Myocardial Repair with Long-Term and Low-Dose Administration of a Nitric Oxide Synthesis Inhibitor: Myofibroblasts, Type III Collagen and Fibronectin

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Objective - To study the healing process of the myocardium in hypertensive rats undergoing inhibition of nitric oxide synthesis.

Methods – Two groups of animals were studied: one received L-NAME, 12mg/kg/day, and the other was a control group. The presence of type III collagen, fibronectin, and α-smooth muscle actin-positive cells was assessed by immunohistochemistry.

Results – Fibronectin was seen in both early and late lesions, while type III collagen was seen mainly in areas of incomplete healing, situated among myocytes and around the intramyocardial branches of the coronary arteries. Areas representing early and late lesions showed a population of spindle-shaped cells. Immunohistochemistry showed that these cells were positive for α-smooth muscle actin.

Conclusion – In the myocardium of hypertensive rats, the α-smooth muscle actin-positive cells are related to the accumulation of type III collagen and fibronectin in the areas of myocardial damage.

Keywords: myofibroblasts, nitric oxide, myocardial remodeling, α-smooth muscle actin

The discovery of the role of nitric oxide (NO) in the maintenance of vascular tonus led to the development of an experimental model for hypertension induced by the chronic inhibition of the NO synthase (NOS), the enzyme responsible for the synthesis of NO from the amino acid L-arginine. This experimental model for hypertension, known as “NO-deficient hypertension”, inspired a series of studies aimed at defining the structural and functional features of the heart in this kind of hypertension.

The N\textsuperscript{G}-nitro-L-arginine methyl ester (L-NAME) is one of the most well known NOS inhibitors and is commonly used in experimental models. The continuous use of this compound leads to the development of high blood pressure as a result of generalized vasoconstriction, reduction in the intracellular levels of cGMP, and morphological changes in the renal microvasculature.

The use of high doses of L-NAME leads to the development of marked hypertension in rats. The effects of this kind of NOS inhibition were studied using stereology to examine the structural changes in the myocardium. Significant changes, such as an increase in the size of myocytes, and interstitial and perivascular fibrosis, were shown.

Left ventricular hypertrophy (LVH) is one of the most common manifestations of HBP. When LVH is caused by HBP, it involves 2 different, but interrelated, processes. The first process is myocyte hypertrophy and the second is an increased synthesis of fibrillar collagens, mainly type I and type III. Myocyte death has been suggested as one of the factors that accounts for the increased amount of collagen (substitutive fibrosis). However, this topic is controversial.

The extracellular matrix (ECM) is the myocardial compartment mostly involved in the process of myocardial healing following the loss of myocytes. At the site of the lesion, the ECM consists of macromolecules that are responsible for the formation of the fibrin-fibronectin network and for the invasion by neutrophils, monocytes, macrophages, fibroblasts and phenotypically transformed fibroblasts (myofibroblasts [MFs]).
MFs show features of both fibroblasts and smooth muscle cells, and the characterization of MFs is based on ultrastructural criteria. The production of collagen by MFs is regulated by autocrine and paracrine signs. MFs are also related to the production of cellular fibronectin. The main components of the myocardial ECM are type I and III fibrillar collagen and fibronectin. Changes in the amount and distribution of these proteins are mainly related to changes in heart function; thus, these changes may also affect myocardial compliance.

According to previous studies of cardiac hypertrophy in rats caused by pressure overload, the presence of fibronectin in ischemic myocardial areas precedes the development of collagen. In addition, other experimental studies have shown that the general myocardial healing process is characterized by an initial accumulation of fibronectin which, in the late stages of this process, is associated with an increase in collagen production.

The objective of this report is to study the healing process of the myocardium during the experimental inhibition of NO synthase.

**Methods**

Male and female Wistar rats weighing 250-300g were used in this study. The rats were divided into 2 groups: a control group and an L-NAME group, having 10 and 14 animals, respectively. The rats were fed a standard diet and unrestricted water. The rats in the L-NAME group were given the NO synthase inhibitor (N\(^\text{3}\)-nitro-L-arginine methyl ester hydrochloride, Sigma Co, lot 44H0102) at a dosage of 12mg/kg/day for 15 weeks. The blood pressure (BP) in the tail was measured weekly with the use of a plethysmograph. After the rats were anesthetized with ethyl ether, their hearts were exposed and a high volume of KCl was injected in the left ventricle (LV). This procedure induced heart arrest in diastole.

A series of fragments of the free left ventricular wall were obtained and fixed by immersion in a solution of 4% formalin and phosphate buffer (pH 7.2) at room temperature. The samples were subsequently dehydrated in increasing concentrations of ethanol, embedded in paraffin and cut in sections of 5µm thickness. The sections were then stained with hematoxylin-eosin and Masson’s trichrome.

The tissue blocks employed for light microscopy were also used for immunohistochemistry. Paraffin sections of 5µm thickness were applied to glass slides, which were pretreated with Silano to warrant a better adherence of the section to the glass slide during the various stages of the technique. The presence of type III collagen, fibronectin and α-smooth muscle actin-positive cells (α-SMA) was examined with the use of the avidin-biotin peroxidase method. Protein digestion with 1% trypsin was performed only to assess the presence of type III collagen. The sections were incubated with rabbit polyclonal antibodies specific for type III collagen (Pharmigen, AB757), in the dilution of 1:200. They were also incubated with fibronectin (Pharmigen, AB1942), in the dilution of 1:300 and with mouse monoclonal antibody specific for α-SMA (DAKO, M851), in the dilution of 1:200. Peroxidase activity was shown in a solution of diaminobenzidine tetrahydrochloride (Sigma Co., 5mg) in 10ml of TRIS at pH 7.0, containing 200µl of 10% hydrogen peroxide at room temperature. The positive control for type III collagen was performed through the observation of the positivity of the tunica adventitia of the intramyocardial branches of the coronary arteries of the rats in the control and L-NAME groups. For fibronectin, this same control was performed with the use of kidneys from control rats, in which the glomerular positivity was observed. The negative control was performed omitting the primary antibody.

The differences in BP levels between the L-NAME and control groups were tested with the Student’s t test, with a level of significance of 0.05.

**Results**

In the control group, BP remained unchanged during the entire experiment. In the L-NAME group, however, BP increased gradually up to 150.0 mmHg after the 10th week of the experiment (tab. I).

At light microscopy, the myocardium of the animals undergoing NO synthase inhibition showed multiple areas of myofibrillar degeneration, necrosis and fibrosis, greatly evidenced in the free wall of the LV. After 100 days using L-NAME, necrotic myofibers characterized by acidophilic stain of the cytoplasm, loss of striations and, sometimes, focal groups of lymphocytes, were observed in all specimens studied. Neutrophils were sometimes observed, especially in early lesions. A diffuse increase in the interstitial collagenous connective tissue next to the vessels was frequently seen. In contrast, the animals in the control group did not show any sign of myocardial damage.

In the myocardium of the control group, the immunoreactivity to type III collagen was identified as a delicate staining of the cytoplasm, loss of striations and, sometimes, focal groups of lymphocytes, were observed in all specimens studied. Neutrophils were sometimes observed, especially in early lesions. A diffuse increase in the interstitial collagenous connective tissue next to the vessels was frequently seen. In contrast, the animals in the control group did not show any sign of myocardial damage.

<table>
<thead>
<tr>
<th>Weeks</th>
<th>Control</th>
<th>L-NAME</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before the administration of L-NAME</td>
<td>99.4±1.0</td>
<td>99.6±1.7</td>
<td>0.78</td>
</tr>
<tr>
<td>After the administration of L-NAME</td>
<td>99.5±0.7</td>
<td>114.5±1.3</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>4th</td>
<td>99.9±0.3</td>
<td>127.9±2.6</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>10th</td>
<td>100.8±0.5</td>
<td>142.9±2.6</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>13th</td>
<td>101.0±2.1</td>
<td>150.0±1.8</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>14th</td>
<td>101.5±2.4</td>
<td>150.0±2.1</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>15th</td>
<td>102.5±2.6</td>
<td>150.0±2.2</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

(mean ± standard deviation; p indicates the likelihood of the difference between the groups to be significant.)
positive immunoreactivity to type III collagen, spreading among the viable muscle fibers, was similarly noted. The myocardium of the L-NAME group showed an increased amount of type III collagen among the muscular fibers and within the tunica adventitia of intramyocardial coronary arteries. Type III collagen and fibronectin were noted in the various foci of early and late myocardial lesions (figs. 1a-d).

There was an increased and uniform distribution of fibronectin in the foci of myocardial lesions filled with nonmuscular cells, i.e., endothelial cells, fibroblasts, MFs, neutrophils and macrophages. These foci were morphologically characterized as having early lesions. There was also a clear distribution of fibronectin in the foci of incompletely healed late lesions.

Type III collagen showed a more uniform distribution in late lesions; however, it was also present in early lesions, although randomly distributed. An increased intercellular space and accumulation of type III collagen and fibronectin in noninfarcted myocardial areas of L-NAME rats were also noted.

Concomitantly, numerous spindle-shaped cells with morphological features of interstitial fibroblasts and positivity for α-SMA (Fig. 1e) were seen in early and late lesions. The vascular smooth muscle cells were also α-SMA-positive and, in the healthy myocardium, they were the only cells with positivity for this antibody (fig. 1f).

Discussion

The collagenous component of the healthy myocardium is part of a system that contains a series of constituents of the ECM (heart interstitium). Its main components are type I and III collagens, organized in a tridimensional network around cardiac myocytes. These elements are responsible for the viscoelastic properties of the myocardium, which are mainly related to the type and proportions of fibrillar proteins and glycoproteins, as well as to the interaction of these proteins with the myocytes. An increase in the content or transformation of the structure of these fibrillar components in relation to cardiac muscle fibers could affect myocardial compliance.

The role of the myocytes and of the cardiac interstitium in the dysfunction of the heart is still controversial. However, previous studies have shown an increase in the volume fraction of collagen in LVH as a result of pressure overload.

The inhibition of NO biosynthesis using high doses of L-NAME induces a significant elevation of the blood pressure, LVH, myocardial necrosis, vascular damage, and interstitial fibrosis. The current results with the low-dose and long-term administration of L-NAME showed that the pressure levels were not so high as those observed in other studies, but myocardial abnormalities were similar to those in the model with high-dosage and short-term administration of L-NAME. When NOS is experimentally inhibited, most myocardial abnormalities are probably the result of a compensatory response to the overload of the circulatory system. However, the extensive myocardial fibrosis noted in these cases does not seem to be related solely to the ventricular overload, but rather to the use of L-NAME, probably as a result of the myocardial ischemia inherent to this experimental model.

In chronic heart failure, changes in the composition of the connective tissue are mainly related to systolic and...
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Diastolic dysfunction, whereas in acute heart failure, the changes occur mainly in the myocytes. Clinical and experimental studies have shown that the ventricular dysfunction induced by pressure overload relates more to the duration of the overload and to the nature of the stimulus than to the extent of the hypertrophic process.

Maintenance of the hypertensive stimulus for 15 weeks induced myocardial remodeling, which showed cellular elements of the inflammatory cell infiltrate and deposits of connective tissue. This latter may have occurred as a result of the synthesis of the inflammatory cells and/or from their growth factors. Myocardial lesions with loss of myocytes, characterized by the presence of foci of myocardiolysis and necrotic myofibers, were also observed.

According to previous studies, there is an association between the responses of the ECM and the cardiomyocytes when the stimulus to the overload is sustained. However, it has not yet been clarified if the changes in the components of the ECM are merely a response to the necrosis of myocytes and/or if these changes induce cellular loss.

In the present study, immunostaining for fibronectin was observed in early myocardial lesions. This immunostaining was noted mainly in areas of myocyte loss, probably as a result of the diffusion of fibronectin that originated from platelets or of the diffusion of plasma into necrotic cardiac myocytes. Fibronectin was also present in late lesions, although the healing tissue does not usually show this protein.

Many functions have been attributed to the increased content of fibronectin in myocardial infarction, including the chemotactic action. Fibronectin plays a role as a sustaining network for the growth and migration of endothelial cells and fibroblasts, and it contributes to platelet aggregation. The presence of fibronectin in myocardial lesions is also related to the angiogenic process that occurs during the healing of the infarction. It may also work as a temporary matrix for deposition and remodeling of other ECM proteins, mainly collagen.

Type III collagen was noted in early lesions or in areas of incomplete healing, as well as in intermuscular spaces and in the tunica adventitia of the L-NAME animals. In accordance with previous studies, the presence of type III collagen in areas of interstitial fibrosis is important for the maintenance of the cell-to-cell relation and for the distribution of the mechanical forces during myocyte contraction.

The elongated cells noted in areas of myocardial lesions may be remnants of endothelial cells from infarcted areas or even MFs contributing to the local synthesis of ECM proteins.

Cardiac response to pressure overload is characterized by the genetic reexpression of a series of fetal proteins of the ECM. Previous studies have shown that the components of the ECM may modulate the phenotypic features of the fibroblasts, increasing or decreasing the expression by these cells of α-SMA, the typical actin isoform of smooth muscle cells. The presence of MFs during the deposition of connective tissue suggests the regulatory function of these cells in the remodeling of the ECM, mainly through the production of fibronectin and type III collagen.

The experimental model of NOS inhibition shows myocyte hypertrophy, a factor that could account for the expression of α-SMA in MFs. As described in previous studies, in addition to growth factors (mainly transforming growth factor β), the mechanical deformation is a significant factor that accounts for the expression of α-SMA in fibroblasts. Willems et al. believe that the abundance of myofibroblasts in myocardial lesions is related to the maintenance of myocardial compliance, preventing the rupture of the affected area during the rhythmic contractile cycles of the heart.

References

Pessanha et al
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