ANG II-induced cardiac molecular and cellular events: role of aldosterone

Wenyuan Zhao,1 Robert A. Ahokas,2 Karl T. Weber,1 and Yao Sun1
Division of Cardiovascular Diseases, Departments of 1Medicine and 2Obstetrics
and Genecology, University of Tennessee, Health Science Center, Memphis, Tennessee
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Zhao, Wenyuan, Robert A. Ahokas, Karl T. Weber, and Yao Sun. ANG II-induced cardiac molecular and cellular events: role of aldosterone. Am J Physiol Heart Circ Physiol 291: H336–H343, 2006. First published February 17, 2006; doi:10.1152/ajpheart.01307.2005.—Chronic elevation of circulating ANG II is associated with cardiac remodeling in patients with hypertension and heart failure. The underlying mechanisms, however, are not completely defined. Herein, we studied ANG II-induced molecular and cellular events in the rat heart as well as their links to the redox state. We also addressed the potential contribution of aldosterone (ALDO) on ANG II-induced cardiac remodeling. In ANG II-treated rats, and compared with controls, we found: 1) the expression of proinflammatory/profibrogenic mediators was significantly increased in the perivascular space and at the sites of microscopic injury in both ventricles; 2) macrophages and myofibroblasts were primary repairing cells at these sites, together with increased fibril collagen volume; 3) apoptotic macrophages and myofibroblasts were evident at the same sites; 4) NADPH oxidase (gp91phox) was significantly enhanced at these regions and primarily expressed by macrophages, whereas superoxide dismutase and catalase levels remained unchanged; 5) plasma 8-isoprostane levels were significantly increased; and 6) blood pressure was significantly elevated. Losartan treatment completely prevented cardiac oxidative stress as well as molecular/cellular responses and normalized blood pressure. Spironolactone treatment partially suppressed the cardiac inflammatory/fibrogenic responses and redox state. Thus chronic elevation of circulating ANG II is accompanied by a proinflammatory/profibrogenic phenotype involving vascular and myocardial remodeling in both ventricles. Enhanced reactive oxygen species production at these sites and increased plasma 8-isoprostane indicate the involvement of oxidative stress in ANG II-induced cardiac injury. ALDO contributes, in part, to ANG II-induced cardiac molecular and cellular responses.

As a circulating hormone, endocrine properties of ANG II are integral to the regulation of blood pressure and volume homeostasis. However, chronic elevation of circulating ANG II, such as occurs with congestive heart failure and hypertension, is inappropriate and has adverse consequences. For example, such neurohormonal activation has been related to a diverse number of vascular diseases, including atherosclerotic coronary artery disease, cardiac perivascular fibrosis, and diabetes (6, 8). An important vascular effect of ANG II is its induction of oxidative stress and a proinflammatory phenotype. Type I ANG II (AT1) receptor blockade can prevent the unfavorable vascular effects by ANG II through hypertension-dependent and -independent mechanisms (1, 18, 22). Our previous studies have demonstrated that, in addition to vascular remodeling, elevation of circulating ANG II levels also leads to extensive microscopic myocardial repair/remodeling in both left and right ventricles in rats (21, 22). However, ANG II-induced myocardial molecular/cellular events and their relation to oxidative stress have not been fully explored. ANG II stimulates aldosterone (ALDO) release from the adrenal cortex. ALDO increases sodium reabsorption, water retention, and potassium and magnesium loss. However, it also produces a variety of other actions that lead to progressive damage in the heart, vasculature, and kidneys (15, 16, 23). Although ANG II has been considered the major mediator of cardiovascular damage, it has been suggested that ALDO may mediate and exacerbate the effects of ANG II. Our previous studies have shown that circulating ALDO levels were significantly increased (6- to 7-fold) in ANG II-infused rats (21). Studies have demonstrated that ALDO contributes to ANG II-induced vascular remodeling (17). Whether ALDO is contributory to ANG II-induced myocardial injury/remodeling, however, has not been fully elucidated.

In the current study, using the ANG II-infused rat models, we sought to detect cardiac expression of proinflammatory/profibrogenic mediators, cells involved in cardiac repair/remodeling and apoptosis, and the appearance and quantity of fibrous tissue as well as their links to the redox state, including reactive oxygen species production and antioxidant defense capacity. We also sought to determine whether ALDO contributes to ANG II-induced cardiac pathological changes.

Materials and Methods

Animal model. Eight-week-old male Sprague-Dawley rats (Harlan, Indianapolis, IN) were used in this study. The following four animal groups were included (n = 8 in each group): 1) untreated age-matched rats served as controls; 2) rats received ANG II (9 μg/h) by implanted minipump for 4 wk; 3) rats on the same dose of ANG II also received an ALDO receptor antagonist spironolactone (150 mg·kg−1·day−1) given by gavage for 4 wk; and 4) rats on the same dose of ANG II also received an AT1 receptor antagonist, losartan (10 mg·kg−1·day−1), given by gavage for 4 wk. Systolic blood pressure was measured at week 3 by the tail cuff method, as previously reported (23). Animals were killed at 4 wk. Blood was collected by cardiac puncture, and plasma was isolated by centrifugation for determining 8-isoprostane levels. Hearts were removed, rinsed in cold normal saline, weighed, frozen in isopentane with dry ice, and kept at −80°C. Serial cryostat coronal sections were prepared for the following studies. This study was approved by the University of Tennessee Health Science Center Animal Care and Use Committee.

In situ hybridization. The localization and optical density of cardiac intercellular adhesion molecule (ICAM)-1 and monocyte chemotactic protein (MCP)-1, transforming growth factor (TGF)-β1, type I collagen and tissue inhibitors of matrix metalloproteinases (TIMP)-1 and TIMP-2, and NADPH oxidase (gp91phox subunit) mRNAs were
detected by quantitative in situ hybridization. In brief, cardiac sections (16 μm) were fixed in 4% formaldehyde for 10 min, washed with PBS (pH 7.4), and incubated in 0.25% acetic anhydride in 0.1 M Tris-HCl for 10 min. Sections were then hybridized overnight with [35S]dATP-labeled DNA probes for ICAM-1, MCP-1, TGF-β1, TIMP-1, TIMP-2, type 1 collagen, and gp91phox at 45°C. The hybridized sections were then washed, dried, and subsequently exposed to Kodak Biomax X-ray film. After exposure, film was developed, and sections were stained with hematoxylin and eosin. Quantification of mRNA optical density was performed using a computer image analysis system (NIH Image, 1.60; see Ref. 23).

**Immunohistochemistry.** The appearance, population, and localization of macrophages, lymphocytes, myofibroblasts, and gp91phox positive cells in the rat heart were detected by immunohistochemistry. Cardiac sections (6 μm) were air-dried, fixed in 10% buffered formalin for 5 min, and washed in PBS for 10 min. Sections were then incubated with the primary antibody against ED1 (macrophages), CD4 (lymphocytes), α-smooth muscle actin (myofibroblasts; Sigma, St. Louis, MO), and gp91phox (kindly provided by Dr. M. Quinn, Montana State University) for 1 h at room temperature. Sections were then incubated with IgG- and peroxidase-conjugated secondary antibody (Sigma) for 1 h at room temperature, washed in PBS for 10 min, and incubated with 0.5 mg/ml diaminobenzidine tetrahydrochloride 2-hydrate + 0.05% H2O2 for 5 min. Negative control sections were incubated with secondary antibody alone. All sections were counterstained with hematoxylin, dehydrated, mounted, and viewed by light microscopy (27).

**TUNEL.** Apoptosis was detected by TUNEL technique using an Apop Tag Fluorescein kit (Intergen, Norcross, GA). In brief, cardiac sections (6 μm) were fixed in 1% paraformaldehyde, followed by incubation with TdT enzyme. Sections were then incubated with antidigoxigenin conjugated with fluorescein. Sections were counterstained with propidium iodide, mounted, and viewed by fluorescence microscopy (9).

**Cardiac morphology.** Cardiac sections (6 μm) were prepared to determine the fibrillar collagen accumulation by collagen-specific picrosirius red staining and observed by light microscopy as previously reported (22). Collagen volume fraction of each section was determined using a computer image analyzing system (NIH image, 1.60), as previously reported (22).

**Western blot.** Cardiac superoxide dismutase (SOD) and catalase levels were determined by Western blot. In brief, cardiac tissue was homogenized in lysis buffer and then separated by 12% SDS-PAGE. After electrophoresis, samples were transferred to polyvinylidene difluoride membranes and incubated with the primary antibody against MnSOD or catalase. Blots were subsequently incubated with peroxidase-conjugated secondary antibody. After being washed, blots were developed with the enhanced chemiluminescence method. The amount of protein detected by each antibody was measured by a computed image analysis system (19).

**Enzyme immunoassay.** Plasma 8-isoprostane levels were detected to reflect levels of systemic oxidative stress using a commercially available enzyme immunoassay kit (Cayman Chemical, Ann Arbor, MI). Briefly, 100 μl of standard or plasma samples were placed in a 96-well plate that was precoated with mouse monoclonal antibody after purification using a C-18 solid-phase extraction cartridge. Thereafter, 50 μl of 8-isoprostane tracer and 8-isoprostane antiserum were added to each well and incubated for 18 h at room temperature. After washing with wash buffer, 200 μl of Ellman’s reagent containing the substrate of acetylcholinesterase was added. The plates were read at 412 nm, and the values of plasma 8-isoprostane levels were calculated from a curve drawn using standard concentrations of 8-isoprostanes (12).

**Statistical analysis.** Statistical analysis of the ratio of heart weight to body weight, in situ hybridization, Western blot, systolic blood pressure, and plasma 8-isoprostane findings was performed using ANOVA. Values are expressed as means ± SE with P < 0.05 considered significant. Multiple group comparisons among controls and each group were made by Scheffé’s F-test.

**RESULTS**

**Heart-to-body weight ratio and blood pressure.** Compared with controls, the heart-to-body weight ratio was significantly increased in ANG II-treated animals, which was prevented by losartan treatment and partially reduced (P > 0.05) by spironolactone treatment. Systolic blood pressure was significantly elevated in ANG II-infused animals. Losartan completely blocked the hypertensive effect of ANG II, whereas spironolactone slightly (P > 0.05) suppressed ANG II-induced hypertension (Table 1).

**Cells involved in cardiac repair/remodeling and apoptosis.** Our previous studies have shown that ANG II infusion leads to perivascular and myocardial microscopic injury in both ventricles of the rat heart, followed by perivascular fibrosis and microscopic scars (21). By immunohistochemistry, we observed accumulated macrophages in the perivascular space and at sites of myocardial injury (Fig. 1, B and C) in ANG II-treated animals, whereas lymphocytes and neutrophils were rare at these sites (data now shown). Myofibroblasts were found accumulated in the same regions (Fig. 1, E and F) and colocalized with perivascular fibrosis and microscopic scars (Fig. 1, H and I). By TUNEL, we observed apoptotic inflammatory cells and myofibroblasts at microscopic injuries in perivascular (Fig. 1K) and microscopic injury (Fig. 1L). These cardiac cellular responses were not seen in untreated controls (Fig. 1, A, D, G, and J). The cellular response and appearance of fibrosis in the heart were completely prevented by the cotreatment with losartan but only attenuated by spironolactone (data not shown).

**Gene expression of proinflammatory/profibrogenic mediators.** As detected by quantitative in situ hybridization (see Fig. 2), low-optical-density ICAM-1 (A), MCP-1 (C), TGF-β1 (E), type I collagen (G), TIMP-1 (I), and TIMP-2 (K) mRNAs were found uniformly distributed in both right and left ventricles of control rats. At 4 wk of ANG II treatment, gene expression of ICAM-1 (B), MCP-1 (D), TGF-β1 (F), type I collagen (H), TIMP-1 (J), and TIMP-2 (L) were markedly increased within the perivascular space of intramural coronary arteries and at sites of microscopic injury in both ventricles. Cotreatment with losartan prevented, whereas spironolactone suppressed, such ANG II-induced gene expressions of cardiac proinflammatory/profibrogenic mediators (Fig. 3) as well as collagen volume fraction (Fig. 4).

**Cardiac oxidative stress.** NADPH oxidase catalyzes the one-electron reduction of molecular oxygen to superoxide radicals. Plasma 8-isoprostane levels were calculated from a curve drawn using standard concentrations of 8-isoprostanes (12).

<table>
<thead>
<tr>
<th>Group</th>
<th>Control</th>
<th>ANG II + Los</th>
<th>ANG II + Spi</th>
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<tr>
<td>Heart/body wt, mg/g</td>
<td>2.99 ± 0.06</td>
<td>4.12 ± 0.16*</td>
<td>2.95 ± 0.08†</td>
</tr>
<tr>
<td>Blood pressure, mmHg</td>
<td>103 ± 14</td>
<td>235 ± 9*</td>
<td>98 ± 8†</td>
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Values are means ± SE. Los, losartan; Spi, spironolactone. P < 0.05 vs. control group (*) and vs. ANG II group †. |
Fig. 1. Cells involved in cardiac repair and apoptosis in response to ANG II infusion. ED-1-positive macrophages are rarely seen in the normal heart (A). In ANG II-treated animals, macrophages are accumulated at the perivascular space (B, arrows) and with microscopic injury (C, arrows). α-Smooth muscle actin-positive myofibroblasts are not present in the perivascular space and myocardium in the normal heart (D, arrow: vascular smooth muscle cells). In ANG II-treated animals, myofibroblasts are accumulated in the perivascular space (E, arrows) and at sites of microscopic injury (F, arrows). Compared with normal heart (G), collagen is accumulated within the perivascular space (H) and at sites of myocardial injury (I, red). Apoptotic cells are not observed in the normal heart (J). Apoptotic inflammatory cells and myofibroblasts are evident in the perivascular space and the sites of microscopic injury (K and L, arrows). A-C and G-L: magnification ×400. D-F: magnification ×100.
anion and is a major source of reactive oxygen species production. SOD catalyzes the dismutation of O$_2$ to H$_2$O$_2$, and catalase further metabolizes H$_2$O$_2$ to water and oxygen. Oxidative stress is enhanced by an imbalance between reactive oxygen species production and antioxidant reserve. To investigate whether oxidative stress is associated with ANG II-induced cardiac damage, we analyzed the expression of NADPH oxidase (gp91phox) by in situ hybridization and immunohistochemistry and SOD and catalase content by Western blot. Compared with the normal heart (Fig. 5A), gp91phox mRNA was largely increased within the perivascular space, and microscopic injury in both ventricles (Fig. 5B) in ANG II-treated animals and cells expressing gp91phox at these sites were primarily inflammatory cells (Fig. 5, D and F). Losartan prevented, whereas spironolactone attenuated, cardiac gp91phox gene expression (Fig. 6). Cardiac SOD and catalase protein levels, however, remained unchanged in ANG II-treated animals compared with controls (Fig. 7).

**Plasma 8-isoprostane levels.** Plasma 8-isoprostane is a biomarker for systemic oxidative stress (4). Compared with control animals, plasma 8-isoprostane levels were significantly increased in ANG II-treated animals. Cotreatment with either losartan or spironolactone prevented the elevation of circulating 8-isoprostane (Fig. 8).

**DISCUSSION**

Herein, we addressed the molecular and cellular events that appear within the right and left ventricles of the rat heart in response to chronic elevation of circulating ANG II as well as their links to oxidative stress and ALDO. This study yielded several findings. First, this study has demonstrated that ANG II induces the proinflammatory phenotype, which is a necessary requisite to the accumulation of fibrous tissue in the heart. The current study has confirmed the previous findings that elevated circu-
ANG II leads to cardiac repair/remodeling represented as perivascular fibrosis and microscopic scars in rats and mice (6, 21, 27). This study has further explored the expression of proinflammatory and profibrogenic mediators as well as cells involved in cardiac repair/remodeling at these sites. Adhesion molecules and chemokines are contributory to inflammatory cell infiltration in the injured tissue and trigger the inflammatory response (14, 28). In ANG II-treated animals, we observed enhanced expression of ICAM-1 and MCP-1 at the perivascular space and sites of microscopic injury in both left and right ventricles, which is accompanied with accumulated macrophages. Macrophages are responsible for cytokine release, phagocytosis, and induction of oxidative stress in the repairing tissue. This study has shown enhanced cardiac TGF-β1 expression, which is spatially coincident with macrophages and myocardial/vascular injury. TGF-β has been demonstrated to promote the differentiation/proliferation of myofibroblasts and stimulates collagen synthesis. Myofibroblasts, phenotypically transformed fibroblasts, are not present in the normal tissue but appear in the repairing tissue and play a key role in fibrous tissue formation (5, 24). In ANG II-treated animals, abundant myofibroblasts were observed at sites of cardiac repair and are colocalized with enhanced collagen expression. The repairing cells, including macrophages and myofibroblasts, are transient cells in the heart and are finally eliminated through apoptosis when the healing is completed. TGF-β also stimulates TIMP synthesis, which inhibits collagen degradation by matrix metalloproteinases and leads to collagen accumulation (10, 11). TIMP expression is tightly controlled at the transcription level (26). In ANG II-treated animals, gene expression of TIMP-1 and TIMP-2 is significantly increased at sites of cardiac injury.

Fig. 3. Effect of losartan (Los) or spironolactone (Spi) on ANG II-induced cardiac TGF-β1 (A), type I collagen (B), TIMP-1 (C), and TIMP-2 (D) gene expression. CTL, control. *P < 0.05 vs. controls. #P < 0.05 vs. ANG II group.

Fig. 4. Cardiac collagen volume fraction in response to ANG II infusion with or without cotreatment of losartan or spironolactone. *P < 0.05 vs. controls. #P < 0.05 vs. ANG II group.
Thus the imbalance of cardiac collagen synthesis and degradation results in cardiac fibrosis in ANG II-treated animals. The second finding of this study is the coupling of the ANG II-induced myocardial injury to oxidative stress. Elevation of circulating ANG II has been demonstrated to induce vascular NADPH oxidase expression (27). This finding has been confirmed in the current study. We observed markedly increased NADPH oxidase (gp91phox) mRNA levels in perivascular space, and cells expressing gp91 phox are primarily macrophages. This study has further detected enhanced gp91phox expression at sites of myocardial microscopic injury in ANG II-treated animals. Again, cells expressing gp91phox at sites of microscopic injury are primarily inflammatory cells. Cardiac SOD and catalase, however, remained unchanged in ANG II-treated rats. These observations suggest that ANG II leads to cardiac oxidative stress by enhancing reactive oxygen species production at sites of cardiac injury. The colocalization of reactive oxygen species production and cardiac injury suggests involvement of oxidative stress in ANG II-induced cardiac inflammatory/fibrogenic responses. Accumulated evidence has
shown that oxidative stress stimulates cardiac damage, endothelial dysfunction, apoptosis, and collagen synthesis in the heart and contributes to the pathogenesis of myocardial remodeling and failure (2, 13, 20). Furthermore, plasma 8-isoprostane was found significantly increased in ANG II-treated animals, indicating the occurrence of systemic oxidative stress.

Our third finding demonstrated that ALDO partially mediates the ANG II-induced cardiac proinflammatory/profibrogenic phenotype. We used cotreatment with losartan, an AT1 receptor blocker, or spironolactone, an ALDO receptor antagonist, to estimate the relative importance of each hormone in myocardial injury. Cotreatment with losartan totally prevented the appearance of cardiac molecular and cellular events. Losartan also completely abrogated cardiac and systemic oxidative stress and normalized blood pressure. In ANG II-treated rats, cardiac injury and subsequent fibrosis appear in both hypertensive left ventricle and nonhypertensive right ventricle. Moreover, it has been shown that the antihypertensive agent hydralazine normalizes ANG II-induced hypertension, but it only reduces, not prevents, the vascular and myocardial effect of ANG II (7). These findings suggest that ANG II-induced cardiac remodeling is related to both hypertension and local effects of ANG II. ANG II has been demonstrated to directly damage the myocardium via several mechanisms, such as increased sarcolemmic permeability and death of myocytes or increased permeability and destruction of coronary microvascular endothelial cells (6). Furthermore, cardiac ANG II production is enhanced at sites of vascular and nonvascular injury in ANG II-treated animals (21). Locally generated ANG II has been indicated to stimulate cardiac inflammatory/fibrogenic responses in an autocrine/paracrine manner (3).

Although ANG II has been considered the major mediator of cardiovascular damage, it has been suggested that ALDO may mediate and exacerbate the damaging effects of ANG II. It has been demonstrated that ALDO contributes to ANG II-induced vascular remodeling (17). This finding has been confirmed in the current study. We observed attenuated vascular remodeling in rats receiving spironolactone. The current study has further indicated that cotreatment with the high dose of spironolactone also suppressed ANG II-induced myocardial molecular/cellular responses. These observations suggest that ALDO contributes, in part, to ANG II-induced myocardial repair/remodeling. The protective role of spironolactone on cardiac damage is hypertension independent, since spironolactone did not suppress blood pressure in ANG II-treated animals.

In summary, chronic ANG II treatment is accompanied by a proinflammatory/fibrogenic phenotype involving the intramural coronary circulation and myocardium of both right and left ventricles and is based on induction of oxidative stress at these vascular and nonvascular sites of injury. The proinflammatory cardiac phenotype is requisite to the appearance of fibrosis at these sites. Losartan blocked, whereas spironolactone suppressed, ANG II-induced cardiac molecular and cellular responses, indicating the involvement of ALDO in ANG II-induced cardiac injury.

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GRANTS

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