Angiotensin-Converting Enzyme Inhibition and Angiogenesis in Myocardium of Obese Zucker Rats

Jorge E. Toblli, Gabriel Cao, Graciela DeRosa, Federico Di Gennaro, and Pedro Forcada

Background: Obesity, hypertension, and non-insulin-dependent diabetes mellitus (NIDDM) are associated with microvascular rarefaction in the myocardium and this contributes to increase cardiovascular morbidity and mortality. At present, controversial data exist in medical literature regarding the specific role of angiotensin-converting enzyme (ACE) inhibitors concerning angiogenesis in different tissues. The present study was designed to determine the possible beneficial effects of an ACE inhibitor perindopril on myocardial angiogenesis in an animal model of obesity, hypertension, and NIDDM, such as the obese Zucker rat (OZR) and control lean Zucker rats (LZR).

Methods and Results: Ten-week-old male OZR (fa/fa) and LZR (Fa/fa) were used in this study: OZR group (G1, n = 10), OZR with perindopril group (G2, n = 10); LZR group (G3, n = 10). For 6 months, G2 received a daily dose of 3 mg/kg of perindopril, by gavage, and G1 and G3 received an equal volume of vehicle throughout the experiment. After 6 months of treatment, all rats were killed, hearts were harvested for pathology studies, including immunohistochemistry, using monoclonal antibodies against rat endothelial cell (RECA-1) and endothelial nitric oxide synthase. At the end of the study, OZR treated with perindopril presented: 1) lower blood pressure (BP) (127 ± 3.2 v 152.4 ± 3 mm Hg, P < .01) and 2) lower heart weight/100 g body weight (0.22 ± 0.02 v 0.36 ± 0.04 g, P < .01) than OZR untreated. Moreover, OZR that received perindopril showed higher: 1) myocyte density (2044 ± 67 v 847 ± 91 myocytes/mm², P < .01) and 2) capillary density (1348 ± 118 v 436 ± 78 capillaries/mm², P < .01); higher amount of: 1) vascular endothelial growth factor (VEGF) in the myocardium (P < .01) and higher percentage of capillaries with positive immunostaining for eNOS (P < .01), compared with untreated OZR. There was a correlation between both the amount of VEGF in myocardium and the number of capillaries (r = 0.88; P < .01) and VEGF and eNOS expression in myocardial capillaries (r = 0.93; P < .01) in OZR treated with perindopril. Finally, OZR that received P showed an improvement in insulin/glucose ratio (P < .01) when compared with untreated OZR.

Conclusions: These results suggest that ACE inhibition by perindopril improves myocardial angiogenesis in this animal model of human metabolic syndrome. The pathway that involves bradykinin, eNOS, and VEGF could be involved in this effect; however, because no additional antihypertensive treatment group was included in our study, the BP-lowering effect cannot be excluded. Am J Hypertens 2004;17:172–180 © 2004 American Journal of Hypertension, Ltd.

Key Words: Angiogenesis, myocardium, obesity, diabetes, angiotensin-converting enzyme inhibitors.

It is well known that hypoxia is a potent stimulus to develop new vessels by increasing a number of angiogenic factors. Moreover, angiogenesis is recognized as an essential mechanism of tissue repair and has a major role in the physiologic adaptation to ischemia.

Nevertheless, under pathologic conditions such as hypertension, obesity, and diabetes mellitus, the angiogenic response could be insufficient or blunted. Linked to this, a variable degree of microvascular rarefaction has been reported in all aforementioned diseases.2–5

The renin angiotensin system (RAS) plays a role in the angiogenic process and therefore, any potential therapeutic intervention on this regard is important. However, the effect of angiotensin-converting enzyme (ACE) inhibitors on microvascular rarefaction is controversial at present. Due to the well-known vasculogenic effect of angiotensin...
II, the blockade of its production or action might be expected to induce vascular rarefaction. Nevertheless, the administration of ACE inhibitors, especially those with high tissue affinity, have shown proangiogenic properties by increasing microvessel density independently of the lowering of arterial blood pressure (BP) in different tissues in experimental models.\textsuperscript{6–8} As a result, it has been suggested that the principal mechanism of action of ACE inhibitors on microvessel structures may be related to the stimulation of the bradykinin receptor.\textsuperscript{9}

The obese Zucker rat (OZR) is an accepted model of obesity, insulin resistance state, glucose intolerance or mild diabetes mellitus, and hyperlipidemia.\textsuperscript{10,11} Some reports suggest that OZR do not become hypertensive,\textsuperscript{12} yet most studies using this rodent strain, including a recent report from our group, state that they present at least mild-to-moderate arterial hypertension.\textsuperscript{13–16} These conflicting findings could result from different measurement techniques, from differences in the age or sex of the rats studied, or the level of sodium intake.\textsuperscript{17} In summary, OZR is an attractive experimental model to investigate a number of cardiovascular and metabolic abnormalities related to the human syndrome X. The aim of the present study was to determine the possible beneficial effects of an ACE inhibitor perindopril on myocardial angiogenesis in this representative animal model of the human syndrome X.

**Methods**

All the experiments were approved by the Hospital Aleman Ethic Committee and the Teaching and Research Committee and followed the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Ten-week-old male Zucker rats, obese (fa/fa) (OZR) and lean (Fa/fa) (LZR), (Charles River Laboratories, Wilmington, Massachusetts) were housed in individual cages at 21°C ± 2°C and a 12-h light/darkness cycle (7 AM to 7 PM). Rats were divided into three groups: OZR group (G1, n = 10), OZR with perindopril group (G2, n = 10), and LZR group (G3, n = 10). All the animals were allowed to drink tap water, and fed standard rat chow ad libitum. For 6 months, G2 received a daily dose of 3 mg/kg of perindopril, by gavage, whereas G1 and G3 received an equal volume of vehicle (normal saline solution) throughout the experiment. Dose treatment was adjusted each week based on calculation of the body weight for each animal. At baseline and throughout the experiment, systolic BP (SBP) was measured monthly, by tail-cuff plethysmography. After 6 months of treatment all rats were euthanized under anesthesia (sodium thiopental 40 mg/kg, intraperitoneally). The heart was rapidly excised, weighed, and portions of the left ventricle were harvested for light microscopy (LM), high-resolution light microscopy (HRLM), and immunohistochemistry studies.

**Biochemical Procedures**

After a 14-h fast, rat blood samples were collected at baseline, and at the end of the experiment. Plasma glucose, cholesterol, and triglycerides levels were measured by standard methods with an Automatic Analyzer (Hitachi 911, Tokyo, Japan). Serum insulin was determined by a solid phase two-site immunoassay with monoclonal antibodies (DRG Instruments GmbH, Marburg, Germany).

**Heart Processing and Examination**

Fragments from the left ventricle and interventricular septum were fixed in phosphate-buffered 10% formaldehyde (pH 7.2) and embedded in paraffin for the LM study. For HRLM, samples were fixed with 2% glutaraldehyde in 0.1 mol/L phosphate buffer (pH 7.4) and processed for epoxy embedding (Polybed R812; Fluka, Warrington, PA). Thin sections were stained with uranyl acetate and lead citrate. All observations in LM and HRLM were performed using a light microscope Nikon E400 (Nikon Instrument Group, Melville, NY).

**Immunohistochemical Staining**

Immunostaining was carried out as previously described.\textsuperscript{18} To evaluate the number of capillaries in myocardium, a mouse monoclonal antirat endothelial cell IgG antibody-1 (RECA-1) (Abcam Ltd, Cambridge, UK), which reacts with rat endothelial cell antigen, at the dilution 1:200, was used. Vascular endothelial growth factor (VEGF, C-1) was detected by a mouse monoclonal IgG2a anti-VEGF antibody (sc-7269, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) at a dilution 1:100. The endothelial nitric oxide synthase (eNOS) expression was studied using a rabbit polyclonal anti-eNOS IgG antibody (sc-653, NOS3; Santa Cruz Biotechnology) at a dilution 1:100.

**Morphologic Analysis**

In every heart, the analysis of capillary and cardiac myocyte densities was performed using the semithin sections (HRLM). The numerical density of myocytes and coronary capillaries was determined by counting the total number of myocytes and vessels ±8 μm with positive immunostaining for RECA-1, within the confines of each of 20 random 1.13 mm\textsuperscript{2} adjacent fields, viewed at ×400 magnification and both were expressed as mean per millimeter squared. All measurements were carried out using an image analyzer Image-Pro Plus ver. 4.5 for windows (Media Cybernetics, LP., Silver Spring, MD). Positive area for VEGF was assessed on 20 consecutive microscopic fields at ×400 magnification, where each field represents 1.13 mm\textsuperscript{2}, resulting in a total explored area of 22.6 mm\textsuperscript{2}. The eNOS was expressed as a percentage of positive immunostaining on a total of 150 vessels (range 7 to 9 μm) following the direction of the epicardium to the endocardium, at ×400 magnification and data were averaged.
when appropriated.

Table 2. Parameters at the end of the experiment

<table>
<thead>
<tr>
<th>Mean ± SD</th>
<th>G1 (n = 10) OZR</th>
<th>G2 (n = 10) OZR with Perindopril</th>
<th>G3 (n = 10) LZR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat body weight (g)</td>
<td>596.5 ± 47.4*</td>
<td>576.3 ± 39.0*</td>
<td>380.7 ± 33.4</td>
</tr>
<tr>
<td>Glycemia (mmol/L)</td>
<td>13.3 ± 0.9*</td>
<td>12.7 ± 0.7*</td>
<td>5.0 ± 0.6</td>
</tr>
<tr>
<td>Insulin (pmol/L)</td>
<td>755.6 ± 119.1*</td>
<td>509.0 ± 87.6*</td>
<td>46.3 ± 16.2</td>
</tr>
<tr>
<td>Insulin/glucose ratio</td>
<td>57.1 ± 8.4*</td>
<td>39.7 ± 6.6*</td>
<td>9.4 ± 3.9</td>
</tr>
<tr>
<td>Triglycerides (mmol/L)</td>
<td>10.6 ± 1.9*</td>
<td>7.8 ± 2.0*</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>Cholesterol (mmol/L)</td>
<td>5.1 ± 0.6*</td>
<td>3.9 ± 0.7*</td>
<td>1.6 ± 0.2</td>
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</table>

* v G3 P < .01.
† v G2 P < .01.

Because the OZR groups showed no statistical differences with respect to lipid levels (cholesterol and triglycerides) at the end of the experiment, those OZR, which received perindopril, presented lower values (Table 2).

BP Levels

At baseline, all groups of obese and lean rats presented similar SBP levels. However, although untreated OZR (G1) presented a significant (P < .01) increase in SBP throughout the study, OZR treated with perindopril showed significantly lower levels of SBP, not different from the ones of the LZR group (G3). Fig. 1 illustrates the monthly evolution of SBP in all groups throughout the study.

Morphologic and Immunohistochemical Findings

At the end of the experiment, untreated OZR (G1) showed a total heart weight significantly higher than OZR treated with perindopril (G2), as illustrated in Table 3. In addition, when heart weight was related to total body weight in all groups, once again untreated OZR (G1) presented a higher heart-to-body weight ratio than the other groups, which showed very similar data between them (Table 3). In concordance with cardiac hypertrophy, myocyte density per area (number of myocyte per millimeter squared) was decreased, whereas myocyte diameter was increased in untreated OZR (G1) when compared with the other groups (G2 and G3), which displayed similar myocyte densities...
and myocyte diameters (Table 3). Moreover, concerning capillary density, untreated OZR (G1) presented significantly lower values in comparison with the rest of the groups (G2 and G3), as shown in Table 3 and illustrated in Fig. 2A. The myocyte-to-capillary ratio was markedly increased in untreated OZR (G1), indicating substantial capillary rarefaction in this group. In opposition, OZR that received perindopril (G2) presented a greater number of capillaries per area (Fig. 2B), with a myocyte-to-capillary ratio not different from that of the LZR group (G3). The VEGF amount was reduced in myocardium from untreated OZR (G1), whereas the OZR + perindopril group (G2) presented a significant increase, even higher than that observed in LZR (G3), as shown in Figs. 3A and 4. Moreover, it is worth mentioning that there was a positive and significant ($P < .01$) correlation between the amount of VEGF in myocardium and the number of vessels in all groups (OZR $r = 0.84$; OZR + perindopril $r = 0.88$; LZR $r = 0.80$). This suggests a strong relationship between these two variables. The eNOS immunostaining in myocardial vessels was markedly lower in animals from the G1 group (OZR) compared with the other groups (G2 and G3). On the other hand, OZR with perindopril animals (G2) showed a significantly higher expression of eNOS, which was also higher than that found in LZR (G3), as represented in Fig. 3B and illustrated in Fig. 5.

Finally, despite a positive correlation between the amount of VEGF in myocardial tissue and the percentage of capillaries with positive immunostaining for eNOS in lean and in both obese groups, the untreated OZR group (G1) showed the lowest, although significant ($P < .01$) value of Spearman rho, whereas those that received perindopril (G2) presented a better correlation coefficient (OZR $r = 0.78$; OZR + perindopril $r = 0.93$ and LZR $r = 0.85$).

### Table 3. Morphologic and immunohistochemical parameters

<table>
<thead>
<tr>
<th></th>
<th><strong>G1 (n = 10)</strong></th>
<th><strong>G2 (n = 10)</strong></th>
<th><strong>G3 (n = 10)</strong></th>
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<tbody>
<tr>
<td>Heart weight (g)</td>
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<td>Heart weight (g)/100 g BW</td>
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<td>Myocyte density*</td>
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<td>Myocyte diameter (μ)</td>
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<tr>
<td>Capillary density†</td>
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<tr>
<td>Myocyte/capillary ratio</td>
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BW = body weight.

* Number of myocytes/mm².

† Number of capillaries/mm².

‡ v G2 and G3 $P < .01$.

§ v G3 $P < .01$. 

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**FIG. 1.** Monthly evolution of systolic blood pressure obtained by tail-cuff method. Values are expressed as mean ± SD. OZR = obese Zucker rat; LZR = lean Zucker rat; P = perindopril; SBP = systolic blood pressure.

**FIG. 2.** High-resolution light microscopy (1 μm sections). **(A)** Myocardial section from G1 (OZR) rat with small number of capillaries (arrows) per area (toluidine blue-fucsin; magnification, ×400). **(B)** Myocardial section from G2 (OZR with perindopril) rat, with a greater number of capillaries per area compared with animals from G1 (OZR) (toluidine blue-fucsin; magnification, ×400).
Discussion

In the present study, although OZR presented high BP and substantial reduction in myocardial capillary density along with a significant decrease in both VEGF in the myocardium and eNOS expression in myocardial capillaries, this was reversed in the myocardium in addition to BP control in those OZR that received treatment with perindopril. The beneficial effect of perindopril was characterized by a marked reduction in total heart weight, together with an improvement in the capillary density and in concordance with a more adequate myocyte-to-capillary ratio. We also observed an increase in VEGF and eNOS expression and a greater correlation between capillary density and the amount of VEGF in myocardium in this group of rats. In addition, OZR given perindopril presented some improvement in the insulin resistance state, as shown by the insulin-to-glucose ratio.

A possible limitation of this study could be that there was no additional antihypertensive treatment group with the same BP level as OZR treated with perindopril. Therefore, we cannot rule out the possibility that the BP lowering could be part of the beneficial effects. However, we can speculate that the effect on angiogenesis could not merely be due to a decrease in BP. This statement is supported by other studies using an ischemic leg model in hypertensive and nonhypertensive rats, where the researchers, using a low-dose ACE inhibitor (subantihypertensive dose) alone or in combination with the diuretic indapamide, induced an early and substantial increase in capillary density in comparison with the vasodilator hydralazine, without significant changes in BP. 19,20 Moreover, both VEGF and eNOS were upregulated in the ischemic hindlimb treated by the ACE inhibitor.

In the present study, as well as in our previous experiments, 16 we observed a significant cardiac enlargement in untreated OZR. Insulin resistance has been related to the development of arterial hypertension and heart failure. 21 Furthermore, selective resistance to insulin signaling in the vasculature, as well as abnormalities of glucose-transporter-4 (GLUT-4) protein, have been well documented, not only in skeletal muscle but also in the myocardium of OZR. 22-25 In addition, an increase in serum fatty acids and, consequently, their accumulation in cardiac myocytes resulting in cardiac enlargement and dysfunction, has been reported in obese hyperinsulinemic rats. 26 On the other hand, those therapeutic interventions that ameliorate the

FIG. 3. A) Bar graph expressing area, in square millimeters, with positive immunostaining for vascular endothelial growth factor (VEGF) in myocardium in all groups. Values are presented as mean ± SD. OZR = obese Zucker rat; LZR = lean Zucker rat; P = perindopril. *P < .01 versus other groups; **P < .01 versus G2. B) Bar graph illustrating more than 150 vessels (range, 7 to 9 μ) explored from epicardium to endocardium, the percentage of myocardial capillaries with positive immunostaining for eNOS in all groups. *P < .01 versus other groups; **P < .01 versus G2.
insulin resistance such as insulin-sensitizing agents, thiazolidinediones, and ACE inhibitors have been associated with an improvement in cardiac hypertrophy.\textsuperscript{27,28} The administration of ACE inhibitors is frequently associated with the enhancement of whole-body insulin sensitivity in a variety of insulin-resistant animal models or in insulin-resistant humans with essential hypertension.\textsuperscript{29–33} In the present study, OZR receiving perindopril showed a lower insulin-to-glucose ratio in comparison with untreated OZR, which suggests an improvement in the insulin resistant state. Therefore, it is not surprising that in the present study, OZR that received perindopril showed a remarkable reduction in cardiac dimensions relative to untreated OZR, suggesting that part of this beneficial effect was due to some improvement in the insulin-resistant state.

Although angiotensin II is involved in the mechanism of insulin resistance by inhibiting insulin receptor substrate-1/phosphatidylinositol 3-kinase activation,\textsuperscript{34} and consequently, the beneficial effect of ACE inhibitors on insulin resistance could be elicited by decreasing angiotensin II tissue concentration, in the past years evidence using various animal models, including diabetic rodents, has demonstrated that the most likely mechanism is due to stimulation of the bradykinin synthesis pathway.\textsuperscript{35,36}

Development of arterial hypertension has been strongly associated with the insulin-resistant state in OZR, probably throughout a defect in the dopamine receptor in renal proximal tubules, which is caused by increased levels of plasma insulin,\textsuperscript{14} and by the impairment in endothelium-dependent relaxation.\textsuperscript{37} Therefore, an improvement in insulin resistance by ACE inhibition could secondarily help to control high BP and, as a result, prevent cardiac hypertrophy in these animals, as was observed in our study.

Recently, Chou et al\textsuperscript{38} have shown that insulin-resistant and glucose-intolerant states may decrease the expression of VEGF, one of the most potent angiogenic factors in the myocardium in OZR. A potential mechanism for decreased VEGF expression in the myocardium in insulin-resistant states is the loss of insulin-induced VEGF expression. Studies have shown in different cell types that insulin can increase VEGF mRNA expression.\textsuperscript{39} Therefore, it is possible that part of the angiogenic effect observed in our experiment could be mediated by this mechanism.

A number of reports indicate that the proangiogenic action of angiotensin II takes place through the angiotensin type 1 (AT1) receptor, which involves activation of VEGF/eNOS-related pathway, and this effect is abolished or attenuated when either AT1 receptor antagonist or a VEGF neutralizing antibody is used.\textsuperscript{40–43} In contrast,
when the AT2 receptor is stimulated, a negative modulation of ischemic-induced angiogenesis through an activation of the apoptotic process has been reported. On the other hand, the angiogenic properties of ACE inhibitors have been well-demonstrated in animal models with microvascular rarefaction, but never in rodents with high BP in addition to obesity, hyperglycemia, hyperlipidemia, and insulin resistance, which constitute an interesting experimental model similar to the well-known human metabolic syndrome X.

Despite effective ACE inhibition, angiotensin II can still be generated in the heart. This occurs more often in humans than in rats, by a non-ACE pathway that is a chymase-dependent mechanism. A local subhypertensive angiotensin II concentration has been reported as an angiogenic stimulus; therefore, this effect could contribute to explain part of the beneficial effect of ACE inhibition in our study.

Previous reports have implicated a role for NOS in angiogenesis. At present, the mechanisms by which nitric oxide promotes angiogenesis is not totally understood. Nitric oxide is an endothelial survival factor, inhibiting apoptosis and enhancing endothelial cell proliferation by increasing the expression of VEGF. Nitric oxide synthase expressions were increased with perindopril treatment in patients with coronary heart disease. Data in the present study are partially in concordance with the report from Silvestre et al. where they found that the proangiogenic effect of ACE inhibition is mediated by a bradykinin B2 receptor pathway and associated with the upregulation of eNOS content.

Finally, we suggest that a mechanism of perindopril is responsible for reversing vascular rarefaction in the myocardium of OZR, which should be mediated by enhancing eNOS expression throughout the bradykinin pathway and, as a result, an increase in VEGF expression. This assumption is mainly supported by the high correlation observed between the percentage of vessels with positive immunostaining for eNOS and the amount of VEGF, along with a positive and also significant relationship between the amount of myocardial VEGF per area and capillary density, in OZR treated with perindopril. However, because no additional antihypertensive treatment group was included in our study, we cannot eliminate the possibility that the BP reduction could also contribute to this effect.

In conclusion, we believe that the present study provides information addressing the usefulness of some types of ACE inhibitors, such as perindopril, to achieve an improvement in myocardial angiogenesis, which might also be the scenario in humans with multiple cardiovascular risk factors.

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References


