Effects of Enalapril on the Vascular Wall in an Experimental Model of Syndrome X

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Evidence links the insulin resistance syndrome with endothelial dysfunction. Previously, we have described a decreased endothelial nitric oxide synthase (eNOS) activity in both aortic endothelium and cardiac tissue, and an increased proliferation of aortic primary cultured vascular smooth muscle cells (pC-VSMCs), obtained from fructose-fed rats (FFR), an experimental model of syndrome X. Because the participation of the renin-angiotensin system (RAS) in this model is still unclear, the present study examined the effect of chronic administration of an angiotensin converting enzyme (ACE) inhibitor enalapril (E) on pC-VSMCs proliferation and eNOS activity in a conduit artery (aorta) and in resistance vessels (mesenteric vascular bed) from fructose-fed rats. Male Wistar rats were used: Control, FFR, Control + E, and FFR + E (n = 8 in each group). After 8 weeks, tissue samples were obtained and 10% fetal calf serum (FCS) proliferative effect was examined in pC-SMCs of aortic and mesenteric arteries by [3 H]thymidine incorporation. The eNOS activity was estimated in endothelial lining from both origins by conversion of [3 H]arginine into [3 H]citrulline. The FFR aortic and mesenteric pC-VSMCs showed a significantly increased 10% FCS-induced [3 H]thymidine incorporation compared to controls. The FFR aortic and mesenteric endothelium eNOS activity was significantly decreased. Chronic treatment with E abolished the increased proliferation and restored eNOS activity. These data confirm that changes in VSMCs proliferation and endothelial dysfunction at different levels of the vascular system are involved in syndrome X, and that the inhibition of angiotensin II production can revert those changes, suggesting an important role for RAS and possibly kinins, in the physiopathologic mechanism of this model of syndrome X. Am J Hypertens 2002;15:872–878 © 2002 American Journal of Hypertension, Ltd.

Key Words: Vascular smooth muscle, nitric oxide, fructose-fed rats, insulin resistance, hypertension, enalapril.

Various cardiovascular risk factors and disease states similar to those seen in type 2 diabetic patients also seem to cluster in nondiabetic individuals. This cluster of risk factors has been called syndrome X, also known as metabolic cardiovascular syndrome or insulin resistance syndrome.1–3 Changes in the vascular wall could be involved in the cardiovascular alterations associated with this state. Endothelial dysfunction, either as a cause or a consequence, is now known to be associated with the spectrum of cardiovascular diseases.4 Vascular smooth muscle cell (VSMC) migration and proliferation into the vascular wall is associated to the progression of atherosclerosis and, together with thrombosis and vasospasm, lead to vascular occlusion and myocardial infarction.5

Feeding carbohydrate-enriched diets to normal rats has been shown to induce insulin resistance and hyperinsulinemia associated with an elevation of blood pressure (BP).6 Fructose feeding in rats (FFR) provides a model of dietary-induced insulin resistance, which has been used to examine interactions in the cluster of metabolic alterations known as syndrome X that includes hyperinsulinemia, insulin resistance, hypertension, and dyslipidemia.7 Insulin resistance related to this model could probably be due to a defect in insulin receptor activity and changes in the postreceptor cascade of insulin actions.8 The mechanism of fructose-induced hypertension is still not clear. Hyperinsulinemia could activate the sympathetic system, which in turn could elevate the BP.6 An impaired response to endothelium-dependent vasodilators in FFR has also been demonstrated.9 A growing body of evidence indicates that locally generated vasoactive substances such as angioten-
sin (Ang) and nitric oxide (NO) are important determinants of the natural history of vascular disease. Recent evidence suggests that endothelial NO production could be decreased in FFR at both renal and vascular levels. Alterations in both endothelial production of NO and VSMC growth could be associated with the initiation or progression of the atherosclerotic process and to vascular changes in hypertension. Even when changes in the cardiovascular production of vasoactive agonists including Ang II, as well as changes in the AT1 receptor density have been reported in this experimental model, the role of renin-angiotensin system (RAS) in this model is still unclear.

One of the objectives of the present study was to examine the effects of an experimental model of syndrome X on the endothelial nitric oxide synthase (eNOS) activity and its association with changes in the proliferative behavior of primary cultured VSMCs obtained from the aorta and mesenteric vascular bed as representatives of a conduit artery and resistance vessels. Angiotensin converting enzyme (ACE) inhibitors have been reported to exert potent antiatherosclerotic and antihypertensive effects in several experimental models of vascular disease. Therefore, it is of interest to examine also the effect of chronic administration of enalapril on primary cultured smooth muscle cells (pC-VSMCs) proliferation and eNOS activity in the aorta and mesenteric vascular bed from FFR. In addition, cardiac eNOS activity was examined.

**Methods**

**Animals and Experimental Design**

All procedures were performed according to Institutional Guidelines for Animal Experimentation. Male Wistar rats (25 to 30 days old) were fed a standard commercial chow diet ad libitum and housed during the experimental period of 8 weeks in a room under conditions of controlled temperature (20°C), humidity, and 12-h light/dark cycle.

Animals were randomly divided into four groups: group I: control rats with free access to tap water; group II: control + enalapril-fed rats with free access to tap water receiving enalapril (10 mg/kg/day) during the last 4 weeks of the study; group III: FFR (n = 8) rats receiving fructose (Parafarm, Buenos Aires, Argentina) in their drinking water as a 10% (w/v) solution during 8 weeks; and group IV: FFR + E, fructose-fed rats receiving enalapril (10 mg/kg/day) in the drinking water during the last 4 weeks of the study (n = 8 in each group).

**Relative Heart Weight**

To evaluate the development of cardiac hypertrophy in treated rats, each animal was weighed before killing under ether anesthesia at the end of experiment. Then the heart was removed from the great vessels, dropped in chilled Hank’s buffered saline solution (HBSS) and blotted with tissue paper to remove as much blood as possible and weighed. Total heart weight was corrected as the ratio heart weight (milligrams) per 100 grams of total body weight (HW/BW ratio).

**Systolic BP Measurement**

Systolic BP was monitored indirectly in conscious, prewarmed, slightly restrained rats by the tail-cuff method and recorded on a Grass model 7 polygraph (Grass Instruments Co., Quincy, MA).

**Biochemical Determinations**

After overnight fasting, blood samples for glucose, triglycerides, and cholesterol determinations were taken from all rats by the tail-bleeding method at the end of the experimental period. The plasma metabolite concentrations were determined using commercial kits by enzymatic colorimetric methods (Wiener Lab., Rosario, Argentina).

**Intraperitoneal Glucose Tolerance Test**

Three days before the end of the experimental period, an intraperitoneal glucose tolerance test was performed. Rats, fasted overnight, were anesthetized with pentobarbital and glucose was challenged intraperitoneally (2 g/kg). Blood samples were taken by the tail-bleeding method at 0, 30, 60, and 90 min after injection to determine plasma glucose concentration (glucose oxidase-peroxidase method) and total area under the curve was calculated as millimoles per liter per 90 min.

**Cell Cultures**

Aortic and mesenteric smooth muscle cell (SMC) cultures were performed according to a method previously described. At the end of the experimental period, the animals were killed by decapitation under ether anesthesia, and thoracic aorta and mesenteric vascular bed were aseptically excised and placed in chilled HBSS with antibiotic mixture for further dissection. Aortic SMCs were obtained by enzyme dispersion with 1.5 mg/mL collagenase (Class II, Worthington Biochemical Corp., Lakewood, NJ) in F-12 modified Eagle’s medium (MEM) with 10% fetal calf serum (FCS; GEN S.A., Buenos Aires, Argentina). Mesenteric SMCs were isolated by digestion with an enzyme mixture containing 2 mg/mL collagenase, 0.15 mg/mL elastase, 2 mg/mL bovine serum albumin, and 0.35 mg/mL soybean trypsin inhibitor in Ham’s F12 medium. After a 2- to 3-h period in an oscillating water bath at 37°C, isolated SMCs from each aorta and from each mesenteric bed were independently grown in 10% FCS/MEM F-12, incubated at 37°C under humid 5% CO2-air conditions. Primary cultures were obtained by seeding 2 x 10^4 cell/well in a 24-well plaque for [3H]thymidine incorporation.

**Characterization of Cultured VSMCs**

Cultured aortic and mesenteric SMCs exhibited the characteristic hill-and-valley growth pattern upon reaching...
confluence. They were identified by the presence of positive staining with antiswine muscle α-actin (Sigma Immunoclonics, St. Louis, MO). The presence of factor VIII was investigated to assess the complete removal of endothelial cells from the vessels. Staining with antifactor VIII antibodies (Sigma Immunoclonics) was negative.

**Measurement of DNA Synthesis**

To evaluate cell proliferation, aortic and mesenteric SMCs were seeded at equal density and allowed to grow until 80% confluence was reached. Cells were made quiescent by replacing the medium with 0.1% FCS/F-12 MEM for 48 h. Then they were incubated for 24 h with 10% FCS/ F-12 MEM. A nonstimulated control group was incubated with 0.1% FCS/F-12 MEM. Cells were pulsed with 1.0 μCi/mL [3H]thymidine (New England Nuclear, Boston, MA) during the last 3 h of the incubation period. After washing the cells with cold phosphate buffer solution, DNA was precipitated with 10% trichloroacetic acid and finally solubilized with 0.1% sodium dodecylsulfate and 0.1 mol/L sodium hydroxide. Aliquots of the extract were counted in a scintillation counter. Data from the proliferation assays are expressed as dpm per micrograms of protein (Lowry microassay) in the cell lysate or as percent of the respective control group (0.1% FCS/F-12 MEM) and were obtained in all cases as the mean from values of three wells for each aorta or each mesenteric vascular bed.

**Measurements of eNOS Activity**

The eNOS activity was measured by the conversion of L-[3H]arginine to L-[3H]citrulline, as previously described.12,13,19 Excised thoracic aorta (40 mm) were isolated and cut longitudinally. The endothelial cells were removed with a plastic scraper and then sonicated in a buffer (pH 7.4, 37°C) containing 50 mmol/L Tris, 20 mmol/L N-[2-hydroxyethyl]piperazine-N’-[2-ethanesulfonic acid (HEPES), 250 mmol/L sucrose, 1 mmol/L diethiothreitol, 10 μg/mL leupeptin, 10 μg/mL soybean trypsin inhibitor, 5 μg/mL aprotinin, and 0.1 mmol/L phenyl methyl sulfonyl fluoride. Mesenteric vessels were homogenized on ice for four 15-sec intervals with a polytron homogenizer and then sonicated in the same buffer. Heart tissue from left ventricle myocardium was also homogenized on ice for four 15-sec intervals with a polytron homogenizer and then sonicated in the same buffer. After centrifugation of the homogenates (1000 g, 5 min, 4°C) and determination of protein content (Bradford method), aliquots of 50 μL were added to 100 μL of a cocktail reaction buffer containing 50 mmol/L Tris, 20 mmol/L HEPES, 1 mmol/L diethiothreitol, 10 μmol/L flavin adenine dinucleotide (FAD), 50 μmol/L riboflavin 5’-monophosphate (FMN), and 10 μCi/mL L-[2,3-3H]arginine (New England Nuclear), and incubated for 30 min at 37°C in a shaking bath in the presence of 10 μg/mL calmodulin and 3 mmol/L CaCl2 or with 3 mmol/L ethylene glycol-bis (2-aminoethyl)tetra-acetic acid (EGTA) in absence of Ca2+/calmodulin. The reaction was stopped by adding 1 mL of cold distilled water and the mixture applied to an anion-exchange chromatography column containing Dowex AG 50W-X8 (200 to 400 Mesh) resin previously saturated with 50 μL of 100 mmol/L L-citrullin and 2 mL of 50 mmol/L Tris, 20 mmol/L HEPES buffer (pH 7.4) and eluted with 2 mL of distilled water. Concentration of the specifically eluted L-[3H]citrulline was determined by liquid scintillation counting. The calcium-dependent NOS activity was calculated as the difference between activity in the presence and absence of Ca2+/calmodulin. Values were corrected to the amount of protein (Bradford method) present in the homogenates and the incubation time (cpm per milligram of protein per minute). Each rat aorta, mesenteric vascular bed, or heart tissue was processed and eNOS activity measured independently.

**Reagents**

Unless otherwise noted, reagents were purchased from Sigma Chemical Co., St. Louis, MO. Enalapril was generously provided by Roemmers Laboratories (Buenos Aires, Argentina).

**Statistical and Data Analysis**

Data are expressed as mean ± SEM. The statistical significance comparing data between groups was assessed by one-way ANOVA followed by Newman-Keuls post test. A P value of < .05 was considered significant.

**Results**

Chronic fructose administration induced several alterations included in the cluster of risk factors of syndrome X. Compared to control group, at the end of the experimental period, FFR showed a nonsignificant increase in fasting levels of glucose and a significant increase in triglycerides (P < .001), without changes in total cholesterol and HDL-cholesterol levels (Table 1). Long-term treatment with enalapril did not modify any of these metabolic variables. The area under the curve of glycemia during the 120 min of the glucose tolerance test was significantly greater in FFR than in control rats (P < .001) (Fig. 1), evidence that these rats developed glucose intolerance. Chronic treatment with enalapril reduced this area in both control and FFR. There were no significant differences in body weight between the experimental and control groups.

Fig. 2 (left panel) shows the evolution of systolic BP measured during the experimental period. The systolic BP of FFR increased significantly from the fourth week (P < .01) and remained elevated until the end of the 8-week experimental period (P < .001). The enalapril administration during the last 4 weeks significantly reduced systolic BP values even lower than those observed in control rats.
without enalapril in both FFR and control rats. The FFR also showed a greater relative heart weight than control rats \((P < .05)\) (Fig. 2, right panel). Treatment with enalapril significantly reduced FFR and control rat HW/BW ratio to values lower than those of control rats without treatment.

To study cell proliferation, only primary cultures of aortic and mesenteric SMCs were used. The proliferative effect of 10% FCS on aortic and mesenteric SMC was assessed by the incorporation of \(^{[3}\text{H}]\text{thymidine}\) into newly synthesized DNA. A significant increased proliferation was found in FFR primary cultured aortic and mesenteric SMCs, compared to control rat cells (Fig. 3A and C). When the proliferation was estimated as the ratio of \(^{[3}\text{H}]\text{thymidine}\) incorporated by 10% FCS-stimulated cells to its own 0.1% FCS-nonstimulated cells, the results showed the same changes (Fig. 3B and D), which confirmed that aortic and mesenteric SMCs from FFR present an altered proliferative behavior in primary culture. Chronic treatment with enalapril was able to reduce significantly \(^{[3}\text{H}]\text{thymidine}\) incorporation of both primary cultured aortic and mesenteric SMCs from FFR (Fig. 3), without effect on control cells.

Fig. 4 shows that eNOS activity in aortic (Fig. 4A) and mesenteric (Fig. 4B) homogenates decreased significantly in the FFR group. There was no significant difference in \(\text{Ca}^{2+}\)/calmodulin-independent NO synthase activity in homogenates among groups (data not shown). Long-term treatment with enalapril was able to restore the eNOS activity in FFR aortic and mesenteric homogenates. Fig. 4C shows that FFR cardiac eNOS activity was also significantly lower than that of control rats, and enalapril administration induced an increase of this variable in control rats. The enalapril did not produced any significant effect on eNOS activity in control rats.

**Discussion**

In the past two decades some concepts have been aiding in our understanding of the etiology of cardiovascular disease associated with type 2 diabetes: the cluster of insulin resistance, glucose intolerance, dyslipidemia, and hypertension known as syndrome X and the recognition of the crucial role played by the endothelium in orchestrating the impact of a variety of cardiovascular risk factors on vasculature in a wide range of vascular diseases including atherosclerosis. A growing body of evidence indicates that locally generated vasoactive substances such as Ang II and NO are important determinants of the vascular

**Table 1.** Metabolic characteristics at the end of the 8-week period

<table>
<thead>
<tr>
<th>Variable</th>
<th>Control (C)</th>
<th>Control+Enalapril</th>
<th>FFR</th>
<th>FFR+Enalapril</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>410.0 ± 8.2</td>
<td>408.2 ± 13.3</td>
<td>418.3 ± 9.1</td>
<td>410.1 ± 20.5</td>
</tr>
<tr>
<td>Basal glycemia (mmol/L)</td>
<td>4.93 ± 0.04</td>
<td>5.41 ± 0.71</td>
<td>7.32 ± 1.62</td>
<td>7.13 ± 0.83</td>
</tr>
<tr>
<td>Plasma triglycerides (mmol/L)</td>
<td>0.54 ± 0.05</td>
<td>0.73 ± 0.10</td>
<td>1.62 ± 0.23</td>
<td>1.74 ± 0.15</td>
</tr>
<tr>
<td>Total plasma cholesterol (mmol/L)</td>
<td>0.90 ± 0.21</td>
<td>0.99 ± 0.07</td>
<td>0.89 ± 0.06</td>
<td>1.06 ± 0.07</td>
</tr>
<tr>
<td>Plasma HDL-cholesterol (mmol/L)</td>
<td>0.40 ± 0.02</td>
<td>0.47 ± 0.03</td>
<td>0.34 ± 0.03</td>
<td>0.38 ± 0.02</td>
</tr>
</tbody>
</table>

**FFR** - fructose-fed rat.

Enalapril (10 mg/kg/day) was administered during the last 4 weeks. Data are expressed as mean ± SEM \((n = 8\) in each group).
The initiation or progression of the atherosclerotic process could be associated with alterations in both endothelial production of NO and VSMC growth, which are in turn influenced by Ang II. Thus, the main objective of the present study was to examine the effects of the pharmacologic intervention in the RAS through inhibition of ACE with enalapril, on the activity of eNOS and its association to changes in the proliferative behavior of VSMCs in primary culture, obtained from a conduit artery as aorta, and from resistance vessels as the mesenteric vascular bed, in an experimental model of syndrome X.

In agreement with data previously reported on this model, present results show that chronic administration of fructose in the drinking water induced glucose intolerance, hyperglycemia, hypertriglyceridemia, and circulatory changes, including cardiac hypertrophy and increased systolic BP. It has been proposed that a 50% magnesium deficiency, which appears when fructose is given in a modified diet (50% w/w), leads to the development of hyperinsulinemia and hypertension. In the present study, fructose was given in drinking water (10%), all experimental and control rats received the same commercial chow, and these conditions induced a lower total dietary magnesium of near 12%. On the other hand, a positive correlation between serum magnesium levels and RAS activity and its modification by converting enzyme inhibitors has been observed. Therefore, we assumed that magnesium deficiency could only partially participate in the production of the observed changes in this experimental model.

Some metabolic and hemodynamic changes reverted by chronic treatment with enalapril. In agreement with a previous report, hypertriglyceridemia and cholesterol values were not modified by enalapril administration. It is interesting to note that the relative heart weight was reduced by enalapril in both treated groups, although bradykinin has been demonstrated to induce proliferation of cardiomyocytes. This might indicate that left ventricular hypertrophy depends on BP levels at a higher extent than on local growth factors. On the contrary, vascular hypertrophy seems to be mediated mainly by local growth modulators and less by BP levels.

Macrovascular disease, including endothelial dysfunction and changes in VSMC migration and proliferation, has been recognized as the major cause of mortality in individuals with type 2 diabetes. In atherosclerotic lesions, arterial SMC change from a contractile to a synthetic phenotype characterized by active proliferation. A similar phenotype modulation, characterized by an active synthesis of DNA, occurs in vitro when isolated VSMCs are grown in culture. Several lines of evidence suggest that NO inhibits VSMCs proliferation, suggesting that signal transduction pathways regulated by NO may be important in VSMC phenotypic modulation. The NO stimulates the production of cGMP, which, in turn, regulates several functions of VSMCs such as smooth muscle relaxation. The major receptor protein for cGMP in VSMCs is the cGMP-dependent protein kinase (PKG), a serine/
threonine kinase that catalyzes the phosphorylation of important proteins that regulate intracellular Ca\(^{2+}\) levels and relaxation of vascular smooth muscle. It has recently demonstrated that PKG also plays a major role in the regulation of the phenotype and morphology of VSMCs. The expression of PKG is highly variable in VSMCs. When adult rat aortic SMCs are subcultured in vitro, PKG expression is reduced to nearly undetectable levels. Coincident with the loss of expression of PKG, VSMCs assume the more synthetic phenotype.\(^{29}\) However, primary cultured VSMCs, as we used in our proliferation experiments, still maintain characteristics derived from the in vivo cell environment, where changes in NO production have been demonstrated. In addition, other investigators have previously reported changes in proliferation of primary cultured aortic SMCs obtained from animal models of diabetes.\(^{30}\) 

Our results indicate an increased proliferative state in response to an unspecified stimulus such as 10\% FCS in aortic and mesenteric SMCs in primary culture isolated from FFR. The VSMCs not only increased their DNA synthesis, assessed by the incorporation of \(^{3}H\)thymidine, but also showed an increase in the relative cell number (data not shown). Chronic ACE inhibition led a proliferative behavior to a level similar to that observed in control VSMCs, supporting a principal role for RAS in the mechanisms involved in the pathogenesis of this alteration. On the other hand, the chronic ACE inhibition was also able to restore eNOS activity. One important physiologic role of the arginine–NO pathway is to protect the cardiovascular system against pathophysiologic insults that can lead to chronic disease such as hypertension, stroke, and atherosclerosis.\(^{31}\) Evidence suggests a critical role for the balance between angiotensin and NO pathways as a regulator of SMC growth.\(^{32}\) Nitric oxide has been shown to inhibit VSMCs proliferation and migration in vitro as well as in vivo.\(^{33,35}\) In agreement with these findings, inhibition of eNOS caused accelerated atherosclerosis in experimental models.\(^{36}\) Major risk factors for atherosclerotic vascular disease, such as diabetes and hypertension, have been associated with impaired NO activity.\(^{37}\) Recently published experimental data support this finding in the FFR experimental model.\(^{12,13}\)

Angiotensin II is now being regarded as a causal factor in the development of hypertension. It is a vasoconstrictor and trophic factor that mediates contractile and proliferative actions, mainly by stimulating AT1 receptors. There are two different pharmacologic ways of interfering with Ang II-mediated actions: ACE inhibition and AT1 receptor blocking. The first inhibits Ang II production and at the same time, prolongs the half-life of bradykinin by inhibition of kinase II and by increasing the activity of the kallikrein-kinin system.\(^{15}\) This pharmacologic intervention induces a beneficial effect on vascular structure and function, attenuates endothelial dysfunction, and enhances NO release from vascular endothelium, contributing to the reduction of vascular structural alterations. A previous work\(^{38}\) showed that chronic antihypertensive treatment in vivo with losartan significantly changes the VSMCs proliferative status in cultures until passage.\(^{5}\) These researchers signaled that at higher passages, cells began to slowly lose their phenotypic differences compared with the untreated group. They did not observe changes in response to captopril treatment, but observed changes were associated with a long-term treatment with losartan. In contrast, we observed changes in proliferative status after long-term treatment with enalapril. In our experiments, chronic treatment with enalapril was able to restore the Ca\(^{2+}\)/calmodulin-dependent eNOS activity in aortic and mesenteric homogenates from FFR to control values. This effect may be due in part to the fact that bradykinin is a potent inducer of NO generation and, therefore, the blocking kinin degradation by ACE inhibitors is an effective mean of augmenting endothelial generation of NO.\(^{39,40}\)

Undoubtedly, RAS contributes to long-term homeostasis of BP and is involved in the pathophysiology of hypertension. Pharmacologic interventions in the RAS has helped to demonstrate its role in cardiovascular disease. It is desirable that antihypertensive drugs not simply reduce BP but also protect against the local effects of high BP and the structural remodeling in blood vessels, the heart, the kidney, and the brain.

In summary, the experimental model of chronic administration of fructose induced glucose intolerance and led to the development of hypertension. Our data indicate that, in this model, VSMCs obtained from a conduit artery and from resistance vessels, in primary culture, showed an increased proliferative behavior when stimulated. These changes were associated with a decrease in aortic and mesenteric endothelial Ca\(^{2+}\)/calmodulin-dependent NO generating activity. Because long-term treatment with enalapril reverted proliferative changes and was able to restore NO-generating activity in aortic and mesenteric endothelium, present results provide further evidence of the role of the RAS and possibly kinins on vascular alterations arising from a hyperglycemic condition.

These findings support the hypothesis that, at the early phase of development of syndrome X, RAS is involved in changes in endothelial function or VSMCs proliferation, which in turn may be associated with the onset or progression of the atherogenic process and the hypertensive vascular changes.

References


