ABSTRACT

Steven J. Blumenthal. THE ROLE OF THYROID HORMONES AND MYOCARDIAL ALPHA- AND BETA-ADRENERGIC RECEPTORS IN THE DEVELOPMENT OF HYPERTENSION IN THE SPONTANEOUSLY HYPERTENSIVE RAT (SHR). (Under the direction of Samuel G. Iams, Ph.D.). Department of Biology, January 1981.

Cardiac membrane preparations from developing euthyroid and hypothyroid spontaneously hypertensive rats (SHR) and Kyoto Wistar (WKY) rats (0 to 125 days of age) were analyzed in an attempt to correlate biochemical changes with the known functional changes occurring with the development of hypertension in the SHR. Several membrane constituents which have been shown to influence myocardial contractility and may reflect sympathetic nervous system alterations were measured in myocardial preparations from SHR and WKY rats at various ages. These constituents included the apparent numbers of alpha- and beta-adrenergic receptors, adenylate cyclase activities and $Na^+,K^+$- and $K^+,Ca^{++}$-ATPase activities. The apparent number of both alpha- and beta-adrenergic receptors decreased as the rats aged. Isoproterenol-stimulated adenylate cyclase activities were significantly higher ($P<0.05$) in the prehypertensive SHR when compared to WKY rats but declined to similar values as hypertension appeared. Both $Na^+,K^+$- and $K^+,Ca^{++}$-ATPase activities were higher in the SHR, when compared to WKY rats ($P<0.05$) at both pre- and post-hypertensive stages. Although not significant, the apparent numbers of cardiac alpha-and beta-adrenergic receptors were slightly elevated in the SHR. This, as well as the increased adenylate cyclase activity stimulated by isoproterenol during the prehypertensive stage may contribute to the onset of hypertension in

the SHR. The elevated ATPase activities measured in the SHR may also be partially responsible for the altered reactivity of the SHR myocardium, resulting in hypertension. Treatment of both strains with methimazole resulted in hypothyroidism, lowered blood pressure below control values, and completely prevented the development of hypertension in the SHRs up to 125 days. The apparent number of both alpha- and beta-adrenergic receptors were similar in euthyroid and hypothyroid WKYs and SHRs. Membrane preparations from hypothyroid animals showed a decrease in all adenylate cyclase and $Na^+,K^+$- and $K^+,Ca^{++}$-ATPase activities when compared to euthyroid controls. Hypothyroidism effectively prevents the onset of hypertension, suggesting these enzymic parameters play an integral part in the genesis of hypertension in the SHR.

# THE ROLE OF THYROID HORMONES AND MYOCARDIAL ALPHA-AND BETA-ADRENERGIC RECEPTORS IN THE DEVELOPMENT OF HYPERTENSION IN THE SPONTANEOUSLY HYPERTENSIVE RAT (SHR)

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# THE ROLE OF THYROID HORMONES AND MYOCARDIAL ALPHA-AND BETA-ADRENERGIC RECEPTORS IN THE DEVELOPMENT OF HYPERTENSION IN THE SPONTANEOUSLY HYPERTENSIVE RAT (SHR)

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For my parents Harry and Ilse

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# INTRODUCTION

Hypertension is the most common pathological condition found in individuals over age 35 (1). It is also the most important factor in the predisposition to other cardiovascular and cerebrovascular diseases (2). Approximately 90% of those afflicted are said to have essential hypertension (1), a condition which develops without preceding organic lesions and whose cause is unknown.

Hemodynamic studies (3) with labile and established hypertensive man indicate that there is a transition from a state of increased cardiac index and normal total peripheral resistance to one characterized by normal cardiac index and increased total peripheral resistance. The increased cardiac index observed in labile hypertension may be an initiating factor in the development of essential hypertension. According to the total body autoregulation theory (4) the inappropriately elevated cardiac index results in overperfusion of the body with regard to its metabolic requirements. This results in vasoconstriction which returns the cardiac index to normal but results in increased total peripheral resistance and increased arterial pressure.

The spontaneously hypertensive rat (SHR) is considered an excellent model for the study of human essential hypertension. The SHR, which is maintained as an inbred strain, was derived from normotensive Wistar-Kyoto (WKY) stock (5). Blood pressure in the SHR increases with age reaching levels approaching 200 mm Hg systolic, and thus surpassing the highest levels of 150 mm Hg obtained for WKY controls. An elevated arterial blood pressure is detectable in the SHR by 30 days of age (6).

Aging SHRs display a transition of hemodynamic characteristics, similar to those observed in human essential hypertension (7). Hemodynamic studies (8) performed in unanesthetized SHRs have shown that there is a transient elevation in cardiac index in young, prehypertensive SHRs, aged 32 to 41 days, concomitant with normal total peripheral resistance. By 80 days, cardiac index has returned to normal while total peripheral resistance and arterial pressure are elevated. The transitory increase in cardiac index observed in prehypertensive SHRs may play a role in the development of hypertension and may be due to altered sympathetic nervous system influence on the heart (8). The sympathetic nervous system's influence on the heart and blood vessels may also be a controlling factor in the established stage of hypertension.

Sympathetic nerve fibers which innervate both the myocardium and vascular smooth muscle release norepinephrine from their postganglionic endings. The cardiovascular system is also, in part, under the control of epinephrine and norepinephrine, hormones secreted by the adrenal medulla. Sympatho-adrenal stimulation of the cardiovascular system increases blood pressure by increasing the rate and contractility of the heart, thereby increasing cardiac output and by increasing resistance to flow by constriction of the peripheral vasculature (total peripheral resistance).

The specific action a hormone has upon a target tissue is mediated by the interaction of a molecule of a hormone with a receptor molecule located on the cell membrane or within a cell of the target tissue. In the case of the catecholamine hormones (norepinephrine and epinephrine), this interaction of hormone and receptor activates cellular membrane-bound enzymes, which causes changes within the cell which may ultimately

result in a measurable change of a physiological parameter such as cardiac output or total peripheral resistance. Changes in these parameters may be manifested as a change in blood pressure. Ahlquist (9) proposed that catecholamines exert their actions through two different types of receptors. He named them alpha and beta receptors based on two distinct potency series that he observed while testing the ability of a series of catecholamine agonists to elicit several physiological responses. Norepinephrine and epinephrine have somewhat different effects on these two receptors. Norepinephrine mainly activates alpha receptors but also activates the beta receptors to a slight extent as well. Epinephrine has a higher affinity for beta receptors but will also activate alpha receptors. Beta stimulation will increase cardiac output and decrease total peripheral resistance. Alpha stimulation will increase total peripheral resistance. Since both norepinephrine and epinephrine can initiate alpha and beta responses, the relative effects of norepinephrine and epinephrine on cardiovascular tissue are determined by the types and numbers of receptors present.

A great deal of work has centered upon the sympathetic nervous system's involvement in the cause and maintenance of hypertension in the SHR. Numerous experiments indicate that altered central nervous system control of the sympathetic nervous system and altered peripheral sympathetic nervous system activity may be involved. Pithing and transection experiments demonstrate the importance of the central nervous system in the tonic maintenance of hypertension (10). Cutting the neural connections between the hypothalamus and mesencephalon (11) or sympathetic denervation (12) will lower blood pressure to a greater extent in the SHR than

the WKY. Sympathetic nerve activity recorded from the splanchnic nerves is increased in the SHR. Severing of these nerves in the SHR and WKY reduces arterial pressure to the same level indicating increased peripheral sympathetic tone in the SHR (10, 13). Sympathetic nervous system activity increases along with increased mean arterial pressure as the SHR ages (14, 15). Pressor responses due to increased sympathetic neural firing are greater, and may be induced more easily in the SHR than WKY by electrical stimulation of the posterior hypothalmus (16, 17). These pressor and neural responses are decreased by chronic treatment with the antihypertensive drugs clonidine or hydralazine (18). Ganglion blockage with hexamethonium reduces sympathetic nervous system activity and hence total peripheral resistance and arterial pressure to the same level in the SHR and WKY (13, 19).

Pharmacologic depletion of norepinephrine in adrenergic nerves via 6-hydroxydopamine administration lowers blood pressure in the SHR (20, 21). Long term treatment of the neonate SHR with 6-hydroxydopamine completely prevents the development of hypertension (21, 22, 23). Since 6-hydroxydopamine does not cross the blood-brain barrier of adult rats it is likely that the site of action whereby hypotension is achieved is the peripheral sympathetic nervous system (21). However, the administration of 6-hydroxydopamine into the lateral ventricle will also lower blood pressure in the SHR (24) and will prevent its development (25, 26), thereby suggesting a central nervous system component as well. Inhibition of sympathetic nervous system activity by the administration of L-dopa following peripheral inhibition of l-amino acid decarboxylase will decrease blood pressure and heart rate in the SHR (27). Immunosympathectomy will also

lower blood pressure and prevent the development of hypertension in the SHR (28, 29).

Consistent with the evidence indicating that hypertension may be due in part to increased central and peripheral sympathetic nervous system activity are the findings of increased plasma norepinephrine levels (30, 31, 32) and dopamine-β-hydroxylase activity (30, 32, 33) in young SHRs.

Although altered central and peripheral sympathetic nervous activities may play a role in the development of hypertension in the SHR, another possible cause may be intrinsic differences in the vascular and/or myocardial tissues of the SHR. Various tissues in the SHR exhibit altered responsiveness to sympathomimetic agents. There is an elevation of sympathetic tone, a hyperresponsiveness to exogenous catecholamines and an increased vascular reactivity in the SHR (35). The intact SHR myocardium does not exhibit the increase in heart rate and cardiac output observed in normotensive rats in response to either small, infused doses of norepinephrine (36) or the specific beta-adrenergic agonist isoproterenol (37). Atria isolated from SHRs exhibit less chronotropic and ionotropic responses to isoproterenol than those of normotensive rats (38). Isolated papillary muscles of SHRs exhibit slow isometric relaxation rates and reduced responsiveness to isoproterenol (39). These results suggest that alterations in the beta-adrenergic receptor mechanisms may exist in the SHR myocardium. Pharmacological blockage studies with the SHR tend to support this premise.

Treatment of SHRs with the beta-adrenergic antagonists propranolol (40, 41, 42), alprenolol (40, 42), atenolol (41), nadolol (41), pindolol (41), sotalal (42), and cP2405 (42) are reported to lower blood pressure

and heart rate. Chronic administration of propranolol (100 mg/Kg/day) will prevent the development of hypertension in the SHR (43). Chronic treatment of neonates with low doses (1 or 5 mg/Kg/day) of propranolol will also significantly lower the blood pressure of SHRs but not that of WKYs (44). Similarly chronic treatment of the SHR from an early age with propranolol or H93/26 (AB Hassle, Molndal, Sweden), a cardioselective $B_1$-adrenergic antagonist will prevent the development of hypertension and structural changes in resistance vessels while similar treatment of SHRs with established hypertension has far less influence (45).

Alpha-adrenergic receptor blockage has also been studied in the SHR. Blockage by phenoxybenzamine, phentolamine or E-643, a quinazoline compound, will reduce blood pressure in SHRs with established hypertension, most likely by reducing vascular tone in peripheral blood vessels (46). Hypotension induced by alpha-receptor blockage is greater in the SHR than WKY (47, 48).

In addition to the sympathetic nervous system, various endocrine organs are also suspected of playing a role in the development and maintenance of human essential hypertension and hypertension in the SHR. Hypertension often accompanies hyperthyroidism (49) and can be induced by the administration of thyroid hormones (50). The thyroid gland has been extensively studied in the SHR although it's exact role and activity are a matter of contention. The results of numerous experiments are often contradictory and have been interpreted various ways by several investigators.

Some workers have suggested that the thyroid gland is hyperactive in the SHR. Increases in area of follicle cells, and irregularity in

follicle shape occur in the prehypertensive SHR thyroid gland (51). These changes become more drastic and thyroid weight increases as hypertension becomes more severe. SHRs also have larger thyroid glands than normotensive controls (51, 52, 53, 54, 55). It has been suggested that in the prehypertensive stage of the SHR increased numbers of pituitary basophils secrete increased amounts of thyroid-stimulating hormone (TSH) which results in hyperactivity of the thyroid which raises blood pressure (51, 56). Hyperfunction of TSH activating hypothalamic nuclei in SHRs has been demonstrated employing histochemical techniques (56). Further evidence of a hyperactive hypothalamic-hypophyseal-thyroid axis comes from reports of elevated levels of TSH in the circulation (54, 55, 57, 58, 59, 60, 61, 62, 63) and in the pituitary (51, 54) of the SHR. The release of TSH mediated by thyrotropin releasing hormone (TRH) is also exaggerated in the SHR and appears to be altered by 15 days of age (62). Recognizable thyroid activity is reported to begin between 16 and 40 days of age in the SHR and is elevated 1.2 to 1.4 times over controls at 40 days and thereafter (64). Interestingly, the rate of increase in blood pressure in SHRs is greatest between 40 and 60 days (5, 65). Specifically, the uptake of radioiodide, cyclic AMP-dependent protein kinase activity (54) and acid phosphatase activity (66) are also increased in SHR thyroids.

Radiothyroidectomy or methylthiouracil treatment will prevent the development of hypertension in the prehypertensive SHR and reduce its severity in SHR with established hypertension (67). Conversely, the administration of thyroid powder will increase the development and severity of hypertension (68) and reverse the protective effects of radiothyroidectomy (69) in the SHR. Identical results are obtained employing

surgical thyroidectomy and thyroid hormones replacement therapy (70). We have also reported that continuous treatment of the SHR from birth with methimazole (MMI), an antithyroid compound, will prevent hypertension, lower heart rate, and significantly increase plasma epinephrine and norepinephrine (71). Similarly, long term treatment of the SHR with propylthiouracil will also reduce blood pressure (64, 72).

Experiments have shown that the thyroid is necessary for the development of hypertension in the SHR; nevertheless, there is a convincing body of experimental evidence suggesting that the thyroid in the SHR is hypoactive. It has also been suggested that an abnormality exists in the SHR pituitary-thyroid axis since thyroid hormones appear to be less effective in depressing TSH synthesis and secretion than in normotensive rats (54, 61, 62). This abnormality has been detected in 15 day old SHRs, an age which precedes the establishment of hypertension. The increased circulating levels of TSH (54, 55, 57, 58, 59, 60, 61, 62, 63) and increased thyroid weight (51, 52, 53, 54, 55) observed in the SHR may be interpreted as an indication of hypothyroidism. It is well documented that a hypothyroid status results in increased secretion of TSH which will stimulate growth of the thyroid gland and the secretion of thyroid hormones. Plasma levels of thyroxine ($T_4$) (51, 52, 54, 55, 57, 58, 63) and triiodothyronine ($T_3$) (55, 61, 62) are reported to be low in the SHR indicating a hypothyroid status. Although the original histological studies of the SHR thyroid were interpreted as an indication of a hyperactive gland (51), these same parameters may also be interpreted to signify a reduction in thyroid hormone secretion (73). Light and electron microscopic investigations of SHR thyroids suggest defects at the thyroid level which

may result in a hypothyroid state (72). SHRs display a heterogeneous colloid indicating an imbalance between colloid storage and hormone secretion. This colloid resists breakdown even following propylthiouracil treatment, suggesting a defect in the ability of the thyroid follicle cell to break down and resorb colloid. The unexpected appearance of non-dilated rough endoplasmic reticulum in the presence of elevated circulating levels of TSH suggests an inhibition of protein synthesis which may play a role in the decreased responsiveness of the SHR thyroid to TSH. This rough endoplasmic reticulum will dilate following propylthiouracil treatment thereby supporting the contention that SHR thyroids will only respond to highly elevated TSH levels. The activity of proteolytic enzymes necessary for the breakdown of colloid is reduced and the colloid is more resistant to proteolytic enzyme action in SHR thyroids (54). The uptake and rate of release of $^{131}I$ from SHR thyroids is less than that of normotensive controls (53, 54) suggesting a hypoactive gland.

While there is controversy as to whether the SHR exhibits hyper- or hypothyroidism there are also reports of normal T4 (60) and T3 (57, 58, 60, 62, 63, 72) concentrations in the SHR.

Thyroid hormones exert a permissive action on the biological responses mediated by catecholamine hormones (i.e., lipolysis, cardiac contractility). These responses are modulated by the thyroid status of the organism (74, 75, 76). Hyperthyroidism results in a state of increased adrenergic activity while hypothyroidism leads to a decrease in adrenergic sensitivity. The heart's response to catecholamine stimulation is particularly sensitive to varying thyroid status. Ventricular ejection time is increased in hypothyroidism (77). Hyperthyroidism is associated with a decreased myocardial

ejection time (77), and an increase in myocardial contractility (78, 79), myosin ATPase activity (80), and myocardial phosphorylase activation by catecholamines (81, 82, 83, 84, 85). Some of these effects appear to involve alterations in the beta-adrenergic receptor mechanism. Specifically, isolated fetal mouse hearts in organ culture exhibit increased sensitivity to β-adrenergic stimulation when exposed to thyroid hormones (86). Cardiac β-adrenergic receptors are less sensitive following propylthiouracil treatment (87, 88, 89, 90). The increased contractility observed in hyperthyroidism can be reduced by β-adrenergic blockage with propranolol (91, 92, 93).

Alpha-adrenergic responses are also affected by variations in thyroid hormone levels. Rats treated with propylthiouracil display increased positive ionotropic (87, 94, 95) and chronotropic (87, 94) responses to α-adrenergic stimulation. It has been suggested that α- and β-adrenergic receptors represent allosteric configurations of a common macromolecule which may be modulated by thyroid hormones (88, 96, 97). The inotropic response to β-adrenergic stimulation is decreased while the α-adrenergic response is increased in atria from hypothyroid rats (88, 98, 99). This appears to be a reciprocal relationship since the opposite effects are observed with thyroxine treatment (88, 99).

Recently the number of adrenergic receptors have been measured directly by methods employing the direct binding of radioligands to cellular fractions of responsive tissues. Tritiated dihydroergocryptine ([$^3$H]-DHE) and dihydroalprenolol ([$^3$H]-DHA) are ligands which have been successfully employed for the assessment of cardiac alpha-(100) and beta-(101) adrenergic receptors respectively. These ligands bind to sites

which have characteristics of either alpha-or beta-adrenergic receptor binding sites. Binding is rapid, reversible, saturable, stereospecific and of high affinity.

The regulation of adrenergic receptors by thyroid hormones has been demonstrated utilizing these methods. The number of myocardial alpha receptors has been reported to be lower in hyperthyroid rats (102, 103, 104) and either decreased (102, 104, 105) or not changed in hypothyroid rats (103). Thyroid hormone replacement therapy to thyroidectomized rats reduced the number of alpha-adrenergic receptors (106). Hyperthyroid rats exhibit an increased number of myocardial beta-adrenergic receptors (102, 104, 105, 107) while hypothyroidism results in a decreased number of beta-adrenergic receptors (102, 104, 105, 108). The number of beta-adrenergic binding sites in cultured rat ventricle slices (109) or heart cells (110, 111) is increased during incubation with thyroid hormones.

Thyroid hormones may also modulate the number of adrenergic receptors in an indirect manner by affecting the circulating levels of catecholamines. Total plasma catecholamines are low in hyperthyroidism and elevated in hypothyroidism (112). It has been suggested that the number of hormone receptor molecules varies inversely with circulating levels of the hormone, thereby providing an effective means of hormone receptor regulation (113). Chronic exposure of tissues to catecholamines will decrease the number of β-adrenergic receptors (114, 115). This relationship may possibly be observed in hypothyroid SHRs which exhibit increased plasma epinephrine and norepinephrine and decreased heart rate and arterial pressure (71).

Direct radioligand binding methods have been employed to assess the

number of beta-adrenergic receptors in the hearts of SHRs. Myocardial membranes from SHRs with established hypertension are reported to possess either a lower (116, 117) or equal (118) concentration of beta-adrenergic receptors while prehypertensive have an increased number (118).

Several enzymatic constituents of the myocardium may also have a role to play in the development and maintenance of hypertension. The activity of adenylate cyclase, the enzyme coupled to the beta-adrenergic receptor, has been measured in various preparations from SHRs with established hypertension and has been found to be decreased related to the activity measured in normotensive animals of a similar age (119, 120, 121, 122, 123, 124). Cardiac adenylate cyclase activity decreases with propylthiouracil treatment or thyroidectomy (125, 126, 127). Since hypothyroidism tends to attenuate hypertension, a decrease in this enzyme may be a factor in the decreased reactivity of the hypothyroid SHR myocardium. Thyroid hormone alters $Na^+,K^+$-ATPase activity in myocardial and other tissues (104, 125, 128, 129). The physiological significance of these changes is not known, but there may in fact be a correlation between $Na^+,K^+$-ATPase activity and the development or maintenance of hypertension. Intracellular $Ca^{++}$ concentrations are involved in the inotropic and chronotropic effects of the myocardium. Intracellular free $Ca^{++}$ concentration is partially regulated by $K^+,Ca^{++}$-ATPase of the sarcoplasmic reticulum. Alterations in the activity of this enzyme may be responsible for abnormal distributions of intracellular $Ca^{++}$. Inducing hypothyroidism effectively decreases $K^+,Ca^{++}$-ATPase activity of the sarcoplasmic reticulum (104, 130, 131). Modifications in the activity of sarcoplasmic reticulum in transporting calcium could be one possible explanation for an alteration in the con-

tractile properties and reactivity of the SHR myocardium. SHR myocardium sarcoplasmic reticulum $K^{+}$,$Ca^{++}$-ATPase activities are greater than those of WKY rats (132).

The present study was undertaken to determine if, during the development of hypertension, changes occur in the number of myocardial alpha- and beta-adrenergic receptors, the activity of adenylate cyclase and/or ATPase enzymes which could account for the altered cardiac index observed in the pre-hypertensive SHR. Since the reactivity of the sympathetic nervous system and the myocardium appears to be altered by thyroid hormones, these parameters were also assessed in hypothyroid animals to determine if this status influenced the development of hypertension in the SHR and if so which parameters were affected.

# MATERIALS AND METHODS

## Materials

Dihydroalprenolol hydrochloride, levo -[propyl-1,2,3-$^{3}$H] - (51 Ci/mmol, 47.4 Ci/mmol), dihydro-α-ergocryptine, 9, 10-[9, 10-$^{3}$H(N)]- (38.8 Ci/mmol), adenosine 3', 5'-cyclic phosphate [2,8-$^{3}$H]- and adenosine 5' - triphosphate [α-$^{32}$P] were obtained from New England Nuclear. DL-propranolol, methimazole, bovine serum albumin, and neutral alumina were obtained from Sigma Chemical Corporation. A23187 was obtained from Calbiochem. Dowex AG 50W-X8 ion exchange resin (200-400 mesh, hydrogen form) was obtained from Bio-Rad. The following compounds used in this study were donated by the indicated companies: phentolamine (Ciba), D-propranolol and L-propranolol (Ayerst), and D-isoproterenol, D-epinephrine and D-norepinephrine (Winthrop). Total and free L-thyroxine (3,5,3',5'-L-tetra-iodothyronine) (T4) and total L-triiodothyronine (3,3',5-triiodothyronine) (T3) radioimmunoassay kits were purchased from Clinical Assays Division of Travenol Laboratories, Inc.

## Treatment of Animals

SHR and WKY rats were bred and maintained in the animal facility of the School of Medicine, East Carolina University. Dames were individually caged, provided with standard laboratory rat chow and tap water ad libitum and maintained on a 14:10, light:dark cycle. At parturition (day 0) litter size was adjusted to 6-10 pups in order to insure that during development the nutritional status of all groups would be relatively consistent, since this parameter has a direct effect on body weight. Only male rats were used in this study to avoid any possible differences which

might arise due to sex. Litters were chosen at random to be either control or methimazole treated groups. Hypothyroidism was induced in neonates by adding 0.01% methimazole to the drinking water of lactating dames. Methimazole passes in the milk and is made available to the nursing pups. Systolic blood pressure was measured with a Friedman:Freed microphonic manometer and tail cuff on conscious, restrained rats 30 days or older after gentle prewarming. This non-invasive method affords reliable and reproducible blood pressure measurements. Core temperatures were monitored using a rectal temperature probe in order to insure that blood pressures were not elevated due to overheating. At the time of sacrifice (0, 5, 10, 20, 30, 40, 50, 60, 100, 125 days of age) rats were weighed and decapitated. Each particular experimental group (age, strain, treatment) was repeated 2 or 3 times. The blood serum from each group was collected, pooled and stored below -25°C until thyroid hormone assays were performed. The hearts were rapidly removed, placed in ice-cold homogenization buffer (250 mM sucrose/50 mM Tris HCl, pH 7.5 at room temperature), rinsed in a change of buffer to remove blood and weighed to the nearest milligram. The atria, great vessels and fat were dissected away and the ventricles also weighed to the nearest milligram.

Preparation of Membranes

The pooled ventricles of each group were homogenized in ice cold homogenization buffer (10 ml/gm) by three 30-second pulses of a Brinkmann Polytron PT-10 tissue homogenizer with the rheostat set at position 5. The homogenate was centrifuged at 4°C, 1000 X g for 10 minutes. The pellet, containing connective tissue and cellular debris, was discarded

and the supernatant was centrifuged at 4°C at 40,000 X g for 30 minutes. The supernatant was discarded and the pellet was resuspended in homogenization buffer to a final concentration of approximately 8 mg protein/ml. The membrane preparation was divided into aliquots and stored below -25°C until enzymic assays were performed. Protein from ventricular membranes was determined by the method of Lowry et al. (133) using bovine serum albumin as a standard.

Beta-adrenergic Receptor Binding Assay

Beta-adrenergic receptor binding was assayed with tritiated dihydroalprenolol, [$^3$H]-DHA, by a method similar to that of Alexander et al. (101). Approximately 200 µg of membrane protein and varying concentrations (0.5 - 10.0 nM) of [$^3$H]-DHA, made up fresh in distilled deionized water, were placed in 13 x 100 mm borosilicate culture tubes on ice. The final incubation volume of all tubes was adjusted to 200µl with incubation buffer (50mM Tris-HCl/5mM $MgCl_2$, pH 7.4 at room temperature). The binding reaction was carried out for 20 minutes in a 37°C shaking water bath and was then terminated by placing the tubes on ice. Non-specific binding was determined by duplicate incubation in the presence of 100 µM DL-propranolol. Membrane-bound radioligand was separated from unbound radioligand by rapid filtration on Whatman GFC glass fiber filters. All tubes and membranes were rinsed five times with 5 ml of ice cold washing buffer (75 mM Tris/0.25 mM $MgCl_2$, pH 7.65 at room temperature). The filters containing the membrane-bound radioligand were air dried overnight and then placed in 10 ml of Toluene/Triton X-100 scintillation fluid. The radioactivity was measured by liquid scintillation spectrophotometry with a Beckman LS 9000 liquid scintillation spectrophotometer. The apparent

number of beta receptors was assessed for rats in groups aged 0, 5, 40, 50, 60, 100 and 125 days by employing a saturable dose of 10 nM [$^3$H]-DHA. In all other age groups the apparent number of beta receptors was determined by Scatchard plot analysis (134).

Alpha-adrenergic Receptor Binding Assay

Alpha-adrenergic receptors were assessed with tritiated dihydroergocryptine, [$^3$H]-DHE, (100) using methods similar to those described for [$^3$H]-DHA binding with the following exceptions: a saturable concentration of 4nM [$^3$H]-DHE was employed for each sample, binding took place at 30°C for 30 minutes and non-specific binding was determined in the presence of 100μM phentolamine- HCl.

Adenylate Cyclase Assays

Adenylate cyclase activity was assessed by measuring the conversion of [$\alpha$-$^{32}$P] ATP to cyclic [$^{32}$P] AMP as described by Queener et al. (135) with slight modification of the incubation mixture. The reactions were carried out in borosilicate culture tubes (12 x 75 mm) in a final volume of 0.1 ml which contained: [$\alpha$-$^{32}$P]ATP, 1 mM (0.5 to 1.5 μCi); Tris/HCl, 37.5 mM (pH 7.5); $MgCl_2$, 9 mM; $CaCl_2$, 50 μM; cyclic AMP, 0.5 mM (included to minimize the effects of phosphodiesterase); dithiothreitol, 0.1 mM; phosphocreatine, 5 mM; and creatine phosphokinase, 0.5 units. The reactions were initiated by the addition of enzyme protein (30-50 μg), incubated at 37°C for 10 minutes, and terminated by the addition of 0.1 ml of a solution containing ATP, 40 mM; cyclic AMP, 5 mM; and 0.03μCi of cyclic [8-$^3$H]AMP to allow subsequent calculations of cyclic AMP recovery. The isolation of cyclic AMP formed during the reaction was by slight

modification of the technique of Salomon et al. (136) which combined the techniques originally reported by Krishna et al. (137) and White et al. (138). Deionized water (1.7 ml) was added to the terminated adenylate cyclase reaction mixtures which were subsequently poured onto columns (0.9 x 5 cm) of Dowex AG 50W-X8 ion exchange resin (200-400 mesh, hydrogen form). The column effluent resulting from sample application was discarded. Additional deionized water (3 ml) was applied to the column and the effluent was also discarded. Cyclic AMP was then eluted with deionized water (6 ml), and the effluent was allowed to pass directly onto columns of neutral alumnia (1.2 gm). Imidazole, (100 mM, pH 7.6, 8 ml) was added to the alumina columns, and the effluent was collected into scintillation vials for subsequent determination of radioactivity in a liquid scintillation spectrometer. Recovery of cyclic [8-$^{3}$H]AMP was routinely 70 to 80%.

ATPase Assays

ATPase activities were measured at 37°C by monitoring the release of inorganic phosphorus from 3 mM Tris ATP (139). Total $Na^{+},K^{+}$-ATPase activity was unmasked by pretreating the cardiac membranes with sodium dodecyl sulfate (SDS) according to the established protocol of Besch et al. (140). Briefly, freshly thawed membrane preparations (4-8 mg/ml) were diluted 1:10 in imidazole-HCl buffer (pH 7.1) containing 3.8 mM SDS. After preincubation for 20 minutes at room temperature, 40 µl of the diluted suspension was added to previously prepared reaction tubes of 1 ml of incubation medium containing 50 mM histidine, 3 mM $MgCl_2$, 100 mM NaCl and 10 mM KCl, pH 7.4. $Na^{+},K^{+}$-ATPase activity was the portion of total activity inhibited by 8 mM ouabain. The ouabain-sensitive $Na^{+},K^{+}$-

ATPase activity accounted for 70% or more of the total ATPase activity measured in SDS pretreated membranes. $K^+,Ca^{++}$-ATPase activity was measured in freshly thawed membranes not pretreated with SDS (141). This activity was assessed in a medium containing 50 mM histidine (pH 7.4), 3 mM $MgCl_2$, 50 μM $CaCl_2$, 90 mM KCl, 5 mM $NaN_3$ and 5.2 μM A23187 (ionophore/protein ratio of 0.2 - 0.3 μg/μg). The ionophore A23187 was added to assure that the full activity of $K^+,Ca^{++}$-ATPase was not limited by intravesicular accumulation of $Ca^{++}$. $K^+,Ca^{++}$-ATPase activity was taken as the activity inhibited by 1 mM Tris EGTA under conditions otherwise identical to those above.

Serum thyroid hormone levels

Circulating thyroid hormones may exist either bound to plasma proteins or unbound (free). Serum free and total T4 and T3 levels were measured by competitive binding radioimmunoassay using procedures outlined by Clinical Assay Division of Travenol Laboratories, Inc. In the total thyroid hormone assays serum samples were incubated with either $[^{125}]$I-T4 or $[^{125}]$I-T3 tracer hormone in tubes coated with either rabbit anti-T4 or anti-T3 antibodies. The incubation mixtures used in the total T4 assays contained 8-anilo-1-napthalene sulfonic acid and salicylate to displace any T4 which was bound to binding proteins. Serum hormone and $[^{125}I]$ tracer may both bind to the antibody which is immobilized on the tube's inner wall. The amount of radioactivity remaining associated with the tube following the incubation and decanting of the reaction mixture is inversely proportional to the amount of serum hormone. The degree to which the serum hormone displaces the $[^{125}I]$ tracer hormone from binding

to the antibody was quantified by counting the tubes in a gamma counter. The amount of T4 and T3 was determined by interpolation from a standard curve based on known standards. Free T4 was determined by first incubating samples in rabbit anti-T4 antibody coated tubes. The free T4 becomes immobilized on the tube while the serum fraction is removed. This is followed by a second incubation with [$^{125}$I] T4 tracer and quantification the same as that used for determining total serum samples.

Statistical Analysis

At separate ages, differences in body weight, ventricle weight to body weight ratio (mg/100 gm) and blood pressure were determined within the same strain, between treatments and between strains, with the same treatment by the Student's t-test. A P value of 0.05 or less was accepted as a statistically significant difference.

Differences in T3, free T4, total T4 [$^{3}$H]-DHE binding, [$^{3}$H]-DHA binding, adenylate cyclase activities and ATPase activities were determined by analysis of covariance within the same strain, between treatments and between strains, with the same treatment with age as the covariant. A P value of 0.05 or less was accepted as statistically significant.

# RESULTS

## Characterization Of Adrenergic Receptor Binding In Ventricular Membrane Preparations Using Radioligands

In order to assure that the radioligands employed in this study bound to molecules with the characteristics of alpha- or beta-adrenergic receptors, several experiments were performed to characterize this binding. [$^3$H]-Dihydroergocryptine ([$^3$H]-DHE) and [$^3$H]-dihydroalprenolol ([$^3$H]-DHA) were used to measure alpha- and beta-adrenergic receptors, respectively. The distinction between alpha- and beta-adrenergic receptors is based upon two distinct potency series of various catecholamine agonists to elicit various physiological responses as well as the ability of several antagonists to specifically block these responses. Since this specificity originates at the level of the receptor molecule, the capability of adrenergic agonists and antagonists to compete with the radioligands employed in this study for occupancy of receptor binding sites should reflect the same order of potency and specificity observed biologically. Additionally, since the l-isomers of catecholamine agonists and antagonists are more potent than the d-isomers in eliciting their effects, receptor binding should also display this stereospecificity. The specificity and stereospecificity of [$^3$H]-DHE binding to alpha-adrenergic binding sites is shown in Table 1. Epinephrine is more potent than norepinephrine in competing for binding sites. Both agonists display stereospecificity, the l-isomers being more potent than the d-isomers in competing for binding sites. Phentolamine, an alpha-adrenergic antagonist inhibited binding. Propranolol, a beta-adrenergic antagonist and isoproterenol, a beta-adrenergic agonist displayed no stereospecificity in their very weak

effects. The specificity and stereospecificity of [$^3$H]-DHA binding to beta-adrenergic binding sites is shown in Table 2. The decreasing order of potency for the catecholamine agonists isoproterenol, epinephrine, and norepinephrine is demonstrated as well as their stereospecificity. Propranolol competed very strongly for binding sites and displayed stereospecificity. Phentolamine did not inhibit binding. Since there is a limited number of adrenergic receptors in any tissue at any one time the binding of radioligands to the receptors should be a saturable process. Binding of [$^3$H]-DHE to alpha-adrenergic receptors and [$^3$H]-DHA to beta-adrenergic receptors is a saturable process (Figure 1). In vivo and in vitro the effects of adrenergic agonists and antagonists are rapid. When studied directly radioligands should also bind to adrenergic receptors rapidly. The rapidity of [$^3$H]-DHE and [$^3$H]-DHA binding to their respective receptor sites in rat ventricular membranes is illustrated in Figure 2. These experiments demonstrate that in rat ventricular membrane preparations the radioligands [$^3$H]-DHE and [$^3$H]-DHA bind to alpha- and beta-adrenergic receptors, respectively.

Table 1. Influence of Adrenergic Agents on the Specific Binding of [$^3$H]-DHE to Rat Ventricular Membrane Alpha-adrenergic Receptor Binding Sites

| Agent added | [$^3$H]-DHE bound (fmol/mg protein) |
|---|---|
| none | 85.6 |
| Phentolamine | 57.4 |
| (—)-Propranolol | 72.6 |
| (+)-Propranolol | 73.9 |
| (—)-Epinephrine | 57.0 |
| (+)-Epinephrine | 70.3 |
| (—)-Norepinephrine | 59.7 |
| (+)-Norepinephrine | 68.2 |
| (—)-Isoproterenol | 70.1 |
| (+)-Isoproterenol | 65.5 |

Binding was determined in the presence 3.8 nM [$^3$H]-DHE and 1μM concentrations of the agents added. Binding conditions were the same as those described in Methods. Each value is the mean of six determinations.

Table 2. Influence of Adrenergic Agents on the Specific Binding of [$^3$H]-DHA to Rat Ventricular Membrane Beta-adrenergic Receptor Binding Sites

| Agent added | [$^3$H]-DHA bound (fmol/mg protein) |
|---|---|
| none | 56.8 |
| Phentolamine | 67.9 |
| (—)-Propranolol | 24.2 |
| (+)-Propranolol | 49.3 |
| (—)-Epinephrine | 49.8 |
| (+)-Epinephrine | 55.2 |
| (—)-Norepinephrine | 54.5 |
| (+)-Norepinephrine | 54.9 |
| (—)-Isoproterenol | 24.8 |
| (+)-Isoproterenol | 42.3 |

Binding was determined in the presence of 7.4 nM [$^3$H]-DHA and 1μM concentrations of the agents added. Binding conditions were the same as those described in Methods. Each value is the mean of six determinations.

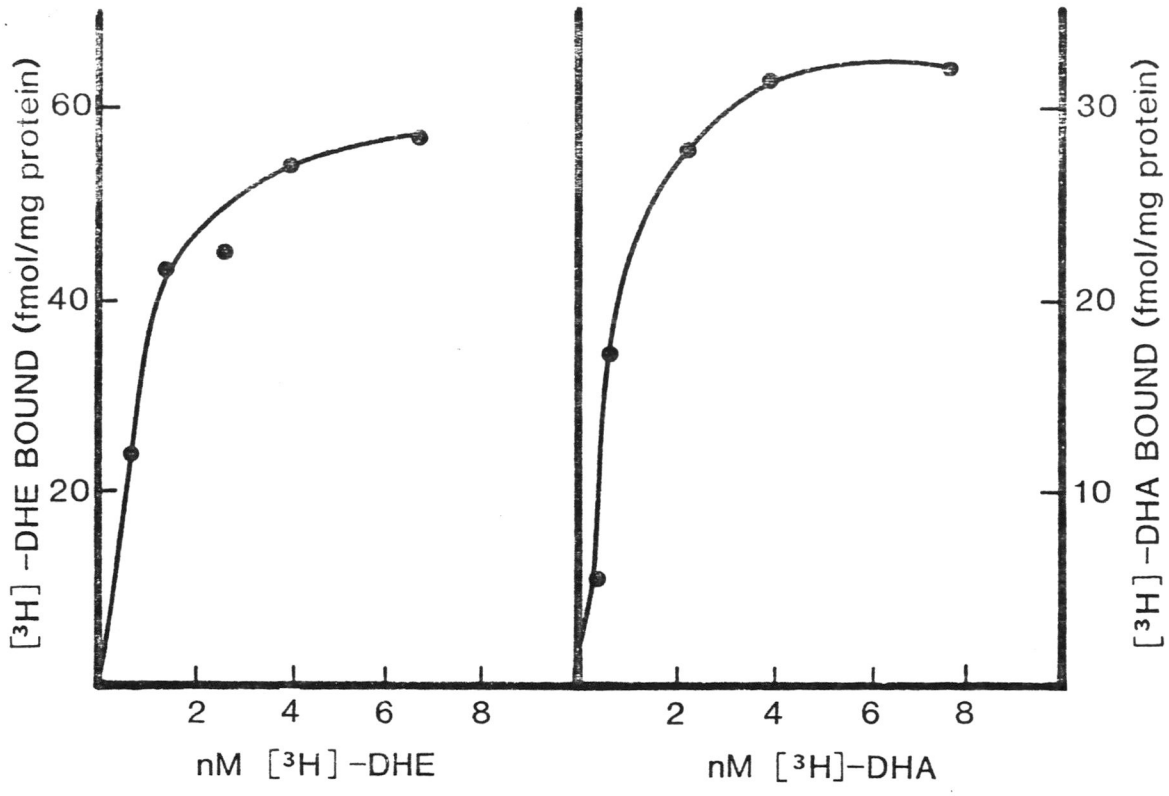


FIGURE 1. Saturation of Specific [$^3$H]-DHE Binding to Alpha-adrenergic Receptors and [$^3$H]-DHA Binding to Beta-adrenergic Receptors of Rat Ventricular Membranes

Specific [$^3$H]-DHE binding was the binding displaceable by 100μM phentolamine and was saturable at approximately 7 nM [$^3$H]-DHE. Specific [$^3$H]-DHA binding was the binding displaceable by 100μM DL-propranolol and was saturable at approximately 6nM [$^3$H]-DHA. Results are from typical experiments with rat ventricle membranes. Binding conditions were the same as described in Methods. Each value is the mean of triplicate incubations.

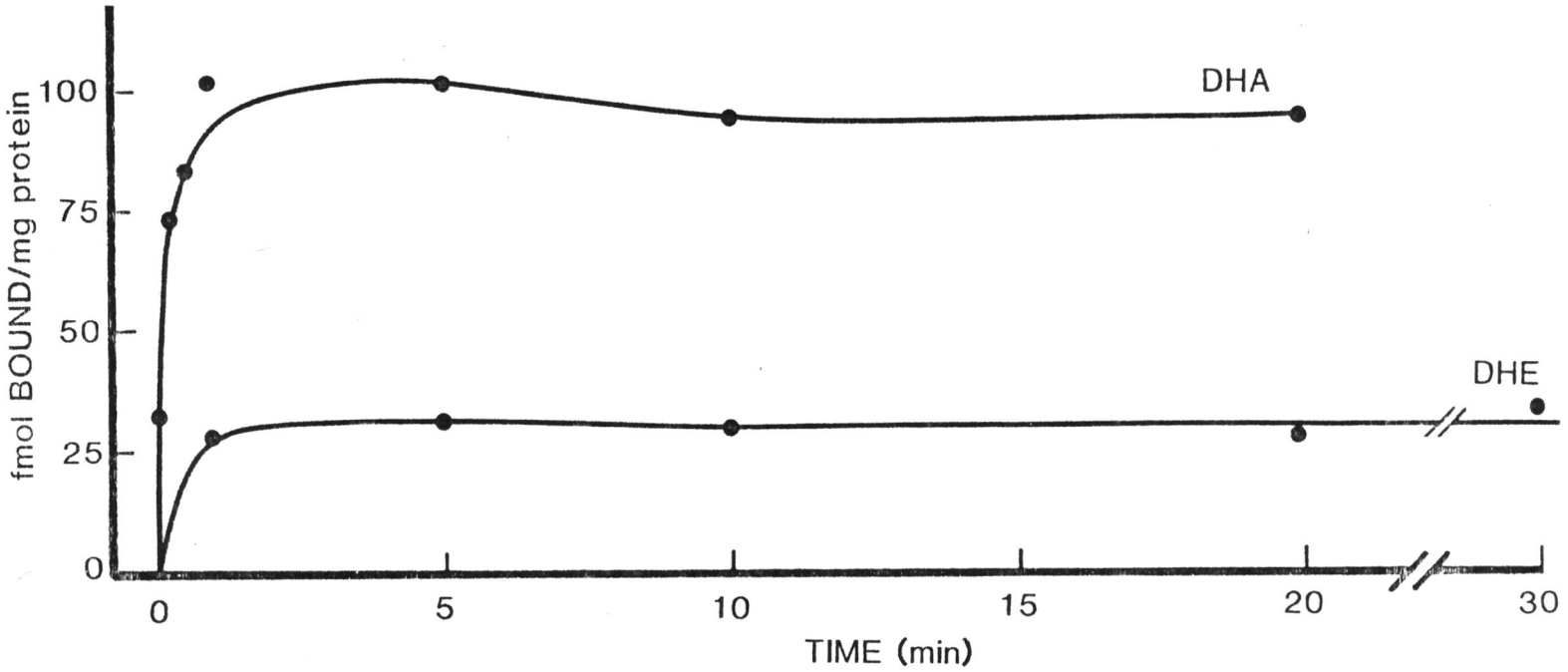


FIGURE 2. Rapidity of Specific [$^3$H]-DHE Binding to Alpha-adrenergic Receptors and [$^3$H]-DHA Binding to Beta-adrenergic Receptors of Rat Ventricle Membranes

Specific [$^3$H]-DHE binding was the binding displaceable by 100μM phentolamine. Specific [$^3$H]-DHA binding was the binding displaceable by 100μM DL-propranolol. Rat ventricular membranes were incubated for varying lengths of time. Each value is the mean of triplicate incubations.

## Thyroid Hormone Levels

Serum triiodothyronine (T3) levels for control neonates were below the range of the assay until the rats of both strains reached 10 days of age (Table 3). The maximum levels were reached by 30 days of age, 1.22 - 1.50 ng/ml, and dropped slightly by 125 days where the WKYs were lower than the SHRs. Serum levels for free and total thyroxine (T4) reached adult levels by 20 days of age and were similar in both strains (Table 4). Methimazole treatment lowered total T3 below the range of the assay in all ages of the SHR except age 20 days, while measurable levels below controls existed for most ages after 10 days in the WKY (Table 3). Methimazole significantly lowered ($P<0.05$) free T4 and total T4 in both strains (Table 4).

## Gravimetric Determinations

Body weights of SHRs were significantly lower ($P<0.05$) than those of WKY rats at 5 and 10 days of age (Table 5). Methimazole treatment significantly lowered ($P<0.05$) body weight in SHRs at 5, 30, 100 and 125 days of age and in WKY rats at all ages tested. The ventricle to body weight ratio of the SHR was significantly greater ($P<0.05$) than the WKY rat at 0, 5, 10, 30, 100 and 125 days of age. In both the SHR and WKY rat, the ventricle to body weight ratio increased until 5 days of age and decreased thereafter. Methimazole treatment significantly lowered ($P<0.05$) the ventricle to body weight ratio in the SHR at 10 and 30 days of age and the WKY rat at 10 and 20 days of age.

## Blood Pressure

Blood pressures in the control SHRs were significantly higher ($P<0.05$) than in the control WKY rats at all ages measured (Table 6). At

125 days, the oldest age measured, systolic blood pressures for SHRs and WKY rats averaged 187±4 and 135±2 mmHg respectively. Methimazole treatment significantly lowered blood pressures ($P<0.05$) in the SHRs and WKY rats at all ages (132±6 verses 100±2). Hypothyroid SHRs exhibited blood pressures similar to or lower than those associated in normotensive control WKY rats.

Alpha- and Beta-adrenergic Receptor Binding

The variation between groups in apparent numbers of both alpha- and beta-adrenergic receptor binding was greatest in the prehypertensive period (0 to 30 days) during the development of hypertension.

Alpha-adrenergic receptor binding sites decreased with age in the membrane preparations assessed from both SHRs and WKY rats (Table 7). At 0 days of age [$^3$H]-DHE bound to ventricle preparations of control SHRs and WKY rats were 112 and 173 fmol/mg protein, respectively, while by 125 days of age binding had decreased to 23 and 20 fmol/mg protein respectively. Although not statistically significant, alpha-adrenergic receptor densities were higher in SHR versus WKY at all ages except 0 days of age. Alpha-adrenergic receptor binding was not significantly altered by methimazole treatment in either SHRs or WKYs. Beta-adrenergic receptor binding sites also decreased with age in the cardiac membranes assessed from SHRs and WKY rats (Table 8). The decreases in [$^3$H]-DHA binding from 0 to 125 days of age were 82 to 37 fmol/mg protein for the SHRs and 72 to 32 fmol/mg protein for the WKY rats. Since a dramatic drop in beta-receptor density between 30 and 100 days of age was initially noted, this was explored further at 40, 50 and 60 days of age. It appeared

that the density of beta receptors in the developing SHR underwent a dramatic decrease around the age of 30-40 days. During this period [$^3$H]-DHA binding decreased from 80 to 42 fmol/mg protein. Although not statistically significant, beta-adrenergic receptor densities were higher in SHRs at all ages tested except 5 days of age. Beta-adrenergic receptor binding was not significantly altered by methimazole treatment in SHRs or WKY rats.

Adenylate Cyclase Activities

Basal cardiac adenylate cyclase activities decreased with age in a similar fashion with the decrease in alpha- and beta-adrenergic receptor densities; however, a more gradual decrease was observed rather than the abrupt drop seen in beta receptor density at 30-40 days in the SHR. Although not statistically significant, basal activity of the prehypertensive SHR was higher than that activity measured in the WKY rats (Table 9). This role was reversed as hypertension developed and at 60-100 days of age, the basal cyclase activity of the SHRs was slightly lower than that assessed in the WKY rats. Guanine nucleotide-stimulated adenylate cyclase activities also showed an age dependent decrease with the activities of membranes from SHRs being slightly higher than those of WKY rats during the prehypertensive stage and slightly lower at 100 days of age (Table 9).

Adenylate cyclase is coupled to the beta-adrenergic receptor and is activated by binding of the catecholamine hormones to the beta-adrenergic receptor. Isoproterenol, a synthetic beta-adrenergic agonist may be used to bind to the beta-adrenergic receptor and thereby stimulate the catechol-

amine hormone-sensitive adenylate cyclase activity. Isoproterenol-stimulated adenylate cyclase activities assessed in cardiac membranes from SHRs were significantly higher ($P<0.05$) than those activities assessed from WKY rats at early ages (0-30 days). This increase was also reversed by 100 days of age where the hormone-stimulated activity (1μM isoproterenol) in the SHRs was 16% lower ($P<0.05$) than in the WKY rats. The per cent stimulation produced by isoproterenol (guanine nucleotide stimulated/isoproterenol stimulated adenylate cyclase activity X 100) was also greater in the prehypertensive SHR cardiac preparations when compared to the WKY and was also reversed at 100 days of age (Table 9). The increase in all adenylate cyclase activities of both strains at 5 days of age correlated with the increase in heart wt/body wt at this age (Table 5).

Methimazole treatment significantly lowered ($P<0.05$) all cardiac adenylate cyclase activities tested in membranes from SHRs. This included basal and Gpp(NH)p stimulated activities (data not shown). As seen in Figure 3, methimazole effectively lowered the catecholamine stimulated adenylate cyclase activities of the SHR myocardial preparations to values slightly lower than those values assessed in normotensive control WKY rats. Although adenylate cyclase activities were also lowered by methimazole treatment in the WKY rats, they were not significant. This included basal and Gpp(NH)p stimulated (data not shown) as well as isoproterenol stimulated activities (Figure 3).

ATPase Activities

The activity of the $Na^+,K^+$-ATPase was significantly higher ($P<0.05$)

in cardiac membranes prepared from SHRs when compared to WKY rats at all ages tested except 5 days. The activities of this enzyme did not decrease with age as was seen with receptor densities and adenylate cyclase activities. $Na^+,K^+$-ATPase activities were significantly decreased ($P<0.05$) in cardiac preparations from both strains of rats treated with methimazole (Figure 4). The activity of the $K^+,Ca^{++}$-ATPase was significantly higher ($P<0.05$) in cardiac membranes prepared from SHRs when compared to activities from membranes of WKY rats (Figure 5). This ATPase activity of the sarcoplasmic reticulum also did not decrease with age as was seen with the adrenergic receptors and adenylate cyclase activities. Methimazole treatment also decreased $K^+,Ca^{++}$-ATPase activities ($P<0.05$) in both strains of rats (Figure 5).

TABLE 3. Serum T3 (ng/ml) in Control and Methimazole (MMI) Treated SHRs and WKY Rats*

| | | AGE (Days) | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Strain | Treatment | 0 | 5 | 10 | 20 | 30 | 100 | 125 |
| SHR | Control | 0.00 | 0.00 | 0.26 | 0.88 | 1.50 | 1.13 | 0.80 |
| SHR | MMI† | | 0.00 | 0.00 | 0.88 | 0.00 | 0.00 | 0.00 |
| WKY | Control | 0.00 | 0.03 | 0.52 | 1.11 | 1.22 | 1.06 | 0.62 |
| WKY | MMI | | 0.00 | 0.00 | 1.10 | 0.31 | 0.64 | 0.00 |

* Serum pooled from 6 to 18 rats

† Significantly different from SHR Control; $P<0.05$

TABLE 4. Serum T4 in Control and Methimazole (MMI) Treated SHRs and WKY Rats*

| AGE (Days) | Free (ng/ml) | | | | Total (µg/100ml) | | | |
|---|---|---|---|---|---|---|---|---|
| | SHR Control | SHR MMI† | WKY Control | WKY MMI†† | SHR Control | SHR MMI† | WKY Control | WKY MMI†† |
| 0 | 0.14 | | 0.06 | | 0.69 | | 0.56 | |
| 5 | 0.40 | 0.06 | 0.23 | 0.12 | 1.70 | 1.05 | 1.38 | 1.17 |
| 10 | 0.53 | 0.02 | 0.63 | 0.08 | 2.53 | 1.24 | 2.65 | 1.33 |
| 20 | 0.92 | 0.70 | 1.08 | 0.61 | 4.00 | 3.44 | 4.20 | 3.38 |
| 30 | 0.50 | 0.06 | 0.49 | 0.15 | 4.56 | 1.49 | 3.25 | 0.85 |
| 100 | 0.47 | 0.00 | 0.36 | 0.00 | 5.59 | 1.94 | 3.99 | 1.05 |
| 125 | 0.61 | 0.00 | 0.39 | 0.00 | 5.46 | 2.36 | 4.37 | 1.39 |

* Serum pooled from 6 to 18 rats

† Significantly different from SHR Control; $P<0.05$

†† Significantly different from WKY Control; $P<0.05$

TABLE 5. Body Weight (gm) and Ventricle:Body Weight Ratio (mg/100gm) of Control and Methimazole (MMI) Treated SHRs and WKY Rats

| Strain | Treatment | AGE (Days) 0 | 5 | 10 | 20 |
|---|---|---|---|---|---|
| SHR | Control | | | | |
| | Body wt. | (24) 5±0.1 | (23) 9±0.2† | (32) 14±0.2† | (16) 29± 1 |
| | Ventricle/body wt. | (24) 574± 30† | (23) 634± 13† | (32) 551± 12† | (16) 466±12† |
| SHR | MMI | | | | |
| | Body wt. | | (21) 8±0.3* | (30) 14±0.3 | (15) 30± 1 |
| | Ventricle/body wt. | | (21) 591± 26 | (30) 448± 10* | (15) 458±15 |
| WKY | Control | | | | |
| | Body wt. | (18) 5±0.1 | (20) 10±0.3 | (17) 17±0.4 | (14) 30± 2 |
| | Ventricle/body wt. | (18) 418± 10 | (20) 563± 14 | (17) 470± 11 | (14) 504±10 |
| WKY | MMI | | | | |
| | Body wt. | | (24) 9±0.1† | (33) 15±0.5† | (14) 24± 1† |
| | Ventricle/body wt. | | (24) 572± 20 | (31) 415± 12† | (14) 457±13† |

Values are mean ± S.E.; numbers in parentheses are sample size

* Significantly different from SHR Control; $P<0.05$
† Significantly different from WKY Control; $P<0.05$

TABLE 5. (Continued)

| Strain | Treatment | AGE (Days) 30 | 100 | 125 |
|---|---|---|---|---|
| SHR | Control | | | |
| | Body wt. | (16) 70± 2 | (5) 295± 9 | (5) 309±11 |
| | Ventricle/body wt. | (16) 420± 8† | (5) 302± 4† | (5) 334± 9† |
| SHR | MMI | | | |
| | Body wt. | (13) 55± 1* | (6) 108± 3* | (6) 119± 4* |
| | Ventricle/body wt. | (13) 356± 9* | (6) 323±31 | (6) 318±14 |
| WKY | Control | | | |
| | Body wt. | (15) 66± 2 | (5) 295±11 | (5) 321± 5 |
| | Ventricle/body wt. | (15) 388± 7 | (5) 267± 1 | (5) 271± 4 |
| WKY | MMI | | | |
| | Body wt. | (13) 41± 3† | (7) 151± 4† | (7) 111± 3† |
| | Ventricle/body wt. | (13) 414±30 | (7) 216± 5† | (7) 233± 7† |

Values are mean ± S.E.; numbers in parentheses are sample size

* Significantly different from SHR Control; $P<0.05$
† Significantly different from WKY Control; $P<0.05$

TABLE 6. Systolic Blood Pressure (mmHg) in Control and Methimazole (MMI) Treated SHRs and WKY Rats

| Strain | Treatment | AGE (Days) 30 | 100 | 125 |
|---|---|---|---|---|
| SHR | Control | (15) 123±2† | (5) 192±3† | (5) 187±4† |
| SHR | MMI | (13) 99±2* | (6) 122±2* | (4) 132±6* |
| WKY | Control | (14) 103±4 | (5) 134±4 | (5) 135±2 |
| WKY | MMI | (12) 80±5† | (7) 106±4† | (7) 100±2† |

Values are mean ± S.E.
Numbers in parentheses are sample size

* Significantly different from SHR Control; $P<0.05$

† Significantly different from WKY Control; $P<0.05$

TABLE 7. [$^3$H]-DHE Bound (fmol/mg protein) to Ventricle Preparations from Control and Methimazole (MMI) Treated SHRs and WKY Rats

| | | AGE (Days) | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Strain | Treatment | 0 | 5 | 10 | 20 | 30 | 100 | 125 |
| SHR | Control | 112* | 89±16 | 61±17 | 97±32 | 54±13 | 36±2 | 23±3 |
| SHR | MMI | | 94± 4 | 79± 5 | 61± 5 | 54± 8 | 26±2 | 30±3 |
| WKY | Control | 173±6 | 76±18 | 54* | 53±12 | 32± 9 | 32±4 | 20±1 |
| WKY | MMI | | 70±26 | 64±23 | 113±49 | 42± 6 | 30±5 | 22±3 |

Each value represents mean ± S.E. for 2 to 3 pooled preparations

* Represents 1 preparation

TABLE 8. [$^3$H]-DHA Bound (fmol/mg protein) to Ventricle Preparations from Control and Methimazole (MMI) Treated SHRs and WKY Rats

| | | AGE (Days) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Strain | Treatment | 0 | 5 | 10 | 20 | 30 | 40 | 50 | 60 | 100 | 125 |
| SHR | Control | 82±10 | 63± 6 | 92±15 | 77±26 | 80±21 | 42±3 | 47±3 | 37±4 | 30±3 | 37±4 |
| SHR | MMI | | 68±33 | 87± 5 | 86± 7 | 41± 6 | | | | 24±2 | 33±2 |
| WKY | Control | 72±12 | 85± 3 | 73± 9 | 65±13 | 45±11 | 50±2 | 48±3 | 33±2 | 23±1 | 32±1 |
| WKY | MMI | | 72±14 | 81±32 | 84±28 | 65± 4 | | | | 34±5 | 27±2 |

Each value represents mean ± S.E. for 2 to 8 pooled preparations

TABLE 9. Adenylate Cyclase Activities from Ventricle Preparations of SHRs and WKY Rats (pmol Pi produced/mg protein/minute)

| Strain | Activity Measured | AGE (Days) 0 | 5 | 10 | 20 | 30 | 40 | 50 | 60 | 100 |
|---|---|---|---|---|---|---|---|---|---|---|
| SHR | Basal | 67±12 | 59± 6 | 40± 7 | 39± 6 | 33±3 | 32±5 | 25±2 | 22±1 | 17±1 |
| WKY | Basal | 47± 1 | 54±12 | 47± 0 | 33± 2 | 29±0 | 26±2 | 22±1 | 24±2 | 23±2 |
| SHR | Gpp(NH)p (1 μM) | 80±14 | 94± 1 | 79± 8 | 74±12 | 54±0 | 53±1 | 50±7 | 43±2 | 39±0 |
| WKY | Gpp(NH)p (1 μM) | 60± 4 | 76±20 | 67± 3 | 68± 0 | 51±1 | 47±3 | 44±2 | 42±1 | 42±1 |
| SHR | Isoproterenol (1 μM ISO) | 144±30 | 172± 7 | 133±20 | 123±24 | 91±2 | 83±3 | 73±8 | 62±3 | 55±1 |
| WKY | Isoproterenol (1 μM ISO) | 96± 2 | 124±31 | 106± 3 | 99± 8 | 79±2 | 72±1 | 68±3 | 64±2 | 65±2 |
| SHR | % Stimulation by ISO | 79± 7 | 83± 8 | 66±14 | 65± 8 | 70±6 | 57±5 | 46±7 | 44±6 | 41±3 |
| WKY | % Stimulation by ISO | 60± 8 | 62± 0 | 59± 0 | 46±11 | 56±5 | 54±6 | 56±5 | 52±2 | 55±0 |

Activities were assessed as described in Methods

Each value represents the mean ± S.E. for 2 to 8 pooled preparations

Gpp(NH)p - 5' Guanylylimidophosphate

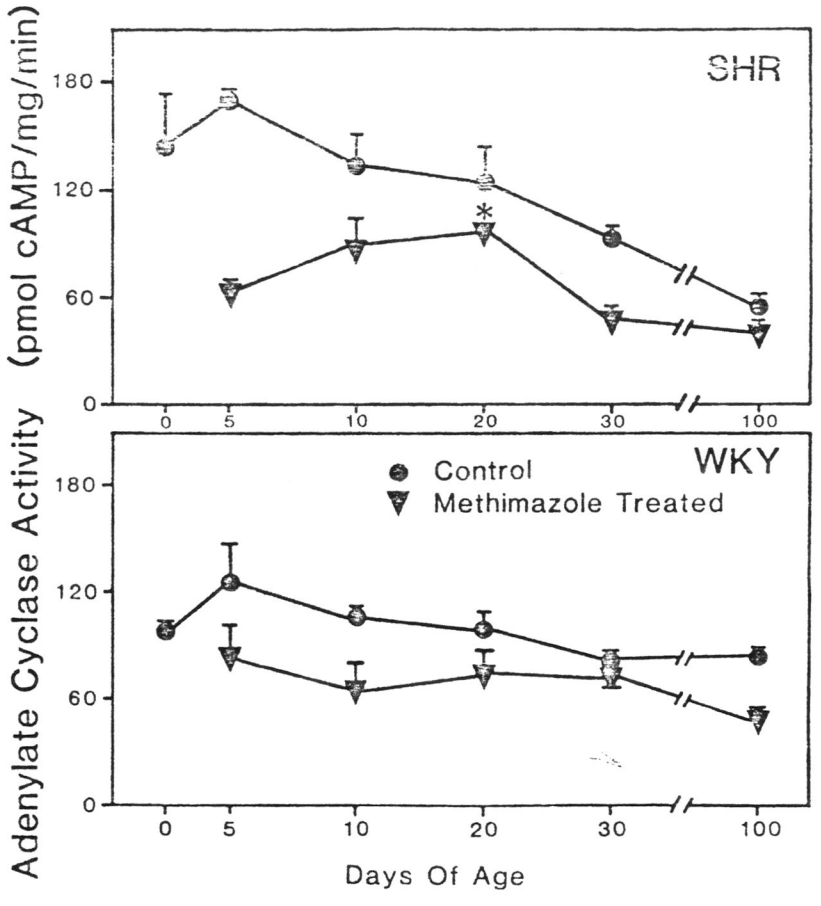


FIGURE 3. Effect of Methimazole on Isoproterenol-Stimulated Adenylate Cyclase Activities in Ventricular Preparations of SHRs and WKY Rats. Cardiac membrane protein (40μg) was incubated with 1μM Gpp(NH)p as described in Methods. Methimazole treatment significantly lowered ($P<0.05$) the isoproterenol-stimulated activities in SHR myocardial preparations at all ages tested. Each point represents the mean ± S.E. of 2 to 8 pooled preparations.

* only one determination

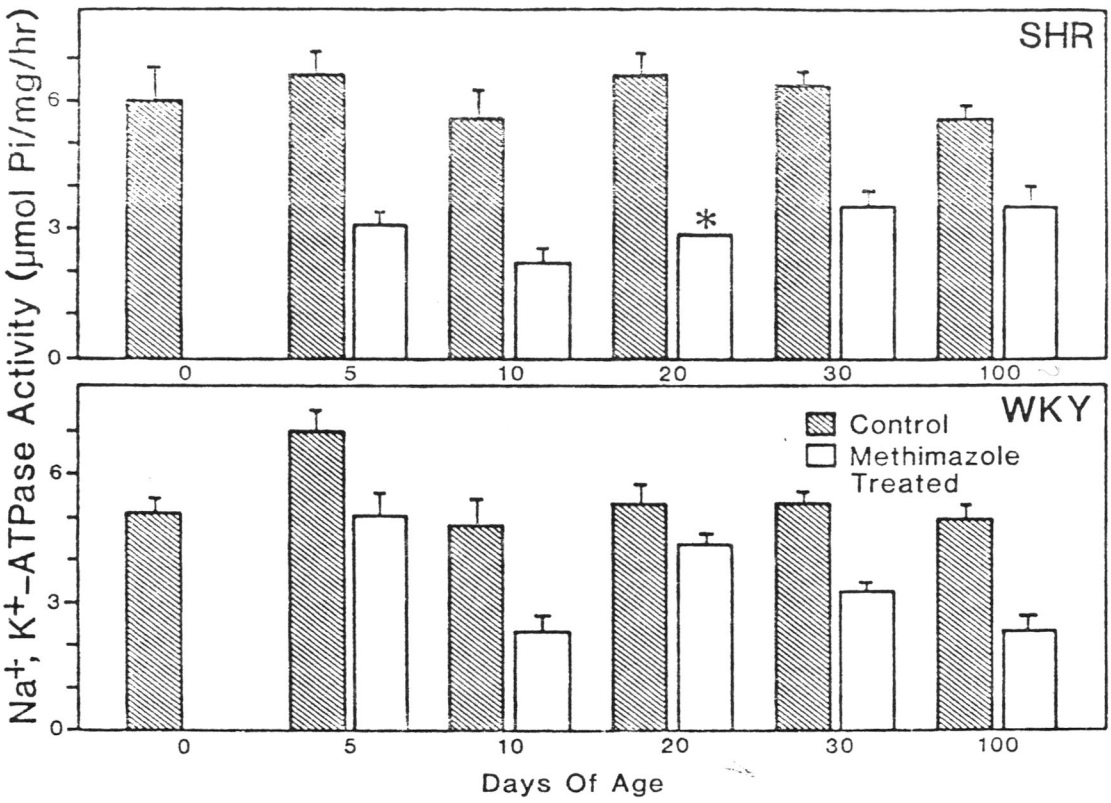


FIGURE 4. Effect of Methimazole on $Na^+,K^+$-ATPase Activities of Ventricular Preparations from SHRs and WKY Rats. Activities were assessed as described in Methods and are those activities measured after unmasking all activity using an optimal concentration of SDS. SHR activities are significantly higher ($P<0.05$) than corresponding activities in the WKY rats at all ages tested except 5 days. Methimazole significantly lowered ($P<0.05$) the $Na^+,K^+$-ATPase activities at all ages tested of both strains. Each bar represents the mean ± S.E. of 2 to 8 pooled preparations.

* only one determination

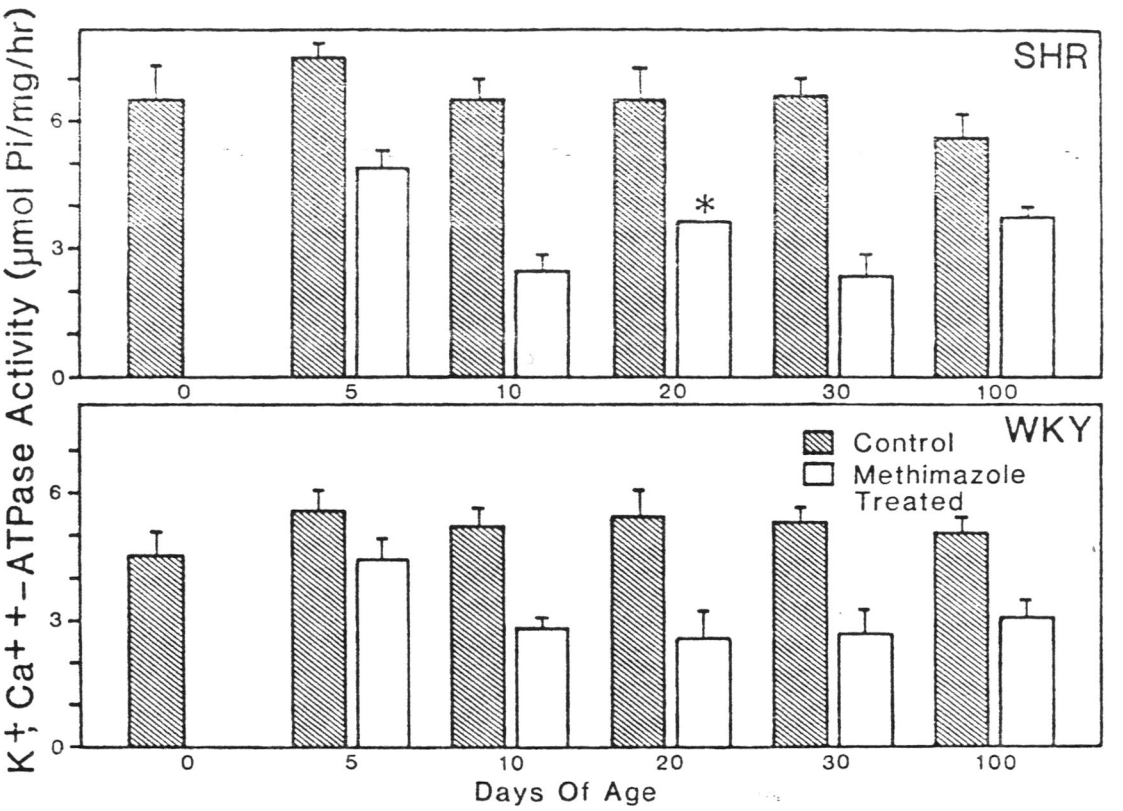


FIGURE 5. Effect of Methimazole on $K^+$,$Ca^{++}$-ATPase Activities of Ventricular Preparations from SHRs and WKY Rats. Activities were assessed as described in Methods and are those activities measured in the presence of the ionophore A23187. Activities in the SHR are significantly higher than corresponding activities in the WKY rats at all ages tested. Methimazole significantly lowered ($P<0.05$) the $K^+$,$Ca^{++}$-ATPase activities at all ages tested of both strains. Each bar represents the mean ± S.E. of 2 to 8 pooled preparations.

* only one determination

# DISCUSSION

Cardiac hemodynamic changes involving the sympathetic nervous system have been proposed to be a possible cause of hypertension in the SHR. The results of this study demonstrate that indeed the reactivity of the sympathetic nervous system appears to be altered. This is reflected in the relative numbers of alpha- and beta-adrenergic receptors and the adenylate cyclase activities measured. The myocardial membranes from pre-hypertensive SHRs had higher basal, guanine nucleotide and isoproterenol stimulated activities than the corresponding WKY cardiac preparations. Inducing hypothyroidism, which appears to result in a state of decreased myocardial adrenergic sensitivity, failed to effectively decrease the apparent number of alpha- or beta-adrenergic receptors. However, myocardial adenylate cyclase and $Na^+,K^+$-ATPase activities of the sarcolemma as well as the $K^+,Ca^{++}$-ATPase activities of the sarcoplasmic reticulum were attenuated in hypothyroid animals of both strains.

This study demonstrated that the age-dependent decreases in myocardial alpha- and beta-adrenergic receptors of the SHR correlates well with the pattern of hemodynamics found in the development of hypertension. During the prehypertensive period when cardiac output is elevated (8) the numbers of alpha- and beta-adrenergic receptors are highest. Cardiac output decreases as hypertension becomes established (8) concomitant with decreases in alpha- and beta-adrenergic receptors. This is true for the WKY as well as the SHR; however, there appears to be a trend for the total apparent numbers of both alpha- and beta-adrenergic receptors to be higher in the SHRs, especially during the prehypertensive period. Several investi-

gators have employed direct radioligand binding to assess the number of beta-adrenergic receptors in hearts of SHRs with established hypertension. Woodcock (116) and Limas (117) found the total number of receptors to be lower in the SHR myocardium when compared to normotensive animals. The results of this study did not demonstrate this. In older animals, a similar number of myocardial beta-adrenergic receptors was found in the SHR and the WKY. This is in agreement with the work of Frieswick, et al. (118) who also reported no differences in myocardial beta-adrenergic receptors in adult SHR and WKY rats.

The reported increased cardiac index observed in early stages in the development of hypertension (8) could be indicative of an increased beta-adrenergic reactivity. If so, this may be reflected in increased activities of myocardial adenylate cyclase at early ages. Adenylate cyclase is coupled to the beta-adrenergic receptor. Binding of the catecholamine hormones to the beta-adrenergic receptor stimulates the activity of adenylate cyclase. Adenylate cyclase converts ATP to cyclic AMP which stimulates phosphorylating enzymes and protein kinases within the myocardial cell. This results in a variety of biochemical changes within the cell, the result of which is an overall increase in the myocardial cell's contractile activity. This may be manifest in an increased contractility and cardiac output of the heart. These data confirm this. While no increase in cardiac beta-adrenergic receptors was found in the prehypertensive SHR when compared to control WKY rats, striking alterations in the adenylate cyclase activities were seen. All activities (both strains) were highest at the youngest ages and decreased with time. There was a sharp rise in all activities at 5 days of age. This correlated with an

increase of the heart wt/body wt also seen at this age and probably reflected an increased growth phenomenon at this particular age. At early ages before hypertension was manifested basal-, guanine nucleotide- and isoproterenol-stimulated activities were consistently higher in the SHR compared with normal WKY rats.

The most interesting finding was observed with the beta-adrenergic stimulated adenylate cyclase activity. This activity was 50% higher at birth in the SHR, when compared to the WKY rat. This contribution in the prehypertensive phase could account for the state of higher cardiac index first seen in the development of hypertension (8). This altered reactivity of the myocardium may play a key role in the genesis of hypertension in the SHR.

The decrease in cardiac index as hypertension becomes established in the SHR appears to be associated with a decreased sympathetic responsiveness and beta-adrenergic reactivity. This study confirms previous reports that cardiac adenylate cyclase stimulation by beta agonists is depressed in adult hypertensive rats (119, 120, 121, 122, 123) when compared to normotensive controls. It would appear from these data that hormone stimulation of adenylate cyclase became less sensitive with the onset of hypertension. This enzymatic activity, which is elevated in prehypertensive SHRs, decreases as hypertension begins to manifest itself. In 100 day old rats isoproterenol-stimulated adenylate cyclase activity was 16% lower in membrane preparations from SHRs as compared to WKY animals. Basal and guanine nucleotide-stimulated, adenylate cyclase activities decreased with age in both the WKY and SHR to similar levels by 100 days of age.

Controversy remains regarding the activity of the thyroid gland in

the SHR. The results of this study suggested no differences in thyroid hormone levels between the developing SHR and WKY rat which is in agreement with previous reports (57, 58, 60, 62, 63, 72).

Hyperthyroidism appears to result in a state of increased adrenergic activity while hypothyroidism appears to lead to a decrease in adrenergic sensitivity (74, 75, 76). The heart's response to catecholamine stimulation seems to be particularly sensitive to varying thyroid status.

If development of hypertension in the SHR results from some abnormality or overactivity of the sympathetic nervous system, then inducing hypothyroidism to lower this overactivity in the SHR would be expected to decrease or prevent hypertension. It might also be expected that hypothyroidism would decrease adrenergic sensitivity to catecholamines. In this study methimazole treatment produced hypothyroidism but did not alter the number of alpha- or beta-adrenergic receptors in the WKY or SHR. This is in contrast to reports by other authors who state that the hypothyroid state effectively lowered the apparent number of adrenergic receptors in strains of rats different from those employed in this study (102, 103, 106, 108). While no change in receptors was found, a significant decrease in adenylate cyclase activities in membranes prepared from hypothyroid animals was seen. These results agree with those of various investigators who showed a decrease in cardiac adenylate cyclase activity with propylthiouracil treatment or thyroidectomy (125, 126, 127). This study demonstrated that the catecholamine-stimulated adenylate cyclase activity of the SHR can be reduced below control levels (normotensive WKY rats) by methimazole treatment until 100 days of age. This treatment also prevented the development of hypertension for at least 240 days (unpublished

data).

Decreases in adenylate cyclase activities due to methimazole treatment were more dramatic in the SHRs than the WKY strain. This was probably due to higher levels of activity in SHR preparations as compared to WKY membranes not subjected to methimazole treatment. Methimazole effectively lowered the isoproterenol-stimulated adenylate cyclase activities in both strains to approximately the same levels. Sharma (20) suggested that thyroid hormones modulate guanine nucleotide stimulation and regulation of the adenylate cyclase enzyme. This could also account in part for the reduced sensitivity to adrenergic stimulation seen in hypothyroidism. These data demonstrate a definite trend whereby the SHR $Na^+,K^+$-ATPase activities were consistently higher than similar activities assessed in the WKY myocardial membranes. This is in apparent disagreement with data presented by Limas (117) who showed that $Na^+,K^+$-ATPase activities are similar in the SHR and WKY cardiac membranes. They used membrane preparation containing a significant amount of sarcolemmal vesicles, and total activity was not assessed. In order to assess total enzymic activity in these preparations, these vesicles were rendered permeable to various substrates and inhibitors by preincubation with the detergent SDS (140). Although administration of thyroid hormone has been shown to increase $Na^+$, $K^+$-ATPase activities in different tissues, $Na^+,K^+$-ATPase activity of cardiac tissue in a variety of species remained unchanged following $T_4$ injections (unpublished data). Propylthiouracil treatment or thyroidectomy will depress the activity of the $Na^+,K^+$-ATPase or [$^3$H]-ouabain binding in membrane preparations (104, 129). In the present study methimazole treatment significantly lowered the activities of the $Na^+,K^+$-ATPase in both the

SHR and WKY myocardial preparations. This decreased myocardial activity may not reflect a change in the sympathetic nervous system but may be due to other non-specific alterations. Further investigation is needed to determine how this altered enzymic activity relates to hyper- or hypotension.

Intracellular $Ca^{++}$ concentrations are involved in the inotropic and chronotropic responses of the myocardium. Alteration in intracellular free $Ca^{++}$ concentration, regulated by the $Ca^{++}$ pump of the sarcoplasmic reticulum may cause abnormal distributions of the intracellular $Ca^{++}$. These data demonstrated that SHR sarcoplasmic reticulum $K^{+},Ca^{++}$-ATPase activities were greater than the corresponding activities in WKY rats. Similar results were also observed by Aoki et al. (132) using adult SHRs. These investigators also demonstrated a decreased $Ca^{++}$ binding in the SHR sarcoplasmic reticulum and postulated that the sarcoplasmic reticulum of the SHR, being leaky and not able to accumulate $Ca^{++}$ properly, would cause not only the $Ca^{++}$ pump to maintain a higher rate but would cause intracellular free $Ca^{++}$ concentration to be increased. The increase in free $Ca^{++}$ could possibly induce a high tone in vascular muscle resulting in an elevation in blood pressure. In this study increased $K^{+},Ca^{++}$-ATPase activities in the pre-hypertensive and post-hypertensive SHR myocardium were found. This mechanism may be partially responsible for the genesis of hypertension as well as the maintenance of it. In agreement with others, these data indicate that inducing hypothyroidism effectively decreases $K^{+},Ca^{++}$-ATPase activity of the sarcoplasmic reticulum (130, 131). It appeared that methimazole treatment attenuated the enzymic activities examined in this study.

The pattern of changing ventricle:body weight ratios observed in the SHR and WKY has previously been reported to occur in other strains of rats (142). The increased ventricle:body weight ratios of the SHR indicated a hypertrophied myocardium since SHR body weights were significantly lower than those of WKYs only at 5 and 10 days of age. Persistence of this hypertrophy in hypothyroid SHRs which exhibit significantly lower blood pressure at ages normally associated with established hypertension indicates that hypertrophy of the myocardium itself is not necessarily a determinant of hypertension.

The results of this study indicated that the reactivity of the sympathetic nervous system appears to be altered in the development and maintenance of hypertension in the SHR. Furthermore, although circulating levels of thyroid hormones were comparable to those found in normotensive WKY rats depression of thyroid activity by methimazole treatment lowered certain myocardial enzymatic activities and blood pressure.

The observed decreases in both alpha- and beta-adrenergic receptors correlate well with the changing pattern of hemodynamics which are reported to occur in the aging SHR (8). Although not statistically significant, there is a definite trend for adrenergic receptors to be greater in the prehypertensive stage of hypertension and lower in the established stage of hypertension in the SHR. Further studies with greater numbers of individual rats need to be performed to determine conclusively if differences in the numbers of adrenergic receptors are a significant factor in spontaneous hypertension. A dramatic decrease in beta-adrenergic receptor density was observed between 30 and 40 days of age in the SHR. During this period in the development of hypertension cardiac output is

reported to decrease in the SHR while total peripheral resistance increases (8). Studies of adrenergic receptor densities in resistance vessels of aging SHRs would be of interest, especially during this transition period.

Basal, guanine nucleotide and isoproterenol-stimulated adenylate cyclase activities all showed decreases with age similar to the decreases observed with adrenergic receptors. Perhaps the finding of greatest interest was that isoproterenol-stimulated adenylate cyclase activities were significantly higher in prehypertensive and lower in established hypertensive SHRs. Again it would be illustrative to determine adenylate cyclase activities in resistance vessels of aging SHRs since it would be expected that this activity might be altered during the ages when total peripheral resistance is elevated.

Methimazole treatment significantly lowered all adenylate cyclase activities and blood pressure in the SHR to levels slightly below those observed in WKY animals. Methimazole treatment did not significantly lower adenylate cyclase activities in the WKY. These data indicated that an increased reactivity to sympathetic nervous system stimulation in the SHR may be due to differences intrinsic to the myocardium of the SHR.

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# APPENDIX

## Thesis Related Publications

Iams, S.G., and S.J. Blumenthal. The effect of methimazole (MMI) on the blood pressure and plasma catecholamine levels in the spontaneously hypertensive rat (SHR). The Physiologist 22: 60, 1979.

Blumenthal, S.J., M.M. McConnaughey, and S.G. Iams. Cardiac beta-adrenergic receptor binding, adenylate cyclase, $Na^{+}$,$K^{+}$-ATPase and $K^{+}$,$Ca^{++}$-ATPase activities in the developing spontaneously hypertensive rat (SHR). Endocrinology Abs. 106: 169, 1980.

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