Aortic Smooth Muscle Cell Proliferation and Endothelial Nitric Oxide Synthase Activity in Fructose-Fed Rats

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The aim of this study was to evaluate the proliferative behavior of vascular smooth muscle cells in primary culture (pC-SMC) and the endothelial nitric oxide synthase (eNOS) activity in the endothelial lining of the aorta of fructose-fed rats (FFR). This is an experimental model of syndrome X, a cluster of cardiovascular risk factors including hyperinsulinemia, insulin resistance, and hypertension that has been suggested to be of pathophysiologic importance for the development of atherosclerosis.

Male Wistar rats were used: Control (n = 12) and FFR (n = 12). After receiving fructose in drinking water (10% w/v) during 8 weeks, biochemical parameters, systolic blood pressure (SBP) and relative heart weight (RHW) were determined. The proliferative effect of 10% fetal calf serum (FCS) was examined in aortic pC-SMC by [3 H]thymidine incorporation and by cell counting. Ca2+/calmodulin-dependent NOS activity was estimated in aortic endothelial lining and in heart tissue homogenates by conversion of [3 H]arginine into [3 H]citrulline.

Fructose-fed rats showed hyperinsulinemia (P = .0263), altered glucose tolerance test (P < .001), higher SBP (P < .0001), and RHW (P = .0145), compared to control rats. These animals also showed an increase of 10% FCS-induced [3 H]thymidine incorporation (P < .0001) and cell number of aortic pC-SMC (P = .0049) and decreased eNOS activity in both aortic endothelium (P = .0147) and cardiac tissue (P < .0001).

These data support the hypothesis that syndrome X is associated to changes in SMC proliferation and endothelial dysfunction, which could be involved in the onset or progression of the atherogenic process. Am J Hypertens 2001;14:1135–1141 © 2001 American Journal of Hypertension, Ltd.

Key Words: Vascular smooth muscle cell proliferation, endothelial nitric oxide synthase, fructose-fed rats, insulin resistance, hypertension, syndrome X.

Hypertension and type 2 diabetes mellitus are highly prevalent associated diseases in Western societies. Because epidemiologic evidence supports a link between hyperinsulinemia and blood pressure (BP), there is a surge of interest in this association, in particular with regard to the possible underlying mechanisms. Various cardiovascular risk factors and disease states similar to those 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). Again, insulin resistance and hyperinsulinemia are often assumed as primary and central features of this syndrome. It is important to note that insulin resistance is also a common feature of the microvascular angina associated with endothelial dysfunction and coronary spasms.
migration and proliferation into the vascular wall are associated to the progression of atherosclerosis and together with thrombosis and vasospasm lead to vascular occlusion and myocardial infarction.8

Feeding carbohydrates-enriched diets to normal rats has been shown to induce insulin resistance and hyperinsulinemia associated with an elevation of BP.9 Fructose feeding in rats (FFR) provides a model of dietary-induced insulin resistance that has been used to examine interactions among the cluster of metabolic alterations known as syndrome X.10 Recent evidence suggests that endothelial nitric oxide (NO) production could be decreased in FFR at both vascular11,12 and renal13 levels.

Because initiation or progression of an atherosclerotic process could be associated with alterations in both endothelial production of NO and SMC growth, the main objective of the present study was to examine the effects of an experimental model of syndrome X on the activity of aortic endothelial NO synthase and its association to changes in the proliferative behavior of primary cultured SMC obtained from the same artery.

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 an experimental period of 8 weeks in a room under conditions of controlled temperature (20°C), humidity and a 12-h light/dark cycle. Animals were randomly divided into two groups: control rats (n = 12), with free access to tap water; FFR rats (n = 12) receiving fructose (Parafarm, Buenos Aires, Argentina) in their drinking water as a 10% (w/v) solution during 8 weeks.14

Systolic BP Measurement

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

Biochemical Determinations

After overnight fasting, blood samples for glucose, triglycerides, and insulin determinations were taken from all rats by the tail-bleeding method at the end of the experimental period. The plasma glucose and triglyceride concentrations were determined using commercial kits by enzymatic colorimetric methods (Wiener Lab, Buenos Aires, Argentina). The plasma insulin was determined by radioimmunoassay using monoclonal antibody to human insulin (Diagnostic Product Co., Los Angeles, CA).

Glucose Tolerance Test

Three days before the end of the experimental period, an intraperitoneal glucose tolerance test was performed. After an overnight fast, 2 g/kg glucose was administered intraperitoneally to pentobarbital-anesthetized rats. 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 millimole per liter per 90 min. Food was removed at approximately 5:30 pm, and studies commenced at approximately 8:30 am the next morning (approximately 15 h). Standard light/dark cycles were maintained by the animal facility with lights on from 6:00 am to 6:00 pm and lights off for the remaining 12 h.

Cell Cultures

Cultures were performed according to a method previously described.15 At the end of the experimental period, the animals were killed by decapitation under ether anesthesia, and the thoracic aorta and heart were aseptically excised and placed in chilled Hank’s Balanced Salt Solution with an antibiotic mixture for further dissection. Aortic SMC were obtained by enzyme dispersion with 1.5 mg/mL collagenase (Class II, Worthington Biochemical Corp., Lakewood, NJ) in F-12 (Ham mixture) modified Eagle’s medium (MEM) with 10% fetal calf serum (FCS) (GEN S.A., Buenos Aires, Argentina) after a 2- to 3-h period in an oscillating water bath at 37°C. Isolated SMC from each aorta 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 either 6 to 8 × 10^3 cell/well in a six-well plate (for cell counting) or 2 to 4 × 10^4 cell/well in a 24-well plate (for [3H]thymidine incorporation).

Characterization of Cultured SMC

Although cultured aortic SMC exhibited the characteristic hill-and-valley growth pattern upon reaching confluence, they were identified by the presence of positive staining with antismooth muscle α actin (Sigma Immunochemicals, 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 Immunochemicals) gave negative results.

Measurement of DNA Synthesis

For measurements of cell proliferation, aortic SMC 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 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 before the end 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 were 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 the cases as the mean of values from three wells for each aorta.

**Cell Number Determination**

Cell counts were performed to verify that the effects observed with the [3H]thymidine assay were reflective of changes in growth. After 48 h of incubation in the presence of 0.1% FCS or 10% FCS, cells were trypsinized and counted using a hemocytometer (Neubauer chamber). The relative cell number was expressed as a ratio of the average stimulated (10% FCS) to nonstimulated (0.1% FCS) amount.

**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 the experiment. Hearts were treated rats, each animal was weighed before killing under ether anesthesia at the end of the experiment. Hearts were removed from the great vessels, dropped in chilled HBSS and blotted with tissue paper to remove as much blood as possible and weighed. Total heart weight was corrected as the ratio cardiac weight (in milligrams) per 100 grams of total body weight.

**Measurements of NO Synthase Activity**

Endothelial NO synthase activity was measured by the conversion of L-[3H]arginine to L-[3H]citrulline, as previously described.12 Excised thoracic aortas (40 mm) were isolated as described and cut longitudinally. The endothelial cells were removed with a plastic scraper and then sonicated in a buffer (pH 7.4) containing 50 mmol/L Tris, 20 mmol/L HEPES, 250 mmol/L sucrose, 1 mmol/L dithiothreitol, 10 μg/mL leupeptin, 10 μg/mL soybean trypsin inhibitor, 5 μg/mL aprotinin, and 0.1 mmol/L phenylmethylsulfonyl fluoride. After centrifugation of the homogenates (10000 g, 5 min, 4°C) and determination of protein content (Bradford method), aliquots of 50 μL were added to 100 μL of a reaction buffer containing 50 mmol/L Tris, 20 mmol/L HEPES, 1 mmol/L dithiothreitol, 1 mmol/L NADPH, 0.1 mmol/L tetrahydrobiopterin, 50 μmol/L FAD, 50 μmol/L 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 EGTA in absence of Ca2+/calmodulin. The reaction was stopped by adding 1 mL of cold distilled water and the mixture applied to a 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 NO synthase 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 protein per minute).

For the measurement of endothelial NO synthase in cardiac tissue, samples from frozen left ventricle (−70°C) were homogenized with a glass stirrer in the same buffer used for the aortic endothelium assay, adjusting the homogenate concentration to 100 mg of tissue per milliliter of buffer. After centrifugation (1000 g, 5 min, 4°C) and determination of protein content (Bradford method), aliquots of 50 μL were processed as indicated above.

**Reagents**

Otherwise noted, reagents were purchased from Sigma Chemical Co., St. Louis, MO.

**Statistical and Data Analysis**

Data are expressed as mean ± SEM. The statistical significance comparing data from the two groups was assessed by unpaired t test and unpaired Welch’s corrected t test when variances were different. Two-way ANOVA was used when comparing glucose tolerance test curves. A P value < .05 was considered significant.

**Results**

The chronic administration of fructose as a 10% drinking solution during 8 weeks was able to induce several alterations included in the cluster of risk factors called syndrome X. Fructose-fed rats ate less chow than controls (16.2 ± 0.5 g vs 18.2 ± 0.4 g, P < .0071). Fructose solution intake by FFR (45.9 ± 2.7 mL) was higher (P < .0001) than water consumption by controls (28.9 ± 1.1 mL). Derived from these data, estimated FFR caloric intake was higher than controls (53.3 ± 2.1 kcal/day vs 39.4 ± 0.9 kcal/day; P < .0001). Fructose-fed rats showed at the end of the experimental period a significant increase in fasting levels of glycemia (P = .002), triglyceridemia (P = .0202), and insulinemia (P = .0263) (Table 1). The analysis of the glucose tolerance test and the comparison between areas under the curve of glycemia during 90 min from control and experimental groups (Fig. 1) shows that FFR developed glucose intolerance assessed by the significantly increased area values in the treated animals (P < .0001).

Table 1 also shows the evolution of the systolic BP levels measured during all the experimental period. The BP of FFR increased significantly from the fourth week (P = .0014) and remained elevated up to the end of the 8 weeks of the experimental period. Even when the BP levels did not reach those corresponding to a severe hypertension, there was a highly significant difference com-
pared to the control levels \((P < .0001)\). Cardiac hypertrophy developed in the experimental model. As shown in Table 1, values of the index heart weight/body weight were significantly higher \((P = .0145)\) in the experimental group than in controls.

When the proliferative effect of 10% FCS on aortic SMC was assessed by the incorporation of \[^3\text{H}\]thymidine into newly synthesized DNA, a significant difference was found between FFR and control primary cultured aortic SMC, considered as the \[^3\text{H}\]thymidine incorporated per microgram of protein in the cell lysate (Fig. 2a) or considered as the ratio of \[^3\text{H}\]thymidine incorporated by 10% FCS-stimulated cells to 0.1% FCS-stimulated cells (Fig. 2b). The effects observed with the \[^3\text{H}\]thymidine assay were reflective of changes in growth. The ratio of the average stimulated (10% FCS) to nonstimulated (0.1% FCS) cell number showed a similar pattern that confirm that aortic SMC from FFR present an altered proliferative behavior in primary culture (Fig. 2c).

Table 1. Metabolic characteristics at the end of the 8-week period and blood pressure at 0, 4, and 8 weeks

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Sham ((n = 12))</th>
<th>FFR ((n = 12))</th>
<th>(P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>410.0 ± 5.8</td>
<td>418.3 ± 6.4</td>
<td>.3470 (NS)</td>
</tr>
<tr>
<td>Heart weight (mg)</td>
<td>923.8 ± 17.1</td>
<td>1006.7 ± 22.7</td>
<td>.008</td>
</tr>
<tr>
<td>Relative heart weight (mg/100 g)</td>
<td>225.0 ± 2.9</td>
<td>239.0 ± 5.1</td>
<td>.0145</td>
</tr>
<tr>
<td>Fasting insulin (pg/mL)</td>
<td>2.3 ± 0.4</td>
<td>7.8 ± 2.5</td>
<td>.0263</td>
</tr>
<tr>
<td>Fasting triglycerides (mg/dL)</td>
<td>119 ± 12</td>
<td>161 ± 15</td>
<td>.0204</td>
</tr>
<tr>
<td>Fasting glycemia (mmol/L)</td>
<td>88.9 ± 0.23</td>
<td>131.9 ± 8.4</td>
<td>.0002</td>
</tr>
<tr>
<td>Systolic blood pressure</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>110.5 ± 1.6</td>
<td>111.5 ± 0.8</td>
<td>.2919 (NS)</td>
</tr>
<tr>
<td>4 weeks</td>
<td>108.7 ± 1.3</td>
<td>121.5 ± 3.3</td>
<td>.0014</td>
</tr>
<tr>
<td>8 weeks</td>
<td>107.5 ± 1.6</td>
<td>130.5 ± 2.0</td>
<td>&lt;.0001</td>
</tr>
</tbody>
</table>

FFR = fructose-fed rats; NS = not significant.

Data are mean ± SEM from 12 rats in each group. A significant difference was assessed by Welch-corrected unpaired \(t\) test.

Fig. 3a shows that the activity of the Ca\(^{2+}\)/calmodulin-independent NO synthase from the aortic endothelial lining decreased significantly in the experimental group \((P = .0147)\). There were no significant differences in Ca\(^{2+}\)/calmodulin independent NO synthase activity in endothelial homogenates among the two groups (data not shown). Fig. 3b shows the same data obtained from cardiac tissue homogenates. The Ca\(^{2+}\)/calmodulin-dependent NO synthase from cardiac tissue homogenates decreased significantly in the experimental group \((P < .0001)\).

Discussion

Two concepts have recently emerged that aid in the understanding of the cause 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 the impact of a variety of
risk factors on vasculature in a wide range of cardiovascular diseases including atherosclerosis. Because initiation or progression of the atherosclerotic process could be associated with alterations in both endothelial production of NO and SMC growth, the main objective of the present study was to examine the effects of an experimental model of syndrome X on the activity of aortic endothelial NO synthase and its association to changes in the proliferative behavior of SMC in primary culture obtained from the same artery. In addition, we observed changes in the relative heart weight and the activity level of NO production in cardiac tissue homogenates.

The present data show that chronic administration of fructose in the drinking water induced glucose intolerance and increased systolic BP levels. It has been previously suggested that mechanisms involved in this model of experimental diabetes included insulin resistance, low insulin-stimulated lipogenesis, and low glucose oxidation probably due to a defect in the insulin receptor activity and changes in the postreceptor cascade of insulin actions. The

FIG. 2. Proliferative behavior of aortic smooth muscle cells in primary culture from control (light bars) and FFR (dark bars) groups, stimulated with 10% fetal calf serum (FCS) or 0.1% FCS (considered as a no-stimulating condition), expressed as dpm of [3H]thymidine incorporated per microgram of protein (a) or as a ratio between [3H]thymidine incorporated per microgram of protein when cells were incubated with 10% FCS and 0.1% FCS (b). Panel c shows data obtained by counting the primary cultured smooth muscle cells and expressed as a ratio of cell number when cells were incubated with 10% FCS and 0.1% FCS. Each bar represents the mean ± SEM of triplicate wells from six rat aortas. A significant difference (P < .05) was assessed by Welch-corrected unpaired t test. Other abbreviation as in Fig. 1.

FIG. 3. Activity levels of Ca$^{2+}$/calmodulin-dependent nitric oxide synthase, expressed as cpm per milligram of protein in aortic endothelial lining (a) and in cardiac tissue homogenates (b) from control (n = 6) and FFR (n = 6) group. A significant difference was assessed by Welch-corrected unpaired t test. eNOS = endothelial nitric oxide synthase; other abbreviation as in Figs. 1 and 2.
precise mechanisms by which carbohydrate-enriched diets increase BP is still not clear. Hyperinsulinemia could activate the sympathetic system, which in turn will elevate the BP. It has been also demonstrated that FFR showed an impaired response to endothelium-dependent vasodilators. In addition, changes in cardiovascular production of vasoactive agonist as well as changes in its receptor density have been reported.

Macrovascular disease, which includes endothelial dysfunction and changes in SMC migration and proliferation, has been recognized as the major cause of mortality in people 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 occurs in vitro when isolated SMC are grown in culture and it is characterized by an active synthesis of DNA.

We observed an increased proliferative response in aortic SMC in primary culture isolated from FFR in response to an unspecific stimulus such as 10% FCS. Aortic SMC not only increased their DNA synthesis, assessed by the incorporation of [3H]thymidine, but also showed an increase in the relative cell number. It has been suggested that hyperinsulinemia, as a consequence of the insulin-resistant state, could stimulate SMC to proliferate, leading to acceleration of atherosclerosis. In this experimental model we determined the presence of hyperinsulinemia at the end of the experimental period, but in our in vitro experiments, insulin was not used as proliferative stimulus. In additional experiments, incubations with either 100 or 500 μU/mL insulin for 24 h were not able to induce differences in aortic SMC [3H]thymidine incorporation between FFR and controls (data not shown).

Changes in proliferative response of primary cultured aortic SMC from rats turned both diabetic and hypertensive by this particular experimental procedure has not been previously reported. Several evidences suggest a critical role for the NO/endothelial NO synthase pathway as a negative regulator of SMC growth in vivo. Functional and anatomic abnormalities of the vascular endothelium are commonly associated with diabetes. Both hyperglycemia and dyslipidemia contribute to endothelial dysfunction. Hyperglycemia results in impairment of endothelial cell NO production, perhaps through activation of protein kinase C in endothelial cells. Activation of protein kinase C and associated reduced NO production predispose to increased production of vasoconstrictor prostaglandins, endothelin, glycated proteins, endothelium adhesion molecules, and platelet and vascular growth factors, which cumulatively enhance vasmotor tone and vascular permeability, growth, and remodeling.

Nitric oxide has been shown to inhibit vascular smooth muscle cell proliferation and migration in vitro as well as in vivo. In agreement with these findings, inhibition of the Ca2+/calmodulin NO-producing enzyme caused accelerated atherosclerosis in experimental models. Major risk factors for atherosclerotic vascular disease, such as diabetes and hypertension, have been associated with impaired NO activity. Recently published experimental data support this finding in the FFR model.

Associated with an increase in BP in FFR is also an increase in heart weight. Cardiac hypertrophy is secondary to the elevation in BP, as it has been previously demonstrated in experimental models of hypertension. Although the mechanisms of cardiac hypertrophy in FFR is not certain, results of previous studies suggest that angiotensin II through the AT1 receptors could be involved in this process.

In summary, the experimental model of chronic administration of fructose in drinking water induced glucose intolerance and led to the development of hypertension and cardiac hypertrophy. Our data indicate that primary cultured aortic SMC showed an increased proliferative behavior in response to an unspecific stimulus. These changes were associated with a decrease in the Ca2+/calmodulin-dependent NO synthase activity at both the aortic endothelium and myocardial levels. These findings support the hypothesis that at an early phase of development of syndrome X the changes in endothelial function or SMC proliferation may be related to the onset or progression of the atherogenic process.

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References


