Myocardial Fibrosis in DOCA-Salt Hypertensive Rats
Effect of Endothelin ET<sub>A</sub> Receptor Antagonism

Fatima Ammarguellat, PhD; Isabelle Larouche, BSc; Ernesto L. Schiffrin, MD, PhD

**Background**—To test the hypothesis that endothelin-1 contributes to cardiac fibrosis, cardiac collagen deposition was studied in deoxycorticosterone acetate–salt (DOCA-salt) hypertensive rats, in which the endothelin system is activated. The effects of the ET<sub>A</sub>-selective endothelin receptor antagonist A-127722 were evaluated.

**Methods and Results**—A-127722 (30 mg/kg per day) was administered for 4 weeks. Myocardial fibrosis was evaluated after Sirius red F3BA staining. Systolic blood pressure was 103±1.6 mm Hg in unilaterally nephrectomized rats (Uni-Nx), 202±3.2 mm Hg in DOCA-salt rats (P<0.01 versus Uni-Nx), and 182±3.1 mm Hg in ET<sub>A</sub> antagonist–treated DOCA-salt rats (P<0.01 versus DOCA-salt or Uni-Nx). In DOCA-salt rats, interstitial and perivascular collagen density was increased in the subendocardial and midmyocardial regions of the left ventricle (3- to 4-fold, P<0.05), whereas in subepicardial myocardium, the increase was predominantly perivascular. The ET<sub>A</sub> antagonist prevented cardiac fibrosis in DOCA-salt rats. Procollagen I and III mRNA, which were increased in hearts of DOCA-salt rats, were normalized by ET<sub>A</sub> antagonist treatment. TGF-β<sub>1</sub> mRNA and TGF-β<sub>1</sub> protein increased at 1 week in DOCA-salt rats and were lowered in ET<sub>A</sub> antagonist–treated rats.

**Conclusions**—ET<sub>A</sub> receptor–mediated collagen deposition in hearts of DOCA-salt rats results from increased procollagen synthesis associated with an initial increment in expression of TGF-β<sub>1</sub>. These results support the hypothesis of a role for endothelin-1 in cardiac collagen deposition in mineralocorticoid hypertension, which may have pathophysiological and pharmacological implications in hypertensive heart disease. ([*Circulation*. 2001;103:319-324.])

**Key Words:** collagen • myocardium • growth substances

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Cardiac remodeling in hypertension is characterized by increased cardiomyocyte volume and collagen deposition.1,2 Deposition of collagen (specifically types I and III) in the myocardial interstitium and around coronary arteries has been demonstrated in renovascular hypertension, after angiotensin II or aldosterone infusion, and in aging spontaneously hypertensive rats.3-6 Collagens I and III are synthesized by interstitial and pericoronary fibroblasts.7 Cardiac fibroblast activity is regulated by various factors, including hemodynamic (coronary perfusion, ventricular overload) and humoral (aldosterone and angiotensin II) factors.4,8,9

Involvement of endothelin-1 (ET-1) in hypertensive models such as deoxycorticosterone acetate (DOCA)–salt hypertension has been suggested by vascular overexpression of ET-110,11 and blood pressure reduction in response to endothelin antagonists.12 ET-1 promotes growth of cardiomyocytes in vitro,13-16 induces collagen synthesis by cardiac fibroblasts,16,17 and has been shown to influence development of left ventricular hypertrophy.18,19 In DOCA-salt hypertension, ET-1 is overexpressed in the heart, although overexpression appears to be limited to endothelial cells and the endocardium.20

The aim of this study was to analyze (1) the effects of DOCA-salt hypertension on cardiac collagen deposition and (2) the effects of the ET<sub>A</sub>-selective endothelin antagonist A-127722 on collagen density in this model, in which ET-1 plays a role.21 We tested the hypothesis that ET-1 stimulates myocardial collagen deposition in DOCA-salt hypertensive rats via the activation of ET<sub>A</sub>-receptors. We also evaluated whether changes in collagen deposition reflect changes in collagen synthesis, and the potential role of TGF-β<sub>1</sub> in our findings. The blood pressure and vascular effects of A-127722 in these DOCA-salt rats were previously published by us22 and will be mentioned only briefly.

**Methods**

**Animal Experiments**

The study was approved by the Animal Care Committee of the Clinical Research Institute of Montreal and conducted in accordance with recommendations of the Canadian Council of Animal Care. DOCA-salt hypertension was induced by the method of Ormsbee and Ryan,23 as already described.21 Male Sprague Dawley rats (Charles River, St Constant, Quebec, Canada) weighing 200 g were unilaterally nephrectomized (Uni-Nx) under sodium pentobarbital anesthesia (40 mg/kg). Silicone rubber impregnated with DOCA (Sigma...
Chemical Co) (200 mg per rat) was implanted subcutaneously, and rats were offered 1% saline to drink. Control rats also underwent Uni-Nx but received a silicone rubber implant without DOCA and tap water to drink. The ETA endothelin receptor antagonist A-127722 was administered in the drinking water (30 mg/kg per day) for 4 weeks. Systolic blood pressure was measured weekly by the tail-cuff method with the rats under slight restraint after they had been warmed. Readings were recorded on a model 7 polygraph fitted with a 7-P8 preamplifier and PCPB photoelectric pulse sensor (all from Grass Instruments Co). The average of 3 pressure readings was obtained. Rats were killed by decapitation at the end of the experiment, and the heart was fixed in Bouin’s solution. To study the time course of some of the changes, additional groups of DOCA-salt rats and ET₄ antagonist–treated DOCA-salt rats were investigated at 1 and 2 weeks of DOCA and salt administration.

Collagen Quantification
The hearts fixed in Bouin’s solution were processed for paraffin embedding in an automated system (Shandon Citadel tissue processor). Serial sections (5 μm) of the median part of the left ventricle were obtained. Tissue sections were dewaxed with ethanol and stained with Sirius red F3BA (0.5% in saturated aqueous picric acid) (Aldrich Chemical Co). Collagen density was evaluated throughout the inner third (subendocardial myocardium), the middle third (midmyocardium), and the outer third (subepicardial myocardium) of the circumference of the left ventricle. From each of 3 nonconsecutive serial sections (which allowed convergence of results), 10 fields in each region of the heart (magnification ×20) were recorded. The severity of cardiac fibrosis was evaluated after Sirius red staining with the use of an image analysis system (Northern Eclipse 5.0, EMPIX Imaging Inc). A single investigator blinded to the experimental groups performed the analysis.

Reverse Transcription–Polymerase Chain Reaction Analysis of Procollagen I and III mRNA
Total RNA was extracted from the left ventricle with Trizol reagent (Gibco-BRL). mRNA (0.5 μg) was reverse transcribed in a final volume of 20 μL with MMLV reverse transcriptase (Gibco-BRL) and 1 μg oligo(dT) primer. Single-strand cDNA (4 μL) was used for polymerase chain reaction (PCR) to amplify a 405-bp fragment of procollagen (procollagen I) cDNA with the complementary antisense primer GTTTACAGGAAACGAGCGG and the sense primer CGATGGATCCAGTTCGAGTA at an annealing temperature of 56°C. For a 447-bp fragment of procollagen (procollagen III) cDNA, the antisense primer was CCACTCTCTGAACTCTGTTAAGTG, the sense primer CACCCCTGAACTCAAGAGTGG, and the annealing temperature 58°C. For a 290-bp fragment of TGF-β₁ cDNA, the antisense primer was CAACGCCATCTATGAGAAAACC, the sense primer GGCTCAAGACCTACAGAGGTG, and the annealing temperature 52°C. For a 192-bp fragment of TGF-β₂ cDNA, the antisense primer was CAAGCTCATCTAAGAAACC, the sense primer AAGCTCTGATTCGCTTCAT, and the annealing temperature 55°C. For a 172-bp fragment of TGF-β₃ cDNA, the antisense primer was ATGTGTTGTCCAC-CAC, the sense primer TATGATGACATCAAGAAAGTGG, and the annealing temperature 56°C. PCR was performed with Taq polymerase (Gibco-BRL) for 22 cycles for procollagens and 30 cycles for TGF-β₁ and GAPDH. Reverse transcription (RT)-PCR products were subjected to electrophoresis on a 1.5% agarose gel, and ethidium bromide–stained bands were analyzed densitometrically.

Western Blot Analysis of TGF-β₁ Protein
Protein was extracted from frozen tissue in lysis buffer containing PBS, sodium deoxycholate 0.5%, SDS 0.1%, sodium orthovanadate 1 mmol/L, PMSF 1.0 mmol/L, Nonidet-P40 1%, and aprotinin 1 μg/mL. Protein concentration was determined with the BioRad protein assay (Bio-Rad Laboratories Inc). TGF-β₁ was immunoprecipitated from 2 mg protein with a specific antibody (Santa Cruz Biotechnology Inc) at a dilution of 1:200. Samples were electrophoresed in a 10% SDS-polyacrylamide gel at 60 V for 2 hours and transferred onto a PVDF membrane at 100 V for 1 hour. Membranes were incubated with the specific antibodies to TGF-β₁ at a dilution of 1:1000 overnight at 4°C. Horseradish peroxidase–conjugated rabbit IgG (1/5000, from Santa Cruz Biotechnology) was used as second antibody. Bands were visualized by chemiluminescence (kit from Boehringer Mannheim) and quantified by densitometry.

Statistical Analysis
The means of collagen density from 3 sections of each area of each heart were evaluated. Results are expressed as mean±SEM. Statistical significance was assessed by 1-way ANOVA followed by a Student-Newman-Keuls test. Differences were considered significant at a value of P<0.05.

Results
Blood Pressure and Body and Heart Weights of Rats
As already reported,22 the systolic blood pressure of DOCA-salt hypertensive rats was significantly elevated after 4 weeks (Table 1). Treatment with A-127722 resulted in slightly but significantly lower blood pressure in DOCA-salt hypertensive rats than in untreated DOCA-salt rats. Body weight of DOCA-salt hypertensive rats was significantly lower than that of normotensive control rats, as we have found in previous studies.10,12 Relative heart weights were similar in treated and untreated DOCA-salt hypertensive rats and higher than in normotensive rats.

Effect of Endothelin Antagonist on Collagen Deposition
There was a dramatic increase in interstitial and perivascular myocardial collagen in the subendocardial and midmyocardial regions of the left ventricle of DOCA-salt rats compared with Uni-Nx controls (Figures 1 and 2). In the subepicardial myocardium, in contrast, collagen density was increased predominantly around blood vessels. Treatment with A-127722 prevented collagen deposition in the left ventricle of DOCA-salt rats.

Expression of Procollagen Type I and III mRNA and TGF-β₁ mRNA and Protein
Figure 3 shows that in DOCA-salt rats, levels of procollagen type I and III mRNA in the left ventricle were 1.6-fold higher than in Uni-Nx rats (P<0.01). In DOCA-salt rats treated with the ET₄ receptor antagonist, levels of procollagen I and III mRNA were similar to basal levels.

In DOCA-salt rats, there was a trend toward an increase in TGF-β₁ mRNA at 1 week that did not achieve significance,
whereas in ET\textsubscript{A} antagonist–treated rats, there was a significant decrease compared with DOCA-salt rats \( (P < 0.05, \text{Figure 4}) \). No change in TGF-\( \beta \)\textsubscript{1} mRNA levels could be demonstrated in the heart of any of the groups investigated at 2 and 4 weeks (Table 2). TGF-\( \beta \)\textsubscript{1} protein was expressed in the left ventricle as the 55-kDa full-length precursor, as previously shown by others.\textsuperscript{24} In hearts of DOCA-salt rats after 1 week of hypertension, TGF-\( \beta \)\textsubscript{1} protein levels were 2.5-fold higher than in hearts of control rats (Figure 5). In ET\textsubscript{A} antagonist–treated rats, cardiac TGF-\( \beta \)\textsubscript{1} protein was significantly lower than in DOCA-salt rats. In the second and fourth weeks of hypertension, no statistically significant elevation was detectable in DOCA-salt hypertensive rats (Table 2).

**Discussion**

The present study investigated collagen deposition and its mechanism in the heart of DOCA-salt hypertensive rats and the role that ET-1 may play in cardiac fibrosis in this model of hypertension. In this model, which has an endothelin-dependent component,\textsuperscript{10–12,21,22} collagen deposition was dramatically increased interstitially and perivascularly in subendocardial and midmyocardial areas of the left ventricle, but predominantly around blood vessels in the subepicardium. Although development of left ventricular hypertrophy was unaffected by the ET\textsubscript{A}-selective antagonist A-1227722, cardiac collagen deposition was dramatically reduced. In association with this, procollagen I and III mRNAs were increased in DOCA-salt rat hearts, a rise that was abrogated by the ET\textsubscript{A} antagonist. In the first week, cardiac TGF-\( \beta \)\textsubscript{1} expression was increased in DOCA-salt rats and was significantly decreased by the ET\textsubscript{A} antagonist. These data suggest a critical role of the activated endothelin system in cardiac fibrosis in mineralocorticoid hypertension, resulting from increased collagen synthesis via an ET-1–induced TGF-\( \beta \)\textsubscript{1}–dependent component.

**Effect of ET-1 on Cardiac Collagen Versus Left Ventricular Hypertrophy**

The potential involvement of ET-1 in some models of experimental hypertension, such as DOCA-salt hypertension,
has been well demonstrated.\textsuperscript{10–12,21,22} In the heart, ET-1 overexpression occurred mainly in the endothelium of blood vessels in DOCA-salt hypertensive rats. ET-1 produced in blood vessels of the heart may stimulate interstitial fibroblasts to produce collagen\textsuperscript{16,17} but could conceivably have only minor effects on cardiomyocytes. Blood pressure was lowered only moderately in endothelin antagonist–treated rats. More effective blood pressure lowering may be necessary to have a significant effect on cardiac hypertrophy, as discussed below. Alternatively, development of left ventricular hypertrophy and cardiac fibrosis may obey different determinants. Whereas blood pressure elevation may be critical for development of cardiac hypertrophy, it may have less influence on collagen deposition. Cardiac fibrosis may result in this experimental paradigm, mainly from stimulation of cardiac interstitial fibroblasts by ET-1, whereas left ventricular hypertrophy may develop in the absence of ET-1 stimulation if blood pressure remains elevated, as it did despite ETA antagonism in the present study. Similar findings have been reported recently in this same model when the rats were treated with inhibitors of the renin-angiotensin system: reversal of cardiac fibrosis without change in cardiac hypertrophy.\textsuperscript{25}

**Relationship of Cardiac Fibrosis and Left Ventricular Hypertrophy**

Cardiac hypertrophy does not necessarily translate into increased collagen deposition. Collagen content is increased in pressure-induced hypertrophy in animal models\textsuperscript{26,27} and in humans.\textsuperscript{28} However, volume overload in rats\textsuperscript{29} or humans\textsuperscript{30} induces hypertrophy in the absence of changes in collagen deposition.

**TABLE 2. Time Course of TGF-β\textsubscript{1} mRNA and Protein Expression in the Left Ventricle of DOCA-Salt Hypertensive Rats Treated With ETA-Selective Endothelin Receptor Antagonist**

<table>
<thead>
<tr>
<th>Time</th>
<th>Uni-Nx</th>
<th>DOCA-Salt</th>
<th>DOCA-Salt A-127722</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGF-β\textsubscript{1} mRNA/GAPDH</td>
<td>0.57±0.06</td>
<td>0.64±0.08</td>
<td>0.47±0.08*</td>
</tr>
<tr>
<td>Week 2</td>
<td>1.20±0.12</td>
<td>1.14±0.13</td>
<td>1.14±0.15</td>
</tr>
<tr>
<td>Week 4</td>
<td>0.87±0.10</td>
<td>0.77±0.11</td>
<td>1.14±0.08*</td>
</tr>
<tr>
<td>TGF-β\textsubscript{1} protein (arbitrary units)</td>
<td>2.5±1.0</td>
<td>6.6±2.1†</td>
<td>3.2±1.0</td>
</tr>
<tr>
<td>Week 2</td>
<td>1.1±0.2</td>
<td>1.1±0.1</td>
<td>1.3±0.3</td>
</tr>
<tr>
<td>Week 4</td>
<td>6.0±1.7</td>
<td>8.1±0.6</td>
<td>7.0±0.6</td>
</tr>
</tbody>
</table>

*P < 0.05 vs DOCA-salt hypertensive rats; †P < 0.01 vs other groups.

**Figure 3.** Expression of procollagen type I and III mRNA in left ventricle of DOCA-salt hypertensive rats and effect of ET\textsubscript{A} antagonist A-127722, both expressed as percentage of Uni-Nx controls. RT-PCR was performed, and mRNA levels were normalized to GAPDH internal control. Left, Representative blots. Right, Mean±SEM (n=6). *P<0.01 vs other groups.

**Figure 4.** Expression of TGF-β\textsubscript{1} mRNA in left ventricle of DOCA-salt hypertensive rats and effect of ET\textsubscript{A} antagonist. RT-PCR was performed, and mRNA levels were normalized to GAPDH internal control. Top, Representative blots. Bottom, Mean±SEM (n=6). *P<0.05 vs DOCA-salt+ET\textsubscript{A} antagonist.

**Figure 5.** Expression of TGF-β\textsubscript{1} protein in left ventricle of DOCA-salt hypertensive rats and effect of ET\textsubscript{A} antagonist. Top, Representative Western blots. Bottom, Mean±SEM (n=3). *P<0.05 vs DOCA-salt+ET\textsubscript{A} antagonist; **P<0.01 vs Uni-Nx.
deposition. Pathophysiological conditions that cause synthesis and accumulation of collagen can thus be dissociated from conditions that activate cardiomyocyte hypertrophy. Indeed, in the present study, we found that endothelin antagonism completely abrogated the increase in collagen content in the hearts of DOCA-salt rats but did not significantly affect cardiac hypertrophy. Cardiac hypertrophy in DOCA-salt rats may have a component associated with collagen deposition via endothelin-mediated mechanisms and one independent of both collagen deposition and ET-1 stimulation.

Localization of Collagen Deposition in the Heart
ET-1–dependent fibrosis occurred in DOCA-salt hypertensive rats predominantly interstitially and perivascularly in the midmyocardial and subendocardial regions of the left ventricle and predominantly perivascularly in the subepicardium. The distribution of increased interstitial collagen is strikingly similar to the localization of changes in arterioles and capillaries in the DOCA-salt hypertensive rat as an expression of ET-1–mediated effects. Increased density of small arterioles 20 μm in lumen diameter and capillary rarefaction were found mainly in the subendocardial myocardium of DOCA-salt hypertensive rats, which was corrected by treatment with an ET<sub>A</sub> antagonist. There may be a common mechanism for cardiac fibrosis and vascular abnormalities in DOCA-salt rats: They both appear to be a consequence of vascular overproduction of ET-1. Increased arteriolar density could lead to increased coronary resistance through lengthening of arteriolar segments. Decreased capillary density together with fibrosis may compromise oxygen and nutrient supply to cardiac myocytes, contributing to hypoxia in this more vulnerable area of the myocardium. Hemodynamic (including pressure) and metabolic variables probably participate, together with ET-1 and presumably other hormonal stimuli, to induce vascular growth and rarefaction in this region of the heart of DOCA-salt hypertensive rats, whereas ET-1 appears to play a fundamental role in fibrosis, as shown by its prevention under ET<sub>A</sub> receptor blockade.

Synthesis of Collagen Explains Increased Collagen Deposition in DOCA-Salt Rats
Expression of procollagen I and III mRNA in the heart of DOCA-salt rats was increased compared with control rats, indicating enhanced collagen synthesis. ET<sub>A</sub>-receptor antagonist treatment blunted collagen overexpression, suggesting that increased collagen synthesis was mediated via activation of ET<sub>A</sub> receptors on cardiac fibroblasts. ET-1 activates the procollagen I promoter, reflected in this study by the increased collagen deposition. TGF-β<sub>1</sub> protein levels were increased in the heart of DOCA-salt rats in the first week, returning to normal thereafter. ET<sub>A</sub> antagonism prevented the elevation of TGF-β<sub>1</sub>, implicating TGF-β<sub>1</sub> in the fibrotic response. Not surprisingly, no increase of TGF-β<sub>1</sub> was found in the second and fourth weeks of hypertension. Indeed, a significant rise of the TGF-β<sub>1</sub> 55-kDa precursor has been documented only in the initial week of pressure overload. After cardiac irradiation, TGF-β<sub>1</sub> mRNA rose only initially in the first week and returned to normal later. Interestingly, there was no correlation between TGF-β<sub>1</sub> protein and mRNA. Together, these data demonstrate an ET-1–induced TGF-β<sub>1</sub>–dependent increase in cardiac collagen deposition in the heart of DOCA-salt rats. Preliminary data (not shown) demonstrate the absence of significant change in collagen-degrading activity (matrix metalloproteinase-2) in DOCA-salt rats or under ET<sub>A</sub> antagonism, in agreement with the previously demonstrated absence of significant changes of cardiac matrix metalloproteinase expression in this hypertensive model.

Cardiac Fibrosis in DOCA-Salt Hypertensive Rats: Relationship With Other Endocrine Systems
The mechanism for increased ET-1 production in DOCA-salt hypertension remains unclear. Recent studies have suggested that vasopressin, the levels and effects of which are enhanced in DOCA-salt hypertension, may play a role in stimulation of ET-1 expression. A V<sub>1</sub> vasopressin antagonist reduced blood pressure and vascular remodeling of DOCA-salt hypertensive rats similarly to the effects of ET<sub>A</sub> antagonists. This was associated with abrogation of vascular overexpression of ET-1 mRNA. Genetically vasopressin-deficient Brattleboro rats do not develop DOCA-salt hypertension and are unable to upregulate ET-1 expression. The mechanism for vasopressin increase in DOCA-salt rats may be related to changes in serum osmolality and action on the hypothalamus. This may require the effect or presence of high salt and mineralocorticoids. Alternatively, mineralocorticoids may potentiate the effects of vasopressin on ET-1 expression in blood vessels and heart. Mineralocorticoids, particularly aldosterone, induce cardiac hypertrophy and fibrosis in rats. Whether aldosterone exerts its effects via ET-1, as DOCA does in the DOCA-salt rat, remains to be determined. It is of interest to note the recently concluded Randomized Aldactone Evaluation Study (RALES), in which blockade of aldosterone by spironolactone improved cardiac mortality in heart failure patients, which could be mediated in part via blunting of ET-1 effects on the heart.

In conclusion, in DOCA-salt hypertensive rats, significantly increased interstitial and perivascular collagen deposition was observed in the left ventricular myocardium, which was prevented if rats were treated with an ET<sub>A</sub> receptor antagonist. These results suggest a role for ET-1 in increased procollagen I and III synthesis, leading to cardiac fibrosis in DOCA-salt hypertensive rats, which may have pathophysiological implications in hypertension by contributing to myocardial stiffness and contractile dysfunction. In association with this response, there is an initial increase in TGF-β<sub>1</sub> that may play an adjuvant role in the ET<sub>A</sub> receptor–induced cardiac fibrosis. Blockade of ET<sub>A</sub> receptors may exert beneficial cardiac effects in forms of hypertension, such as the DOCA-salt rat, in which the endothelin system is activated, and perhaps in other forms of cardiac disease, by blunting cardiac fibrosis and remodeling.

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