5 µl/hr while the 4 week pumps had a delivery rats of 2.5 µl/hr.

Spironolactone was first dissolved in DMSO, 20mg/100µL. The mixture was then added to 0.9ml of olive oil (Gerald S. Doyle Limited, St. John's, NF). The drug solution was injected subcutaneously into SHRsp to achieve a dose of 20mg/kg/day (Brilla et al., 1993). Vehicle control SHRsp were also studied. These SHRsp were injected with the above vehicle minus spironolactone.

2.4.3.4 Experimental Groups

SHRsp without stroke aged 9.5 to 12.5 weeks were sampled according to the methods outlined in chapter 1. The plasma aldosterone values derived from this group were plotted against age. In addition SHRsp with stroke (typically greater than 12 weeks of age) and age matched SHRsp treated with captopril (50

mg/kg/day, o.) from 6 weeks of age were also sampled. The changes in plasma aldosterone levels in prestroke verses poststroke SHRsp and captopril treated SHRsp were compared.

Stroke development and mortality were assessed in SHRsp treated with captopril (50 mg/kg/day, o.) or in SHRsp concurrently treated with captopril plus aldosterone from 6 weeks of age. Other SHRsp were treated with aldosterone in the absence of captopril treatment from 6 weeks of age. A group of SHRsp were also treated with spironolactone (20 mg/kg/day, s.c.) from 6 weeks of age. In addition, other groups of SHRsp were treated with aldosterone vehicle infusions or spironolactone vehicle injections.

2.4.3.5 Statistical Analysis

The method of statistical analysis used to describe BPs and mortality rate changes was described in Chapter 1 within section 1.5.3.5. Plasma aldosterone levels derived from multiple groups were analyzed using a one way analysis of variance to determine if a significant difference existed. Subsequently, a student's t-test compensated for multiple comparisons using the Bonferroni method was used to determine which groups differed significantly from each other. Changes in plasma aldosterone levels with age were analyzed using linear regression analysis coupled with the determination of a Pearson product of correlation (r value). All tests were performed using Minitab Statistical Software (State College, PA, USA).

2.4.4 RESULTS

2.4.4.1 Alterations in Plasma Aldosterone in SHRsp

The alterations in plasma aldosterone levels in prestroke SHRsp are outlined in Figure 4. Plasma aldosterone levels elevated with age in prestroke SHRsp. Levels obtained from prestroke SHRsp (aged 9.5-12.5 weeks) versus age showed a significant positive correlation. Using the line of best fit as a point of reference, at the youngest age investigated (9.5 weeks), plasma aldosterone levels were approximately 0.25 nmol/l. This value increased to approximately 3.9 nmol/l by 12.5 weeks of age. The results suggest that the plasma levels of this hormone rise with age during the prestroke time period examined.

The mean plasma aldosterone levels in the above prestroke SHRsp was 2.0 nmol/l. This value is plotted in Figure 5 (Group 3). As shown in Figure 5, poststroke SHRsp (10.5 to 13.5 weeks of age) had significantly higher mean plasma aldosterone levels of 11.4 nmol/l. Captopril treated SHRsp had plasma aldosterone levels of about 0.50 nmol/l which were below those levels observed in both pre and poststroke nontreated SHRsp. Captopril treatment equally suppressed plasma aldosterone levels in SHRsp at ages where the animals did not develop stroke (18- 20 weeks) as well as in animals that eventually did develop stroke (35 to 40 weeks of age). As we will subsequently demonstrate, the coinfusion of aldosterone (16μg aldosterone/day/rat, s.c.) into captopril treated rats abolished the ability of captopril to retard stroke development. Captopril plus

Figure 4. Alterations in plasma aldosterone with age in untreated SHRsp aged 9.5 to 12.5 weeks prior to the development of hemorrhagic stroke. The results suggest that plasma aldosterone may inappropriately rise with age in SHRsp ($r = 0.62$, $P < 0.01$, $n = 15$ SHRsp).

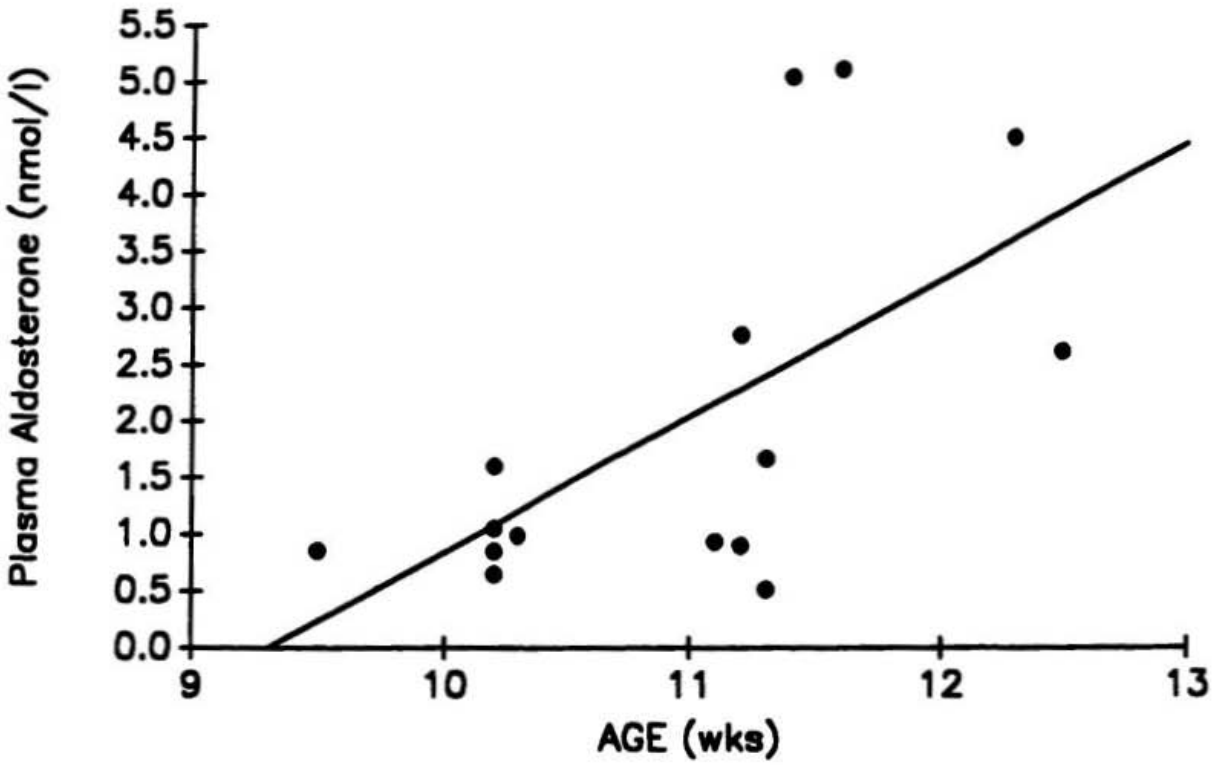
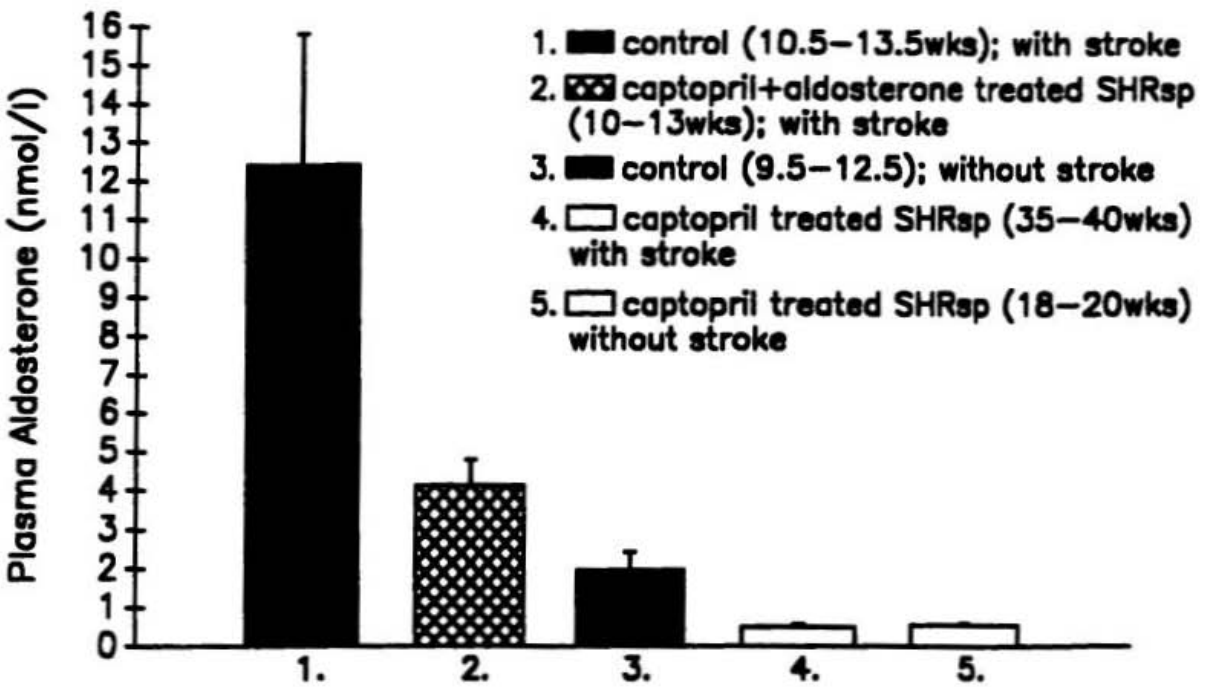

Plasma Aldosterone (nmol/l)
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
9
10
11
12
13
AGE (wks)

Figure 5. Plasma aldosterone levels in untreated SHRsp and captopril treated SHRsp with and without aldosterone supplement. The results show that plasma aldosterone values were higher in untreated poststroke vs prestroke SHRsp. Captopril treatment (50 mg/kg/day, orally) significantly reduced plasma aldosterone whereas the subcutaneous infusion of aldosterone (0.66 µg/hr) into captopril treated SHRsp raised plasma aldosterone to levels between those present in pre and poststroke SHRsp. Statistics - One-way analysis of variance of groups 1 to 5, $P<0.001$; 1vs3, 1vs4, 1vs5, 2vs4, 2vs5, 3vs4 and 3vs5 all significantly different at $P<0.05$, t-test compensated for multiple comparisons using Bonferroni correction (n values - 1.= 15, 2.= 5, 3. = 15, 4.= 5, 6. = 5 SHRsp).


Plasma Aldosterone (nmol/l)
16
15
14
13
12
11
10
9
8
7
6
5
4
3
2
1
0
1.
2.
3.
4.
5.
1. control (10.5–13.5wks); with stroke
2. captopril+aldosterone treated SHRsp (10–13wks); with stroke
3. control (9.5–12.5); without stroke
4. captopril treated SHRsp (35–40wks) with stroke
5. captopril treated SHRsp (18–20wks) without stroke

aldosterone treated SHRsp (sampled between the ages of 10.5 to 13.5 weeks of age, all with evidence of stroke) were found to have significantly higher plasma aldosterone values when compared to SHRsp treated with solely with captopril (Figure 5). The mean plasma aldosterone value of 6.3 nmol/l was obtained from the captopril plus aldosterone treated group of SHRsp was between the levels observed in untreated prestroke and poststroke SHRsp. Therefore, aldosterone supplementation of captopril treated SHRsp successfully approximated the plasma aldosterone levels typically observed in non captopril treated SHRsp.

2.4.4.2 The Effect of Aldosterone Supplementation During Captopril Treatment on Mortality and Stroke Development in SHRsp

The results of this experiment are outlined in Figure 6A and 6B. As shown in Figure 6A, SHRsp receiving captopril treatment plus aldosterone infusion had BP's that did not significantly differ from those present in SHRsp receiving no treatment when these groups were compared over common life spans. When compared over common life spans, captopril plus aldosterone treated SHRsp did have higher BP's than SHRsp treated only with captopril. However, at ages past those where a 100% mortality occurred in SHRsp treated with captopril plus aldosterone, SHRsp treated with captopril alone achieved BP's equal to or higher than those of the former group and still survived. As indicated in Figure 6B, the infusion of aldosterone into SHRsp treated with captopril prevented captopril treatment from retarding the onset of stroke development. As previously discussed,

Figure 6. Alterations in systolic blood pressure (A) and mortality associated with hemorrhagic stroke (B) in untreated control and captopril treated SHRsp in the presence or absence of aldosterone infusion. The infusion of aldosterone (0.66 µg/hr, s.c.) into captopril treated (50 mg/kg/day, orally) SHRsp raised plasma aldosterone to levels between those present untreated pre and poststroke SHRsp (see Figure 5). The infusion of aldosterone into captopril treated SHRsp reversed the antistroke effects of captopril treatment. Statistics - Fig. 6A - ANOVA - over the lifespan of A, AvsB -NS, over lifespan of C, AvsC - NS, BvsC - $P < 0.01$; Fig. 6B - Mann-Whitney - AvsC - NS, BvsC - $P < 0.001$ (n values A = 11, B = 7, C = 9).

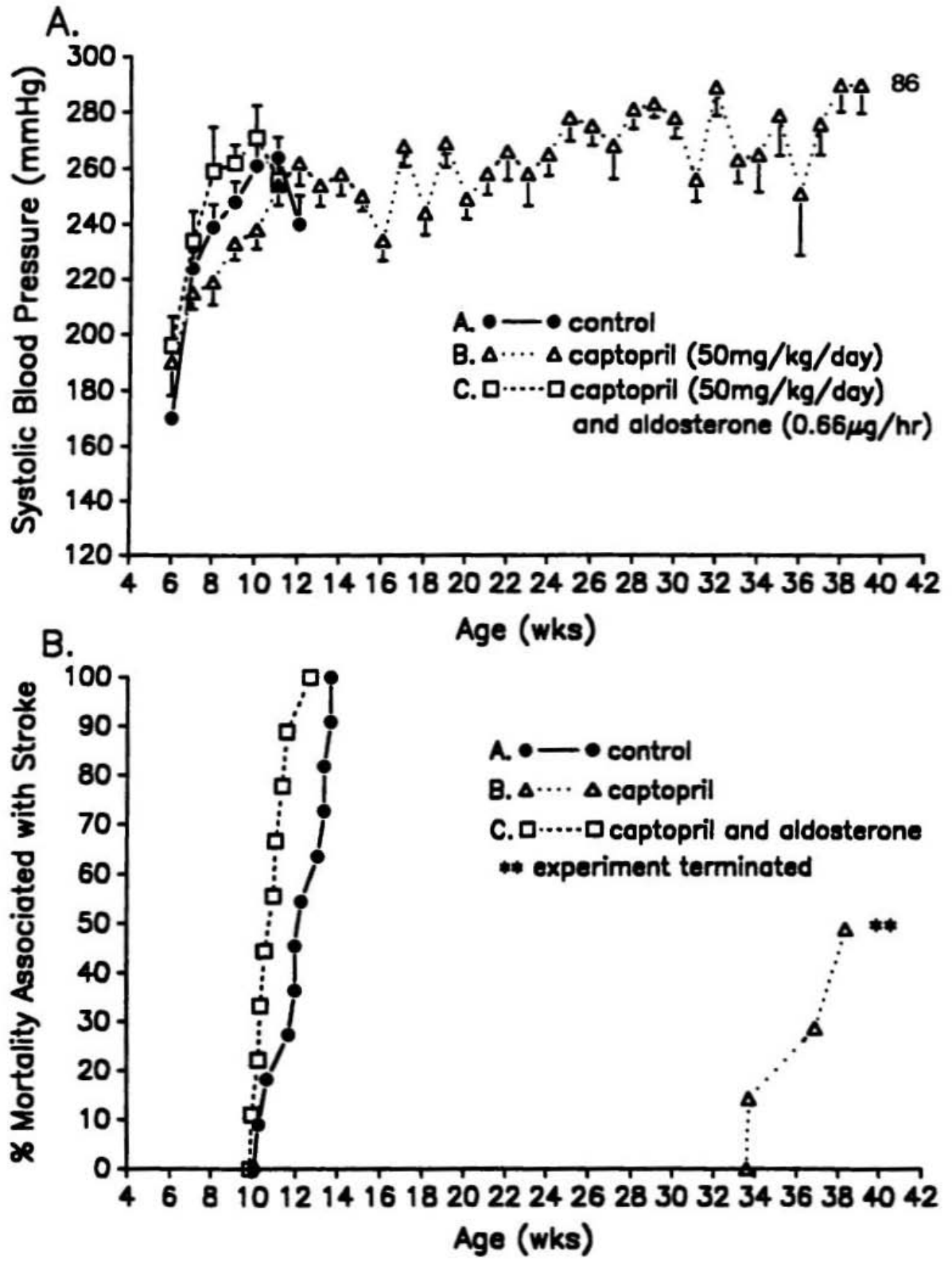
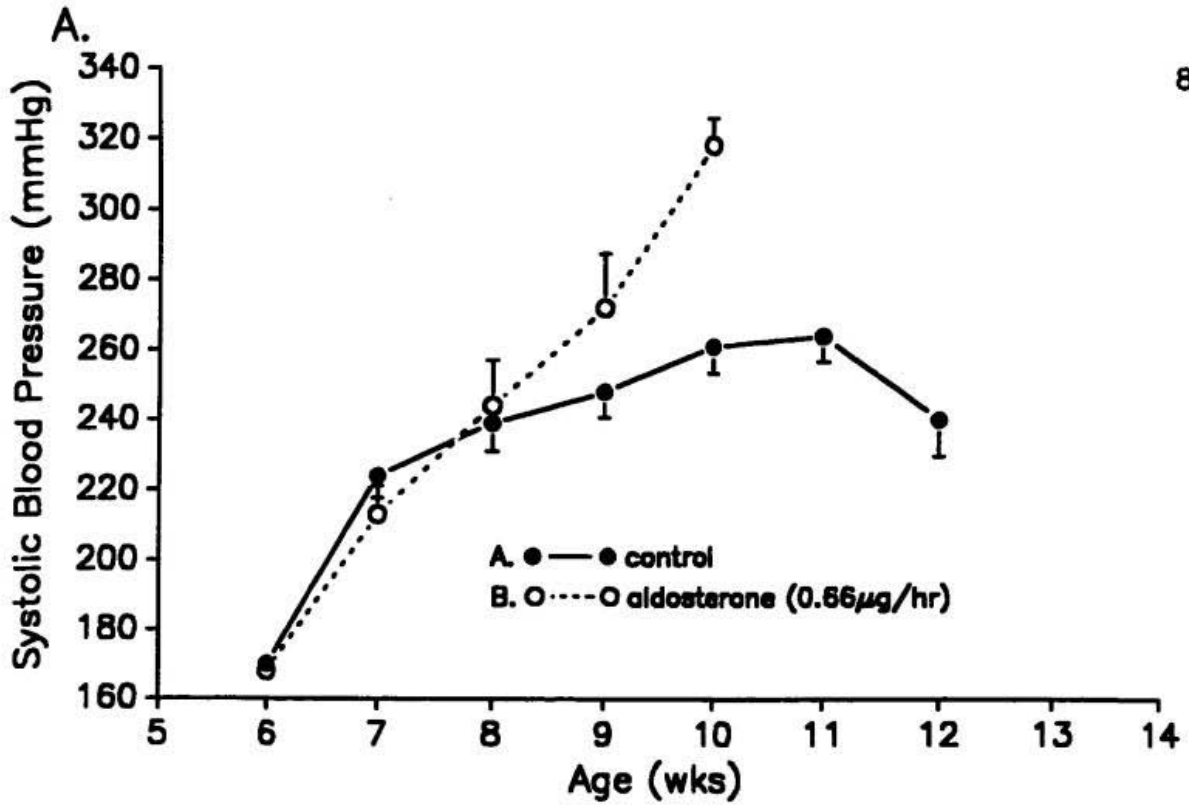
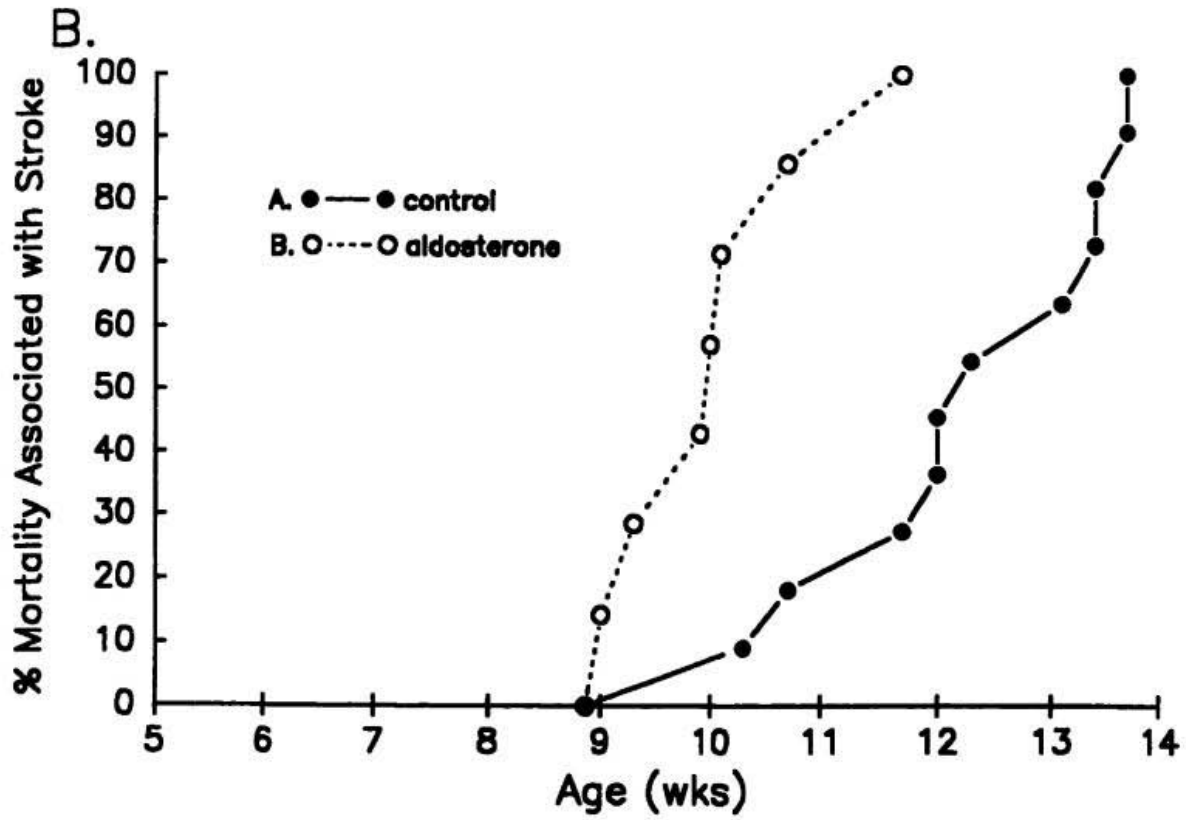

A.
Systolic Blood Pressure (mmHg)
300
280
260
240
220
200
180
160
140
120
86
A. ● — ● control
B. △ ····· △ captopril (50mg/kg/day)
C. □ ····· □ captopril (50mg/kg/day)
and aldosterone (0.66μg/hr)
4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42
Age (wks)
B.
% Mortality Associated with Stroke
100
90
80
70
60
50
40
30
20
10
0
A. ● — ● control
B. △ ····· △ captopril
C. □ ····· □ captopril and aldosterone
** experiment terminated
**
4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42
Age (wks)

Figure 7. Alterations in systolic blood pressure (A) and mortality associated with hemorrhagic stroke (B) in untreated SHRsp and SHRsp treated with aldosterone. Treatment of SHRsp with aldosterone (0.66µg/hr, s.c.) accelerated the onset of stroke development in SHRsp. Statistics -ANOVA- Fig 7A- over overlapping life spans, A vs B - NS; In Fig 7B - Mann Whitney - A vs B - $P < 0.01$ (n values A = 11, B = 7).


A.
Systolic Blood Pressure (mmHg)
340
320
300
280
260
240
220
200
180
160
5
6
7
8
9
10
11
12
13
14
Age (wks)
A. ● — ● control
B. O ---- O aldosterone (0.66μg/hr)


B.
% Mortality Associated with Stroke
100
90
80
70
60
50
40
30
20
10
0
5
6
7
8
9
10
11
12
13
14
Age (wks)
A. ● — ● control
B. O ---- O aldosterone

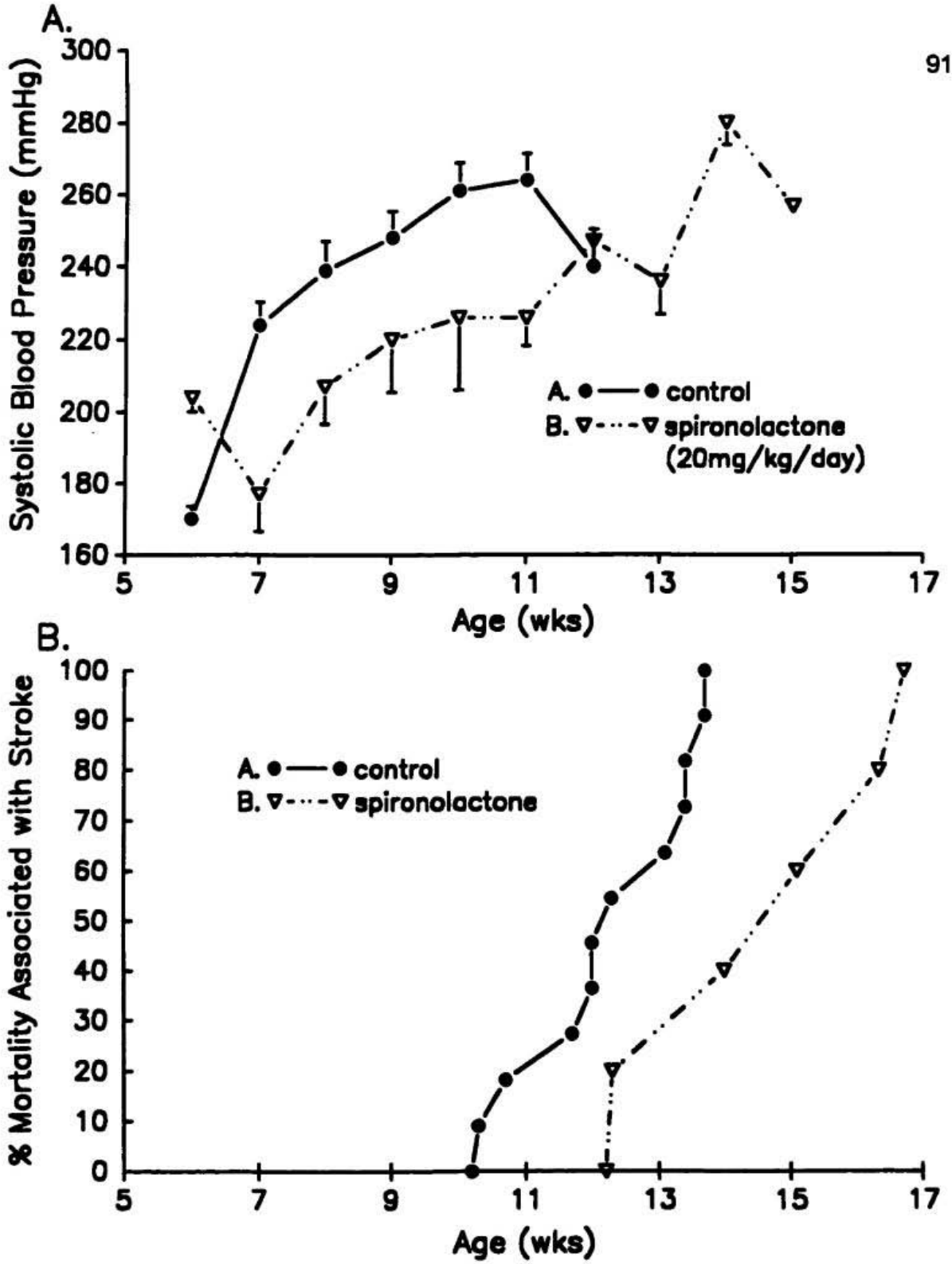
an analysis of plasma aldosterone levels of the SHRsp treated with captopril plus aldosterone infusion (Figure 5) indicated levels that were less than those of untreated SHRsp that developed stroke and greater than those present in comparably aged prestroke SHRsp. The data are consistent with the hypothesis that the ability of captopril to retard the onset of stroke development in SHRsp may be produced by a lowering of plasma aldosterone levels.

The effects of aldosterone treatment in the absence of captopril treatment is outlined in Figure 7A and 7B. When compared to non treated SHRsp over a common lifespan, aldosterone treatment did not significantly modify the BP's of SHRsp (Figure 7A) but did accelerate the onset of mortality associated with stroke (Figure 7B).

2.4.4.3 The Effect of Spironolactone Treatment on Mortality and Stroke Development in SHRsp

The results of these experiments are shown in Figure 8A and 8B. Statistical analysis performed on the BPs recorded over the common lifespans of the spironolactone and control groups indicated that the spironolactone treatment significantly reduced the BPs (Figure 8A) and retarded the onset of mortality associated with stroke relative to that observed in untreated SHRsp. The average age at death of these 2 groups of SHRsp was 14.7 and 12.5 weeks respectively. The magnitude of spironolactone's effect in retarding the onset of mortality in SHRsp was less than that observed in SHRsp treated with captopril.

Figure 8. Alterations in systolic blood pressure (A) and mortality associated with hemorrhagic stroke (B) in untreated control and spironolactone treated SHRsp. Spironolactone (20 mg/kg/day, s.c.) treatment of SHRsp significantly reduced blood pressure but produced only a small increase in the lifespan of the animals. Spironolactone treatment did not duplicate the effects of captopril treatment. Statistics -ANOVA- in Fig 8A - over lifespan of, A, A vs B - $P < 0.001$: in Fig 8B - Mann Whitney - A vs B - $P < 0.05$ (n values A = 11, B = 5).


A.
Systolic Blood Pressure (mmHg)
300
280
260
240
220
200
180
160
5
7
9
11
13
15
17
Age (wks)
A. ● —— ● control
B. ▽ - - - ▽ spironolactone
(20mg/kg/day)
B.
% Mortality Associated with Stroke
100
90
80
70
60
50
40
30
20
10
0
5
7
9
11
13
15
17
Age (wks)
A. ● —— ● control
B. ▽ - - - ▽ spironolactone

2.4.4.4 The Effect of Vehicle Treatment on Mortality and Stroke Development in SHRsp

The potential effects of the infusion/injection vehicles on stroke development within SHRsp were also studied. The injection vehicle for the administration of spironolactone, dexamethasone or deoxycorticosterone was olive oil which was administered at a volume of 0.1 ml/100 g body weight. SHRsp injected daily with the above vehicle died at an average age of 12.2 ± 0.3 weeks (range 11.2 to 13.0 weeks, n = 4). SHRsp infused with polyethylene glycol (aldosterone vehicle) at 0.5 or 2.5 µl/hr by implanted osmotic pumps from 6 weeks of age died at an average age of 13.6 ± 0.5 weeks (range 13.0 - 15.0 weeks). When mortality profiles were compared to those of untreated SHRsp (average age of death, 12.4 ± 0.36 weeks, range 10.3 to 13.7 weeks, n = 11), no significant differences were observed (Mann Whitney rank order test). In other experiments, captopril treated SHRsp (50 mg/kg/day) were injected with olive oil (the deoxycorticosterone vehicle, n = 4) or infused with polyethylene glycol (the aldosterone vehicle) by osmotic pumps (n = 4). None of these animals developed stroke. The latter experiments were terminated when SHRsp reached an average age of 17 weeks which surpassed the age at which a 100% mortality occurred within captopril treated SHRsp receiving either aldosterone infusion (0.66 µg/hr) or deoxycorticosterone injections (40 mg/kg 2X/week). When the alterations in blood pressure with age were statistically analyzed over comparable life spans, no significant differences were observed

between untreated SHRsp, SHRsp treated with captopril alone and SHRsp injected with olive oil or infused with polyethylene glycol vehicle in the presence or absence of captopril treatment (Group effect of treatments, P = 0.436). It was concluded that the acceleration in stroke development observed in SHRsp treated with deoxycorticosterone or aldosterone, the inhibition of the antistroke effects of captopril treatment observed during cotreatment with aldosterone or deoxycorticosterone and the antihypertensive effects observed during spironolactone treatment could not be attributed to the vehicles used.

2.4.5 DISCUSSION

The key finding of the present study was the observation that the reconstitution of plasma aldosterone levels to values observed prior to the suppression of this hormone by captopril treatment negated the antistroke effects of captopril treatment. Plasma aldosterone levels elevated with age in SHRsp after 10 weeks of age and were about 5 times higher in SHRsp that developed stroke when compared to similar aged SHRsp that had not yet developed stroke. This is a remarkable elevation in plasma aldosterone levels. Normotensive Kyoto Wistar rats (WKY, the normotensive strain used to develop SHR strains) typically exhibit 1 nmol/l levels of plasma aldosterone between 5 to 25 weeks of age (Kim *et al.*, 1991). At 12.5 weeks of age prestroke SHRsp had three times and poststroke SHRsp had ten times the plasma aldosterone levels typically observed within the normotensive strain. Captopril treatment of SHRsp suppressed plasma aldosterone

to levels comparable to those previously reported in WKY (Kim *et al.*, 1991) and prevented mortality associated with stroke up to 34 weeks of age. The reinfusion of aldosterone into captopril treated SHRsp to achieve levels present between pre and poststroke nontreated SHRsp totally negated the effects of captopril treatment and caused mortality to be comparable to that observed in untreated SHRsp. These results suggest that under conditions where AII levels should be suppressed by captopril, elevations in plasma aldosterone represent a significant risk factor for stroke development within SHRsp.

2.4.5.1 Reduced Plasma Aldosterone Levels May Account for the Antistroke Effects of Captopril

Untreated control SHRsp had much higher levels of plasma aldosterone levels once hemorrhagic stroke occurred when compared with prestroke control animals. When combined with the observation that plasma aldosterone increased with age prior to stroke, these data suggest that as the SHRsp grow older and approach the age at which they are likely to develop a cerebral hemorrhage, plasma aldosterone levels rise. Once the animals have sustained stroke, the plasma aldosterone levels may undergo an even greater increase. These findings suggest that the high plasma aldosterone levels may have contributed to the development of hemorrhagic stroke. Excessively elevated plasma aldosterone levels may also have played a role in promoting death after stroke developed. Consistent with this possibility, in the studies outlined in Chapter 1, we observed

that some SHRsp older than 30 weeks of age that appeared asymptomatic in terms of physical signs of stroke exhibited evidence of a past cerebral hemorrhage. As previously discussed, typically non treated SHRsp live (on average) for a period of 1.5 weeks after the first signs of stroke are observed. It is likely that some SHRsp may have developed stroke during captopril treatment and unlike nontreated SHRsp survived the stroke. In this regard, Smeda *et al.* (1999a) have observed that captopril treatment started at the first sign of stroke or up to 6 days after stroke in SHRsp prolongs the lifespan of the SHRsp (from 1.5 weeks) to between 4 to 15 weeks after stroke has developed. Aldosterone supplementation, such as that used in the present experiments, nullified the effect of captopril treatment in the above scenarios.

A concern in the present study resided in the fact that a continuous infusion of aldosterone (0.67 ug/ hr) was presented to SHRsp treated with captopril from 6 weeks of age and continued until approximately 11 to 13 weeks of age when the animals died. Over this age range, the animals grew larger which may have resulted in the younger, smaller animals receiving more aldosterone per kg of body weight compare with the older, larger SHRsp. This early over priming of the SHRsp at a young age could have promoted the subsequent development of stroke in SHRsp via a mechanism independent of a subsequent endogenous rise in aldosterone that naturally occurs just prior to stroke development. In a series of more recent investigations from our laboratory (Smeda *et al.*,1999a), captopril

treatment was begun in a group of SHRsp at 10 weeks of age. This corresponds to an age at which plasma aldosterone was shown to increase with time in studies outlined earlier. It was determined that when captopril treatment was begun at 10 weeks of age, there was no mortality associated with stroke through 24 weeks of age when the study was discontinued. Similarly, co-treatment of a group of SHRsp with captopril plus aldosterone beginning at 10 weeks of age showed that the aldosterone negated the antistroke effects of captopril treatment (captopril +aldosterone treated SHRsp mean age at death 16.5±0.8 weeks of age, n=6; untreated SHRsp mean age at death 14.0± 1 weeks of age, n=8; captopril treated SHRsp, no death up to 24 weeks of age, n=7). In other experiments Smeda *et al.* (1999a) observed that although the initiation of aldosterone cotreatment with captopril treatment in 10 week old SHRsp reversed the antistroke effects of captopril cotreatment, the initiation of aldosterone coinfusion in at 23 weeks of age in SHRsp treated with captopril from 10 weeks of age did not significantly alter the lifespan of the SHRsp when compared to SHRsp solely treated with captopril. Both groups lived on average to about 28 weeks of age. This would suggest that elevations in plasma aldosterone in prestroke SHRsp from 10 weeks on, exert a significant impact in enhancing stroke development during captopril treatment, however very likely long term captopril treatment (up to at least 23 weeks of age) may promote a resistance to stroke development in which the antistroke effects of captopril are perpetuated in the presence or absence of high plasma aldosterone

levels. It is also possible that there is an age related window in SHRsp during which elevations in plasma aldosterone may be particularly lethal with respect to stroke development.

Treating SHRsp with aldosterone alone did not significantly change the BPs profile yet it significantly accelerated the mortality rate compared with untreated control SHRsp. The effect on mortality was not dramatic, on average, the aldosterone treated SHRsp died 2.5 weeks earlier than control SHRsp. One might expect aldosterone treated SHRsp to have extreme hyperaldosteronemia, which in turn would be associated with a dramatic increase in mortality rate relative to controls. A possible explanation for the modest impact of aldosterone infusion (in the absence of captopril treatment) on mortality could be due to the fact that untreated control SHRsp died on average at 12.5 weeks of age. This represents a relatively young animal. It is reasonable to speculate that the development of hemorrhagic stroke and subsequent death requires a minimal period of time. Hence it might be difficult to accelerate stroke onset despite the presence of severely high aldosterone levels.

2.4.5.2 The Mechanisms of Aldosterone's Stroke Effects in SHRsp

2.4.5.2.1 The Possibility that Aldosterone Mediates Stroke in SHRsp by Stimulating the Mineralocorticoid Receptor

After observing the relationship between the onset of stroke development and elevations in plasma aldosterone in SHRsp we assumed that aldosterone's

actions would be mediated solely via the stimulation of renal mineralocorticoid receptors. If this were the case, it would present a plausible hypothetical mechanism explaining aldosterone's action. It is clear that a high salt diet accelerates stroke development in SHRsp (Smeda. 1989). We had the conception that elevations in plasma aldosterone in SHRsp may act to promote sodium and water retention by stimulating renal mineralocorticoid receptors. Hence, aldosterone might act in a manner equivalent to feeding the rats excessive salt and thus promote stroke. If this were the case then treating the SHRsp with spironolactone, a mineralocorticoid receptor antagonist should duplicate the effects of captopril treatment. Spironolactone treatment was successful in significantly reducing both BPs and the mortality rate associated with stroke in SHRsp. The intracellular mineralocorticoid receptor is a possible mediator of these effects. However, the effect that spironolactone treatment had on the mortality rate was considerably less robust than that which was observed in the captopril treated SHRsp. Since the BPs and mortality rate of SHRsp were significantly altered by spironolactone treatment compared with controls, we concluded that the drug was active. An explanation considered to account for spironolactone's modest effects on mortality rate was the possibility that the once daily dosing of spironolactone (20 mg/kg/day) was too infrequent to allow the drug to exert its full effects. However, this seems unlikely as canrenone, the spironolactone metabolite and primary mineralocorticoid receptor antagonist has a plasma half life of 16 hours in humans (Range and Dale, 1991).

The possibility was considered that the 20 mg/kg/day subcutaneous dose of spironolactone was insufficient in concentration to block the mineralocorticoid receptors. In this regard, it was noted that the dose used in these investigations was approximately 4 times the per kg oral dose used to treat humans with primary hyperaldosteronism (Young and Klee, 1988). There is evidence that certain effects associated with high plasma aldosterone in rats are not reversed by comparable doses of spironolactone. For example, using a rat model of primary hyperaldosteronism, Brilla *et al.* (1993), demonstrated hypertension, left ventricular hypertrophy and myocardial fibrosis. A 20 mg/kg/day, subcutaneous dose of spironolactone was able to reduce but not normalize blood pressure. It prevented myocardial fibrosis but did not change left ventricular hypertrophy. Alternatively, these same investigators found that increasing the dose of spironolactone to 200 mg/kg/day (subcutaneously) normalized blood pressure and prevented left ventricular hypertrophy and myocardial fibrosis.

A pilot study was undertaken to determine what effect, if any, a larger dose would have on mortality rate in SHRsp. A dose of 150 mg/kg/day, subcutaneous, was used to treat SHRsp and no significant change in mortality rate was noted. In more recent studies (Smeda *et. al.*,1999a) involving the treatment of our SHRsp with a daily dose of spironolactone at 120 mg/kg/day from 10 weeks of age did not greatly alter the onset of stroke development in SHRsp (on the other hand captopril treatment started at the same age did retard stroke and aldosterone coinfusion

reversed these effects). Consequently, it was concluded that spironolactone's modest effect on mortality rate in SHRsp in the present study was likely not the result of an insufficiently large dose of the mineralocorticoid antagonist.

Aside from the above arguments there is other indirect evidence that is consistent with the likelihood that spironolactone would be an ineffective antistroke agent. A wide variety of other diuretic's (furosemide, amiloride, chlorothiazide, acetozolamide) that promote urinary sodium and water loss (in the presence or absence of potassium loss) have been tested in SHRsp and none of these have been able to alter the onset of stroke development in these animals (Smeda and Trachenko, 1991). Ironically, in the latter study furosemide, a diuretic that can enhance AII production accelerated stroke development in SHRsp. If spironolactone was acting at renal mineralocorticoid receptors, promoted salt and water loss into the urine, and if this mechanism was important in altering the onset of stroke, then one might expect that other diuretics could mimic this response. As it stands the general noneffectiveness of diuretics in altering stroke development in SHRsp supports the notion that spironolactone induced diuresis and natriuresis (via mineralocorticiod receptor blockade) is not a mechanism involved in altering the onset of stroke in SHRsp.

2.4.5.2.2 The Possibility that Aldosterone Mediates Stroke in SHRsp by Stimulating Nongenomic Receptor(s)

There is an additional possibility that would explain spironolactone's modest

effects on mortality rate of SHRsp. Aldosterone's proposed pathological role may not be mediated via the stimulation of intracellular mineralocorticoid receptors. If this were the case the system would be spironolactone-insensitive.

There is a possibility that aldosterone may activate surface (nongenomic) membrane receptor(s) in addition to the intracellular mineralocorticoid receptors. One rapid, nongenomic effect of this mineralocorticoid appears to be activation of the phospholipase C (PLC) second messenger cascade in the vascular smooth muscle cells (VSMC's) (Christ *et al.*, 1995b). The significance of these actions within the context of the present investigations is unclear. A speculative effect of an aldosterone mediated PLC system activation is offered below.

An important mechanism in the autoregulation of cerebral blood flow (described in more detail in Chapter 3) is the occurrence of cerebrovascular constriction in response to elevations in blood pressure. It has been proposed that blood flow could be regulated distally by the constriction of the cerebral vasculature under conditions of elevated pressure. The loss of such a function could promote overperfusion of the distal vasculature under hypertensive conditions thereby promoting the formation of cerebral hemorrhage (Smeda, 1992). This autoregulatory mechanism, referred to as pressure dependant constriction (PDC) is thought to be mediated in part by activation of protein kinase C (PKC) (Osol *et al.*, 1991) which can also be activated by PLC activation. If in fact aldosterone activated the PLC pathway in VSMC's, it could have altered vascular tone and PDC.

Furthermore, such an effect would not be mediated via the intracellular mineralocorticoid receptor and therefore would not be blocked by spironolactone. Under prolonged conditions of hyperaldosteronism with or without spironolactone treatment, the PLC pathway may have been chronically hyperactivated, eventually becoming functionally compromised. Under continued severe hypertensive conditions, the PDC response could have failed due to a compromised PLC/PKC system. This may in turn, have promoted overperfusion in the distal vasculature and subsequent hemorrhagic stroke formation.

In addition to PKC activation, VSMC depolarization has also been proposed as a possible mediator of PDC (Nelson and Quayle, 1995). Aldosterone has also been reported to increase the Na^+ /K^+ exchanger activity in VSMC's (Christ *et al.*, 1995a). The elevated plasma aldosterone levels documented in SHRsp may bind to the putative membrane surface receptor. In turn, this may activate the Na^+/H^+ exchanger producing altered ionic fluxes within the VSMC's, a process that could alter PDC by modifying the voltage activating mechanisms that contribute to the initiation of PDC. Such an alteration could also modify cerebral blood flow autoregulation in a manner conducive to the initiation of stroke.

CHAPTER 3: THE EFFECTS OF STROKE AND CAPTOPRIL TREATMENT ON CEREBROVASCULAR FUNCTION IN SHRsp

LITERATURE REVIEW

3.1 THE ROLE OF PRESSURE DEPENDENT CONSTRICTION IN PROMOTING CEREBRAL BLOOD FLOW AUTOREGULATION

Johnson (1986) described autoregulation of blood flow as the tendency of blood flow to remain constant in an organ or tissue despite changes in arterial pressure. One important mechanism that contributes to the maintenance of cerebral blood flow autoregulation is the ability of cerebral blood vessels to respectively constrict or enlarge their lumen diameters in response to increases or decreases in pressure. The blood flow (BF) through a vascular bed is governed by the blood pressure drop across the vascular bed (ΔP, the infusion pressure forcing blood into the organ/tissue [primarily the mean systemic blood pressure, near 100mmHg in humans] minus the back pressure [the postcapillary venule pressure near 15 mmHg] preventing the blood from leaving the organ/tissue) and by the vascular resistance (VR) to blood flow (i.e., $BF = \Delta P/VR$). ΔP is primarily governed by the systemic blood pressure and a major determinant of VR is the lumen diameter size of the arterioles. If the internal diameter of a tube was reduced by 1/2 (increasing VR), flow through the tube would decrease to 1/16 at a constant ΔP. In an organ such as the brain, elevations in blood pressure that might produce an increase in flow are counteracted by an appropriate degree of arterial vascular

constriction which raises vascular resistance to blood flow and helps to maintain constant blood flow through the system despite the presence of elevated blood pressure. The ability of some classes of arteries to constrict to elevations in pressure can be demonstrated in isolated arterial segments under *in vitro* conditions, hence it is a characteristic, that is not mediated by the release of substances from extravascular tissues or nerves and appears to occur in response to vascular stretch produced by elevations in pressure. In isolated arteries, this response has been termed the Bayliss phenomena or response (Bayliss, 1902), the myogenic response (Smeda, 1993) or pressure dependent constriction (PDC) (Smeda, 1992,1993). The ability of an isolated vessel to reduce its lumen diameter in response to elevated intravascular pressure is referred to as PDC throughout this thesis.

3.1.1 The Characteristics and Occurrence of Pressure Dependent Constriction in Vasculature

PDC was first described by Bayliss (1902) who cannulated one end of a canine carotid artery and tied off the other end in a manner that permitted the manipulation of intravascular pressure. It was reported that when the pressure was raised, the artery initially began to swell then it immediately constricted. If the pressure was reduced, relaxation of the artery was noted. More recently, similar observations have been made in a variety of vascular beds. For example, Kuo *et al.* (1990) examined isolated porcine subepicardial arterioles cannulated at both

ends for the manipulation and evaluation of intraluminal pressures. The vessels were visualized using a microscope and video system (video microscopy) providing continuous measurement of the lumen diameter. Dilation was observed when pressures were decreased from 60 to 20 cmH_2O whereas constriction was noted when pressures where increased from 60 to 140 cmH_2O. The authors concluded that PDC may play an important role in the regulation of coronary blood flow.

Osol and Halpern, (1985) used video microscopy to study PDC in posterior cerebral arteries (PCA) isolated from Kyoto Wistar stroke prone spontaneously hypertensive rats (stroke resistant strain) (SHR) and Wistar Kyoto normotensive rats (WKY). These authors reported constriction in response to elevations in pressure and dilation in response to reductions in arteries taken from both strains of rats. However, the PDC in the vessels sampled from SHR was limited to maintaining a constant diameter over a range of pressures whereas WKY vessels showed a reduction in vessel diameter.

Evidence of PDC in human arterioles was reported by Miller *et al.* (1997) using coronary arterioles from patients undergoing cardiac surgery. The coronary arterioles were cannulated, equilibrated at 100 mmHg for 45 minutes and visualized using microscope and video equipment. The investigators reported their results relative to the passive diameter attained in the presence of vasorelaxation with sodium nitroprusside. At 60 mmHg. the arterioles constricted to 55% of their passive lumen diameter. Wallis *et al.* (1996) dissected arterial segments from

cerebral biopsy specimens of patients undergoing resection procedures for neoplasms. The authors explained that vessels were taken from an area as far as possible from the diseased area. All patients were normotensive with no evidence of cardiovascular disease. Arteries were cannulated and mounted in a myograph such that pressure could be manipulated and vessels were allowed to equilibrate at 60 mmHg for 90 minutes. The arterial diameter was found to either decrease or remain constant during a series of pressure steps. The response was lost in Ca^{2+} -free Krebs with EGTA. The investigators concluded that the PDC response may contribute to autoregulation of cerebral blood flow in humans in the face of varying arterial blood pressures.

3.2 THE SIGNAL TRANSDUCTION MECHANISMS PROMOTING PRESSURE DEPENDENT CONSTRICTION

McCarron *et al.* (1997) proposed two mechanisms to explain the signal transduction pathways responsible for the production of PDC. PDC was reported to rely on a stretch-induced activation of protein kinase C (PKC); causing an increased Ca^{2+} sensitivity of the myofilaments within the vascular smooth muscle cells (VSMC). In addition, vascular stretch produced by elevated pressures was thought to promote an increase in intracellular [Ca^{2+}] levels by depolarizing the VSMC, thereby augmenting the opening probability of voltage-dependent Ca^{2+} channels.

3.2.1 Phospholipase C, the Inositol Phosphate and Protein Kinase C Second Messenger System

A common signal transduction mechanism that promotes constriction of VSMC's in response to a variety of vasoconstrictors such as vasopressin, norepinephrine and 5-hydroxytryptamine involves the activation of phospholipases. Phospholipase C (PLC) is an important activator of vasoconstriction (Horowitz *et al.*, 1996; Takuwa, 1996). PLC activation primarily promotes the breakdown of membrane phosphotidyl inositol, which through a number of intermediate steps forms inositol triphosphate (IP_3) and diacylglycerol (DAG). IP_3 releases intracellular sacroplasmic stores of Ca^{2+}, elevating intracellular Ca^{2+} which activates actin and myosin interaction and promotes vasoconstriction. DAG activates PKC which through a signal cascade can promote the phosphorylation of caldesmon and or calponin. These latter intermediates act on actin filaments in the VSMC's and inhibit actin to myosin interaction. When phosphorylated, calponin and caldesmon lose their ability to inhibit constriction. Under conditions of PKC activation, low levels of intracellular Ca^{2+} can promote constriction (Nishimura and van Breeman, 1989). In addition, PKC activation can directly open voltage gated Ca^{2+}channels in a voltage independent manner (Fish *et al.*, 1988). The activation of other phospholipases (phospholipase D and phosphatidyl choline dependent PLC) can promote the breakdown of phosphatidyl choline which leads to the formation of

DAG (and PKC activation) in the absence of IP_3 formation (Boarder, 1994; Zannetti *et al.*, 1997; Liu *et al.*, 1999).

Evidence has been reported which supports of a role for various components of the PKC second messenger system in the generation of PDC. For example, using video microscopy, Osol *et al.* (1993) investigated the role of PKC in pressure induced tone in a branch of the rat posterior cerebral artery and mesenteric vessels. The investigators found that indolactam (PKC activator) augmented the degree of tone in cerebral arteries pressurized to 125 mmHg. Conversely, comparably sized mesenteric arteries which do not generate pressure induced tone, were unaffected by indolactam at 125 mmHg. Staurosporin (inhibitor of PKC) produced a dose dependant vasodilation of pressurized cerebral arteries. Staurosporin was also found to be a more potent inhibitor of tone in cerebral arteries at 125 mmHg compared with tone induced by K^+ depolarization. The authors concluded that their findings support a role for PKC in the regulation of pressure dependent vascular tone.

The role of PKC in the maintenance of tone in pressurized human coronary arterioles was examined by Miller *et al.* (1997). Addition of the PKC inhibitor calphostin C resulted in a dose dependent reduction in tone. In arterioles treated with the vasoconstrictor endothelin (to produce a similar degree of constriction) calphostin C produced no change in diameter. Next, these investigators examined arterioles that dilated instead of constricting in response to an acute elevation in

transmural pressure (i.e., lacked PDC). The addition of the PKC activator phorbol 12-myristate 13-acetate permitted PDC to occur in the arterioles in response to elevations in transmural pressure.

In other studies, Osol *et al* (1993) examined the possibility that PLC was involved in the generation of pressure dependent tone in posterior cerebral arteries from normotensive rats. The inhibition of PLC by U-73122 produced a concentration dependent vasodilation. The action of the inhibitor was confirmed by showing that the PLC mediated constriction to serotonin could be significantly attenuated or abolished by U-73122. The authors concluded that their results supported a role for PLC in the maintenance of pressure dependent tone. They explained that the previously reported modulation of cerebral arterial pressure dependent tone by PKC may occur through the activation of PLC which could form DAG, a potent activator of PKC.

It has been reported that stretch of the vascular wall produced when arterial pressure rises may be the stimulus that leads to PDC. Kulik *et al.* (1991) investigated the effects of stretching VSMC's. Smooth muscle cells from the main and proximal branches of the pulmonary artery from adult Sprague-Dawley rats were isolated and cultured on thin silicone sheets. Preliminary studies reportedly showed that cells could remain attached to the sheets with a 20% stretch for at least 1 hour. IP_3 levels in the VSMC's were found to be significantly elevated following a 20% stretch for 25 seconds. The authors concluded the VSMC's can

respond to mechanical stretch with an increase in IP_3. Studies performed by Narayanan *et al.*, 1994, have indicated that DAG and IP_3 elevated in the VSMC's of pressurized canine renal arteries

Vascular smooth muscle cell Ca^{2+} levels were measured during both agonist and pressure induced constriction in arterioles from the rat cremaster muscle (Meninger *et al.*, 1991). These isolated arterioles were cannulated and Ca^{2+} levels were determined using the Ca^{2+} sensitive dye fura-2, imaged via a fluorescence microscope. PDC was induced by a transmural pressure step from 90 to 130 cmH_2O. The vessel diameter was reported to show a transient increase followed by a return to baseline or slightly smaller value. This response was associated with a concurrent 8% increase in vessel wall Ca^{2+}.

3.2.2 Pressure Dependent Membrane Depolarization as a Mediator of Constriction

Changes in the resting membrane potential (Em) of VSMC's has also been proposed as a possible mediator of pressure induced contraction in several arterial preparations (Smeda and Daniel, 1988). Roman and Harder (1993) showed that increasing the transmural pressure from 20 to 120 mmHg produced smooth muscle cell membrane depolarization from -57 mV to -38 mV in isolated perfused canine renal arcuate arteries. The relationship between transmural pressure and resting membrane potential was also investigated using cannulated canine interlobular arteries (Harder *et al.*, 1987). These authors documented membrane depolarization

from a control value of -57 mV at 20 mmHg to -37 mV at 120 mmHg. It was suggested that Em depolarization may allow Ca^{2+} to enter via voltage-gated channels. It was interesting to note however, that while the membrane potential was shown to be significantly depolarized at each pressure step relative to a control value at 20mmHg, the ability to maintain near constant diameter with increasing transmural pressure did not begin until pressures greater than 60 mmHg were applied. This suggests that VSMC depolarization alone is unable to account for the ability of canine interlobular arteries to maintain their diameter under conditions of elevated pressure.

Depolarization of VSMC membrane potential during pressurization has also been investigated in the cerebral circulation (Harder, 1984). Cat middle cerebral arteries (MCA) were investigated using glass microelectrodes and visualized with microscopic and photographic equipment. Vessel diameter and smooth muscle Em were documented during manipulations of transmural pressure. When the transmural pressure was increased from 20 to 90 mmHg, the vessel constricted and the membrane potential altered from -52 mV. Increasing transmural pressure further to 140 mmHg also resulted in vessel constriction as well as further VSMC depolarization to a value of -44 mV. Reducing Transmural pressure from 140 to 60 mmHg caused the membrane potential to return to a more negative value of -62 mV.

A comparison of membrane potential values at various transmural pressure's

was made using cannulated middle cerebral arteries from SHR and WKY normotensive rats by Harder *et al.* (1985). At 0 mmHg the membrane potentials recorded in the vessels from SHR and WKY were not significantly different from each other. However, at transmural pressure values greater than 40 mmHg and up to 100 mmHg, the VSMC from SHR cerebral vessels were more depolarized relative to those from WKY rats. These investigators also reported that MCA's from SHR possessed a greater degree of active tone relative to WKY. It was suggested that the VSMC membrane from SHR's may respond to elevations in transmural pressure in an exaggerated fashion. Also, that this greater degree of membrane depolarization may allow more Ca^{2+} entry in vessels from SHR compared with WKY. Similar findings were reported by Miller *et al.* (1997) using human coronary arterioles. Pressurized vessels from hypertensive patients were shown to possess greater pressure induced vasoconstriction relative to vessels from normotensive patients.

Recently, the ion responsible for carrying the depolarizing current has been investigated using cannulated rat PCA's. Using microelectrodes, two Cl^- channel blockers were found to produce VSMC hyperpolarization and dilation of arteries pressurized to 80 mmHg. These same agents had no effect on membrane potential when pressure dependant tone was absent. These investigators reported that activation of Cl^- channels in VSMC's may produce an outward Cl^- current leading to depolarization which increases the entry of Ca^{2+} into VSMC's through voltage

dependant Ca^{2+} channels (Nelson *et al.*, 1997).

3.2.3 The Involvement of Voltage Gated (Dihydropyridine Sensitive) Ca^{2+} Channels in Mediating Pressure Dependent Constriction

Karibe *et al.* (1997) examined the role of cytosolic Ca^{2+} and PKC in pressure induced contraction. Branches of the femoral artery isolated from Wistar rats were cannulated and visualized with a video digitizing system. Cytosolic Ca^{2+} levels were assayed using the Ca^{2+} sensitive dye, fura-2. The small skeletal muscle arteries were shown to dilate and immediately return to baseline diameter values when the transmural pressure was increased from 40 to 100 mm Hg. A concurrent 33% increased in cytosolic Ca^{2+} relative to baseline was also reported. Next, these authors treated the vessels with the inhibitors of PKC, H7 and staurosporin which abolished the pressure induced contraction but did not block the rise in cytosolic Ca^{2+}. When the skeletal muscle arteries were treated with the dihydropyridine Ca^{2+} channel blocker nifedipine, both the myogenic contraction and the increase in cytosolic Ca^{2+} was abolished. The investigators in this study concluded that an increase in cytosolic Ca^{2+} alone may not be enough to cause myogenic contraction in small skeletal muscle arteries. Also, that an adequate PKC activity may be necessary to couple the increase in cytosolic Ca^{2+} to smooth muscle contraction.

Further studies of the effects of dihydropyridine Ca^{2+} channel blockers on PDC were conducted using nimodipine (Haws and Heistad, 1984). Cat pial

arteries were viewed via craniotomy and diameter changes were documented with a microscope and video equipment. A transient rise in mean arterial pressure of 20 and 40mmHg was accomplished by partial ligation of the thoracic aorta. Pressure changes were measured proximal to the ligation. Under control conditions, pial arteries respectively showed a 6.6 and 12.1% reduction in diameter in response to a 20 and 40mmHg mean arterial pressure step. Intravenous nimodipine caused the pial vessels to dilate (Haws and Heistad, 1984).

The actions of a dihydropyridine Ca^{2+} channel antagonist on PDC were also investigated in the cerebral circulation by McCarron *et al.* (1997). These investigators studied cannulated PCA's from WKY rats using a microscope and video equipment. Using a 30 to 70 mmHg pressure step, vessel diameter returned to resting values following an initial distension, under control conditions. This ability to maintain diameter in the face of increasing pressure was abolished by the two dihydropyridine Ca^{2+} channel blockers (nimodipine and (-) 202 791) as well as by the removal of extracellular Ca^{2+}. These results were offered as confirmation that PDC requires Ca^{2+} entry through, dihydropyridine sensitive voltage-dependent Ca^{2+} channels.

3.2.4 The Involvement of Stretch Activated Dihydropyridine Insensitive Ca^{2+} Channels in Mediating Vascular Constriction

A number of studies primarily (but not exclusively) involving venous tissue

have demonstrated the presence of Ca^{2+} channels that are activated by mechanical stretch which are distinct from the dihydropyridine-sensitive, voltage-dependent Ca^{2+} channel.

Laher *et al.* (1988) found that the generation of mechanical stretch of rabbit facial vein rings by wires induced constriction that was accompanied by an increase in $^{45}Ca^{2+}$ influx into the VSMC's and was not affected by a concentration of the dihydropyridine Ca^{2+} channel blocker (PN 200-110) that significantly reduced tone and $^{45}Ca^{2+}$ influx in vessels contracted by K^+ depolarization. The authors proposed that the influx of $^{45}Ca^{2+}$ occurred through non voltage gated Ca^{2+} channels. In other studies from the same laboratory involving strips rabbit basilar arteries, diltiazem (a voltage gated Ca^{2+} channel blocker) was found to effectively block agonist induced contraction (norepinephrine, 5-hydroxytryptamine and K^+), however it was not effective in altering stretch induced tone (Bevan, 1982). Hwa and Bevan (1986) examined the effects of several different Ca^{2+} blocking agents on K^+ and stretch induced tone in a rabbit ear artery ring preparation. The investigators reported that both K^+ and stretch induced tone were similarly suppressed by reducing the extracellular Ca^{2+} concentrations, by the inorganic Ca^{2+} antagonists, Mn^{2+} and Mg^{2+} as well as by the non-dihydropyridine Ca^{2+} blockers verapamil and diltiazem. In contrast however, the dihydropyridine Ca^{2+} channel blockers nimodipine and nifedipine were not successful in blocking the stretch induced tone. The authors

suggested that a dihydropyridine resistant Ca^{2+} pathway operated by stretch may be involved in promoting stretch induced tone.

Harder (1984) noted that when cerebral arterial rings are stretched between two wires, the Em recorded from VSMC's is not depolarized compared to the resting state, whereas VSMC's from cerebral arteries were shown the depolarize in response to pressurization. It was suggested that dihydropyridine-sensitive voltage-dependant Ca^{2+} channels play a role in constriction of pressurized but not in mechanically stretched arterial vessels. Harder (1984), speculated that threading wires through small arterial rings may cause cellular damage resulting in a situation where VSMC's do not depolarize. McCarron *et al.* (1997) also suggested that the differential effects of dihydropyridine- Ca^{2+} channel blockers on stretch and pressure induced vascular tone could be partially explained by different VSMC membrane potentials under different technical conditions at the time of dihydropyridine- Ca^{2+} channel blocker administration.

3.3 STROKE RELATED ALTERATIONS IN CEREBROVASCULAR PRESSURE DEPENDENT CONSTRICTION IN SHRsp

Smeda (1992, 1994) suggested that the cerebral hemorrhages observed in the brains of SHRsp may result from the loss of the cerebrovasculature's ability to constrict in response to elevations in blood pressure. The loss of this function could promote conditions of elevated pressure and overperfusion of the

down stream vasculature, causing the blood vessels to burst and cerebral hemorrhage to form. In studies, MCA's were sampled from stroke resistant SHR (characteristics discussed in section 1.2.1) as well as pre and poststroke SHRsp. Using video microscopy, the ability of the MCA's to constrict to a 100 mmHg pressure step was used as a measure of PDC . Pressurisation of MCA's revealed that vessels isolated from SHR possessed the greatest degree of PDC while those sampled from poststroke SHRsp lacked the ability to elicit PDC. The loss of PDC preceded stroke development in SHRsp (Smeda, 1992). More recent studies (Smeda and King, 2000) have indicated that there is an age related decline in the ability of the MCA's of prestroke SHRsp to elicit PDC after about 11weeks of age, 1 to 2 weeks prior to stroke.

Laser Doppler studies (Smeda *et al.* 1999b) indicated that under *in vivo* anesthetized conditions, the brain of SHRsp loses its ability to autoregulate cerebral blood flow in animals that develop stroke. Young 10 week old prestroke SHRsp were capable of maintaining near constant cerebral blood flow up to mean arterial blood pressures of 200 mmHg while older (13 week old) poststroke SHRsp exhibited a linear increase in cerebral blood flow when blood pressure was increased from 120 to 260 mmHg in a slow continuous manner. This suggested the presence of extremely high cerebral blood flows in poststroke SHRsp. Of particular importance, the inability to autoregulate cerebral blood flow preceded stroke development in SHRsp. Age matched prestroke SHRsp

littermates to the poststroke SHRsp also exhibited an inability to autoregulate cerebral blood flow. On the basis of these experiments it was suggested that if PDC was important in maintaining cerebral blood flow autoregulation, a loss of PDC could contribute to the loss of autoregulation in poststroke SHRsp. Since both defects preceded stroke development in SHRsp, it was further suggested that a loss of such functions under hypertensive conditions, could promote very high cerebral blood flows in older prestroke SHRsp. It was hypothesized that the high pressures and flows in the cerebral microvasculature could predispose the vasculature to rupture, which in turn could create intracerebral hemorrhages.

Smeda and King (1999) studied the mechanisms promoting the loss of PDC in the MCA's of SHRsp. PDC in the MCA's of young prestroke SHRsp was inhibited by PKC inhibitors such as staurosporin, chelerythrine and bisinolylmaleimide. These inhibitors not only blocked the ability of the MCA's to constrict to pressure but also inhibited the ability of PKC activators (phorbol dibutyrate) to elicit vasoconstriction in the same MCA's (suggesting that the inhibitors were in fact acting as PKC inhibitors). When the MCA's of young prestroke SHRsp with robust PDC responses, older prestroke SHRsp with attenuated PDC, and poststroke SHRsp with no PDC were compared, a direct correlation was observed between the ability of the MCA's to elicit PDC and their subsequent ability to constrict in response to direct pharmacological PKC activation by phorbol esters. These experiments suggested that PKC activation

may be partially responsible for the production of PDC and that defects in the PKC system could contribute to the stroke related loss of PDC in the MCA's of SHRsp.

In other studies, the electromechanical properties of the MCA's were assessed (Smeda and King, 2000). When compared to younger prestroke SHRsp (with MCA's that exhibited PDC), the MCA's of poststroke SHRsp lacked the ability to alter their VSMC Em in response to a 100 mmHg pressure step. These arteries maintained a constant depolarized VSMC Em at all pressures. This feature was further investigated. Patch clamp studies of isolated VSMC's from poststroke SHRsp indicated that the depolarized state of the VSMC's was produced by a very high Cl^- conductance in the cells (Dr. John Smeda, personal communication). Other investigations indicated that an inability to alter VSMC Em in response to pressure shifts was not the only defect observed in the VSMC's of the MCA's. The MCA's VSMC's of poststroke SHRsp also exhibited defective voltage gated Ca^{2+} channels (Smeda and King, 2000). The channel behaved in a manner consistent with the possibility that it was locked in a semi open state but was incapable of reacting to changes in Em. The channel could be blocked by low levels of dihydropyridine antagonists (nifedipine) but acted as if the voltage sensor of the channel was defective. A symptom of this defect was the observation that an elevation in extracellular K^+ which promote VSMC depolarization, open voltage gated Ca^{2+} channels, and promote constriction

could not produce constriction in the MCA's of poststroke SHRsp while pharmacological agonists such as vasopressin that were capable of releasing an internal sarcoplasmic store (likely via an IP_3 mediated release) of Ca^{2+} (Nemenoff, 1998) did constrict the MCA's.

In summary, the above experimental evidence would suggest that PDC in the MCA's of SHRsp likely involves pressure induced VSMC activation of the PKC system. Activation of the latter system could promote constriction by sensitizing the contractile apparatus to Ca^{2+} and or by the direct voltage independent opening of voltage gated Ca^{2+} channels (see section 3.2.1). PDC may also involve the opening of VSMC voltage gated Ca^{2+} channels via pressure dependent VSMC depolarization (see sections 3.2.2, 3.2.3). Defects in the PKC system, and an inability to promote constriction in response to VSMC depolarization possibly because of a defect in the VSMC voltage gated Ca^{2+} channels may account for the loss of PDC in the MCA's of poststroke SHRsp. Of importance to the subsequent experimental sections is that the symptomatology of the above defects is manifested in isolated MCA segments by the observation that the MCA's are unable to constrict in response to PKC activation by phorbol esters or in response to high K^+ induced VSMC depolarization.

EXPERIMENTAL RESEARCH

3.4 THE EFFECTS STROKE AND CAPTOPRIL TREATMENT ON MIDDLE CEREBRAL ARTERY FUNCTION IN SHRsp

3.4.1 THE OBJECTIVES OF THE STUDY

Pressure dependent constriction (PDC) within MCA's becomes compromised in SHRsp a few weeks prior to the development of stroke (Smeda 1992, Smeda and King 2000) and is absent in animals following the development of cerebral hemorrhage. The purpose of this series of *in vitro* experiments was to determine if captopril treatment of SHRsp from weaning preserved the ability of the MCA's to constrict to pressure. If captopril treatment was found to preserve PDC in the MCA's, experiments were designed to determine if functional aspects of the signal transduction mechanisms thought to be involved in promoting PDC were also preserved following treatment. In this regard, the presence of PDC in the presence of a dysfunction within a particular signal transduction mechanism would suggest that the signal transduction mechanism may not be involved in promoting PDC and might be altered as a consequence of hypertension. Because of the evidence implicating both VSMC depolarization induced opening of dihydropyridine-sensitive voltage dependent Ca^{2+} channels and protein kinase C (PKC) activation in the generation of PDC, experiments were designed to test the ability of the MCA's of SHRsp to constrict to both depolarization (via an elevation in external potassium concentration

($[K^+]_o$)) and PKC activation (via phorbol dibutyrate in the presence of nifedipine) in relation to captopril treatment. Finally, vessels were exposed to vasopressin to ensure that any alterations in PDC or responses to phorbol dibutyrate or $[K^+]_o$ were not the result of a generalised failure of vascular smooth muscle (VSMC) contractility in the MCA's.

3.4.2 HYPOTHESES

We tested the hypothesis that captopril treatment of SHRsp from weaning will preserve the ability of the MCA's to constrict to pressure for a duration long after the MCA's of SHRsp not treated with captopril (that have developed stroke) have lost their ability to exhibit PDC. The maintenance of PDC in the MCA's of captopril treated SHRsp will be associated with an ability of the MCA's to constrict in response to PKC activation by phorbol dibutyrate and VSMC depolarization produced by elevated $[K^+]_o$.

3.4.3 MATERIALS AND METHODS

3.4.3.1 Experimental Animal Groups

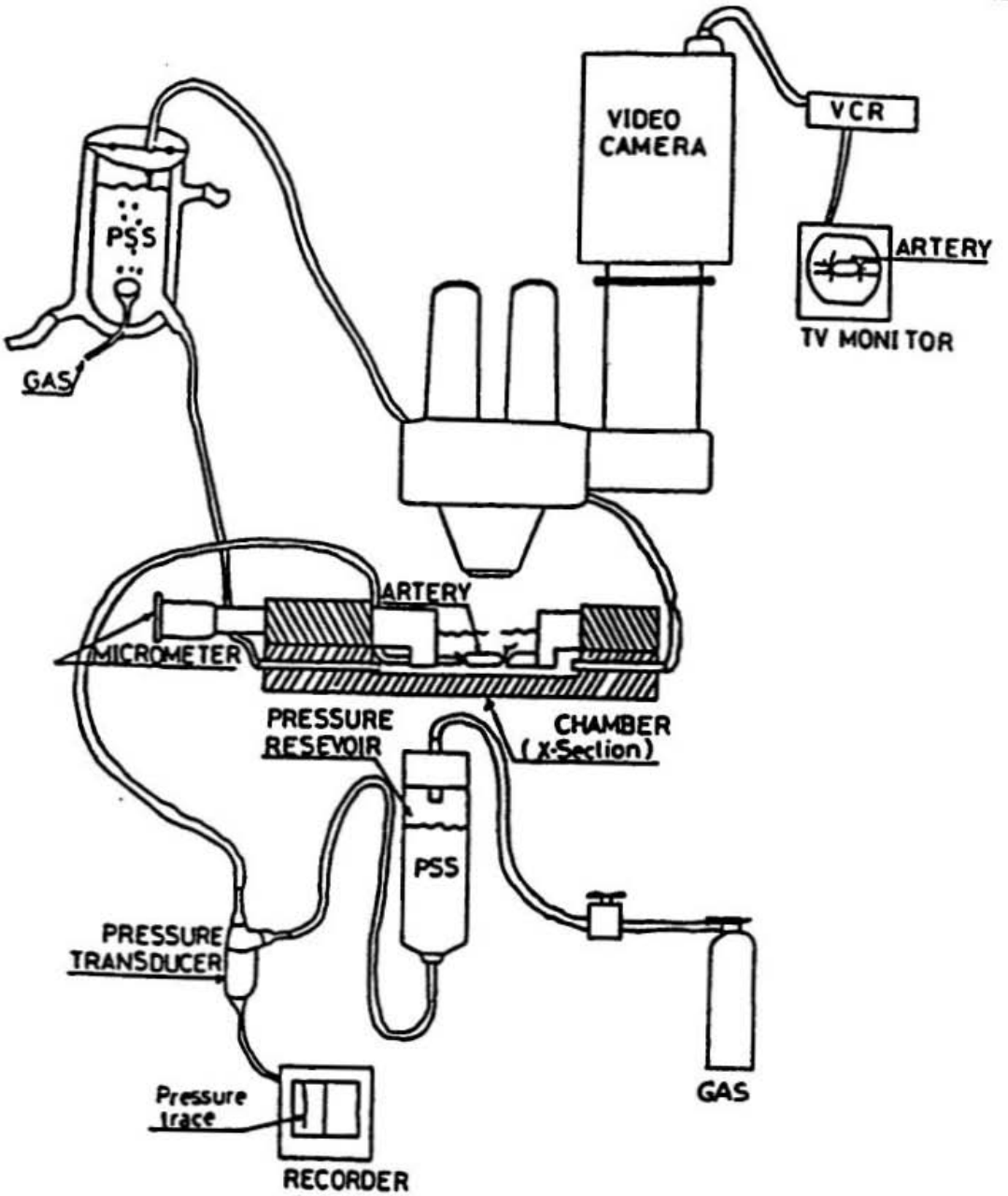
Functional studies on the MCA's were performed using arteries sampled from 4 experimental groups of SHRsp. These consisted of untreated control SHRsp between the ages of 10 to 14 weeks of age that developed hemorrhagic stroke and SHRsp treated with an oral dose of captopril of 50 mg/kg/day from 6 weeks of age that were sampled at 11 to 14 or 18 to 20 weeks of age. None of the later groups of captopril treated SHRsp exhibited behavioural or histological

evidence of cerebral hemorrhage. Finally, a group of 7 captopril treated SHRsp were kept on the oral dose of captopril and 3 of the 7 rats exhibited behavioural signs of stroke and physical evidence of cerebral hemorrhage between 34 to 40 weeks of age. The other rats did not show any behavioural evidence of stroke up to the time the experiment was terminated after 40 weeks of age. Unfortunately, experimental difficulties only allowed us to carry out functional studies on the MCA's of the 3 poststroke captopril treated SHRsp sampled between 34 to 40 weeks of age. The captopril treatment protocol was identical to that previously described in detail in section 1.5.3.2 of Chapter 1. The monitoring of stroke development, the behavioural and physical signs of stroke in SHRsp are discussed in detail in section 1.5.3.4 of Chapter 1.

3.4.3.2 The Assessment of Pressure Dependent Constriction in the Middle Cerebral Arteries of SHRsp

The apparatus used for *in vitro* studies of PDC (Figure 9) was based on a design by Halpern *et al.*, (1984). The 20 ml tissue bath contained in and outflow ports which allowed constant nonrecirculated flow of Krebs saline solution (composition in mM, Na^+ 139, K^+ 4.6, Mg^{2+} 1.2, Ca^{2+} 2.5, Cl^- 120, HCO_3^- 22.1, SO_4^{-4} 1.2, PO_4^{-3} 1.2, and glucose 11.1 at a pH of 7.4) bubbled with 95% O_2/5% CO_2 and maintained at 37°C. The temperature in the bath was measured directly with a temperature probe (Cole-Parmer Instrument Company, Model 8402-10; Quebec, Canada). The flow of Krebs solution was produced by a combination

Figure 9. A schematic diagram of the pressure myograph. The pressure myograph apparatus allowed visualization and quantification of alterations in middle cerebral artery (MCA) diameter in response to vasoactive stimuli. The isolated MCA was mounted onto a micropipette located in the tissue bath containing Krebs saline solution. The lumen of the micropipette and the artery were continuous, filled with Krebs and connected to a gas cylinder in such a way as to allow manipulation of intravascular pressure. Changes in arterial lumen diameter in response to pressure or vasoactive stimuli were recorded on VCR tape and viewed via the television monitor.


VIDEO CAMERA
VCR
ARTERY
TV MONITOR
PSS
GAS
ARTERY
MICROMETER
PRESSURE RESEVOIR
CHAMBER (X-Section)
PSS
PRESSURE TRANSDUCER
GAS
Pressure trace
RECORDER

of gravity due to an elevated main reservoir and a faucet mounted aspirator. Helical coils of plastic tubing carried the Krebs saline through a warming jacket containing circulating water at 37°C. The water was warmed with a heater (Haake, model DI;Berlin) and circulated with a peristaltic pump (Watson - Marlow Limited, H.R. Flow Inducer, England).

SHRsp were anesthetised with a combination of 10 mg Xylazine (Rompun, Bayer Inc., Ontario, Canada) plus 50 mg Ketamine (Ketalean, MTC Pharmaceuticals, Ont., Canada) per kg body weight (i.m). Brains were removed and quickly placed in Krebs saline solution, bubbled with 95% O_2/5% CO_2 and cooled on ice to approximately 4°C. MCA's were carefully dissected away from the surrounding brain tissue and pulled onto the end of a small glass pipette within the chamber such that the artery and pipette lumens were continuous. The glass pipettes (World Precision Instruments Inc., Florida, USA.) were pulled using a micropipette puller (Sutter Instrument Co., California, USA.). The end on which the artery was mounted had a diameter of approximately 90µm. The artery was then secured with a strand of fine suture (10.0 nylon, Alcon Surgical; Texas, USA) tied around the artery and the pipette. The free end of the artery was tied to form a blind sac as Krebs under low pressure (approximately 16mmHg) passed through the lumen to prevent collapse. Both the artery and pipette contained Krebs solution and were connected in a manner that permitted

the manipulation of intravascular pressure.

Manipulation of intravascular pressure was accomplished using a pressurised gas cylinder, pressure adjustment valve and a small pressure reservoir column of Krebs (15ml capacity) connected in series with the lumen of the pipette and the artery under investigation. A sphygmomanometer used for documenting pressure alterations was connected to the system with a "Y" junction between the pressure adjustment valve and the pressure reservoir of Krebs solution.

MCA lumen diameter changes in response to intravascular pressure manipulation and the addition of vasoactive substances were viewed through a microscope at a magnification of 322x (Wild Heerbrugg, M3C; Switzerland) connected to a television camera (Hitachi, CCTV camera, Japan), and VCR (Mitsubishi, HS-2120; Ontario). The specimen was illuminated from below with a fibre optic illuminator (Cole-Parmer Instrument Company, Model 9745-00; Quebec). Changes in lumen diameter were recorded on video tape along with the corresponding time data using a video timer (GYYR, model G88; California). The video images were replayed and quantified at a later time. The scale of image size to actual size was determined using a standardarized 100 μm length grid under the microscope. The relationship was 100 μm = 3.22 cm on the television monitor. Great care was taken to ensure that the lumen diameter measurements were taken from an identical location along the length of artery at

each experimental step. Alterations in vessel diameter were reported as a percent change on lumen diameter according to the following formula:

%change in lumen diameter= [(final diameter ÷ initial diameter) -1] x 100%

The final diameter represents the steady state lumen diameter in response to either a 100mmHg pressure step or the introduction of a vasoactive substance while the initial diameter represents the steady state internal diameter prior to this manipulation.

3.4.3.3 Experimental Protocol

One MCA isolated from each animal was exposed to a pressure step and the phorbol dibutyrate protocol (protocol A) and the other was exposed to the $[K^+]_o$ and vasopressin protocol (protocol B). The protocol performed first (i.e., A vs B) was varied to ensure that any potential effects produced as the result of a differential in time between the removal of the MCA's from brain and the initiation of the experiment did not bias a particular protocol.

3.4.3.4 Protocol A: (Pressure and Phorbol Dibutyrate Mediated Constriction)

1. The MCA was mounted and allowed to equilibrate while pressurised to 100 mmHg for 30 minutes at a bath temperature of 37°C. This time would allow normally functioning vessels to develop pressure dependent tone.

2. The arterial pressure was reduced to near 0 mmHg for 6 minutes to eliminate

PDC. It is believed that the artery perceives this situation as being analogous to conditions of minimal blood flow which causes the artery to relax.

3. The pressure within the MCA was quickly elevated to 100 mmHg for 4 minutes. Measuring the lumen diameter at 1 second post pressurisation is considered to be a representation of the lumen diameter prior to any appreciable PDC. Once the vessel had been pressurised for 4 minutes, a steady state had been reached and the internal diameter was measured again. The values recorded at 4 minutes and 1 second after pressurisation were considered to be the lumen diameters that existed with and without PDC, respectively. These values were used to calculate the percent change in internal diameter in response to the 100mmHg pressure step for the purpose of quantifying the ability of that artery to undergo PDC.

4. The flow of Krebs solution was stopped and nifedipine was added to the bathing solution to attain a final concentration of 3 μM while the artery was maintained at a pressure of 100 mmHg. This dihydropyridine (DHP)-voltage-dependent Ca^{2+} channel antagonist was used to block Ca^{2+} influx in the smooth muscle cells via voltage-dependent Ca^{2+} channels, one of the proposed mediators of PDC. Steady state was reached within 3 minutes and the internal diameter was recorded at that time. Previous studies (Smeda and King, 2000) have indicated that this level of nifedipine maximally vasodilates the MCA's.

5. The PKC activator phorbol dibutyrate was added to the bathing solution in the

presence of 3 μM nifedipine to attain a final concentration of 1 μM. This was done to evaluate the ability of the artery under investigation to constrict to phorbol ester induced PKC activation. Under these experimental conditions, 1 μM levels of phorbol dibutyrate produce maximal possible levels of constriction (attained by phorbol dibutyrate) which are totally inhibited by PKC inhibitors such as staurosporin, bisinolylmaleimide and chelerythrine (Smeda and King 1999). This indicated that constriction was being mediated by PKC activation. The internal diameter measured at the end of the nifedipine step (Step 4) and the diameter recorded following phorbol dibutyrate administration was used to calculate the percent change in internal diameter as a result of phorbol dibutyrate induced PKC activation.

3.4.3.5 Protocol B: (KCl and Vasopressin Mediated Constriction)

1. The MCA was mounted and allowed to equilibrate while pressurised to 100mmHg for 15 minutes at a temperature of 23 °C. The MCA has been shown to lack the ability to constrict to elevations in pressure when maintained at this reduced temperature (Smeda and King 2000).

2. As in step 2 of Protocol A (above) the pressure was reduced to 0 mmHg for 6 minutes and the MCA was pressurised to 100 mmHg for 4 minutes at 23 °C to assure that the artery was incapable of eliciting PDC.

3. The flow of Krebs solution was stopped and KCl was added to the tissue bath to achieve a final $[K^+]_o$ of 84.6 mM. This was done to determine the ability of the

MCA to constrict to K^+ induced depolarization. The internal diameter was measured before and after this step and the percent change in lumen diameter was calculated. Approximately 1.5 minutes was required for a K^+ induced diameter change to reach steady state.

5. The Krebs solution bathing the MCA was flushed and vasopressin was added to a final concentration of 1.2×10^{-7} M. This was done to ensure that any impairment in the MCA's ability to constrict to the vasoactive stimulus under investigation was not simply due to an generalised inability to constrict. In this regard, a significant portion of vasopressin constriction within the MCA's of SHRsp is mediated via the intracellular release of Ca^{2+} from a sarcoplasmic store of Ca^{2+}, independent of PKC activation or Ca^{2+} entry through voltage gated channels (Dr John Smeda, personal communication). Under these conditions, MCA's unable to constrict in response to PKC activation or depolarization can still constrict to vasopressin (Smeda and King, 1999, 2000) demonstrating the presence of a competent contractile apparatus. Vasopressin constriction produced steady state diameter within 4 minutes. The internal vessel diameter was recorded before and after the addition of vasopressin and used to calculate the percent change in lumen diameter.

3.4.3.6 Drugs and Chemicals

All drugs and chemicals were purchased from Sigma Chemical Company (St. Louis, Missouri, USA). KCl (used for VSMC depolarization) was dissolved in distilled water and added to the tissue bath (20 ml) to achieve a final concentration of 84.6mM. Phorbol dibutyrate (a protein kinase C activator) was dissolved first in DMSO (1mg/1.98 ml) and then in distilled water. The solution was then added to the tissue bath to achieve a final concentration of 1 μM. Vasopressin (a vasoconstrictor) was purchased in aqueous solution and was added to the tissue bath to achieve a final concentration of 1.2×10^{-7} M. Nifedipine (a dihydropyridine type voltage-dependent Ca^{2+} channel blocker) was dissolved in absolute ethanol (at 1.73 mM) and was added to the tissue bath to attain a final concentration of 3 μM.

3.4.3.7 Statistical Analysis

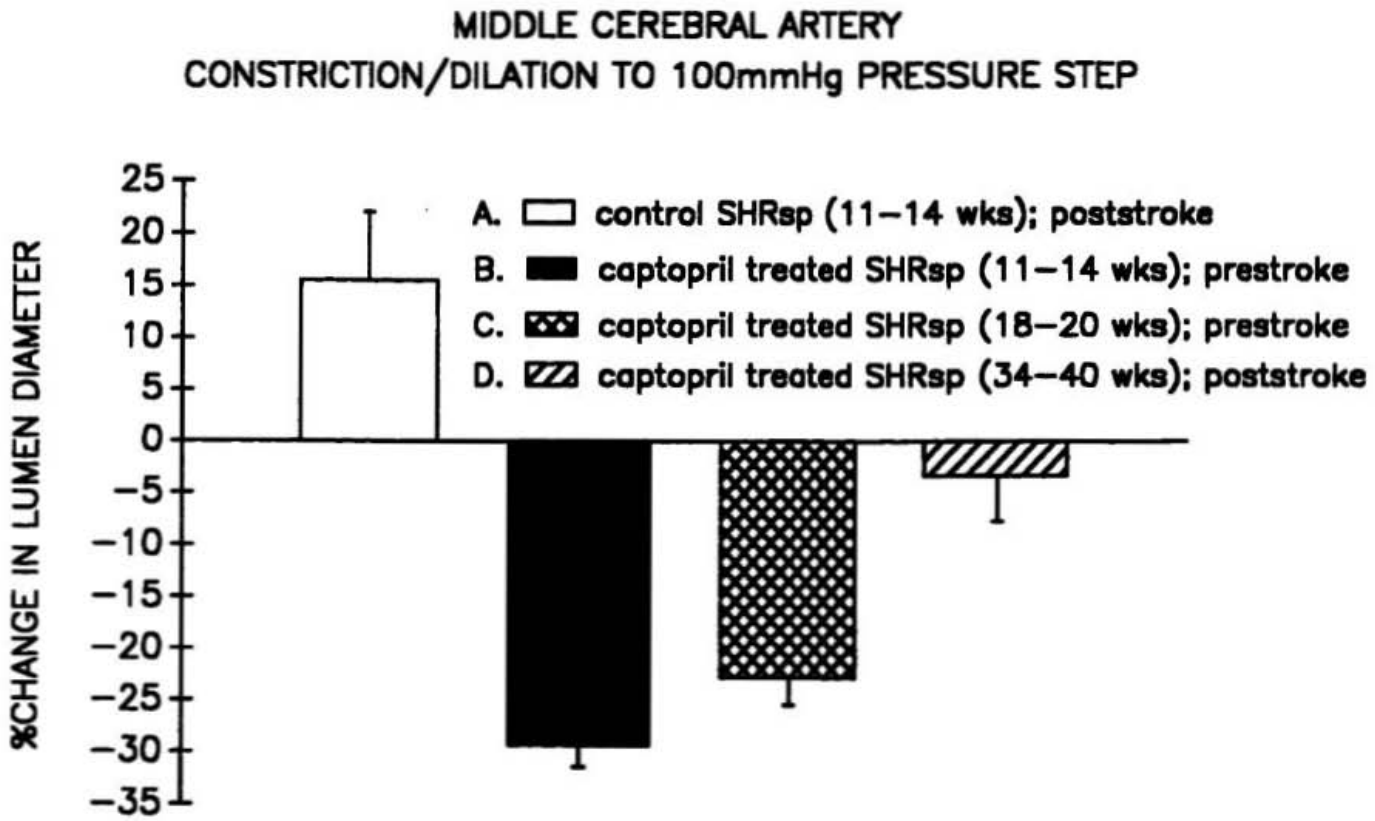
The relationship between percent change in lumen diameter in the 4 experimental groups was investigated using one way analysis of variance (ANOVA) to determine whether or not a significant difference existed among these groups. This was done for each of the studies outlined in protocol's A and B. An unpaired student's t-test compensated for repeated measures using the Bonferroni method was then used to determine which groups differed from each other.

3.4.4 RESULTS

3.4.4.1 The Effects of Captopril Treatment on Pressure Dependent Constriction of the Middle Cerebral Arteries of SHRsp

Figure 10 outlines the alterations in lumen diameter occurring in response to a 100 mmHg pressure step in MCA's sampled from SHRsp in the presence and absence of stroke and or captopril treatment. MCA's sampled from 11-14 week old untreated control SHRsp with stroke dilated when exposed to a 100mmHg pressure step with an average change in lumen diameter of 15.5±6.5%. MCA's isolated from age matched prestroke SHRsp treated with captopril exhibited significant constriction in response to the same pressure step with an average value -29.4±2.0%. A comparable degree of constriction was also observed by pressurising the MCA's from the older (18-20 weeks of age) group of prestroke captopril treated SHRsp. Vessels from this group exhibited a decrease in lumen diameter of -22.7±2.7%, in response to the pressure step. The degree of constriction produced was not significantly different from that observed in MCA's sampled from the younger prestroke captopril treated group. The constriction to pressure observed in the MCA's of the two prestroke captopril treated groups was significantly different from the dilation noted in the poststroke control SHRsp. The oldest group of animals from which MCA's were sampled were treated with captopril up to ages of 35-40 weeks at which time they sustained a stroke. MCA's from this group were shown to constrict only modestly to the

Figure 10. The effect of captopril treatment and on pressure dependent constriction (PDC) in the middle cerebral arteries (MCA's) sampled from SHRsp. PDC was evaluated in response to a 4 minute exposure to a 100 mmHg pressure step. The MCA's isolated from poststroke control animals dilated in response to the pressure step while vessels isolated from age matched prestroke captopril treated SHRsp exhibited PDC. The degree of PDC noted in the 11-14 week old prestroke captopril treated group was not significantly different from that documented in the 18-20 week old prestroke captopril treated animals indicating no significant change in this function occurred over the age range examined. The 34-40 week old captopril treated SHRsp that developed stroke had MCA's which exhibited a small degree of PDC which was not significantly different from that observed in the MCA's of younger untreated poststroke SHRsp. Captopril treatment may retard the age related impairment in PDC typically observed in untreated SHRsp. However, this vascular function may eventually fail in spite of the captopril treatment after stroke eventually develops in these animals. Statistics: One way ANOVA plus Student's t test compensated for multiple comparisons using Bonferroni correction, A vs. B $P<0.05$, B vs. C, A vs. D are NS. (n= values A=7, B=8, C=12, D=3 SHRsp).

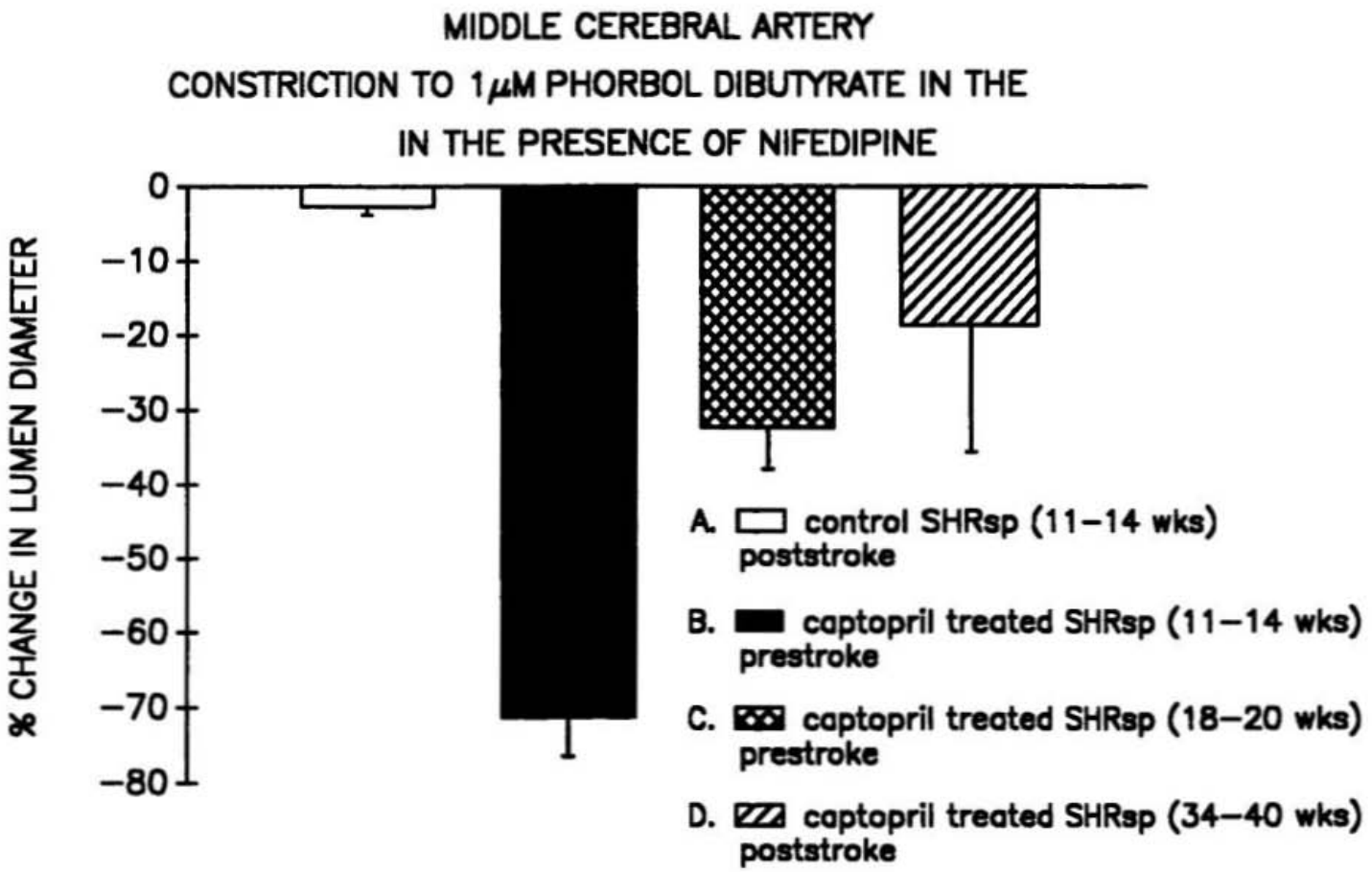

MIDDLE CEREBRAL ARTERY
CONSTRICTION/DILATION TO 100mmHg PRESSURE STEP
%CHANGE IN LUMEN DIAMETER
25
20
15
10
5
0
−5
−10
−15
−20
−25
−30
−35
A. control SHRsp (11−14 wks); poststroke
B. captopril treated SHRsp (11−14 wks); prestroke
C. captopril treated SHRsp (18−20 wks); prestroke
D. captopril treated SHRsp (34−40 wks); poststroke

100mmHg pressure step. The average change in lumen diameter was -3.3±4.4%, not statistically different from the response seen in the poststroke untreated control group, which had died 20 weeks earlier.

3.4.4.2 The Effects of Captopril Treatment on Vasoconstriction Mediated by Protein Kinase C Activation in the Middle Cerebral Arteries of SHRsp

The ability of phorbol dibutyrate to mediate constriction in the presence of nifedipine within the MCA's of SHRsp in the presence and absence of stroke and or captopril treatment are outlined in Figure 11. The MCA's from 11-14 week old untreated poststroke control SHRsp exhibited a minor degree of constriction in response to 1μM phorbol dibutyrate induced PKC activation. The average change in lumen diameter value derived from this group was -2.7±1.1%. MCA's isolated from the age matched captopril treated prestroke SHRsp group exposed to the same concentration of phorbol dibutyrate exhibited a significantly greater -71.4±8.0% constriction than that observed in untreated poststroke SHRsp or older (18-20 week) prestroke SHRsp subjected to captopril treatment (-32.3±5.6% constriction). MCA's isolated from the 35-40 week old poststroke captopril treated animals showed a variable and low ability to constrict to phorbol dibutyrate induced PKC activation, exhibiting an average change in lumen diameter of -18.6±17.0%. This response was weaker than that observed in the two prestroke captopril treated SHRsp groups but was not significantly different from the constriction documented in the MCA's of the untreated poststroke

Figure 11. The effect of captopril treatment on protein kinase C (PKC) mediated phorbol dibutyrate induced constriction of the middle cerebral arteries (MCA's) sampled from SHRsp. Phorbol dibutyrate (1 μM) was introduced into the bath in the presence of nifedipine (3 μM). Previous studies in our laboratory have shown that under the latter conditions the constriction produced by phorbol dibutyrate (in MCA's from SHRsp, Dahl and Sprague Dawley rats) is inhibited by the PKC inhibitors chelerythrine (12 μM) or bisindolylmaleimide (5 μM) suggesting that the response is mediated by PKC activation. Phorbol dibutyrate administration resulted in a marked degree of vasoconstriction in the MCA's of 11-14 week old prestroke captopril treated animals whereas the MCA's of age matched untreated poststroke SHRsp lost the ability to constrict to phorbol dibutyrate. There was a significant decline in the ability of the MCA's from captopril treated SHRsp to constrict to the phorbol ester between 11-14 and 18-20 weeks of age suggesting an impairment in this function with age during captopril treatment. The MCA's of 34-40 week old captopril treated SHRsp that developed stroke exhibited some ability to constrict to phorbol dibutyrate however this was variable and not statistically different from the responses observed in untreated poststroke controls. Statistics: One way ANOVA plus Student's t test compensated for multiple comparisons using Bonferroni correction, A vs. B and B vs. C $P<0.05$ and A vs. D is NS. (n= values A=7, B=8, C=12, D=3 SHRsp).

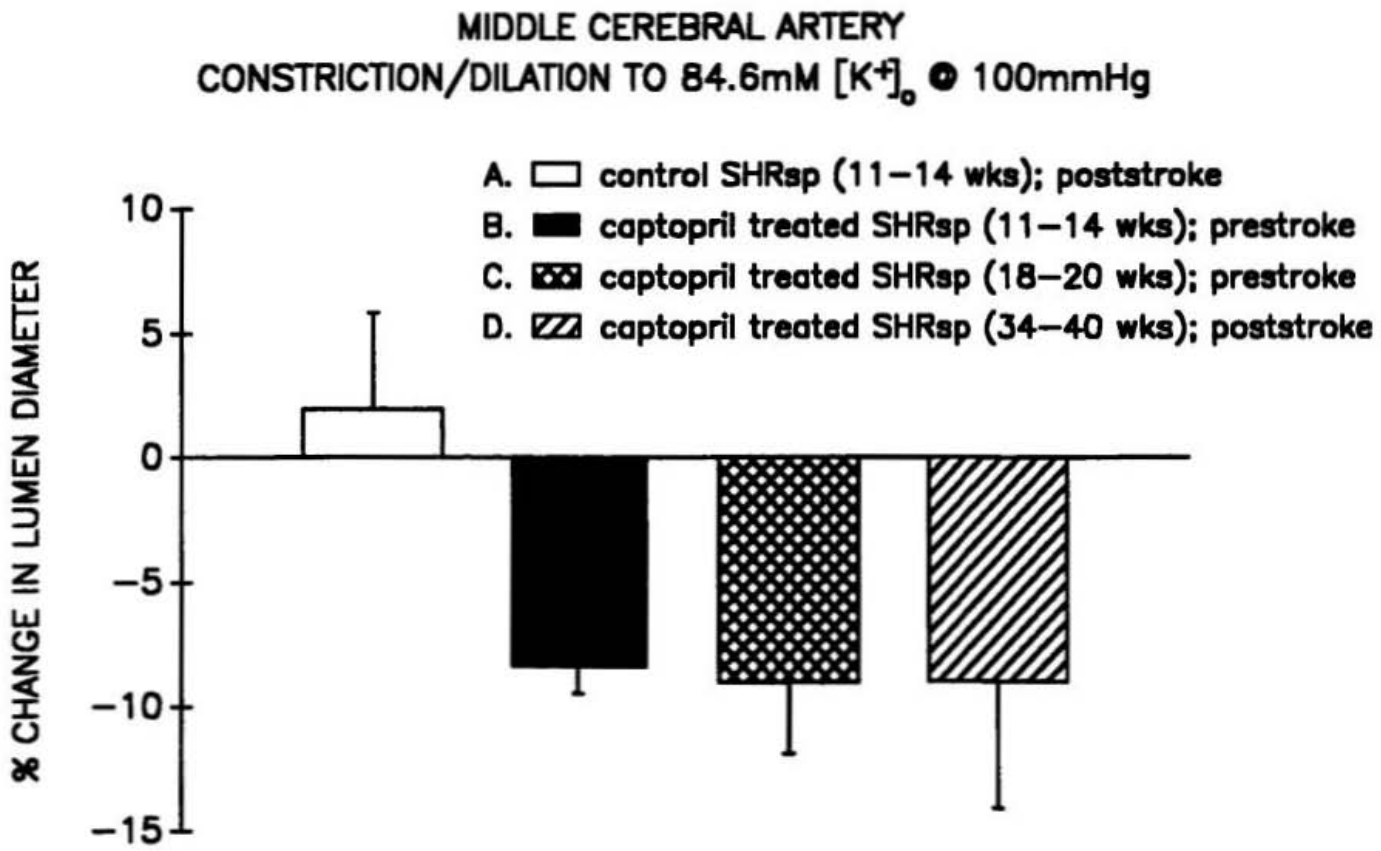

MIDDLE CEREBRAL ARTERY
CONSTRICTION TO 1μM PHORBOL DIBUTYRATE IN THE
IN THE PRESENCE OF NIFEDIPINE
% CHANGE IN LUMEN DIAMETER
0
−10
−20
−30
−40
−50
−60
−70
−80
A. control SHRsp (11−14 wks) poststroke
B. captopril treated SHRsp (11−14 wks) prestroke
C. captopril treated SHRsp (18−20 wks) prestroke
D. captopril treated SHRsp (34−40 wks) poststroke

SHRsp.

3.4.4.3 The Effects of Captopril Treatment on KCl Induced Constriction of the Middle Cerebral Arteries of SHRsp

The ability of high $[K^+]_o$ induced depolarization to mediate constriction within the MCA's of SHRsp in the presence and absence of stroke and or captopril treatment are outline in Figure 12. MCA's isolated from poststroke control SHRsp were unresponsive to 84.6mM $[K^+]_o$ exhibiting an average change in lumen diameter of 1.94±3.9%. In contrast, the MCA's from the young and old prestroke captopril treated groups as well as the poststroke captopril treated animals constricted within a significant difference of each other to high $[K^+]_o$ with average decreases in lumen diameter of -8.4±1.10%, -9.0± 2.9% and -8.91±5.1% respectively. There were no marked differences in the ability of the MCA isolated from the three captopril treated groups to constrict to high $[K^+]_o$. Although the mean constriction to high $[K^+]_o$ observed in the MCA's of captopril treated SHRsp was greater than that observed in untreated poststroke SHRsp, when compensated for multiple comparisons the responses observed between untreated and captopril treated SHRsp did not significantly differ from each other.

Figure 12. The effect of captopril treatment on elevated $[K^+]_o$ induced constriction of the middle cerebral arteries (MCA's) sampled from SHRsp. No statistical differences in the ability of the MCA's to constrict to elevated $[K^+]_o$ was observed when the MCA's of 11-14 week old untreated poststroke controls , 11-14 and 18-20 week old prestroke captopril treated or 34-40 week old poststroke captopril treated SHRsp were compared. These results suggest that the ability of the MCA's to constrict to elevated $[K^+]_o$ may not be significantly effected by captopril treatment. Statistics: One way ANOVA-NS. (n= values A=7, B=5, C=11, D=4 SHRsp).


MIDDLE CEREBRAL ARTERY
CONSTRICTION/DILATION TO 84.6mM $[K^+]_o$ @ 100mmHg
A. control SHRsp (11–14 wks); poststroke
B. captopril treated SHRsp (11–14 wks); prestroke
C. captopril treated SHRsp (18–20 wks); prestroke
D. captopril treated SHRsp (34–40 wks); poststroke
% CHANGE IN LUMEN DIAMETER
10
5
0
−5
−10
−15

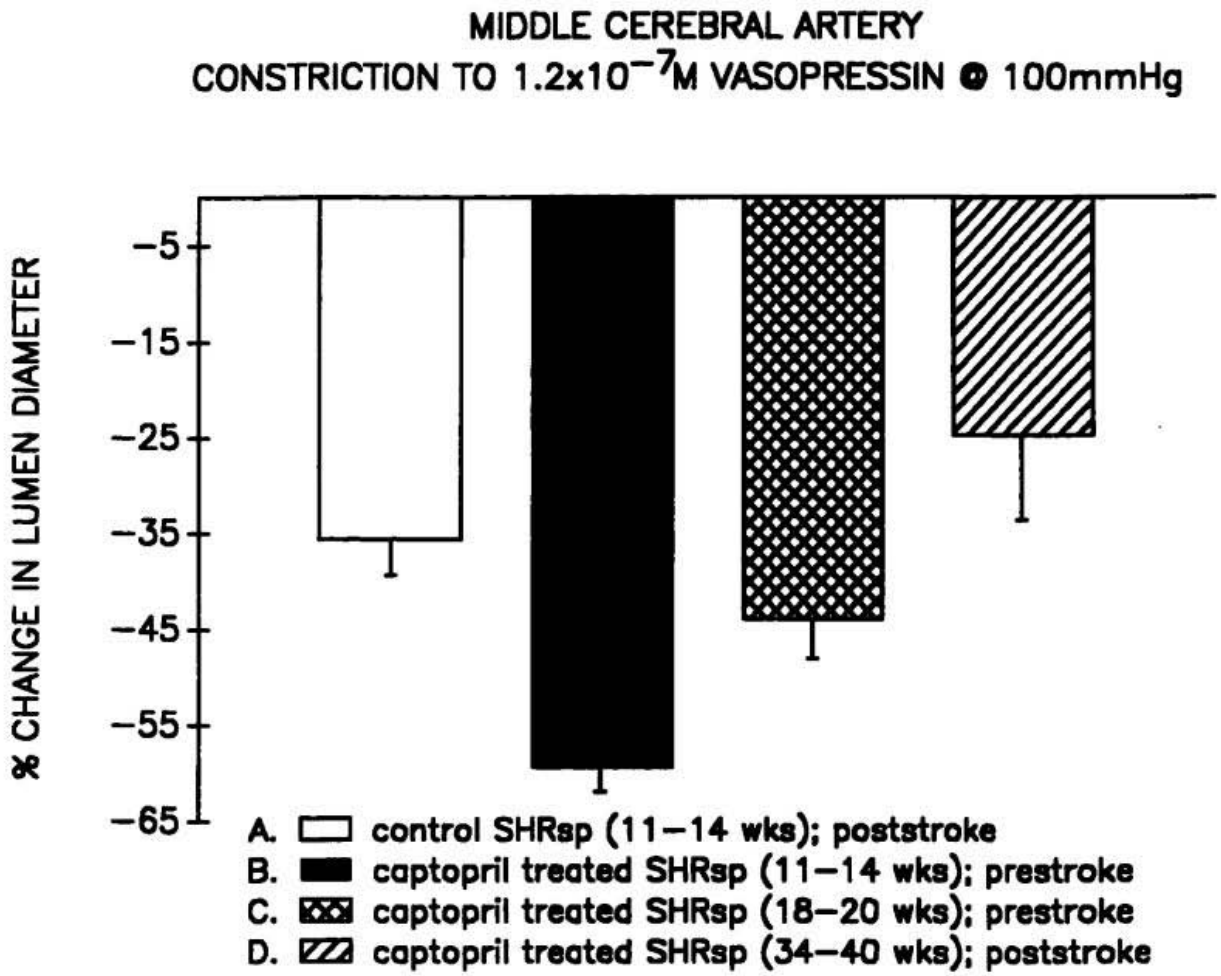
3.4.4.4 The Effects of Captopril Treatment on Vasopressin Induced Constriction of the Middle Cerebral Arteries of SHRsp

The ability of vasopressin to mediate constriction within the MCA's of SHRsp in the presence and absence of stroke and or captopril treatment are outlined in Figure 13. MCA's isolated from the four groups of SHRsp investigated all exhibited a significant ability to constrict to 1.2×10^{-7} M vasopressin. The 11-14 week old prestroke captopril treated SHRsp showed an average change in lumen diameter in response to vasopressin of -59.5± 2.5%. This was significantly greater than the value of -43.9± 4.1% derived from the 18-20 week old prestroke captopril treated animals. The poststroke untreated control SHRsp exhibited a vasopressin induced average change in lumen diameter of -35.6±3.7% significantly less than the 11-14 week old prestroke captopril treated SHRsp but not significantly different from the 18-20 week old prestroke or the 34-40 week old poststroke captopril treated groups.

3.4.5 DISCUSSION

The following key observations were made in the study. SHRsp (that were not treated with captopril) developed stroke between the ages of 11 to 14 weeks and had MCA's that (a) lost their ability to constrict to a 100mmHg pressure step, (b) were unable to constrict to PKC activation by phorbol dibutyrate in the presence of nifedipine and were unable to constrict in response to high potassium ($[K^+]_o$= 84.6mM). The loss of the latter constrictor functions in

Figure 13. The effect of captopril treatment on vasopressin induced constriction of middle cerebral arteries (MCA's) sampled from SHRsp. The results indicate that all vessel groups exhibited a substantial ability to constrict to vasopressin. There were however significant differences between groups. The MCA's of the 11-14 week old untreated poststroke control SHRsp constricted significantly less than the age matched prestroke captopril treated rats. A significant decline in vasopressin induced MCA constriction was noted in the 18-20 week old prestroke captopril treated group when compared with the younger prestroke captopril treated SHRsp. There was no significant difference in the response to vasopressin between the untreated control poststroke SHRsp and 35-40 week old captopril treated SHRsp that developed stroke. The MCA response to vasopressin was studied to ensure that any impairments in the response to pressure or the aforementioned vasoactive substances were not simply the result of a generalized inability to constrict. Statistics: One way ANOVA plus Student's t test compensated for multiple comparisons using Bonferroni correction: A vs. B and B vs. C $P<.0.05$ and A vs. D is NS. (n= values A=7, B=5, C=11, D=4 SHRsp).


MIDDLE CEREBRAL ARTERY
CONSTRICTION TO 1.2x10⁻⁷M VASOPRESSIN @ 100mmHg
% CHANGE IN LUMEN DIAMETER
-5
-15
-25
-35
-45
-55
-65
A. control SHRsp (11–14 wks); poststroke
B. captopril treated SHRsp (11–14 wks); prestroke
C. captopril treated SHRsp (18–20 wks); prestroke
D. captopril treated SHRsp (34–40 wks); poststroke

poststroke SHRsp was not due to a general inability of the arteries to constrict since MCA's sampled from the poststroke SHRsp readily constricted to 1.2×10^{-7} M vasopressin (-35% change in lumen diameter). Vasopressin mediates constriction, in part, via the release of a sarcoplasmic store of Ca^{+2} (Jackson 1996; Nemenoff 1998), a mechanism that is not utilized to promote constriction in response to pressure, PKC activation or VSMC depolarization (discussed later). Captopril treatment of SHRsp prevented stroke development up to 30 weeks of age and attenuated the loss of vasoconstriction in response to pressure, PKC activation and high potassium induced depolarization in SHRsp sampled between 11 to 20 weeks of age.

The present study was limited by the fact that we did not sample a prestroke group of SHRsp at an age prior to stroke development. Therefore, the ability of captopril treatment to preserve vascular function could not be directly related to the degree of vascular function present prior to stroke development in untreated SHRsp. However, shortly after completion of this project, young 9-10 week old prestroke SHRsp were sampled by other members of the laboratory using an identical protocol used to study the MCA's in the present thesis (Dr. John Smeda, personal communication). The MCA's of 9-10 week old SHRsp exhibited a pressure dependent constriction to a 100mmHg pressure step of 26 ±2 % (n=5), produced 67 ±10 % reduction in lumen diameter in response to 1 μM phorbol dibutyrate in the presence of 3 μM nifedipine (n=6) and reduced

their lumen diameter by 44 ±8 % in response to 84.6mM $[K^+]_o$ (n=6). The MCA's of these animals constricted to 1.2×10^{-7} M vasopressin at a level of 50 ±4% (n= 6). In the case of constrictor responses to pressure, PKC activation or vasopressin, the magnitude of the responses observed in the MCA's of the latter group of 9 to 10 week old prestroke SHRsp were similar (not significantly different) to corresponding responses in the captopril treated SHRsp sampled between 11 to 14 weeks of age shown in Figures 10 , 11 and 13. Therefore in the case of these responses, at 11 to 14 weeks of age, captopril treatment of SHRsp preserved the ability of the MCA's to constrict to pressure and PKC activation and maintained vasopressin responsiveness at levels present in SHRsp two weeks prior to stroke development. On the other hand the MCA's of 9 to 10 week old SHRsp constricted to depolarization in response to high potassium to a much greater degree than the MCA's of older SHRsp treated with captopril. This would suggest that constriction in response to potassium depolarization declines with age in the MCA's of SHRsp despite the presence of captopril treatment.

MCA from SHRsp that failed to develop stroke and aged under conditions of captopril treatment showed a small decline in their ability to elicit PDC to a 100 mmHg pressure step and a more substantial decline in their constrictor responses to PKC activation by phorbol dibutyrate. These functions were further attenuated in SHRsp that eventually developed stroke after 34 weeks of age.

However, the average responses to pressure or phorbol dibutyrate were greater in 34 to 40 week old SHRsp that developed stroke during captopril treatment than in younger untreated 11 to 14 week old SHRsp that developed stroke in the absence of captopril treatment. In the case of high $[K^+]_o$ induced constriction, the MCA's of the three captopril treated groups of SHRsp (ages 11 to 40 weeks) exhibited comparable responses regardless of the presence or absence of stroke. The above results would indicate that captopril treatment delays the loss of pressure dependent, and PKC mediated constriction in the MCA's of SHRsp as well as stroke development in the animals. In addition, captopril treatment is of some benefit in preserving this function even in animals that eventually developed stroke during captopril treatment.

3.4.5.1 The Relation Between the Loss of Cerebrovascular Pressure Dependent Constriction and Stroke Development in SHRsp

Studies (Smeda 1992, Smeda and King, 1999, Smeda *et al.* 1999b, Smeda and King, 2000) have indicated that the loss of PDC in the MCA's of SHRsp precedes stroke development and is absent in SHRsp that have developed stroke. The decline in this vascular function starts to occur in prestroke SHRsp after about 11.5 weeks of age and regression analysis of the change in this function with age predicts that PDC would be totally lost in MCA of prestroke SHRsp at about 16 weeks of age, a time which coincides with the age when 100% mortality associated with stroke occurs (Smeda and King,

2000). It was suggested that the loss of PDC could promote a loss of cerebral blood flow (CBF) autoregulation in the MCA perfusion domain, promoting overperfusion in the region, leading to cerebral hemorrhage formation. It is therefore tempting to suggest that by preserving PDC and CBF autoregulatory function, captopril treatment prevents stroke and death in SHRsp. Such a conclusion is premature and in view of recent experiments, not fully correct. Studies (Smeda *et al.* 1999b) involving laser Doppler techniques have indicated that CBF regulation is lost within the perfusion domain of the MCA in SHRsp and that this alteration does precede stroke development in SHRsp. However, it was also observed that the perfusion domain serviced by the posterior cerebral artery (PCA) also lost its ability to autoregulate cerebral blood flow in a manner parallel to the MCA perfusion domain while PDC was not abolished in the isolated PCA arteries studied *in vitro* using a myograph. The conclusions reached were that the loss or presence of PDC in the larger $\simeq$ 200 μm diameter cerebral vessels feeding the microvasculature do not always perfectly predict the loss of CBF autoregulation (which is primarily governed by the microvasculature <40 μM in diameter). It was suggested that the loss of PDC likely extends farther upstream from the microvasculature towards the larger arteries in the case of the MCA verses the PCA vascular tree in SHRsp that have developed stroke. It is possible that if the extent of PDC loss was more extensive in the MCA vascular tree, overperfusion might favour this area over the PCA perfusion domain. In

this regard hemorrhagic lesions do preferentially occur at higher frequencies in the MCA versus the PCA perfusion domains (Smeda, 1992)

In conclusion, although the loss of PDC in the MCA and the other associated aspects of vascular function demonstrated in this section may represent, the type of alterations present in the cerebral microvasculature, this has not been proven. In view of this, one can not conclude with certainty that the preservation of PDC in the MCA's of SHRsp during captopril treatment is the mechanism that is responsible for retarding the onset of stroke development in SHRsp. Despite this, we would expect that the preservation of PDC, even if only in the MCA vascular tree, would significantly reduce overperfusion and retard the onset of cerebral hemorrhage in the region.

3.4.5.2 The Effect of Captopril Treatment on the Mechanisms Promoting Cerebrovasculature Pressure Dependent Constriction

As discussed in the introduction, two general possible inter-related mechanism have been suggested to promote PDC in cerebral and other vascular beds. Elevations in pressure have been suggested to promote PKC activation. In this regard PKC inhibitors inhibit PDC in cerebral vessels from SHRsp and normotensive rats (Osol et al.,1991; Smeda and King, 1999). Likewise, the levels of the PKC activator DAG have been shown to elevate in the VSMC's of renal arteries (Narayanan *et al.*, 1994). Other studies have shown that elevations in pressure promote VSMC depolarization of cerebral blood

vessels, including those of SHRsp (Harder, 1984; Smeda and King, 2000). Such a response will open voltage gated Ca^{2+} channels admitting Ca^{2+} and promoting constriction. Ca^{2+} channel antagonists totally inhibit PDC in cerebral blood vessels. Theories have emerged suggesting that PKC activation in combination with Ca^{2+} influx into VSMC's is responsible for the production of PDC (Hill *et al.*, 1990; Gokina *et al.*, 1999). Differing versions of these theories have suggested that the combined individual effects of Ca^{2+} entry and PKC activation are required to promote PDC and that the inhibition of either of the two individual mechanisms is sufficient to inhibit PDC (Hill *et al.*,1990). Other researchers have suggested that the influx of Ca^{2+} may in fact promote the activation of PKC which in turn activates constriction (Laher and Bevan, 1989). In this scenario the blockage of PKC with inhibitors or Ca^{2+} entry via Ca^{2+} channel blockers would also inhibit PDC.

In the present study, stroke development in SHRsp was associated with a loss of PDC and a defect in the ability of the MCA's to constrict in response to PKC activation (by phorbol dibutyrate) and depolarization (by high $[K^+]_o$). These mechanisms have been previously studied in SHRsp. The ability to constrict in response to elevated pressure was very strongly related to the ability of the MCAs to constrict in response to PKC activation and is inhibited by PKC inhibitors (Smeda and King, 1999). In the present study we were able to study PDC in a 100 mmHg pressure step and responsiveness to 1 μM phorbol

dibutyrate within the same MCAs. Consistent with the observations of Smeda and King (1999), regression analysis indicated a significant correlation between the two functions when the data for all the animal groups were combined.

PDC response (%) = 6.14 + 0.535 phorbol dibutyrate response (%).

Pearson coefficient of correlation (r value) = 0.764, $P<0.001$

Since high $[K^+]_o$ induced constriction was measured under conditions where PDC was inhibited by low temperatures (23°C), the relationship of high $[K^+]_o$ induced constriction to PDC response could not be studied in the same arteries. However, when the left vs the right MCAs from each animal were compared, the observation that a) the MCAs of young, untreated 9-10 week old prestroke SHRsp (sampled after project) and 11-14 and 18-20 week old captopril treated SHRsp exhibited comparable PDC responses (~ 23 to 28%) and quite varying contractile responses to high $[K^+]_o$ (44 to 8%) and b) the further observation that poststroke 30-34 week old captopril treated SHRsp lost their ability to elicit PDC but were still able to constrict to high $[K^+]_o$ at levels comparable to those present in prestroke captopril treated SHRsp (11-20) weeks of age (Figure 13) would suggest that a direct relationship between the ability of the MCA to constrict to pressure and respond to high $[K^+]_o$ induced depolarization does not exist.

The electrophysiological alterations associated with the inability of MCAs to constrict to depolarization have been studied in poststroke SHRsp (Smeda and King, 2000). Whether these defects are a mechanism responsible for the

loss of PDC in poststroke SHRsp remains unresolved. The MCAs of 9-10 week old prestroke SHRsp exhibit pressure dependent depolarization but the change in membrane potential is small in response to a 0 to 100 mmHg pressure step (from -46 ± 2 to 38 ± 1 mV). This degree of depolarization can not account for the substantial degree of constriction produced in the same arteries (42 ± 3%) simply through the opening of voltage gated Ca^{2+} channels. There was also no evidence that Ca^{2+} entry through voltage gated channels triggered an alternative contractile mechanism such as PKC activation, since PKC inhibitors had no effect on depolarization induced constriction in these arteries. After stroke development, the VSMCs of MCAs from SHRsp depolarized and maintained a constant membrane potential with varying pressure (-34 ± 5 vs -35 ± 2 mV, 0 vs 100 mmHg). Patch clamp studies indicate that the depolarization observed in the VSMCs of the MCAs of poststroke SHRsp was mediated by a very large increase in chloride conductance in the VSMCs which could be blocked by chloride channel blockers (niflumic acid or IAA-94). When the MCAs were treated with chloride channel inhibitors, the membrane potentials were normalized; however PDC still was not recovered in the MCAs of SHRsp by such treatment (Dr. John Smeda, personal communication) suggest that this electrophysiological alteration was not solely responsible for the loss of PDC in the arteries. Other studies indicated that high $[K^+]_o$ levels depolarized the VSMCs of the MCAs of poststroke SHRsp; however, the VSMCs behaved as if

the voltage sensor on the voltage gated channel was defective, creating a situation where depolarization could not enhance the opening probability of the voltage-gated Ca^{2+} channel (Smeda and King, 2000). More recent studies suggest that depolarization in response to pressure may occur after constriction has taken place in the MCAs of Sprague Dawley rats and prestroke SHRsp (Smeda and King, unpublished results).

3.4.5.3 Vasopressin Mediated Constriction of the Middle Cerebral Arteries

The purpose of exposing the experimental groups of MCA's to vasopressin was to ensure that any differences in vasoreactivity found in the aforementioned studies were not simply due to a generalized inability to respond to vasoactive stimuli. It was interesting to note that statistically, the MCA response to vasopressin mirrored the phorbol dibutyrate constrictor responses. That is, the 11-14 week old prestroke captopril treated SHRsp possessed MCA's which constricted significantly more to phorbol dibutyrate and vasopressin relative to age matched poststroke control arteries. There was a significant decline in phorbol dibutyrate and vasopressin induced MCA constriction between the 2 prestroke captopril treated groups. Also, the phorbol dibutyrate and vasopressin responses in both poststroke groups were not significantly different from each other, but were reduced compared with prestroke groups. Generally however, the magnitude of the vasopressin induced constriction was larger than that documented in response to phorbol dibutyrate. For example, the

average vasopressin induced constriction including the 4 experimental groups, was 40.9% whereas the average phorbol dibutyrate induced response was 31.1%.

The reason that the phorbol dibutyrate and vasopressin induced responses are similar may be related to the similar mechanisms via which these two vasoactive stimuli exert their constrictive effects. According to Jackson (1996) and Nemenoff (1998), vasopressin receptor activation in the VSMC is thought to activate PLC via a G-protein and thereby generate IP_3 and DAG via the degradation of phosphatidylinositol-4,5 bisphosphate. The IP_3 is thought to cause the release of Ca^{2+} from internal stores. The DAG is thought to activate PKC which in turn, lowers the Ca^{2+} requirements necessary for VSMC contraction. Consequently, the two arms of this second messenger system seem to participate in VSMC activation in response to vasopressin. Alternatively, phorbol dibutyrate is thought to lead to vasoconstriction solely via the PKC arm of this second messenger pathway which may account for the greater vasopressin induced MCA constriction relative to the phorbol dibutyrate induced response.

3.4.5.4 Concluding Remarks Related to the Studies in Chapter 3

Collectively, the observations of MCA function suggest pressure induced depolarization may not be the primary mechanism of action in PDC. The reasons are as follow; a) the magnitude of VSMC depolarization is insufficient to

promote the level of constriction seen; b) the absence of a depolarization induced triggering of PKC; and c) preliminary evidence that pressure induced depolarization may follow constriction in the arteries. This is further reinforced by the observation that the MCAs from 30 to 34 week old captopril treated SHRsp that develop stroke exhibit some constriction to elevated $[K^+]_o$ (Figure 12) but show very little ability to constrict to pressure and the observation that the MCA's from 9-10 week old prestroke SHRsp (sampled after the project) exhibit comparable PDC response to 10 to 14 week old captopril treated SHRsp but substantially higher constrictor responsiveness to elevated $[K^+]_o$.

On the other hand, the ability of MCAs to constrict to PKC activation in response to phorbol dibutyrate is well correlated to the ability of the arteries to constrict to pressure in all the animals tested. This would suggest that PKC activation may be an important signal transduction mechanism involved in mediating PDC. The preservation of this mechanism by captopril treatment may be instrumental in preserving PDC in the arteries. The observation that PKC activation can open voltage gated Ca^{2+} channels in VSMCs in a voltage independent manner under conditions where membrane potentials are clamped (Fish, *et al.*, 1988) could provide an explanation as to why both PKC inhibitors and voltage gated Ca^{2+} channel antagonists can inhibit PDC in the MCAs of SHRsp. If such a mechanism existed in the MCAs of SHRsp, the voltage gated Ca^{2+} channel could promote PDC solely via PKC activation independent of

pressure dependent depolarization.

20-hydroxyeicosatetraenoic acid (20-HETE), an arachidonic (AA) metabolite produced by the cytochrome P-450 4A pathway, has also been proposed as a possible mediator of PDC in rat cerebral vessels (Gebremedhin et al., 2001). This suggestion was based on the authors' in vivo findings including the observation that inhibition of 20-HETE formation with N-methylsolfonyl-12, 12-dibromododec-11-enamide (DDMS) or its vasoconstrictor actions using 15-HETE or 20-hydroxyeicosa-6(Z), 15(Z)-dienoic acid (20-HEDE), impaired the autoregulation of CBF to elevations in arterial pressure. Support for 20-HETE's aforementioned role also included the in vitro observation that PDC in the rat MCA was eliminated by administration of DDMS, 15-HETE or 20-HEDE.

A possible relationship between these findings and those outlined in this thesis is highlighted by reports of an ACE inhibitor mediated increase in 20-HETE formation in renal microsomes. The microsomes were derived from Sprague-Dawley rats treated with captopril (25 mg/kd) or enalapril (10 mg/kg) for one week. While the investigators noted that the relevance of these finding to the antihypertensive actions of ACE inhibitors have yet to be determined, it is reasonable to consider that a captopril treatment mediated increase in 20-HETE levels could play a role in the preservation of PDC noted in our captopril treated SHRsp.

Recent studies indicate that captopril treatment initiated at the first sign of

stroke and up to 6 days following, can prolong the lifespan of SHRsp by approximately 16 weeks (Smeda *et al.*, 1999a). Aldosterone co-infusion with captopril treatment nullified the effect of captopril treatment suggesting that the ability of captopril to prolong life in poststroke SHRsp could be mediated by the suppression of aldosterone release secondary to the inhibition of AII production. Of interest, the treatment of poststroke SHRsp with captopril allowed the MCAs of the animals to regain their ability to constrict to PDC and PKC activation via phorbol dibutyrate in the presence of nifedipine. This would indicate that captopril treatment not only retards the loss of PDC along with the onset of stroke in SHRsp but can repair the loss of PDC after stroke has developed in the animals.

The systemic effects of poststroke captopril treatment important in preventing death in SHRsp are not fully understood. In recent years the brains of SHRsp have been studied using magnetic resonance imaging (MRI), a technique that can detect brain edema, hemorrhage and ischemia under *in vivo* conditions. The earliest brain defect occurring in SHRsp was the onset of cerebral edema (Blezer *et al.*, 1998). This coincided with a marked loss of proteins into the urine in the animals (> 40 mg/day). MRI studies indicated that edematous sites often became hemorrhagic (Takahashi *et al.*, 1994). ACE inhibitor treatment started after the first MRI signs of edema stopped the development of proteinuria and reversed the onset of brain edema (Blezer *et al.*,

1998). Edema formation in the brain in hypertensive patients can promote a condition termed hypertensive encephalopathy which is associated with a loss in cerebral blood flow autoregulation. It is possible that the sites of edema formation may also lose blood flow autoregulatory ability. If this were the case, they may become overperfused. This would not only increase edema formation but it might also place these sites at risk for hemorrhage formation. Hence, by either preventing or reversing edema ACE inhibitors could help prevent the onset or further spread of hemorrhage.

4. A SUMMARY OF THE FINDINGS AND THE GENERAL CONCLUSIONS OF THE ENTIRE STUDY

The results of investigations outlined in Chapter 1 showed that captopril treatment is capable of markedly retarding hemorrhagic stroke related mortality in SHRsp independent of a significant reduction in blood pressure. This in combination with the observation that a non ACE inhibitor antihypertensive agent produced a substantial hypotensive effect with only a very mild effect on mortality rate suggested that captopril's antistroke effects may be related to this drug's specific mechanisms of action. In investigating this possibility, we found that plasma aldosterone levels increased as untreated SHRsp approached the age at which the development of hemorrhagic stroke is likely. We also found that plasma aldosterone levels were reduced in captopril treated SHRsp compared with untreated controls and that supplementing plasma aldosterone in

captopril treated SHRsp via an exogenous source reversed the antistroke effects of this drug. These findings led to the suggestion that a component of the pathogenesis in SHRsp may be mediated by inappropriately elevated levels of plasma aldosterone. An attempt to simulate the effects of captopril treatment with the aldosterone antagonist spironolactone was initiated. Unlike captopril, spironolactone treatment produced a small but significant hypotensive effect as well as a significant, but modest, reduction in the mortality rate. The mild effects of spironolactone treatment on mortality rate were not anticipated and one possible explanation is that aldosterone may not exert a pathological effect in SHRsp via a spironolactone sensitive intracellular mineralocorticoid receptor but instead, may act via the relatively newly described nongenomic pathway. This suggestion however is speculative and the role that such a nongenomic mechanism may play in SHRsp is unclear.

Next, investigations turned to the relationship between MCA function, captopril treatment and mortality associated with hemorrhagic stroke. Recall that untreated SHRsp typically develop hemorrhagic stroke over the age range of 11-14 weeks. This is closely preceded by an impaired ability of the MCA to exhibit PDC, thought in turn, to result from a reduced ability to constrict to PKC activation. We found that MCA's isolated from 11-14 week old prestroke captopril treated SHRsp had retained the ability to constrict to pressurization and PKC activation. In contrast, vessels isolated from age matched poststroke

controls exhibited a markedly reduced ability to constrict to the same vasoactive stimuli. Based on these findings we proposed that captopril treatment may retard the development of hemorrhagic stroke via the preservation of the MCA's ability to constrict to pressurization. Also, this preservation of PDC may be mediated by a captopril induced protection of the PKC signalling cascade. The ability of the MCA to constrict to high $[K^+]_o$ induced depolarization was not related to the loss of PDC.

Once the captopril treated SHRsp reached the age of 18-20 weeks, the ability of the MCA to constrict to PKC activation was significantly reduced relative to younger captopril treated animals. This preceded the development of hemorrhagic stroke in a manner which parallelled observations typically noted in untreated SHRsp. While speculative and in need of further investigation, we further suggest that this impaired second messenger system may subsequently lead to a significant impairment in PDC between 18-20 and 34-40 weeks of age. The end result of this could be that the ability of the MCA to regulate blood flow is compromised. This, in turn, leads to the formation of hemorrhagic stroke in captopril treated SHRsp, parallelling the pathogenesis which occurs in untreated SHRsp, yet at a much older age.

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