Cardiac Microvasculature in DOCA-Salt Hypertensive Rats
Effect of Endothelin ET_A Receptor Antagonism

Isabelle Larouche, Ernesto L. Schiffrin

Abstract—The cardiac abnormalities associated with hypertension include left ventricular hypertrophy and vascular changes. The latter may affect the cardiac microvasculature and predispose to myocardial ischemia. To test the hypothesis that endothelin-1 contributes to changes in the microcirculation of the heart, we studied cardiac microvessels of the deoxycorticosterone acetate–salt (DOCA-salt) model of hypertension in the rat, in which the endothelin system is activated, and the effect of the endothelin-A (ET_A) subtype–selective endothelin receptor antagonist A-127722. A-127722 (30 mg \( \cdot \) kg\(^{-1} \cdot \) d\(^{-1} \)) was administered for 4 weeks. Arterioles (\( \approx 20 \mu m \) in luminal diameter) were identified in the myocardium by use of immunolabeling with an anti–smooth muscle \( \alpha \)-actin antibody, and capillaries with an anti-laminin antibody with nuclear counterstaining by nuclear fast red. Systolic blood pressure was 103±1.6 mm Hg in unilaterally nephrectomized rats (UniNx), 202±3.2 mm Hg in DOCA-salt (\( P < 0.01 \) versus UniNx), and 182±3.1 mm Hg in ET_A antagonist–treated DOCA-salt (\( P < 0.01 \) versus DOCA-salt or UniNx). Arteriolar and capillary densities were altered significantly in the subendocardial myocardium but not in the subepicardial myocardium of the left ventricle. Arteriolar density per square millimeter was 18.1±1.48 in UniNx, 31.9±3.26 in DOCA-salt (\( P < 0.01 \) versus UniNx), and 24.2±1.36 in ET_A antagonist–treated DOCA-salt (\( P < 0.05 \) versus DOCA-salt or UniNx). Capillary density per square millimeter was 2395±148 in UniNx, 1576±107 in DOCA-salt (\( P < 0.01 \) versus UniNx), and 1982±31 in ET_A antagonist–treated DOCA-salt (\( P < 0.01 \) versus DOCA-salt or UniNx). In conclusion, in DOCA-salt hypertensive rats, subendocardial arteriolar growth and capillary rarefaction were observed in the left ventricular myocardium, and both were partially corrected by ET_A receptor antagonism. This suggests a role for endothelin-1 in cardiac arteriolar growth and capillary rarefaction, which may have pathophysiological implications by contributing to myocardial ischemia in hypertension. (Hypertension. 1999;34[part 2]:795-801.)

Key Words: microcirculation ■ arterioles ■ growth ■ capillaries ■ hypertension, experimental ■ myocardium ■ ischemia

Endothelin-1 (ET-1), a potent 21-amino-acid vasoconstrictor peptide produced by the endothelium of blood vessels, has been implicated as a mediator of blood pressure elevation\(^1\) and in the induction of vascular smooth muscle growth.\(^2,3\) These effects of ET-1 are mediated by the endothelin-A (ET_A) subtype of endothelin receptor on vascular smooth muscle cells. The potential involvement of ET-1 in some models of experimental hypertension, such as deoxycorticosterone acetate (DOCA)–salt hypertension, has been suggested by the vascular overexpression of ET-1.\(^4,5\) Overexpression of ET-1 occurs in DOCA-salt hypertensive rats in blood vessels of different organs, including the heart, in which enhanced expression was found in the endothelium of epicardial and intramyocardial blood vessels and in the endocardium.\(^6\)

Hypertension is often associated with cardiac complications. The major cardiac alteration is left ventricular hypertrophy, which may be implicated in the development of coronary insufficiency and arrhythmia and may lead to congestive heart failure.\(^7\) In addition, abnormalities that occur in coronary vessels are aggravated by hypertension. In humans, atherosclerosis at the level of epicardial coronary arteries is accelerated by hypertension and is often complicated by partial or complete occlusion of the vessel lumen and/or plaque rupture with or without thrombosis, leading to myocardial infarction. In addition, microvascular disease typically occurs in the heart in hypertension and may cause angina even in the presence of normal epicardial coronary arteries.\(^8\)

The intramyocardial precapillary coronary vascular tree comprises small coronary arteries and arterioles. Small arteries present significant alterations in hypertension, as demonstrated in different hypertensive models: decreased lumen, increased media-to-lumen ratio, and sometimes increased media cross section.\(^9,10\) We previously showed that small intramyocardial arteries of DOCA-salt hypertensive rats present significant growth, that is, hypertrophic remodeling, with decreased lumen, increased media-to-lumen ratio, and in-
creased media cross section. ET-1 is involved pathogenically in these changes, as shown by their reversal by treatment with endothelin antagonists.\textsuperscript{3,10} Coronary arterioles (lumen diameter \(< 20 \mu m\)) play an important role in resistance to blood flow, and capillaries (lumen diameter \(< 8 \mu m\)) are involved in oxygen/nutrient delivery to the myocardium.\textsuperscript{11–15} Abnormalities of the distribution, density, length, and tortuosity of these small vessels in hypertension may affect coronary vascular resistance and contribute to myocardial ischemia.\textsuperscript{16–19}

The major aim of this study was to investigate (1) the effects of DOCA-salt hypertension on the density of coronary arterioles and capillaries in rat and (2) the effect of the ET\textsubscript{A}-selective endothelin receptor antagonist A-127722 on coronary arterioles and capillaries in this model, in which ET-1 has been shown to be involved.\textsuperscript{1} Specifically, we tested the hypothesis that ET-1 plays a role in myocardial microvascular abnormalities in DOCA-salt hypertensive rats via activation of ET\textsubscript{A} receptors.

\section*{Methods}

\subsection*{Animal Experiments}

The study was approved by the Animal Care Committee of the Clinical Research Institute of Montreal and conducted in accordance with the recommendations of the Canadian Council of Animal Care. DOCA-salt hypertension was induced by the method of Ormsbee and Ryan.\textsuperscript{20} Briefly, male Sprague-Dawley rats (Charles River, St Constant, Québec, Canada) weighing 200 g were unilaterally nephrectomized under sodium pentobarbital anesthesia (40 mg/kg). Silicone rubber impregnated with DOCA (Sigma Chemical Co) (200 mg/rat) was implanted subcutaneously, and rats were offered 1% saline to drink. Control rats (UniNx) were unilaterally ne-

\subsection*{Immunohistochemistry}

To study arterioles and capillaries in the myocardium, the histochemical approach to identify these, described by Sabri et al,\textsuperscript{21} was modified as follows. The antibodies used were a mouse monoclonal antibody against smooth muscle \(\alpha\)-actin (Sigma) and rabbit polyclonal antibody directed against laminin (Sigma). The hearts, fixed in Bouin’s fixative, were processed for paraffin embedding in an automated system (Shandon Citadel tissue processor). Serial sections (5 \(\mu m\)) of the median part of the left ventricle were obtained. The tissue sections were dewaxed with ethanol and then blocked with 10\% normal goat serum or 10\% normal sheep serum for anti-laminin and anti-\(\alpha\)-smooth muscle \(\alpha\)-actin antibodies, respectively, for 1 hour at room temperature to reduce nonspecific binding. Three sections of the left ventricle from each rat were incubated with anti-laminin IgG (1:500) or anti-\(\alpha\)-smooth muscle \(\alpha\)-actin IgG (1:300) for 16 hours at 4\(^\circ\)C. The sections were washed thoroughly 3 times with PBS and incubated with secondary biotinylated antibodies (1:100) for 1 hour at room temperature. After 3 washes with PBS, the sections were developed with 3,3′-diaminobenzidine in Tris-HCl-

\subsection*{H\textsubscript{2}O\textsubscript{2}}

Sections labeled with anti-laminin IgG were also counterstained with nuclear fast red (ICN) for 5 minutes.

\section*{Determination of Arteriolar and Capillary Density}

In every heart, microscopic analysis of arteriolar and capillary density with a video imaging program (Northern Eclipse 5.0, EMPIX Imaging Inc) was performed on 3 nonconsecutive serial sections (which allowed convergence of results) of the median part of the ventricle. Vessel density was evaluated throughout the inner third (subendocardial myocardium) and the outer third (subepicardial myocardium) of the circumference of the left ventricle. From each section, 15 to 35 fields (magnification \times 100 and \times 20 for capillaries and arterioles, respectively) were recorded. Quantitative analyses were performed by a blinded observer. Capillary density was determined in sections labeled with laminin antibody counterstained with nuclear fast red and was based on the quantification of positively labeled structures with \(< 8 \mu m\) lumen size and with 1 nucleus (endothelial cell). Arterioles were quantified in sections that were immunolabeled with smooth muscle \(\alpha\)-actin antibody. Only small blood vessels with a lumen diameter of \(< 20 \mu m\) surrounded by 1 smooth muscle layer that stained positively were counted.

\section*{Data Analysis}

Data per heart were the means of the density of vessels from 3 sections. Data are expressed as mean\(\pm \)SEM. Statistical significance was assessed by 1-way ANOVA followed by a Student-Newman-Keuls test or in the case of proportions by \(\chi^2\) test. A value of \(P<0.05\) was considered significant.

To evaluate the tortuosity of arterioles, the mathematical model proposed by Adair et al\textsuperscript{22} was used. The relationship between sampling probability and sine of the orientation angle is the basis of the method: \(I/sin\) is used as a weighting factor (W) to correct for the decreased sampling incidence caused by orientation of vessels. Vessels are modeled as cylindrical tubes, and their profile is treated as the elliptical intersection of the vessel with the section plane. As a result, \(W = a/b\), where \(a\) is the major axis of the ellipse and \(b\) is the minor axis of the ellipse. Only vessels with \(a/b<2.5\) were used for the calculation, to eliminate the arterioles sectioned along their longitudinal axis.\textsuperscript{21,22} Therefore, \(L_v\) (length density) can be calculated as \(L_v = (S/a/b)/2\pi A_t\), where \(A_t\) is the test area. A series of individual sections are treated as 1 large representative section, and test areas and individual \(W\) values are summed. Tortuosity was assessed quantitatively by use of an anisotropy coefficient calculated as \(L_v/N_a\), where \(N_a\) is numerical density (number of profiles per cross-sectional area). An anisotropy coefficient of 1.0 indicates a total absence of tortuosity, ie, all vessels are parallel to each other, whereas an anisotropy coefficient of 2.0 indicates maximum tortuosity, ie, the orientation of the vessels is entirely random.

\section*{Results}

\subsection*{Blood Pressure and Body and Heart Weights of Rats}

The systolic blood pressure of DOCA-salt hypertensive rats was significantly elevated relative to that of UniNx control rats after 4 weeks (Table 1). Treatment with the endothelin antagonist A-127722 resulted in slightly but significantly lower blood pressure in DOCA-salt hypertensive rats compared with untreated DOCA-salt rats. The body weight of DOCA-salt hypertensive rats was significantly lower than that of normotensive control rats. Relative heart weights were similar in treated and untreated DOCA-salt hypertensive rats and higher than those of normotensive rats, indicating that the endothelin antagonist had no effect on cardiac hypertrophy.

\subsection*{Characterization of the Microvasculature in Subendocardial and Subepicardial Myocardium of the Rat Left Ventricle}

Figure 1 presents the 2 types of small vessels that were characterized by immunohistochemical techniques. Laminin

\section*{Subendocardial and Subepicardial Myocardium of the Rat Left Ventricle}

Figure 1 presents the 2 types of small vessels that were characterized by immunohistochemical techniques. Laminin
TABLE 1. Blood Pressure and Body and Heart Weight of DOCA-Salt Rats Treated With the ET<sub>A</sub>-Selective Endothelin Receptor Antagonist A-127722

<table>
<thead>
<tr>
<th>Parameter</th>
<th>UniNx</th>
<th>DOCA-Salt Hypertensive</th>
<th>DOCA-Salt + A-127722</th>
</tr>
</thead>
<tbody>
<tr>
<td>No.</td>
<td>6</td>
<td>6</td>
<td>8</td>
</tr>
<tr>
<td>Systolic BP, mm Hg</td>
<td>103±1.6</td>
<td>202±3.2*</td>
<td>182±3.1†</td>
</tr>
<tr>
<td>Body weight, g</td>
<td>348.5±3.6</td>
<td>250.8±10.9*</td>
<td>271.8±9.2*</td>
</tr>
<tr>
<td>Heart weight, g</td>
<td>1.16±0.04</td>
<td>1.13±0.04</td>
<td>1.25±0.05</td>
</tr>
<tr>
<td>HW (g)/100 g BW</td>
<td>0.333±0.01</td>
<td>0.451±0.01*</td>
<td>0.460±0.01*</td>
</tr>
</tbody>
</table>

BP indicates blood pressure; HW, heart weight; and BW, body weight. *P<0.01 vs UniNx rats; †P<0.01 vs DOCA-salt hypertensive rats.

Arteriolar and Capillary Density in DOCA-Salt Subendocardial and Subepicardial Myocardium: Effect of A-127722

Arteriolar and capillary densities were not significantly different in the subendocardial myocardium of the left ventricle of DOCA-salt hypertensive rats compared with hearts from Uni-Nx controls (Figure 2, top). In contrast, in the subendocardial myocardium, arteriolar density was higher and capillary density was lower in DOCA-salt compared with that in hearts of Uni-Nx control rats (Figure 2, bottom). A-127722 partially attenuated the changes in subendocardial density of capillaries and arterioles in the DOCA-salt rat. To determine whether changes in arteriolar density could be attributable to a shift in the diameter of arterioles to a greater number of vessels <20 μm in DOCA-salt hearts, which would have artifactually increased the density of vessels counted, the frequency distribution of diameters of arterioles <30 μm was evaluated (Figure 3). There were some significant minor differences in distribution (more arterioles of 10 μm in DOCA-salt rat hearts, fewer of 15 μm), but a χ<sup>2</sup> test showed that proportions of vessels <20 or >20 μm were similar in the 3 groups for lumen diameters of either 15 to 20 μm versus 20 to 25 μm or 10 to 20 μm versus 20 to 30 μm. The increase in arteriolar density in DOCA-salt rat hearts could therefore not be attributed to increased numbers of arterioles <20 μm. Thus, we indeed detected arteriolar growth and capillary rarefaction in the left ventricular subendocardial myocardium of the DOCA-salt hypertensive rat, and both were partially corrected by administration of the ET<sub>A</sub> receptor antagonist A-127722.

Analysis of the tortuosity of arterioles in the subendocardial region of the myocardium, where the density of arterioles was significantly increased, did not show any significant differences in the anisotropy coefficient between groups (Table 2).

Discussion

The present study investigated the microvasculature of the subendocardial and subepicardial myocardium of DOCA-salt hypertensive rats and the potential role that endothelin may play in the changes found in relation to normotensive Uni-Nx control rats. DOCA-salt hypertensive rats exhibited increased arteriolar density and decreased capillary density in the subendocardial layer of the myocardium but not in the subepicardial myocardium, and ET<sub>A</sub>-receptor antagonism partially corrected these microvascular abnormalities. As demonstrated by data in Figure 3, arteriolar density changes could not be attributed to a shift in diameter of arterioles to a greater number of vessels <20 μm in DOCA-salt hearts, which would have resulted in a greater number of arterioles counted, because distribution of arteriolar sizes <20 or >20 μm was similar in the 3 groups for lumen diameters of 15 to 20 versus 20 to 25 μm or 10 to 20 versus 20 to 30 μm.

The site of origin of new arterioles within the microvascular network remains unknown. Although it is widely accepted that capillaries grow by sprouting into the interstitium, an angiogenic sprouting process has not been observed in arterioles. The current hypothesis for arteriolar development is that certain capillaries, selected by presently unknown mechanisms, become invaded with vascular smooth muscle cells and are therefore transformed into arterioles, a process called arteriialization. Arteriialization may occur by several possible mechanisms. Nelhs et al have suggested that pericytes surrounding capillaries may be dormant smooth muscle precursors. These cells may differentiate to smooth muscle cells, resulting in the formation of “meta-arterioles.” Alternatively, vascular smooth muscle cells in preexisting arterioles may migrate downstream to arterialis capillaries. The precise stimuli that initiate the process of arteriialization are as yet unclear. Hypoxia, hemodynamic stresses, and hormonal stimuli may participate to different degrees in the stimulation of arteriolar development. Analysis of the tortuosity of arterioles in the subendocardial region of the myocardium, where the density of arterioles was significantly increased, using the mathematical approach proposed by Adair et al did not show any significant differences in the anisotropy coefficient between groups. Thus, increases in subendocardial arteriolar density, although probably associated with lengthening of arterioles as a result of arteriialization of capillaries, did not result in more tortuous vessels, probably because of the cardiac hypertrophy occurring in DOCA-salt hypertensive rats.

ET-1 elicits a variety of biological effects that include vascular smooth muscle cell contraction and growth. ET-1 has also been shown to induce smooth muscle cell migration in the presence of low concentrations of platelet-derived growth factor or angiotensin II. The potentiation of vascular smooth muscle cell migration by ET peptides was concentration-dependent manner. These results suggest that the endothelin family of peptides, especially ET-1, can induce human coronary artery smooth muscle cell migration in combination with platelet-derived growth factor or angiotensin II, probably via the ET<sub>A</sub> receptor. The partial correction of abnormal arteriolar density by ET<sub>A</sub> receptor antagonism in the present study implicates ET<sub>A</sub> receptors as mediators of effects of ET-1 in the arteriialization process that occurs in the hearts of DOCA-salt hypertensive rats.
Decreased capillary density, or rarefaction, has been described in many vascular beds and different models of hypertension\textsuperscript{16,17} and under the effects of in vivo angiotensin II infusion.\textsuperscript{21} Rarefaction is considered to proceed through a stage of functional rarefaction (reversible) caused by arteriolar spasm and diversion of blood flow away from some capillaries. This may be followed by anatomic rarefaction, with definitive closure of capillaries. One can envisage that the potent vasoconstrictor effect of ET-1 may result in constriction and closure of meta-arterioles, resulting in diversion of blood flow and capillary collapse. This phenomenon may be compounded by the increased resistance to flow resulting from the lengthening of the high-resistance arteriolar tree as a result of the arterialization of capillaries. The end

Figure 1. Arterioles and capillaries in subendocardial myocardium demonstrated by immunolabeling of smooth muscle cells and laminin. Left ventricular sections were immunolabeled with anti-smooth muscle \( \alpha \)-actin and with anti-laminin and counterstained with nuclear fast red for detection of arterioles and capillaries, respectively.
result will be anatomic rarefaction. Blockade of ETA receptors appears to successfully reverse this process, at least in part, suggesting that to some degree, the rarefaction may be functional rather than anatomic at the stage at which intervention with the ETA antagonist was initiated. A potential model for the increased density of arterioles and decreased density of capillaries in the subendocardial myocardium is depicted in Figure 4.

A decrease in capillary density may compromise oxygen and nutrient supply to cardiac myocytes by shortening and reduction of the numbers of capillaries and therefore time capacity of diffusion. Vulnerability to hypoxia is greater in the subendocardial myocardium of the left ventricle than in the subepicardial myocardium.28,29 Interestingly, microvascular abnormalities were localized specifically to the subendocardial region, the more vulnerable area of the myocardium. Moreover, the increase in arteriolar density could lead to an increase in coronary vascular resistance through an increase in the length of the arteriolar segment of the vascular tree (see model in Figure 4), thus contributing to hypoxic events. The localization of the growth (arterioles) and rarefaction process (capillaries) to the subendocardial, more vulnerable, region may suggest that the process is multifactorial. Hemodynamic (including pressure) and metabolic variables probably con-

<table>
<thead>
<tr>
<th>Parameter</th>
<th>UniNx</th>
<th>DOCA-Salt Hypertensive</th>
<th>DOCA-Salt + A-127722</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anisotropy coefficient</td>
<td>1.80±0.04</td>
<td>1.76±0.06</td>
<td>1.71±0.08</td>
</tr>
</tbody>
</table>

TABLE 2. Tortuosity of Subendocardial Arterioles of DOCA-Salt Rats Treated With the ETA-Selective Endothelin Receptor Antagonist A-127722
There is some evidence that ET-1 may induce growth of the heart and contribute to left ventricular hypertrophy\(^1\) and collagen deposition.\(^3\) Cardiac hypertrophy could therefore have a confounding effect on the arteriolar and capillary changes found after endothelin antagonist treatment in DOCA-salt hypertensive rats, because this hypertensive changes found after endothelin antagonist treatment in DOCA-salt hypertensive rats, because this hypertensive model presents rather severe left ventricular hypertrophy. In this and previous studies, we found that although ET-1 is overexpressed in the heart of DOCA-salt hypertensive rats,\(^4\) treatment with either balanced ET\(_A\)/ET\(_B\)\(^3\) or selective ET\(_A\) endothelin receptor antagonists\(^10\) was not associated with improvement of cardiac hypertrophy. This may be explained in part by the fact that in the DOCA-salt hypertensive rat, the increase in ET-1 gene expression occurs in the endothelium of coronary arteries and endocardium rather than the myo-cardium or interstitium.\(^6\) Moreover, blood pressure is lowered only moderately in the endothelin antagonist–treated rats, and more effective blood pressure lowering may be necessary for endothelin antagonism to have a clinically evident effect on cardiac hypertrophy. Consequently, in the absence of differences in cardiac hypertrophy between treated and untreated DOCA-salt hypertensive rats, the microvascular changes found, characterized by increased density of arterioles and decreased density of capillaries in the subendocardial region of the left ventricle, cannot be attributed to cardiac hypertrophy. A limitation of the study is that differences in cellular composition and collagen deposition were not examined and could potentially contribute to the changes found. Remodeling of the heart; apoptosis, which is enhanced in this model, particularly after treatment with endothelin antagonists\(^3;\) and fibrosis could result in alterations in vascular density. Although this cannot be excluded, it would be unlikely that increased arteriolar density and decreased capillary density limited to the subendocardium in the untreated DOCA-salt hypertensive rats would be corrected, in part as a result of a combination of these phenomena in the endothelin antagonist–treated rats. Systolic blood pressure rise was moderately reduced by 20 mm Hg in DOCA-salt hypertensive rats by treatment with the ET\(_A\) antagonist. Although the possibility of a role of lower blood pressure affecting arteriolar growth and capillary rarefaction in the heart cannot be eliminat-


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