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Protective effect of long-term angiotensin II inhibition

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Basso N, Cini R, Pietrelli A, Ferder L, Terragno NA, Inserra F. Protective effect of long-term angiotensin II inhibition. Am J Physiol Heart Circ Physiol 293: H1351–H1358, 2007. First published June 8, 2007; doi:10.1152/ajpheart.00393.2007.—Experimental studies indicate that angiotensin II (ANG II) through its type 1 receptor (AT1) promotes cardiovascular hypertrophy and fibrosis. Therefore, the aim of this study was to analyze whether chronic long-term inhibition of the renin-angiotensin system (RAS) can prevent most of the deleterious effects due to aging in the cardiovascular system of the normal rat. The main objective was to compare two strategies of ANG II blockade: a converting enzyme inhibitor (CEI) and an AT1 receptor blocker (AT1RB). A control group remained untreated; treatment was initiated 2 wk after weaning. A CEI, enalapril (10 mg·kg⁻¹·day⁻¹), or an AT1RB, losartan (30 mg·kg⁻¹·day⁻¹), was used to inhibit the RAS. Systolic blood pressure, body weight, and water and food intake were recorded over the whole experimental period. Heart, aorta, and mesenteric artery weight as well as histological analysis of cardiovascular structure were performed at 6 and 18 mo. Twenty animals in each of the three experimental groups were allowed to die spontaneously. The results demonstrated a significant protective effect on the function and structure of the cardiovascular system in all treated animals. Changes observed at 18 mo of age in the hearts and aortas were quite significant, but each group completely abolished this deterioration. The similarity between the results detected with either enalapril or losartan treatment clearly indicates that most of the effects are exerted through AT1 receptors. An outstanding finding was the significant and similar prolongation of life span in both groups of treated animals compared with untreated control animals.

losartan; enalapril; heart; aorta; life span

THE NATURAL PROCESS OF AGING is related to a progressive modification, and ultimately, a loss of organ function. These alterations are common to all species. In general, there is a correlation between the structural and functional changes associated with aging. In mammals, degenerative processes such as arteriosclerosis, the development of senile plaques in the brain, and the replacement of functional parenchyma by fibroconnective tissue in a variety of organs are considered manifestations of aging (19, 41).

Ultrastructurally, a reduction in the number of cellular organelles such as mitochondria is common in the aging process (13, 21, 36). Lifelong free radical production could play a main role in the reduction of the number and in both structural and functional mitochondria modifications (6, 20, 38). It has been widely postulated that reactive oxygen species (ROS) are causally involved in the aging process (19, 20). In this sense, earlier data (4, 17) have confirmed that nitric oxide synthase (NOS) activity in the aorta and nitric oxide (NO) production diminish with age, whereas chronic long-term administration of angiotensin II (ANG II) inhibitors maintains endothelial NOS activity in old animals. Moreover, the mitochondria from hearts of aged rats chronically treated with ANG II inhibitors were found to have increased NOS activity and decreased hydrogen peroxide formation compared with controls (10).

The renin-angiotensin system (RAS) through its active agent ANG II has a large variety of physiological actions that include arterial blood pressure regulation, sodium and water homeostasis, and stimulation of other endocrine systems. Some of the actions are exerted as an endocrine system. Over the past few decades, a vast amount of information has uncovered a multitude of deleterious cardiovascular and renal effects attributed to excess ANG II. This peptide is now known to act as a paracrine, autocrine, and intracrine factor, whose mechanisms of action range from excitation or injury of whole organs to influence on cellular growth and function, alterations of intracellular signaling pathways, and promotion of extracellular matrix changes. Recently, the deleterious effects of ANG II in the heart have been related to activation of p66shc adaptor protein (18).

The RAS contributes to the pathogenesis of several human diseases including hypertension, congestive heart failure, coronary artery disease, and diabetic nephropathy. Angiotensin converting enzyme inhibitors (CEIs) and ANG II nonpeptide type 1 (AT1) receptor blockers (AT1RBs) are used in the treatment of these diseases. Aging and hypertension are associated with an increased incidence of coronary and cerebrovascular disease and renal failure. Most of the cardiovascular complications are related to alterations in vascular structure and function. The marked enhancement of age-related vascular complications in hypertension suggests an important interaction between the two processes (3, 11). Several reports have demonstrated that the cardiovascular changes observed during aging are similar to those due to high blood pressure (BP) overload with respect to biochemical, mechanical, and electrophysiological properties of the cardiovascular system (2, 5). In rats, both hypertension and age (2) produce an increase in absolute and relative left ventricular weight and myocardial fibrosis (32). On the other hand, the structure and function of the arterial wall also show similar modifications in hypertension and aging. The main vascular structural alteration is enhanced stiffness (15) related to increased media thickness due to smooth muscle cell hypertrophy/hyperplasia and colla-
gen accumulation (31). Endothelial and smooth muscle dysfunction with aging and hypertension has been described in rat aorta (26). The similarities between aging and hypertension, added to the fact that increased BP is usually observed in aged humans and rats, suggest the possibility that hypertension could be a form of accelerated aging. In this sense, some reports have indicated that hypotensive agents like CEIs and AT1RBs could lower BP in normotensive, normoreninemic animals (8, 31). Nonetheless, in mice no hypotensive effect was detected even during chronic treatment with one of the above-mentioned pharmacological agents (22). CEIs could be even more effective in the elderly, based on the favorable effects on cardiovascular fibrosis detected in experimental studies (25). Because the pharmacological effects of the CEI are due to more than one mechanism, mainly inhibition of ANG II formation and blockade of bradykinin metabolism, it was necessary to confirm that these actions are related to blockade of ANG II formation either in the plasma or in the involved tissues. Comparison of the effect of enalapril with an ANG II receptor blocker, which specifically impedes the binding of ANG II to the AT1 receptor, has helped to clarify this matter.

During the last years we have investigated the deleterious effects due to aging on target organs in the normal male Wistar rat. Simultaneously, we have analyzed the protective effect of chronic ANG II inhibition either with a CEI (enalapril) or with an AT1RB (losartan), administered since weaning, on these alterations. The present results describe cardiovascular changes caused by aging in the normal rat and the protective effect observed in this system due to long-term inhibition of ANG II with each of these pharmacological agents. Moreover, we have also determined the influence of each treatment on life span in the normal rat.

MATERIALS AND METHODS

Rats and Experimental Design for Drug Treatments

Male Wistar rats were used and received care in accordance with and under a license granted by our Institutional Animal Care and Use Committee and in compliance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health, revised 1996). Animals (n = 120) were divided at random into three equal groups after weaning: 1) control (tap water to drink), 2) losartan (30 mg·kg⁻¹·day⁻¹ in the drinking water), and 3) enalapril (10 mg·kg⁻¹·day⁻¹) (both drugs from Merck, Rahway, NJ). Rats were housed in groups of five per cage with a 12:12-h light-dark cycle and a temperature range of 20–22°C. Rat chow and drinking material were freely available.

Systolic Blood Pressure Measurement and Cardiac and Aortic Weight

Systolic blood pressure (SBP) was measured once a month over the whole experimental period in conscious rats at 30°C by tail-cuff plethysmography using a pressure transducer (Narco Biosystems, Houston, TX) connected to an oscilloscope. Water intake and body weight were determined twice a week to adjust drug administration. After 6 mo of treatment, 10 animals from each group were killed under pentobarbital anesthesia (40 mg/kg). Similarly, after 18 mo, 10 rats from each group were killed. The heart, the aorta, and the mesenteric artery were excised, cleaned, and weighed. After removal of atria, the right ventricle was separated and weighed.

Tail Length

Tail length as a measure of growth (14) was measured with a standard metric ruler from the anus of the animal to the tip of the tail by the same experimenter to ensure uniformity.

Histological Evaluation

The left ventricle and the same portion of the thoracic aorta were isolated, kept in 10% formaldehyde, and embedded in paraffin for histological determinations. Histological sections were stained with hematoxylin-eosin and Masson’s trichrome. Collagen type III was also evaluated by immunohistochemistry (IHC) in the left ventricle. It was quantified with anti-collagen type III monoclonal antibodies (Biogenex, San Ramon, CA), at a dilution of 1:100. Aortic width was evaluated by IHC using α-actin monoclonal antibodies (Sigma, St. Louis, MO).

Morphometric analyses were carried out in both tissues; these included cardiomyocyte transverse diameter and vessel width. Twenty histological sections of each organ were made, and 10 vascular structures per histological section were analyzed.

Histological sections were analyzed with a Nikon E400 light microscope (Nikon Instrument Group, Melville, NY). Image-Pro Plus 4.5,1,29 software (Media Cybernetics, Silver Spring, MD) was used to evaluate 1) aortic width and aortic area, 2) myocardiocyte diameter and cross-sectional area, and 3) subendocardial and subepicardial fibrosis.

A single investigator blinded to the experimental groups performed the analyses.

Morphometric Techniques

Aortic width. The cross section of the vessel was measured with a perpendicular line going from the external to the internal perimeter. The data obtained are expressed in micrometers.

Aortic area. Four opposite areas of aorta per vessel were measured, excluding the adventitia. The data obtained are expressed in square millimeters.

Myocardiocyte diameter and cross-sectional area. Five images of the myocardium were randomly acquired. Ten consecutive cells of each image, for a total of 50 myocardiocytes per animal, were studied. Myocardiocyte diameter and area were evaluated at ×400.

MYOCARDIOCYTE DIAMETER. Longitudinal and transversal diameters of each cell were obtained and averaged. The results are expressed in micrometers.

MYOCARDIOCYTE CROSS-SECTIONAL AREA. The software evaluated the surface of each cell. The results are expressed in square micrometers.

Cardiac fibrosis evaluation. Masson’s trichrome and collagen III were used to determine cardiac fibrosis (×100). The severity of cardiac fibrosis was evaluated in 10 areas of the myocardium, 5 from the subendocardium and 5 from the subepicardium region. The intensity of the fibrotic process was expressed as a percentage of the total tissue area occupied by stained tissue.

Life Span

The rats remaining in each group were used to determine survival time. All animals were subsequently followed until time of death. The condition of all animals was monitored daily by a veterinarian. During the study, individual rats were euthanized according to the criteria of the veterinarian: tumors that impaired movement, bleeding abdominal or foot ulcers, respiratory infections, or achievement of a moribund state (inability to eat or drink or labored breathing). In all cases where euthanasia was requested, the date of euthanasia was considered as the date of death.
Body weight was significantly decreased only by enalapril treatment (Table 1). Differences were detected immediately after the drug administration began. Growth rate, measured by the length of the tail, was exactly the same in the three groups of rats both at 6 (control 19.2 ± 1.3, losartan 19.0 ± 2.1, enalapril 18.8 ± 1.6 cm) and at 18 (control 20.2 ± 0.3, losartan 20.6 ± 0.1, enalapril 20.5 ± 0.4 cm) mo of age, suggesting that enalapril decreased the white adipose tissue (WAT) accumulation that was observed in normal rats over their entire life. This effect was confirmed by nuclear magnetic resonance and perirenal WAT weighing (data not shown). Food intake was the same in treated and control rats when normalized by body weight (control 7.5 ± 0.3, losartan 6.9 ± 0.4, enalapril 7.2 ± 0.2 g · 100 g body wt⁻¹·day⁻¹).

Water intake decreased over the experimental period, being significantly higher in very young rats (20 ml·100 g⁻¹·day⁻¹ at 1 mo) and stabilizing at ~6 mo of age (10 ml·100 g⁻¹·day⁻¹). It was increased by enalapril administration over the whole experimental period, 31 ± 2% at 6 mo and 28 ± 3% at 18 mo.

Heart, left ventricular, and aortic weights were lower in both groups of treated animals compared with normal control rats at 6 (Table 2) and 18 (Table 3) mo. When organ weight was normalized by body weight, differences disappeared in enalapril-treated rats because of their lower body weight, showing that this relationship was not adequate in this case.

**Systolic Blood Pressure**

SBP increased throughout the study in control rats, whereas animals treated with either enalapril or losartan maintained the BP values registered at 2 mo of age (initiation of SBP measurement) over the whole experimental period (Table 4).
at 16 mo, decreased slightly until 18 mo, remained stable over the next 10 mo, and then began to decline.

DISCUSSION

The most important results obtained in the present study are the equality between the effects of a CEI and an AT\(_1\)RB, clearly demonstrating that chronic inhibition of the RAS, with either pharmacological agent, plays a momentous role in the protection of the cardiovascular system during aging. On the other hand, both compounds substantially prolonged life span. This protective effect was accompanied by maintenance of BP at the same levels registered in young animals, but other tissue mechanisms can also be postulated in this action. Even though we do not have any evidence connecting cardiovascular protection with substantially prolonged life span, both drugs exerted a clearly similar and significant beneficial effect on survival time in treated rats.

The objective of the present study was to analyze the cardiovascular changes due to aging in the normal rat and the protective effect exerted by chronic inhibition of the RAS. In this sense, Michel et al. (33) observed that the CEI perindopril did not prevent the aortic lumen enlargement associated with aging in male normotensive rats but did postpone the increase in medial and intimal thickening; they also found (32) that in normotensive and hypertensive rats treated with perindopril for 1 yr, left ventricular mass and collagen accumulation were significantly reduced compared with untreated animals. The main issue of the present research was to determine whether this protection can be ascribed only to blockade of the RAS or to some other action of CEI (prevention of bradykinin metabolism). Comparison between the effects of the CEI enalapril and the AT\(_1\)RB losartan were performed to clearly establish this possibility.

The results shown in Table 4 have distinctly demonstrated that lifetime inhibition of the RAS with either pharmacological agent prevented the BP increase due to aging in the normal Wistar rat. In humans SBP increases continuously with age, while other experimental reports, either in aged rats or mice (22, 33), showed no changes in BP. CEIs and AT\(_1\)RBs have

<table>
<thead>
<tr>
<th>Group</th>
<th>3rd mo</th>
<th>6th mo</th>
<th>9th mo</th>
<th>12th mo</th>
<th>15th mo</th>
<th>18th mo</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>120±2</td>
<td>139±2</td>
<td>145±2</td>
<td>144±2</td>
<td>138±2</td>
<td>144±2</td>
</tr>
<tr>
<td>Enalapril</td>
<td>110±2*</td>
<td>103±3*</td>
<td>107±5*</td>
<td>106±5*</td>
<td>105±3*</td>
<td>105±4*</td>
</tr>
<tr>
<td>Losartan</td>
<td>114±3*</td>
<td>105±3*</td>
<td>103±4*</td>
<td>100±6*</td>
<td>105±3*</td>
<td>110±6*</td>
</tr>
</tbody>
</table>

Data (in mmHg) are expressed as means ± SE for \( n = 20 \) rats/group. \(*P < 0.05\) with respect to control.

Table 5. Aortic width and area and myocyte diameter and cross-sectional area in 6- and 18-mo-old control and treated normal rats

<table>
<thead>
<tr>
<th>Group</th>
<th>Aortic Width, ( \mu m )</th>
<th>Aortic Area, ( \text{mm}^2 )</th>
<th>Myocyte ( D ), ( \mu m )</th>
<th>Myocyte CSA, ( \mu m^2 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control 6 mo</td>
<td>120±7</td>
<td>0.60±0.01</td>
<td>20.49±0.86</td>
<td>335.8±12.9</td>
</tr>
<tr>
<td>Enalapril 6 mo</td>
<td>104±5</td>
<td>0.52±0.01*</td>
<td>17.12±0.69*</td>
<td>236.2±9.8*</td>
</tr>
<tr>
<td>Losartan 6 mo</td>
<td>107±4</td>
<td>0.53±0.01*</td>
<td>17.82±0.56*</td>
<td>249.4±10.5*</td>
</tr>
<tr>
<td>Control 18 mo</td>
<td>200±9</td>
<td>0.81±0.02</td>
<td>26.2±0.95</td>
<td>548.1±17.6</td>
</tr>
<tr>
<td>Enalapril 18 mo</td>
<td>160±6*</td>
<td>0.68±0.01*</td>
<td>20.4±0.46*</td>
<td>329.8±11.5*</td>
</tr>
<tr>
<td>Losartan 18 mo</td>
<td>163±7*</td>
<td>0.72±0.02*</td>
<td>21.2±0.36*</td>
<td>361.7±12.0*</td>
</tr>
</tbody>
</table>

Data are expressed as means ± SE for \( n = 10 \) rats/group. \( D \), diameter; CSA, cross-sectional area. \(*P < 0.05\) with respect to control.

Fig. 1. Histological aortic photomicrographs at 18 mo. Anti-\( \alpha \)-smooth muscle actin immunolabeling, \( \times 400 \) magnification. Representative photomicrographs show differences regarding aortic width in control, enalapril, and losartan groups.
been shown to be efficacious antihypertensive agents in several forms of experimental and human hypertension that do not have elevated circulating renin levels (29, 40); less information is available in the normal rat. These data confirm results obtained by other authors (8, 33), revealing the important role played by ANG II in regulation of normal BP (24). In fact, both drugs similarly prevented the increase in BP occurring in normal rats during aging. However, only enalapril attenuated the rate of increase in body weight with age, although body growth, measured as increase in tail length, was not impaired. Lower body weight was due to reduced accumulation of visceral and subcutaneous fat and a significant reduction in hepatic steatosis (N. Basso, unpublished observations). Although these effects were not recorded in losartan-treated rats, telmisartan, but not valsartan, also protects against weight gain and hepatic steatosis (42). This effect could be related to its selective peroxisome proliferator-activated receptor-γ-modulating activity.

Aging is accompanied by decreased aortic compliance that is an important promoter of left ventricular hypertrophy. Decreased aortic compliance in aged untreated animals has been detected by their increased vascular mass and by an important thickening of the vessel wall. Similar results have been described by other authors in the male rat; they assumed that this effect is due to hypertrophy of the smooth muscle cells and increased extracellular material deposition, in particular collagen (9).

The present results have shown that these alterations in the aortic wall, already detected in young adult (6 mo old) rats and substantially increased in aged animals, were markedly reduced by chronic ANG II inhibition and that both inhibitors are equally active in the prevention of aortic growth. In this sense, it has been demonstrated that ANG II is a potent trophic factor that promotes cellular hypertrophy and matrix deposition. A recent report has demonstrated that other molecules become altered during aging within the arterial wall including increased converting enzyme, ANG II, AT1 receptors, α-adrenoreceptors, and endothelin 1 (45). Thus most evidence supports the important role played by the RAS in vascular changes due to aging.

The most relevant information obtained in a previous study was the increased NOS activity in the aorta induced by chronic administration of either compound and the finding that NOS activity in the aortic endothelium diminishes with age (17). Moreover, urinary excretion of nitrate + nitrite used as a marker of NO systemic production was significantly higher in treated than in control rats. These results indicate a protective vascular effect due to ANG II inhibition in the adult normal rat through increased NO production (17).

Table 6. Changes in percentage of subpericardial and subendocardial fibrosis and collagen III in hearts of 18-mo-old control and treated normal rats

<table>
<thead>
<tr>
<th>Group</th>
<th>Spe Fibrosis</th>
<th>Sen Fibrosis</th>
<th>Spe Collagen III</th>
<th>Sen Collagen III</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3.73±0.41</td>
<td>8.66±1.21</td>
<td>0.67±0.20</td>
<td>4.81±0.84</td>
</tr>
<tr>
<td>Enalapril</td>
<td>1.49±0.25*</td>
<td>2.30±0.32*</td>
<td>0.26±0.04*</td>
<td>0.26±0.05*</td>
</tr>
<tr>
<td>Losartan</td>
<td>1.18±0.22*</td>
<td>2.24±0.35*</td>
<td>0.29±0.08*</td>
<td>0.35±0.15*</td>
</tr>
</tbody>
</table>

Data (in %) are expressed as means ± SE for n = 10 rats/group. Spe, subpericardial; Sen, subendocardial. *P < 0.05 with respect to control.
Losartan treatment can attenuate several age-related cardiac changes. The protective effects of these compounds include the reduction of heart weight and myocardiosclerosis in aged rats. These results show that cardiac aging is associated with myocyte hypertrophy and increased accumulation of extracellular matrix proteins, particularly collagen within the cardiac interstitium, and both pharmacological strategies completely impaired cardiac remodeling. Previous studies have demonstrated that, in the normal adult rat, treatment with enalapril for 3 wk decreased BP and ventricular weight-to-body weight ratio (30). Furthermore, treatment with either enalapril or losartan over 6 mo induced a reduction in cardiac DNA and collagen concentration as well as in the percentage of fibrosis (16). These effects could be related to a protective action on the myocardium, because cell hypertrophy and collagen accumulation are the main alterations in cardiac structure with age (1).

Previous data indicate that in old animals both treatments doubled the production of NO by submitochondrial particles in heart homogenates, while maximal hydrogen peroxide formation was lower in both treated groups. These results confirmed a protective effect on cardiovascular damage due to aging exerted through inhibition of ANG II and clearly related to diminished ROS (10).

Recently, aging research has focused on the oxidative stress experienced by aging cells because of increased ROS formation. According to the mentioned report (39), there are two reliable means to extend life span of rodents: caloric restriction and knocking out the p66\textsuperscript{Shc} gene. At the same time, the latter confers resistance to oxidative stress (34). It has been suggested that the AT\textsubscript{1} receptor transactivates an intracellular signaling controlled by growth factors and their tyrosine kinase receptors; this downstream mechanism comprises the p66\textsuperscript{Shc} adaptor protein that may play a role in cell senescence. A recent report in p66\textsuperscript{Shc}-knockout mice proposes a fundamental role of this gene in ANG II-mediated cardiac remodeling (18). Other findings suggest that the activation of the RAS may be important for the physiological changes in aged hearts (23); previous data showed that CEsIs attenuate the increment in cardiac tissue apoptotic cells associated with aging (22).

The relationship between ANG II and vascular damage seems to be also linked to oxidative stress. Experimental data demonstrate that increased ROS generation through the activation of NAD(P)H oxidase is an obligatory step in ANG II effects (7, 35, 46). Growing evidence indicates that ROS function as important intra- and intercellular second messengers to modulate signaling cascades that lead to vascular damage in cardiovascular diseases (43); another recent report has stated that ANG II promotes vascular inflammation by inducing premature senescence of vascular smooth muscle cells in vivo and in vitro (27).

An important finding of this study is the prolonged life span of both groups of treated rats, supporting previous results in mice (12). No clear differences in the cause of death could be detected in either control or treated animals. The only evidence of body deterioration in all cases was loss of weight that began later in treated animals. Thus cardiovascular protection cannot be directly linked to extension of maximal life span. This notwithstanding, it has been postulated that p66\textsuperscript{Shc} is part of a signal transduction pathway that regulates stress apoptotic responses and life span in mammals, and the levels of this gene expression correlate with life span duration in mice (44). Oxidative stress is involved in this pathway; thus it can be assumed that inhibition of the RAS has overall antioxidant and anti-inflammatory effects (37) and through these actions could prolong maximal life span in rats. In this sense, resistance to oxidative damage has been postulated as one factor in murine life expansion.

Fig. 3. Histological myocardial photomicrographs at 18 mo. Anti-collagen III immunolabeling, ×400 magnification. Representative photomicrographs show differences regarding myocyte cross-sectional areas and fibrosis in control, enalapril, and losartan groups.

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In conclusion, these studies have demonstrated that chronic long-term inhibition of the RAS with either a CEI or an AT1RB induces an identical and significant protective effect on the deleterious process of aging on the cardiovascular system and a substantial extension in life span in the normal Wistar rat. Protection was determined through functional and structural analysis. Increased activity of NOS in the aortic endothelium and in cardiac mitochondria could indicate a reduction in the mechanisms of oxidative stress even at the mitochondrial level. The sustained decreased oxidative burden may account for the protective effect. ANG II induces excessive release of vascular superoxide radical generated by stimulation of NADPH oxidase, which inactivates NO. Inhibition of ANG II would attenuate oxidative stress and improve vasodilation. The low rate of oxidative stress could be a key to reduction in inflammatory processes and cytokine and growth factor production. This pathway would diminish fibrosis and preserve function and structure in the analyzed target organs and could be involved in the prolonged life span.

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