Expression and Distribution of NOS1 and NOS3 in the Myocardium of Angiotensin II–Infused Rats

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Abstract—Studies have indicated a complex functional interaction between angiotensin (Ang) II and NO in the heart. The purpose of the present study was to examine the protein expression and tissue distribution of NO synthases 1 (NOS1) and 3 (NOS3) in the myocardium of rats that underwent continuous infusion of Ang II at 2 different rates (10 and 40 ng · kg⁻¹ · min⁻¹) for 6 days. Mean arterial pressure increased by ≈15 mm Hg in rats infused with Ang II at 40 ng · kg⁻¹ · min⁻¹, but it remained close to the values observed in saline-infused rats (≈110 mm Hg) when Ang II was infused at 10 ng · kg⁻¹ · min⁻¹. The protein expression of a 160-kDa NOS1 and a 135-kDa NOS3 were found to increase (≈200%) in the myocardium of rats infused with both subpressor and pressor doses of Ang II. Immunohistochemistry studies showed that NOS1 and NOS3 are differentially expressed in myocardial cells. NOS1 was detected in cardiac myocytes and in smooth muscle cells of small and large coronary arteries, whereas NOS3 was detected in the endothelium and in perivascular and interstitial tissues, but NOS3 was not detected in cardiac or smooth muscle cells. Ang II infusion enhanced the tissue immunoreactivity of both isoforms in their specific locations but did not change the distribution throughout the myocardium. Myocardium staining with anti–angiotensin type 1 (AT₁) receptor antibody indicated that AT₁ receptor is expressed in cardiac myocytes, coronary smooth muscle cells, and interstitial and perivascular tissues. Ang II infusion did not change the protein expression and distribution of AT₁ receptor in the myocardium. These results indicate that long-term increases in the circulating levels of Ang II modulate the protein expression of NOS1 and NOS3 and, consequently, the function of the local myocardial NO system. (Hypertension. 2001;37:1423-1428.)

Key Words: angiotensin II ■ nitric oxide ■ nitric oxide synthase ■ heart ■ myocardium

Angiotensin (Ang) II modulates cardiac function and cellular growth in response to physiological and pathological processes.¹ Many of the short- and long-term effects of Ang II on cardiac function and structure are due to its direct action on cardiac myocytes, vascular smooth muscle cells, and cardiac fibroblasts. These effects are mediated through at least 2 different types of receptors, which are broadly distributed in cardiac cells.² However, some of the cardiac effects of Ang II occur through the induction and release of paracrine/autocrine factors, such as transforming growth factor-β, and endothelin-1.³,⁴ In this context, in recent years, experimental evidence has indicated that Ang II and NO influence each other by interacting at various levels of regulation.⁵,⁶ This may have implications not only for the cardiac functions directly influenced by these factors but also for the pathogenesis of processes such as myocardial ischemia and fibrosis.

In general, Ang II and NO exert antagonistic effects in cellular function and growth.⁶,⁷ The cellular mechanisms responsible for this antagonism are not clear. In some systems, this interaction seems to be a simple summation of the effects of Ang II and NO.⁷,⁸ Ang II is able to activate the NO system by inducing the secretion of NO in small and large coronary arteries. Because NO attenuates the vasoconstrictor effect of Ang II, this can cause a negative feedback system to limit the stimulation by Ang II.⁹ The antagonism of NO on Ang II effects is also seen in the growth effect of Ang II on cardiac fibroblasts.¹⁰

The mutual regulatory influence of Ang II and NO seems to extend to gene regulation. Studies performed in angiotensinogen gene–knockout mice and in rat adrenal medulla suggest that Ang II inhibits the expression of NO synthase (NOS)¹.¹¹,¹² In rats, however, long-term infusion of high doses of Ang II increases the expression of NOS1 and NOS3 in the renal cortex but reduces NOS1 expression in the renal medulla.⁸ The influence of Ang II on the regulation of the constitutively expressed isoforms of NOS in the myocardium remains virtually unexplored.

Thus, the present study was designed to examine the effect of long-term increases in circulating levels of Ang II on the expression, and the cardiac tissue distribution of the constitutive isoforms of NOS (ie, NOS1 and NOS3). Experiments...
were also performed to examine the protein expression and the tissue distribution of angiotensin type I (AT₁) receptors in the left ventricle of rats treated or not treated with Ang II.

Methods
The experiments were performed on male Wistar rats (270 to 300 g) obtained from animal facilities of the State University of Campinas (Campinas, SP, Brazil). All procedures followed the university’s guidelines for the use of animals in experimental studies.

Antibodies and Chemicals
Rabbit polyclonal antibodies raised against NOS1, NOS3, and AT₁ were purchased from Santa Cruz Biotechnology. ¹²⁵I-labeled protein A was from Amersham. Ang II was from Calbiochem. All other reagent grade chemicals were from Sigma.

Rat Instrumentation and Arterial Pressure Monitoring
All surgical procedures were performed under aseptic conditions. Rats were anesthetized with a mixture of ketamine (70 mg/kg body wt IM) and diazepam (6 mg/kg body wt IM) and maintained at 37°C. Tygon-tipped polyvinyl cannulas were placed in the lower abdominal aorta and inferior vena cava throughout the femoral artery and vein, respectively. The cannulas were exteriorized at the back of the neck in a 2.5-cm length of stainless steel spring (0.5-cm diameter) attached to a swivel (Instech) at the top of an individual cage that allowed the animal to move freely in its cage while being infused. The animals received single doses of antibiotic (Pentabiótico Veterinário, 100 mg/kg body wt) and were allowed to recover for 5 days before the study. During this period, 0.9% saline was infused continuously through the venous catheter at a rate of 0.5 mL/h. After this period, saline was substituted for Ang II solutions in 2 different concentrations (10 and 40 ng · kg⁻¹ · min⁻¹) in the experimental animals, whereas control animals continued to receive only saline.

Arterial pressure was monitored daily for 6 days for a 1-hour period from 3:00 to 4:00 pm. The amplified signal was beat-to-beat recorded and sampled at 100 Hz with WINDAQ-PRO data acquisition software (DATAQ Instruments).

Tissue Homogenization
At the end of day 6 of Ang II infusion, the animals were anesthetized, hearts were rapidly removed, and the ventricles were minced coarsely and homogenized in ~10 volumes of solubilization buffer (1% Triton-X 100; 100 mMol/L Tris-HCl (pH 7.4); 100 mMol/L sodium pyrophosphate; 100 mMol/L sodium fluoride; 10 mMol/L EDTA; 10 mMol/L sodium vanadate; 2 mMol/L PMSF; and 0.1 mg aprotinin/mL) at 4°C with the polytron operated at maximum speed for 30 seconds. The extracts were centrifuged at 10 000 g for 30 minutes, and the supernatant was used for the assay. Protein concentrations were determined with the Bradford dye binding method. The supernatant was treated with Laemmlí’s sample buffer containing 100 mMol/L dithiothreitol and heated in a boiling water bath for 4 minutes and then subjected to SDS-PAGE (8% bis-acrylamide) in a Bio-Rad miniature gel apparatus (Mini-Protean, Bio-Rad Laboratories). An equal amount of total protein was used for all samples. Electrophoresis of proteins from the gel to nitrocellulose membrane was performed for 90 minutes at 120 V (constant).

Protein Analysis by Immunoblotting
The nitrocellulose membrane was preincubated in blocking buffer (5% nonfat dry milk, 100 mMol/L Tris, 250 mMol/L NaCl, and 0.02% Tween 20) overnight at 4°C. The membrane was then incubated with anti-NOS1, anti-NOS3, or anti-AT₁ receptor antibodies diluted in 10 mL of blocking buffer (3% BSA instead of nonfat dry milk) overnight at 4°C and washed for 60 minutes in blocking buffer without milk or BSA. The blots were subsequently incubated with 2 μCi of ¹²⁵I-labeled protein A (30 μCi/μg) in 10 mL of blocking buffer for 2 hours at room temperature, and then washed again for 30 minutes as described above. ¹²⁵I-labeled protein A bound to the specific antibodies was detected by autoradiography. Band intensities were quantified by optical densitometry of the developed autoradiographs.

Figure 1. Effects of a 6-day infusion of Ang II and saline (n=7) on mean arterial pressure (MAP). Ang II was infused at rates of 10 (n=8) and 40 ng · kg⁻¹ · min⁻¹. *P<0.05 vs values of saline-infused rats.

Tissue Preparation for Immunohistochemistry
Rats were heparinized, deeply anesthetized with pentobarbital sodium, and euthanized with a lethal dose of lidocaine. The ventricles were fixed by overnight immersion with 4% paraformaldehyde in 0.1 mol/L phosphate buffer, pH 7.4, and processed to inclusion in Histotex (Merck). Sections (5 μm) were transferred to poly-L-lysine-coated glass slides. The endogenous peroxidase activity was blocked by treatment with 0.03% H₂O₂ in 0.1 mol/L PBS at room temperature for 30 minutes. The sections were preincubated in blocking buffer (5% nonfat dry milk on 0.1 mol/L PBS) for 45 minutes at 37°C. The sections were extensively rinsed in 0.05 mol/L PBS and incubated with peroxidase-conjugated secondary antibodies (1:100) for 2 hours at 25°C. After washing as above, sections were subjected for 5 minutes to freshly prepared diamobenzidine that contained H₂O₂ (0.8%). Secondary antibody specificity was tested in a series of positive and negative control measurements. In the absence of primary antibodies, application of secondary antibodies (negative controls) failed to produce any significant staining.

Statistical Methods
Data are mean ± SEM of absolute (arterial pressure) or percent (blots) values. Differences among mean values were tested with a 2-way ANOVA for repeated measurements. Bonferroni’s multiple-range test was used as a post hoc analysis if the probability from the F test was <0.05.

Results
Effect of Ang II Infusion on Arterial Pressure
Figure 1 summarizes the effects of saline and Ang II infusion on mean arterial pressure over the course of a 6-day period (saline, n=7; 10 ng · kg⁻¹ · min⁻¹ Ang II, n=8; 40 ng · kg⁻¹ · min⁻¹ Ang II, n=7). The mean arterial pressure of saline-infused rats remained stable at ~110 mm Hg during the experimental period. No significant change was observed in mean arterial pressure of rats infused with Ang II at 10 ng · kg⁻¹ · min⁻¹, but rats infused with Ang II at 40 ng · kg⁻¹ · min⁻¹ showed a sustained increase in mean arterial pressure of ~15 mm Hg compared with saline-infused rats.

Effect of Ang II Infusion on Myocardial Expression of NOS1, NOS3, and AT₁
Representative Western blots and the average values (n=5) of densitometric readings of blots obtained with anti-NOS1
and anti-NOS3 antibodies are shown in Figure 2A and 2B. Single bands of 160 and 135 kDa were observed in blots of myocardial homogenates stained with anti-NOS1 and anti-NOS3 antibodies, respectively. Infusion of Ang II increased the protein expression levels of both NOS1 and NOS3 by \(\approx 200\%.\) Ang II infused at 10 and 40 ng \(\cdot\) kg\(^{-1}\) \(\cdot\) min\(^{-1}\) produced similar increases in the protein expression levels of NOS1 and NOS3.

A single 60-kDa band was observed in Western blots of the myocardium homogenates stained with anti-AT\(_1\) antibody (Figure 2C). Comparable protein expression levels of AT\(_1\) receptor were observed in saline- and Ang II–infused rats.

**Effect of Ang II Infusion on Myocardial Distribution of NOS1, NOS3, and AT\(_1\)**

Figure 3 shows the NOS1 staining in the left ventricle of saline- (Figure 3A and 3C) and 10 ng \(\cdot\) kg\(^{-1}\) \(\cdot\) min\(^{-1}\) Ang II–infused rats (Figure 3B and 3C). NOS1 staining was detected in cardiac myocytes (Figure 3A and 3B, arrows) and in the smooth muscle cells of small and large coronary vessels (Figure 3C and 3D, arrows and asterisks). No significant staining for NOS1 was detected in the endothelium or in the perivascular and interstitial tissues. Remarkable increases of NOS1 staining intensity were observed in cardiac myocytes and in the smooth muscle cells of coronary arteries of rats infused with Ang II. This effect was similar for Ang II infused at rates of 10 and 40 ng \(\cdot\) kg\(^{-1}\) \(\cdot\) min\(^{-1}\) (data not shown).

NOS3 staining was detected (Figure 4A and 4B) in the endothelium (arrowheads) and in perivascular and interstitial tissues (asterisks) of myocardial small and large coronary arteries. Virtually no reaction product was detected within the media of coronary arteries (Figure 4A and 4B) or cardiac myocytes (data not shown). Infusion of Ang II increased the staining intensity of both the perivascular and interstitial tissues and apparently left the endothelium staining unchanged. Again, this effect was similar for both Ang II infusion rates (data not shown).

Figure 5A and B shows the AT\(_1\) receptor staining in the myocardium of saline- and Ang II–infused rats. AT\(_1\) receptor was detected in cardiac myocytes (arrows), in the smooth muscle cells of small and large coronary arteries (arrowheads), and in the perivascular and interstitial tissues. No remarkable difference in the intensity or the distribution of the signal was detected in saline- or Ang II–infused rats (data not shown).

**Discussion**

The present study showed that long-term infusion of Ang II enhances the protein expression of NOS1 and NOS3 in the rat myocardium. This effect was not dependent on the pressor effect of Ang II because similar increases in the myocardial NOS1 and NOS3 protein levels were found in rats infused with subpressor and pressor doses of this hormone. Myocardial staining with specific antibodies against NOS1 and NOS3 confirmed and extended the observation of Western blot experiments to show that NOS1 and NOS3 are differentially expressed in myocardial cells. NOS1 was detected in cardiac myocytes and in smooth muscle cells of coronary arteries, but it could not be detected in the endothelium or perivascular and interstitial tissues. However, NOS3 was detected in the endothelium and perivascular and interstitial tissues, but it was not detected in cardiac myocytes or in coronary smooth muscle cells. Ang II infusion enhanced the tissue immunoreactivity for both NOS1 and NOS3 in their specific locations, but it did not change the distribution of these isoforms in myocardial structures. Finally, the myocardial staining with anti-AT\(_1\) antibody indicated that the AT\(_1\) receptor, the major effector of cardiac effects of Ang II, is expressed in cardiac myocytes, smooth muscle cells of coronary vessels, and perivascular and interstitial tissue. These data are compatible with the notion that Ang II
Figure 3. Light photomicrographs of sections of left ventricle from rats infused with saline (A, C) and Ang II (10 ng·kg\(^{-1}\)·min\(^{-1}\)) stained with anti-NOS1 antibody. These are representative examples of 5 different experiments. A, Cardiac myocytes demonstrating a light positive staining in saline-infused rats. B, Cardiac myocytes of a rat subjected to Ang II infusion, showing a marked staining for NOS1 (arrows). C, A large coronary artery of a rat infused with saline, demonstrating a light staining of smooth muscle cells (arrows). D, High magnification of a large coronary artery of Ang II-infused rat, demonstrating an intense staining of smooth muscle cells (asterisks).
modulates the protein expression of NOS1 and NOS3 and, consequently, the function of the NO system in the myocardial cells.

Although the results of the present study indicate that Ang II may regulate the expression of NOS1 and NOS3 in the rat myocardium, the mechanisms responsible for this phenomenon were not explored in the present study. However, because the effects of Ang II on NOS1 and NOS3 protein expression were demonstrated to be independent of the pressor effect of this hormone, it is reasonable to ascribe the enhanced expression of these NOS isoforms to the action of Ang II on myocardial cells, either directly or indirectly through the local release of autocrine/paracrine factors.

The mechanisms for the regulation of NOS1 and NOS3 expression in the myocardium are virtually unknown. However, the NOS1 promoter region has cis-acting elements such as AP-2, transcriptional enhancer factor-1, cAMP response binding element, and nuclear factor-κB, whereas NOS3 promoter region has Sp1, GATA, and cAMP-responsive element.13 Accordingly, Ang II regulates the activity of transcription factors such as nuclear factor-κB and GATA-4 in vascular smooth muscle cells and cardiac myocytes, respectively.14,15 These mechanisms could be implicated in the regulation of NOS1 and NOS3 expression during Ang II infusion. In addition, Ang II upregulates the cardiac expression of various growth factors such as transforming growth factor-β1, platelet-derived growth factor, and fibroblast growth factor, which potentially can induce gene expression, including the constitutive isoforms of NOS.13

Although NOS1 and NOS3 are known to be expressed in the heart, the cellular distribution of these 2 isoforms is still not clear.13 The anatomic distribution and physiological roles of NOS1 in the heart have been the subjects of relatively few reports to date. Early reports indicated that NOS1 is expressed exclusively in the myocardial neurons.16 More recent studies, however, demonstrated that cardiac myocytes, in addition to the neurons, indeed stain for NOS1.17 Our present data show that in addition to cardiac myocytes, NOS1 is also expressed in smooth muscle cells of coronary arteries. This localization of NOS1 in cardiac and smooth muscle cells may indicate a tissue-specific regulation. Accordingly, it is now well accepted that cardiac, skeletal, and smooth muscle cells express μNOS1, an elongated splice variant of NOS1.18,19 Whether or not the product detected in the present study is the μNOS1 isoform needs further study.
The results of the present study show a remarkable difference in the distribution of NOS1 and NOS3 in the myocardial cells. In addition to the expected endothelial location of NOS3, it was also detected in the interstitial and perivascular tissues. Increases in the staining produced by Ang II infusion were easily detected in the interstitial and perivascular tissues, but no staining could be detected in the endothelium. The reason for the absence of detectable changes in the NOS3 staining in the endothelium could be related to the narrow space occupied by the endothelial cell, which makes conclusions difficult in regard to changes in NOS3 expression by immunohistochemical analysis. The increases of NOS3 protein expression in cardiac interstitial and perivascular tissues induced by Ang II could be due to the well-known effect of angiotensin II on interstitial tissue proliferation and fibrosis.

NOS3 was not detected in significant amounts in cardiac myocytes. Although this result agrees with some of the early studies, it contrasts with more recent studies showing that cardiac myocytes indeed express NOS3. The reason for this discrepancy is not clear but could be related to antibody specificity against certain isoforms of NOS3 in cardiac myocytes.

Finally, we have shown that AT1 receptor is also expressed in cardiac and vascular smooth muscles, as well as in interstitial and perivascular tissues, the structures in which the increases in protein expression of NOS1 and NOS3 were detected. This suggests that Ang II could enhance NOS1 and NOS3 expression via AT1 receptor. However, the mediation via the angiotensin type 2 receptors is also possible.

In conclusion, the present study demonstrates that long-term infusion of Ang II is accompanied by an increase in the protein expression of NOS1 and NOS3 in the cells of rat myocardium, independent of changes in arterial pressure. The present data also provide evidence that NOS1 and NOS3 are differentially distributed in cardiac myocytes, coronary vessels, and interstitial tissue and that these NOS isoforms are upregulated by Ang II in these specific locations. The Ang II upregulation of NOS1 and NOS3 in myocardial cells could account for the impairment of the direct contractile and growth effects of this hormone on vascular smooth muscle cells, cardiac myocytes, and fibroblasts when plasma or tissue levels of Ang II are increased. This may favor functions such as local blood flow, modulation of oxygen consumption, and inhibition of fibroblasts, vascular smooth muscle cells, and cardiac myocyte growth effects of Ang II.

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References
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