Left Ventricular Hypertrophy, Cardiac Myocyte Adaptation, and Collagen/Parenchymal Distribution in Response to Subpressor and Pressor Doses of Angiotensin II in Sprague-Dawley Rats

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INTRODUCTION

Cardiovascular function integrally involves the renin angiotensin system (RAS). Classically, endogenous angiotensin II (A-II), the central product of the RAS, is well known to produce potent vasoconstrictive responses resulting in increases in arterial blood pressure. A growing body of evidence on A-II’s actions, from several labs, supports the conclusion that the peptide is involved in the etiology of hypertension, as well as the pathophysiology of cardiac hypertrophy and remodeling, heart failure, vascular thickening, atherosclerosis and glomerulosclerosis (Yamamoto et al., 2007, Ahmad et al., 2008, Uechi et al., 2008, ). Several studies have shown that A-II stimulates myocardial protein synthesis, while more recent reports have emphasized a difference between the systemic vs. the local generation of A-II (Abrahamsen et al., 2002, Schnee and Hsueh, 2000). The myocardial component of the heart wall consists of myocytes, non-myocyte cells and a coronary microcirculation, which are supported and surrounded by a fibrous network within the interstitial space. Within this network, the major structural protein is collagen. Studies have shown differential effects of A-II on supportive cells than on cardiomyocytes and on fibrillar collagen (Pope et al., 2008). An accumulation of connective tissue in general and fibrillar collagen in particular is present in patients with cardiocyte necrosis and myocardial infarction (Nian et al., 2004, Weber et al., 2004). Evidence has also accumulated to indicate that the structural protein matrix of the interstitium is also an active participant in the hypertrophic process that accompanies left ventricular pressure overload and in patients with end stage cardiomyopathy (Wilke et al., 1996, Elliot 2007, Deschamps and Spinale, 2005). Based on the heterogeneity of the myocardial structure it has been put forward that there are several different cellular responses that occur during the progression of cardiovascular disease. These include non-myocyte cell growth without myocyte hypertrophy; myocyte hypertrophy without non-myocyte growth; and concomitant equal or disproportionate growth of
all cells. Furthermore, the A-II may also be related to calcium intracellular availability as has been suggested for several years now (Bird et. al., 1995, McDonald et. al., 1995). It has previously been established that a single intracardiac administration of retroviral vector containing angiotensin II type receptor antisense (AT,R-AS) prevents the development of hypertension in the spontaneously hypertensive rat model (Reaves et. al., 2003).

The aim of this study was to determine whether cardiomegaly (due to ventricular hypertrophy) induced by chronic subpressor (Sd) and pressor (Pd) doses of A-II can be characterized by significant alterations in morphometry of myocytes and/or quantitative and qualitative changes in collagen underlying a remodeling process.

MATERIALS AND METHODS

Three sets of experiments were performed in this study. The first experimental designed established pressor and subpressor doses of A-II and their systemic effects; and the second was investigated to establish changes in collagen/parenchyma makeup of myocardial tissue in response to A-II and accompanying hypertropic response.

In each set, adult male Sprague-Dawley (SD) rats (Harlan Sprague Dawley, Inc., Indianapolis, Indiana, USA) (n = 144) weighing 290-385 grams were used. Rats were grouped and housed in plastic cages and maintained in an animal facility. The facility was maintained at 23 degrees Celsius, 35-45 % relative humidity and a fixed 12-hour light 12-hour dark cycle. Animals were fed a standard lab chow (Purina # 5001, Ralston Purina Co., St. Louis, MO) and tap water ad libitum.

After a period of acclimatization, rats were anesthetized with Sodium Pentobarbital (60 mg/kg) and mini-osmotic pumps (Alza Corporation, Palo Alto, CA) filled with the specific drug regimen were implanted subcutaneously. These and other animal protocols used in the study were reviewed, approved, and are in accordance with the University Institutional Animal Care and Use Guidelines. Ethical conduct standards were followed thoroughly.

DOSE-DEPENDENT EFFECTS OF ANGIOTENSIN II

In order to study the dose effect of A-II (Sigma Chemical Comp., St. Louis, MO) on blood pressure and heart weight, seven doses (0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0 and 5 microgram per kilogram per day (5.0 µg/kg/day) of the hormone were prepared and put into mini-osmotic pumps before the pumps were subcutaneously implanted into animals. Pumps remained in place throughout the experimental period but had a possible injection span of 14 days. Sham controls received 0.9% physiological saline. At the end of the treatment period, and at the end of the first five days blood pressure was recorded. At the end of 10 days animals and excised hearts were weighed. Hearts were divided into ventricular and atrial components and the sections were weighed. Total heart weight to body weight ratios (HW/BW) were calculated and compared to control animals that received normal saline injections during the same time period. From this data a clear-cut pressor dose (Pd) (5.0 µg/kg/day) was chosen while the subpressor dose (Sd) (1.5 µg/kg/day) was selected as the lowest dose at which blood pressure remained relatively close to the control values but HW/BW was elevated. After the drug treatment period, rats were weighed, anaesthetized with Equithesin, at 0.15 milliliters per 100 grams (ml/100 gm) by intraperitoneal injections (i.p.), and prepared for hemodynamic recording as described below.

COMPARATIVE EFFECTS OF SUBPRESSOR AND PRESSOR DOSES OF ANGIOTENSIN II ON MYOCYTE MORPHOMETRY AND COLLAGEN

In this experiment the animals received either Sd or Pd doses of A-II for fourteen days. The control animals received saline for the aforementioned 14 days. Following the onset of drug regimen, animals were weighed and blood pressures taken daily by the tail-cuff method utilizing standard instrumentation (Iyer and Katovich, 1996). Body weights were determined and recorded. Hemodynamic recordings were done on the eleventh day after onset of A-II infusion. The method for hemodynamic recording is given below. At the end of the hemodynamic recording session animals were removed from the apparatus while under light anesthesia, hearts were excised, rinsed and weighed. Some hearts were utilized for morphometry while others were used for collagen determination.

HEMODYNAMICS

At the end of the drug treatment period, a standard instrumentation procedure (Gerdes et. al., 1992) for the recording of left, ventricular hemodynamic changes was used. Rats were anaesthetized with Equithesin i.p., placed on a 30° heating pad (Delta Isothermal pad, model 39 Dp, Braintree, MA) and prepared for hemodynamic recording. Animals were tracheotomized and a cannula was placed in the trachea. A microtip pressure transducer catheter was used (Millar Instruments, Inc., Houston, TX). The catheter
was attached to a Millar transducer control unit (model TC-100) which was connected to a HSE Elektro-Manometer (Hugo Sachs Elektronik, Hugstetten). A Gould recorder was used for recording all variables. Calibration of pressure was done with a mercury manometer. The pressure transducer catheter was quickly advanced into the right carotid artery and positioned in the left ventricle. When the change in contour and amplitude of the arterial pressure pulse wave to a ventricular type was indicated, ligatures at both the proximal and distal positions of the right carotid artery secured the catheter. Routinely, 15 minutes after the catheter is secured and positioned, when steady state has been reached, the functional parameters were measured. Each recording period lasted approximately 45 minutes to 1 hour.

**CELL ISOLATION**

Animals were sacrificed and hearts were rapidly removed, rinsed in cold saline and weighed. For hearts selected for morphometry analysis the aorta was cannulated and attached by a cannula to a modified Langendorf retrograde perfusion apparatus. Isolated myocytes were obtained according to the techniques of Gerdes et. al., (1992). At the end of the perfusion period the atria were removed and discarded, the right and left ventricular free walls were dissected free of the septum and each of the three pieces placed in separate petri dishes and weighed. Calcium-free Joklik media was then added to the petri plates; the sections were minced into small pieces and the mixture was then poured through nylon mesh to facilitate cell filtration into storage containers.

The isolated cells were stored in 2% glutaraldehyde (Sigma Chemical Comp., St. Louis, MO) until morphological examination. Determination of cell length (the longest end-to-end distance parallel to the longitudinal axis of the cell) was measured with the use of a phase microscope. A minimum of 40 cells from each region of each heart was sized in this manner. Cell volume was determined with a Coulter Channelyzer Model C256 (Coulter Corp. Inc., Hialeah, FL). The Coulter system determines cell volume by measuring the change in electrical resistance accompanying the displacement of electrolyte as cells move through a sized aperture. A correction factor of 1.05 was used to normalize the shape values. Cross-sectional area of the myocyte was calculated by dividing cell volume by cell length.

**TISSUE PROCESSING AND COLLAGEN VOLUME FRACTION**

For hearts undergoing collagen studies following hemodynamic recordings, hearts were rapidly excised, rinsed and weighed. Atria, great vessels, and valvular structures were trimmed away and discarded while ventricles were spared. Ventricles were separated from each other and transmural sections of right ventricle and left ventricle plus septum were saved for specific acidic dye (sirius red F3BA staining) (Orlandi et. al., 2004). The sections were stained with sirius red in saturated aqueous picric acid according to Orlandi et. al. (2004). Collagen volume fraction was assessed using a semi-automate computer-color video assisted procedure as described by Orlandi et. al. (2004). In general, the five micron-thick stained sections were placed in an Olympus field phase-contrast light microscope with a color video camera attached. The microscope was coupled to an image processor that depicted and recorded the specimens in the field. Segments representing connective tissue and parenchyma were identified with the cursor of the image processor. Collagen and parenchyma volume fractions were computed from the cumulative collagen and muscle areas. One section was scanned and from this section, 16 fields (four from each quadrant of a grid system) were randomly selected, identified and the mean collagen volume fraction was calculated. The two-dimensional matrix of the tissue was broken into component picture elements called pixels. Each pixel had a corresponding color intensity (hue range) associated with it. The color was then converted first into an electronic signal, which was set to detect a color yield within the hue range of 55-110. The light intensity produced was consistently established and maintained at a range of 232-240 nanometer (nm).

**COLLAGEN AND PARENCYHIMAL DISTRIBUTION**

The collagen matrix of cardiac or skeletal muscle as being broken down into constituent elements, including an epimysium that surrounds muscle, a perimysium that is an extension of the epimysium serving to separate muscle fiber bundles, and an endomysium or final arborization of the perimysium. The endomysium includes a collagenous weave that surrounds muscle cells (Macchiarelli et. al., 2002, Li et. al., 2002).

Yoshikane et. al. (1992), using type I collagen specific antibodies, found that the endomysial fibrils are composed of type I collagen. Drawing on the birefringent properties of collagen fibers and their differential coloration to polarization microscopy, after staining with the acidic dye sirius red, it was found that the majority of the...
endo-perimysial fibers were composed of thick collagen fiber (Li et al., 2002). The alignment of thick and thin collagen fibers to one another and to cardiac muscle was to identify, morphologically, distinct patterns of collagen accumulation in the interstitium (Deschamps and Spinale, 2005). The present study used this model of accumulation as a base description.

The picrosirius red F3BA technique polarization microscopy was used to enhance collagen fiber birefringence. This methodology indicates several different patterns of myocardial fibrosis as well the collagen types. According to Orlandi et al. (2004), using the picrosirius-polarization technique, collagen type I fibers are represented as thick fibers composed of closely packed thick fibrils, and have an intense birefringence with yellow to red color. Collagen type III are thin fibers composed of loosely packed thin fibrils displaying a weak birefringence of a greenish color. The collagen types III and I course through the interstitial spaces to connect adjacent muscle fibers.

STATISTICAL ANALYSIS

Values presented for all variables are the means ± standard deviation. Student’s t-test and analysis of variance (ANOVA) were used to establish any significant difference between groups of data.

RESULTS

HYPERTROPHY

Table 1 summarizes the results obtained from animals treated with A-II. Six doses of A-II and saline are shown in the table. Shown in the figure are the differences in hearts normalized to body weights (HW/BW) between the treated and the control rats. Average results ± SEM from 7 rats are given for each group in the table. Significant differences in absolute heart weight (HW) and HW/BW occurred with doses above 1.0 µg/kg/day (p < 0.05), while significant elevations in mean arterial pressure (MAP) were observed exclusively with the four highest doses (5.0 µg/kg/day) (p < 0.001). The pressor dose (Pd) from this series of experiments was established to be 5.0 µg/kg/day while the Sd was selected to be 1.5 ug/kg/day.

Table 1: Heart Weight Responses to Chronic Angiotensin II Treatment in Adult Sprague–Dawley Rats. Each animal received angiotensin II dose (1.5 though 5.0 µg/kg/day) doses mini-osmotic pumps. HW = heart weight; HW/BW = heart weight to body weight ratio; MAP = mean arterial pressure; * Indicate significant difference from corresponding controls values, * < 0.05; ** Indicate significantly from corresponding controls and within group, ** < 0.0001; n = 7 per group. Each value represents the mean ± S.E.M.

BLOOD PRESSURE

In Figure 1 results of the Sd and Pd Angiotensin II (A-II) treatment on blood pressure levels are shown. Only mean blood pressure levels and HW/BW values are shown after the treatment period. The values represent percent differences between the tail-cuff blood pressure records for the animals receiving pressor (Pd) or suppressor (Sd) doses, and those receiving saline for 7 days. The mean blood pressure (BP) of the Pd group was significantly elevated (135 millimeters of mercury (mmHg) by 26 % ± 2 (p < 0.05), while that of the Sd was the same as the control level.
for all rats recorded prior to All treatment at 105 mmHg and 107 mmHg, respectively.

**Figure 2**

Figure 1: Systolic Blood Pressure Levels Post-Seven Day Angiotensin II Treatment in Adult Sprague-Dawley Rats. Each animal received subpressor angiotensin II (1.5 µg/kg/day) or pressor dose angiotensin II (5.0 µg/kg/day) doses mini-osmotic pumps. ? = Control, normotensive rats; ? = sterile 0.9% physiological saline administration; ? = Subpressor Dose, angiotensin II administration (1.5 µg/kg/day); ? = Pressor Dose, angiotensin II administration (5.0 µg/kg/day); * Indicate significant difference from corresponding control values, * p < 0.05; n = 7 per group. Each value represents the mean ± S.E.M.

**LEFT VENTRICULAR MYOCYTE MORPHOMETRY**

Table 2 summarizes the morphometric data for left ventricular myocytes. Animals treated with the Pd showed significant increases in cell length (CL) (144 ± 13.0) (p < 0.001), and cell volumes (CV) (28757 ± 4854) (p < 0.001), with only slight elevations in cell width (CW) (5.6 ± 0.55) (p < 0.05), cell length to cell width ratio (CL/CW) (26 ± 3.0) (p < 0.05), and cross-sectional areas (CSA) (200 ± 28) (p <0.05). The Sd did not demonstrate any significant alterations from control in any of these morphometric variables. Myocytes from the septum did not show any significant change in their morphometry, however, CV in this region increased by 17 ± 4 percent.

**COLLAGEN AND PARENCHYMAL DISTRIBUTION**

The fibrillar collagen and parenchymal distribution in the left ventricles are shown in Table 3. The levels were significantly elevated in both the Sd (0.049 ± 0.01) (p < 0.05) and Pd-treated animals (0.065 ± 0.01) (p < 0.001), with a higher increase (195%) occurring in the Pd group. A less amount of parenchyma was seen in both Sd (-15%) (0.620 ± 0.02) (p > 0.05) and Pd-treated (-11%) (0.651 ± 0.02) (p > 0.05) when compared to the controls. A similar level was observed in the septum and the left ventricle free wall since they were not significantly different from each other.
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Figure 4

Table 3: Left Ventricular Fibrillar Collagen and Parenchyma Distribution Following Angiotensin II Administration. Each animal received subpressor angiotensin II (1.5 µg/kg/day) or pressor dose angiotensin II (5.0 µg/kg/day) doses mini-osmotic pumps. Control = sterile 0.9% physiological saline administration; Subpressor dose = angiotensin II administration (1.5 µg/kg/day); Pressor dose = angiotensin II administration (5.0 µg/kg/day); % Difference = percent difference from control; * Indicate significant difference from corresponding control values, * < 0.05; **Indicate significant difference from corresponding control values ** < 0.001; n = 6 per group. Each value represents mean ± S.E.M.

<table>
<thead>
<tr>
<th>Angiotensin II Administration Group</th>
<th>Collagen (mm$^2$)</th>
<th>Parenchyma (mm$^2$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.022 ± 0.007</td>
<td>0.7322 ± 0.01</td>
</tr>
<tr>
<td>Subpressor dose</td>
<td>0.049 ± 0.01*</td>
<td>0.620 ± 0.02</td>
</tr>
<tr>
<td>% Difference</td>
<td>-123.0</td>
<td>-15.0</td>
</tr>
<tr>
<td>Pressor dose</td>
<td>0.001 ± 0.01**</td>
<td>0.651 ± 0.02</td>
</tr>
<tr>
<td>% Difference</td>
<td>-195.0</td>
<td>-11.0</td>
</tr>
</tbody>
</table>

DISCUSSION

It has been demonstrated that the identification of patients at high risk for cardiovascular events is imperative in the reduction of cardiovascular mortality and morbidity (Javed et. al., 2008). The RAS is a major regulatory system of cardiovascular and renal function (Marcheselli and Micieli, 2008). In established hypertension the RAS is often activated (Nilsson, 2008). Renin is a proteolytic enzyme, and its substrate is angiotensinogen. Angiotensin is cleaved to an amino acid peptide (AII) by a converting enzyme, which is found in high concentrations in the lungs. AII acts as a negative feedback regulator of renin secretion.

In the United States, hypertension is a blood pressure condition that reflects increased tone of the arteries and arterioles. The patient without recognizable symptoms is at extreme risk of pathophysiological alterations such as stroke, blindness, diabetic nephropathy, heart and renal disease (Daull et. al., 2007, Ding et. al., 2007). Years of elevated and sustained high blood pressure, may result in vascular and end-organ damage, and unfortunately death. Patients with essential hypertension, which occurs in 90% of patients with hypertension, must take medications for the rest of their lives for the management of hypertension. The relative incidence of hypertension and associated pathophysiological alterations is higher in African- and Latin-Americans, higher in older than in younger adults, and is significantly higher in those with familial hypertension, diabetes mellitus, and hyperlipidemia.

Several investigations over the last several years have established that A-II is a potent stimulus to cardiac enlargement (Schnee et. al., 2000, Abrahamsen et. al., 2002, Yamamoto et. al. 2007). The mechanism and role of A-II-induced vascular endothelial injury is unclear (Yamamoto et. al., 2007). In the present study, using male rats, a dose-related response to the A-II (Table 1) is established. Interestingly, although A-II elevated the blood pressure at high doses, there was cardiac enlargement without the pressor action. Such results are in keeping with the observation that A-II localizes at cellular sites related to protein synthesis and that A-II activates Matrix metalloproteinases (Petén et. al., 1994, Ding et. al., 2007, Daull et al., 2007), which in turn results in myocyte remodeling.

Pressor and subpressor doses of A-II were used in this study to ascertain differences in cardiac tissue responses when animals were chronically exposed to different circulating levels above normal. The use of Alzet mini-osmotic pumps provided a continuous source of A-II infusions. Understandably, it may be hypothesized that extrinsic A-II application could inhibit the conversion pathway for intrinsic A-II production. However, there were no differences in plasma renin activity (PRA) between Pd, Sd and control rats (unpublished data). Also, there were significant alterations in hemodynamics expressed after the completion of A-II Infusions. Such results suggest that there may be catecholamine contribution to hemodynamic changes accompanying the A-II treatment.

Other reports have indicated an A-II adrenergic interdependence in both myocardial (Ahmed et. al., 2008, Uechi et. al., 2008) and renal function (Yousefpour et. al., 2008).
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With this has come the implication that in pathological cardiac hypertrophy, such as with hypertension, where anti-adrenergic and A-II inhibition provide acceptable and effective therapeutic measures, A-II may be playing a more causative role. In related studies to the present one (unpublished data) saralasin, the A-II analog, was clearly effective in eliminating most of the hemodynamic and hypertrophic effects of A-II in the absence of adrenergic blockade; while propranolol or metoprolol did not. Such results confirm a direct effect of A-II in stimulating cardiac enlargement.

Individual myocardial cells have been shown to be non-mitotic beyond the neonatal stage of development; also, there are marked changes in length, width and volume of myocytes that are obtained from the growing heart. However, during non-mitotic cardiac enlargement there is also a clear indication of changes in myocyte morphometry (Gerdes et. al., 1992). Likewise, in the A-II related hypertrophy the morphometric parameters of myocytes were significantly altered (Table 2) with the Pd when compared to the control. The studies by Gerdes et. al. (1992) and Paradis et. al. (2000), have also presented data to show increases in myocyte dimensions of isolated muscle and in whole heart accompanying increases in absolute heart weight. It may be argued then that under circumstances of A-II elevation that the functional behavior of the hypertrophied left ventricle can be attributed to the increased size of the heart, and more specifically to the increased functional capacity of larger and stronger component myocytes. In this study, smaller changes in the morphometric parameters often in the opposite direction of changes observed from the Pd, was seen with the Sd. This occurred with the presence of relatively small but significant elevations in HW/BW and collagen. A rational explanation of this is that the heart responds to the smaller dose of A-II in a specific manner in that it may have begun its hypertrophic process by rearranging and increasing its non-myocyte constituents possibly through an immediate activation of matrix metalloproteinases. This argument is supported by the results expressed as collagen and parenchymal composition in which the collagen content (non-myocyte component) of the heart increased by 123% (Table 3) This increase in collagen was accompanied by a smaller increase of total parenchyma A similar pattern of collagen behavior was also found in rats receiving the Pd where there was a significantly larger proportional increase in myocyte morphometrics.

Angiotensin has been reported to enhance endothelial permeability in small coronary arteries (Sano et. al., 2006). This reasoning would suggest that mitogens (platelet-derived growth factor) could gain entry into the media, as well as into the adventitia of these vessels with collagen accumulation being the end result (Campbell et. al., 1991, Jakob et. al., 2001). A further extension of this line of reasoning would suggest that such mitogens could also gain access to the interstitium and its fibroblast population (Neilson, 2006).

The present results imply that there is a direct relationship between collagen remodeling in the interstitium of the myocardium and the treatment with subpressor or pressor doses of angiotensin II. The significant hypertrophic response that accompanies Pd treatment along with the changes in collagen synthesis and matrix of the interstitium are active participants in the enlargement process of the heart. This is expressed as an accumulation of connective tissue, and fibrillar collagen in particular. These are well-recognized responses to myocardial infarction and myocyte necrosis (Murray et. al., 2008) and are not likely to underlie the present results. Myocardial remodeling invariably occurs in and congestive heart failure (CHF) and is a response to a prolonged cardiovascular stress, which is characterized by a cascade of compensatory structural events. Remodeling of the myocardium interstitium occurs in CHF and likely contributes to the progression of the remodeling process. The myocardial matrix can be considered a biological highway in which a large amount of signaling proteins and structural proteins are being moved within the interstitium, entering and exiting the interstitial space, and docking to cellular components (Deschamps and Spinale, 2005).

In conclusion, the proliferation of fibrillar collagen in the structural remodeling of the cardiac interstitium may be a definable mechanism in A-II induced cardiac enlargement. The progressive and possible extensive nature of this collagen remodeling may lead to more severe pathologic hypertrophy with muscle fiber entrapment. This is apparent with the increase in collagen volume fraction associated with the remodeling of myocardial matrix in established pressure overload. On the other hand, there is limited information on the association between collagen volume fraction and collagen remodeling occurring during the evolutionary period of A-II induced cardiac hypertrophy. These results suggest that in time of cardiac enlargement (hypertrophy) circulating A-II could be a stimulus to cardiac fibroblast
proliferation in both the early and late phases of that process the etiology of hypertension, and a that a local increase is a primary factor of sustained myocardial remodeling. In light of this, the myocardial matrix should be regarded as a complex system of collagen within the cardiac interstitium representing a major determinant of pathological hypertrophy. Further studies and specific strategies that are targeted at modifying activity along this matrix journey will likely alter the course of myocardial remodeling and compensatory heart failure.

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References


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