Chronic angiotensin-(1–7) prevents cardiac fibrosis in DOCA-salt model of hypertension

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Grobe, Justin L., Adam P. Mecca, Haoyu Mao, and Michael J. Katovich. Chronic angiotensin-(1–7) prevents cardiac fibrosis in DOCA-salt model of hypertension. Am J Physiol Heart Circ Physiol 290: H2417–H2423, 2006. First published January 13, 2006; doi:10.1152/ajpheart.01170.2005.—Cardiac remodeling is a hallmark of hypertension-induced pathophysiology. In the current study, the role of angiotensin-(1–7) fragment in modulating cardiac remodeling was examined. Sprague-Dawley rats underwent uninephrectomy surgery and were implanted with a deoxycorticosterone acetate (DOCA) pellet. DOCA animals had their drinking water replaced with 0.9% saline solution. A subgroup of DOCA-salt animals was implanted with osmotic minipumps, which delivered angiotensin-(1–7) chronically (100 ng·kg \(^{-1}\)·min \(^{-1}\)). Control animals underwent sham surgery and were maintained on normal drinking water. Blood pressure was measured weekly with the use of the tail-cuff method, and after 4 wk of treatment, blood pressure responses to graded doses of angiotensin II were determined by direct carotid artery cannulation. Ventricle size was measured, and cross sections of the heart ventricles were paraffin embedded and stained using Masson’s Trichrome to measure interstitial and perivascular collagen deposition and myocyte diameter. DOCA-salt treatment caused significant increases in blood pressure, cardiac hypertrophy, and myocardial and perivascular fibrosis. Angiotensin-(1–7) infusion prevented the collagen deposition effects without any effect on blood pressure or cardiac hypertrophy. These results indicate that angiotensin-(1–7) selectively prevents cardiac fibrosis independent of blood pressure or cardiac hypertrophy in the DOCA-salt model of hypertension.

deoxycorticosterone acetate; blood pressure; cardiac remodeling

Cardiac fibrosis is a major facet of hypertensive cardiac disease, and it interferes with the normal function and structure of the myocardium (8, 61, 62). Increased deposition of basement membrane collagen is a hallmark of the remodeling process, and it results in an increase in cardiac tissue stiffness. This remodeling predisposes the patient to an increased risk of adverse cardiac events, including myocardial ischemia, infarction, arrhythmias, and sudden cardiac death (61). Thus prevention and reversal of cardiac fibrosis are essential in the management of hypertensive heart disease.

The renin-angiotensin-aldosterone system (RAAS) has been suggested to participate in the development of end-organ damage in hypertensive patients (2, 11). Support for this concept comes from clinical trials demonstrating that treatment of hypertensive patients with either angiotensin-converting enzyme (ACE) inhibitors (38, 53) or ANG II type 1 (AT\(_1\)) receptor blockers (41, 60) provides significant protection from, and even reversal of, end-organ damage. Animal studies have also demonstrated that ACE inhibitors and AT\(_1\) receptor antagonists prevent cardiovascular injury (23, 55, 56) as well as protect against renal (3, 55, 57) and cerebral (55–57) injury. The use of these antagonists of the RAAS not only reduces the formation and actions of ANG II but also results in a significant elevation of another fragment of the RAAS, angiotensin-(1–7) [ANG-(1–7)] (17, 25). This peptide, which can be generated locally in the myocardium (47), has been reported to work antagonistically to ANG II and has been implicated in protecting against cardiac pathophysiology (49). Recently, Loot et al. (31) demonstrated that chronic infusion of ANG-(1–7) improved endothelial function and coronary perfusion and preserved cardiac function in an animal model of heart failure. ANG-(1–7) has also been shown to significantly increase cardiac output and stroke volume in rodents (18) and to improve contractile function in isolated perfused rat hearts (46). Using a fusion protein to generate a transgenic rat model that overproduces ANG-(1–7), Santos et al. (49) showed that ANG-(1–7) reduced the induction of cardiac hypertrophy and the duration of reperfusion arrhythmias and improved postischemic function in isolated perfused hearts. Thus ANG-(1–7) may be an important component of the RAAS that has opposing actions to ANG II, and it may provide protective action(s) in the heart and possibly other end organs. ANG-(1–7) also recently has been reported to inhibit the growth of cardiac myocytes in vitro (59), suggesting that elevated levels of ANG-(1–7) may also protect against cardiac hypertrophy in hypertension. Interestingly, blockade of ANG-(1–7) receptors has also been shown to reverse the antihypertensive effects of long-term administration of ACE inhibitors (22). Thus ANG-(1–7) may play a significant role in the beneficial effects on the cardiovascular system attributed to some antihypertensive agents.

Not only has ANG II been implicated in the development of cardiac fibrosis, but so has the aldosterone component of the RAAS (19). It has been demonstrated in experimental studies utilizing uninephrectomized rats that deoxycorticosterone acetate supplementation (DOCA-salt) treatment results in significant cardiac and renal remodeling, including interstitial fibrosis and hypertrophy (15, 69). The purpose of this study was to determine whether chronically elevated levels of ANG-(1–7) alter the development of hypertension, cardiac hypertrophy, and/or cardiac fibrosis in the DOCA-salt model of hypertension.

Materials and Methods

Animals. Male Sprague-Dawley rats weighing between 170 and 250 g were used for this study. Animals were housed in a temperature- and humidity-controlled room in standard shoebox cages and main-
DOCA-salt model. Animals were anesthetized with an intramuscular injection of a ketamine, xylazine, and acepromazine mixture (30, 6, and 1 mg/kg im, respectively) before uninephrectomy. During the surgery, 15 animals were implanted with a subcutaneous pellet of DOCA (40 mg). Seven of these animals were additionally implanted with a subcutaneous osmotic pump that delivered ANG-(1–7) (100 ng·kg⁻¹·min⁻¹) for the duration of the experiment. After surgery (and for the remainder of the experiment), drinking water for these animals was replaced with 0.9% saline solution. Ten animals underwent a sham surgery and were maintained on normal drinking water. Four additional sham animals were implanted with only an ANG-(1–7) pump.

Indirect blood pressure. Systolic blood pressures were determined weekly by an indirect method utilizing a tail-cuff and pneumatic pulse sensor as previously reported (24). Briefly, animals were heated with the use of a 200-W heat lamp for 3–5 min before being restrained in a heated Plexiglas restrainer to which the animals had been conditioned before the experiment. A pneumatic pulse sensor was then attached to the tail distal to a pressure cuff under the control of a Programmed Electro-Sphygmomanometer (Narco). Voltage outputs from the pressure sensor bulb and inflation cuff were recorded and analyzed electronically using a PowerLab signal transduction unit and associated Chart software (ADInstruments). At least three separate indirect pressures were averaged for each animal.

Direct blood pressure/acute ANG II responses. After 4 wk of chronic treatment, animals underwent carotid artery and jugular cannula implantation surgery as previously reported (32). Briefly, animals were anesthetized with a mixture of ketamine, xylazine, and acepromazine (30, 6, and 1 mg/kg im, respectively). A polyethylene cannula (PE-50, Clay Adams) was introduced into the carotid artery for direct blood pressure measurements, whereas a silicone elastomer cannula (Helix Medical) was introduced into the descending jugular vein for acute intravenous injections of drug. Both cannulas were filled with heparin saline (40 U/ml; Sigma) and sealed with stylets. Animals were allowed 24 h to recover before experiments were performed. Direct blood pressure responses to acute intravenous injections of ANG II (5–320 ng/kg) in awake, freely moving animals were recorded by connecting the carotid cannula to a liquid pressure transducer, which was interfaced to a PowerLab (ADInstruments) signal transduction unit. Data were analyzed by using the Chart program that was supplied with the PowerLab system.

Cardiac remodeling. Cardiac remodeling was determined at the end of the direct blood pressure experiment by ventricular hypertrophy and cardiac fibrosis after 4 wk of respective treatments. After the acute ANG II blood pressure experiment, animals were anesthetized by inhaled halothane and then euthanized by decapitation. Ventricular hypertrophy was determined by measuring ventricle mass normalized by body mass. Cross sections of the ventricles were then embedded in paraffin, sectioned at 4 μm, and stained for collagen deposition using Masson’s Trichrome. Single sections from each animal were then viewed and photographed with a Moticam 1000 digital camera (Motic; Richmond, BC, Canada) under ×10 (whole heart) and ×250 (perivascular collagen and myocyte diameter) magnifications. The collagen content of the left ventricle wall was determined by measuring the blue stain density of the left wall (from the septum in the posterior to the septum in the anterior) and was normalized to the red stain density of the same portion of the image. The area of blue-stained collagen surrounding the left anterior descending coronary artery was measured and normalized to the area of the lumen of the vessel. Myocyte cross-sectional diameter was also measured to confirm ventricular hypertrophy results. Color density, perivascular collagen and lumen areas, and myocyte diameter were determined using the ImageJ program from National Institutes of Health (42).

Statistics. Basal direct blood pressure and ventricular hypertrophy (by mass and by myocyte diameter) were analyzed by two-way ANOVA, whereas indirect blood pressures and direct blood pressure responses to graded, acute doses of ANG II were analyzed by two-way ANOVA with repeated measures. Cardiac fibrosis measurements were compared by using the more stringent nonparametric Kruskal-Wallis ANOVA, followed by post hoc Mann-Whitney U tests. Data were considered significantly different with $P < 0.05$.

RESULTS

Indirect blood pressure. DOCA-salt treatment caused a significant increase ($P = 0.006$) in systolic blood pressure over the course of the experiment. ANG-(1–7), when infused simultaneously, failed to prevent these increases ($P = 0.22$) (Fig. 1). ANG-(1–7), when infused alone, did not significantly alter blood pressure when compared with sham-implanted animals ($P = 0.69$; data not shown).

Direct blood pressure/acute ANG II responses. Direct pressure measurements confirmed that whereas DOCA-salt treatment significantly increased pressure ($P = 0.007$), ANG-(1–7) had no effect ($P = 0.20$) on basal systolic blood pressure [sham, 129.2 ± 3.7 (mean ± SE); DOCA, 160.0 ± 5.2; DOCA + ANG-(1–7), 171.6 ± 10.8; ANG-(1–7) alone, 143.8 ± 21.0]. In addition, DOCA-salt treatment significantly potentiated ($P = 0.02$), and ANG-(1–7) infusion had no effect ($P = 0.41$) on, the dose-response curves to acute, graded injections of ANG II (Fig. 2).

Cardiac remodeling. DOCA-salt treatment significantly increased ventricle mass ($P = 0.02$), and ANG-(1–7) failed to prevent this effect ($P = 0.35$) (Fig. 3A). These effects were confirmed by measurement of myocyte diameter, which uncovered a significant increase in size with DOCA-salt treatment ($P < 0.0001$) but no effect of ANG-(1–7) ($P = 0.71$) (Fig. 3B).

Fig. 1. Indirect blood pressure (BP). DOCA-salt treatment led to a significant increase in systolic blood pressure as measured by indirect tail-cuff method ($P = 0.006$). Chronic infusion of ANG-(1–7) had no effect on systolic blood pressure increases due to DOCA-salt treatment ($P = 0.22$). Data are presented as means ± SE.
A significant increase in left ventricular wall fibrosis was observed with DOCA-salt treatment (P = 0.04 vs. sham), and chronic ANG-(1–7) treatment significantly prevented this trend (P = 0.04 vs. DOCA) (Fig. 4). Whereas the increase in ventricular wall fibrosis is only ~4%, this value is most likely diluted by our measurement of collagen in the entire left ventricular wall at ×10 magnification (as opposed to the common method of measuring selected “representative samples” at high magnification). Clearly, from Fig. 4B, analysis of “representative areas” within the left wall would lead to much larger relative changes.

DOCA-salt treatment further resulted in a significant (P = 0.0002 vs. sham) increase in perivascular collagen deposition around the left anterior descending coronary artery, and ANG-(1–7) completely prevented this effect (P = 0.0007 vs. DOCA) (Fig. 5).

**DISCUSSION**

In this study, DOCA-salt treatment resulted in increases in blood pressure, acute pressor responses to ANG II, cardiac hypertrophy, and mid-myocardial and perivascular fibrosis. The ANG-(1–7) fragment had no effect on the elevated blood pressure, acute pressor responses, or cardiac hypertrophy but significantly prevented the cardiac mid-myocardial and perivascular fibrosis in the DOCA-salt hypertensive rat model.

DOCA treatment in uninephrectomized rats drinking 1.0% NaCl has previously been shown to cause hypertension, cardiac hypertrophy, and interstitial and perivascular fibrosis (15, 69). These cardiac effects are mediated via mineralocorticoid receptors (44, 64, 66) and are not observed in animals on a low-salt diet (9). Others (26, 37) have suggested the involvement of AT1 receptors in the fibrosis associated with DOCA-salt treatment. Elevation of cardiac AT1 receptors associated with DOCA-salt treatment may potentiate the well-described fibrotic effect of ANG II. There is evidence that the trigger for cardiac fibrosis in this animal model is coronary vasculitis, because the inflammatory cell infiltration occurs within a few days of DOCA treatment (67).

In a study by Young et al. (69), the cardiac hypertrophy response to DOCA treatment was restricted to the left ventricle, whereas the collagen deposition was indistinguishable between left and right ventricle, thus supporting a humoral, rather than a hemodynamic, etiology. Further support for a humoral etiology for collagen deposition comes from Weber’s (63) laboratory, where spironolactone prevented aldosterone-induced cardiac fibrosis without lowering blood pressure. Additionally, interstitial and perivascular fibrosis can be produced by mineralocorticoid and salt without substantial hypertension or cardiac hypertrophy (69). Young et al. (69) also demonstrated that central infusion of a mineralocorticoid receptor antagonist lowered blood pressure but did not alter the pattern of cardiac fibrosis, further suggesting an independence of fibrosis and blood pressure. Our results also demonstrate that the fibrosis observed in this model was found in both the right and left ventricle walls (right ventricle data not shown), and such fibrosis appears to be independent of both blood pressure and hypertrophy.

The effects of DOCA on cardiac hypertrophy have been postulated to involve ANG II or transforming growth factor-β (TGF-β) (58, 68). Whether these same mechanisms are responsible for the cardiac fibrosis are not clear. Young and Funder (67) demonstrated that animals treated for as few as 8 days with DOCA-salt treatment demonstrated a significant increase in cardiac perivascular collagen deposition, with no changes in heart weight, and that the collagen formation was inhibited by a mineralocorticoid receptor antagonist (67). Further evidence that the cardiac hypertrophy and fibrosis may be independent includes results from Lekgabe et al. (27), who demonstrated that cardiac hypertrophy and the related markers of hypertrophy that were elevated in spontaneously hypertensive rats (SHR) compared with Wistar-Kyoto (WKY) rats were not reversed with chronic relaxin treatment, whereas the fibrosis...
and collagen content in the hearts of the SHR were normalized by relaxin treatment to levels observed in the WKY. In the current study, ANG-(1–7) also showed differential effects on hypertrophy and fibrosis in the DOCA-salt model of hypertension, suggesting different mechanisms may exist for these two end points of cardiac remodeling.

Nehme et al. (35) reported that both aldosterone and ANG II receptor antagonists significantly reduced the cardiac fibrosis induced by coronary artery ligation in male Wistar rats. The effects of the aldosterone receptor antagonist (spironolactone) were independent of changes in mean blood pressure. This form of experimental myocardial infarction in rats has been shown to activate the RAAS (39, 51). Spironolactone also has been shown to reduce arterial collagen production, independent of changes in systemic blood pressure in the SHR (6) and in an N\textsuperscript{ω}-nitro-L-arginine methyl ester plus ANG II with salt model of hypertension (45). As in the current study, Nehme et al. (35) also reported that these treatments did not prevent the cardiac hypertrophy induced in this myocardial infarction model. This is further evidence that the mechanisms for cardiac hypertrophy and fibrosis may be independent.

Generally, the actions of ANG-(1–7) are opposite of those attributed to ANG II (48). ANG-(1–7) has been reported to have intrinsic vasoactive effects in some studies (10, 22, 43, 50), whereas these effects are absent in other studies (14, 54, 65, 70). Bayorh et al. (5) demonstrated that the hypotensive effects of ANG-(1–7) were not observed in Dahl animals on a low-salt diet, and Eatman et al. (16) noted a gender difference in the vasodilatory action of ANG-(1–7) in Dahl rats. Neves et al. (36) recently reported that the vasodilatory effects of ANG-(1–7) are modulated during the estrous cycle. Thus the contrasting findings of ANG-(1–7) on the vasculature may be related to the differences in animal models studied, dose of ANG-(1–7), vascular bed studied, or the experimental condition (in vitro vs. in vivo) in which the peptide is evaluated. In the current study, at the dose of ANG-(1–7) infused, there was no effect on the basal blood pressure of normotensive Sprague-Dawley animals or those animals made hypertensive with

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**Fig. 4.** Left ventricle wall fibrosis. A: DOCA-salt treatment caused a significant increase in collagen deposition in left ventricle wall. Chronic ANG-(1–7) significantly prevented this increase. Data are presented as means ± SE. *P < 0.05 vs. sham; †P < 0.05 vs. DOCA. B: representative images of mid-myocardium at ×250 magnification, stained with Masson’s Trichrome. Blue color indicates collagen fibers.

**Fig. 5.** Perivascular fibrosis. A: DOCA-salt significantly increased perivascular collagen around left anterior descending coronary artery. Chronic ANG-(1–7) significantly prevented this effect. Data are presented as means ± SE. *P < 0.05 vs. sham; †P < 0.05 vs. DOCA. B: representative images of perivascular collagen surrounding left anterior descending coronary artery stained with Masson’s Trichrome. Blue color indicates collagen fibers.
DOCA-salt treatment. There are situations, such as in models of endothelial dysfunction (28), where the vasodilatory effects of ANG-(1–7) have been shown to be blunted. This may be the reason for a lack of effect in our hypertensive model. In the current study, no reduction in the pressor response to acute administration of ANG II was observed. Benter et al. (7) reported that infusion of ANG-(1–7) did not alter the pressor responses to ANG II or phenylephrine in WKY animals; however, it did reduce the response to these agents in the SHR. A higher dose of ANG-(1–7) was utilized in the Benter study compared with ours. Although there was no observable effect on blood pressure at the dose of ANG-(1–7) used in the current study, there was a significant attenuation of cardiac fibrosis.

The mechanism of action of ANG-(1–7), an active peptide fragment of the RAAS, is currently controversial. It has been reported to act as an angiotensin receptor antagonist and an ACE inhibitor, as well as altering prostaglandins and the bradykinin-nitric oxide system. These mechanisms have been exhaustively reviewed by Santos et al. (48) and others. The mechanisms may involve the reported mas receptor (59) or direct interaction with other angiotensin receptors (48).

There are several mechanisms by which ANG-(1–7) may decrease collagen deposition in models of hypertension. First, ANG-(1–7) could reduce blood pressure, which would decrease hemodynamic-dependent profibrotic signaling. Our results would not support this mechanism, because changes in blood pressures and pressor responses to acute ANG II were not observed by indirect or direct methods during chronic ANG-(1–7) treatment (Figs. 1 and 2). Second, ANG-(1–7) could directly inhibit the secretion of collagen or the activation of the fibroblasts or their differentiation into myofibroblasts. Third, ANG-(1–7) may increase collagen degradation by activating matrix metalloproteinases or by inhibiting tissue inhibitors of matrix metalloproteinases. Finally, ANG-(1–7) may inhibit the actions of various profibrotic factors such as nor epinephrine, angiotensin II, TGF-β, and/or endothelin-1, as ANG-(1–7) infusion has been reported to result in a reduction of cardiac ANG II levels (34). Future studies will be aimed at dissecting the possible mechanisms by which ANG-(1–7) reduces cardiac fibrosis in hypertension and to determine if this anti-fibrotic action also occurs in non-hypertensive fibrotic conditions.

This study also supports the idea that ANG-(1–7) has direct physiological actions, rather than simply being the result of metabolized ANG II. Our in vivo results are supported by a recent in vitro study (21). In this study, the authors examined angiotensin fragment binding in cultured adult rat cardiac fibroblasts, and showed that ANG-(1–7) binds to two separate sites that are unaffected by the AT1 receptor antagonist valsartan and the AT2 receptor antagonist PD-123, 319. Importantly, these authors showed that within the concentration ranges studied for binding, ANG-(1–7) inhibited collagen formation as measured by [3H]proline incorporation, and also decreased mRNA expression of various growth factors including TGF-β1, endothelin-1, and leukemia inhibitory factor. Pretreatment of the cardiac fibroblasts with ANG-(1–7) inhibited ANG II-induced collagen protein. Together, the current in vivo study and the in vitro study by Iwata et al. (21) strongly support the hypothesis that ANG-(1–7) actively regulates cardiac fibrosis.

These findings further indicate the significant role that ANG-(1–7) plays in the heart.

Our results parallel those of Loot et al. (31), who demonstrated cardioprotective effects of ANG-(1–7). In the current study, we utilized one-fourth of the dose of ANG-(1–7) that these authors employed. We did not determine the plasma or cardiac levels of ANG-(1–7) in the current study. It is conceivable that utilization of higher concentrations of ANG-(1–7) may result in greater cardioprotection, and it may also uncover effects on hypertrophy.

The results of the current study fit well with the findings of our recent study (20), in which systemic overexpression of ACE2 [the most important enzyme involved in ANG-(1–7) production] in angiotensin-infused hypertensive rats resulted in blood pressure-independent decreases in cardiac fibrosis. This study supports the concept that ACE2 mediates many (or most) of its protective effects through the production of ANG-(1–7). Myocardial infarction increases ACE2 expression in rats and humans (12). Averill et al. (4) also reported that ANG-(1–7) immunoreactivity was significantly increased in the ventricular tissue surrounding an area of myocardial infarction and absent in the remodeled area of infarction. Thus there is evidence suggesting that the ACE2 enzyme and the formation of ANG-(1–7) play significant roles in modifying cardiac remodeling.

Recently, Keidar et al. (25) demonstrated that the mineralocorticoid aldosterone significantly increased cardiac ACE and decreased ACE2 mRNA and activity levels in both humans and mice, whereas aldosterone antagonists produced the opposite responses. These data would suggest that ACE2 levels and the subsequent generation of ANG-(1–7) may be the mechanism by which mineralocorticoid receptor blockers improve cardiac function in myocardial infarct patients (40).

Several investigators (1, 33, 52) have demonstrated that chronic infusion of ANG II results in the development of hypertension, cardiac hypertrophy, and fibrosis. Functional ANG II receptors have been documented in cardiac fibroblasts (13), and ANG II-stimulated expression and secretion of collagen is completely abolished by AT1 and not AT2 receptor antagonism in vivo and in vitro (29, 30). Because ANG-(1–7) is significantly elevated with the use of AT1 antagonists (17), it is possible that some of the anti-fibrotic effects attributed to AT1 antagonism are mediated via actions of ANG-(1–7). In a preliminary study using a chronic ANG II-infusion model of hypertension, we observed a similar prevention of fibrosis (unpublished data) as was seen in the DOCA-salt model in the current paper, which further supports an anti-fibrotic effect of ANG-(1–7).

The development of cardiac fibrosis is a major complication of hypertensive cardiac disease. The increased deposition of basement membrane collagen is a hallmark of the remodeling process. This remodeling can predispose a patient to increased risk of adverse cardiac events, and therefore any reduction or reversal of cardiac fibrosis would facilitate the management of hypertensive heart disease. Our results suggest that ANG-(1–7) may be a beneficial component of the RAAS that provides this cardioprotection during hypertension, and any maneuver that would increase cardiac levels of ACE2 and subsequently ANG-(1–7) may be used as an effective therapeutic intervention in hypertensive heart disease patients.
ANGIOTENSIN-(1–7) PREVENTS CARDIAC FIBROSIS

GRANTS

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