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Clinical and Experimental Hypertension, 00(0): 1–7, (2010)

ISSN 1064-1963 print/1525-6006 online
DOI: 10.3109/10641963.2010.503297

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Nitric Oxide Modulates Reactivity to Angiotensin II in Internal Mammary Arterial Grafts in Hytertensive Patients Without Associated Risk Factors

Claudio Joo Turoni,[1] Rodrigo O. Marañón,[1] Víctor Proto,[2] Ramón Herrera,[2] María Peral de Bruno[1]

[1]*Departamento de Fisiología, INSIBIO-Universidad Nacional de Tucumán, Argentina,* [2]*Centro Modelo de Cardiología S.R.L. Tucumán, Argentina*

Abstract

We investigated the effects of extraendothelial nitric oxide (NO) on angiotensin II (Ang II) reactivity in internal mammary artery (IMA) rings, as well as the impact of hypertension without associated risk factors in this response. Vascular reactivity, NO levels, and resting membrane potentials were determined in hypertensive (HT) and normotensive (NT) IMA rings. Only rings with endothelial dysfunction were included. Ang II produced a dose-dependent contraction that was higher in HT rings. Response to Ang II was potentiated by N□-nitro L-arginine methyl ester (L-NAME) in NT but not in HT rings. The antioxidant agents tempol and diphenyleneiodonium (DPI) reverted the hyperreactivity to Ang II in HT rings. Extraendothelial NO was present in both NT and HT rings. However, NT rings showed higher values. L-NAME and S-methyl-L-thiocitrulline inhibited NO release in all cases. L-arginine reverted this inhibition. Both tempol and DPI increased NO release in both NT and HT rings. The number of vascular smooth muscle cells (VSMC) and anti-α-actin positive areas were lower in HT than in NT rings, without variations in wall thickness or wall/lumen ratio. With regard to resting membrane potential, we found in HT rings that the depolarization induced by Ang II was abolished by tempol. These findings suggest that extraendothelial NO counterregulates Ang II contractility in IMA rings; however, its action could be altered in hypertensive situations even though the patients did not have associated risk factors. We suggest two mechanisms: increased oxidative stress and a decreased ability of nNOS in VSMC to produce NO.

Keywords: coronary arteries, bypass graft, endothelial dysfunction, nitric oxide (NO), angiotensin II, hypertension

INTRODUCTION

The internal mammary artery (IMA) is the most frequently used vessel in coronary artery bypass graft surgery (CABG), since its utilization yields high short- and long-term graft patency rates. However, the mechanisms involved are not well understood. It has been suggested that nitric oxide (NO) plays a pivotal role in the early and long-term results of IMA graft patency. He and Liu demonstrated that IMA showed higher NO values than other vessels used in CABG (radial arteries) (1). Furthermore, Rakicia et al. showed that IMA presented major endothelium-dependent and NO-mediated vasodilation compared to venous grafts (2). The regulatory effect of the endothelium has been shown to be impaired in animal and human hypertension (3). Endothelial dysfunction, which is characterized by impairment of NO bioavailability, is an important risk factor for both hypertension and cardiovascular disease and may represent a major link between these conditions (4).

Angiotensin II (Ang II) is implicated in the control of vascular resistance through a vasocontractile effect. It has been shown that Ang II increases NO production in both animals (5, 6) and human endothelial cells (7); however, in the pathologic states Ang II decreases NO bioavailability through increases in the production of reactive oxygen species (ROS). In vitro, ROS has been shown to combine with NO, which results in quenching and a reduction in its biological activity (8). During early atherosclerotic plaque formation, the Ang II-AT1 receptor blocked improvement of endothelial function (9). In hypertension, Ang II leads to impaired endothelial relaxation (10). In our laboratory, using isolated vessels from spontaneously hypertensive rats (SHR), we demonstrated that Ang II increases ROS through the activation of NADPH oxidase (11). On the other

Address correspondence to María Peral de Bruno, INSIBIO - Universidad Nacional de Tucumán, Balcarce 32 (4000), Tucumán-Argentina. E-mail: mariaperal@ arnet.com.ar

Received 18 November 2009; revised 16 February 2010; accepted 25 February 2010.

hand, in SHR, it was reported that superoxide anion, through a decrease of endothelial NO, modify the vasoconstrictor response to Ang II (12); however, the role and interactions between NO and Ang II in IMA in the context of endothelial dysfunction is not well understood.

It is known that NO is produced by three distinct isoforms of NO synthase (NOS): inducible NOS (iNOS) and constitutive NOS: neural (nNOS) and endothelial (eNOS) (13). Under physiological conditions, eNOS appears to dominate; however, in some arterial beds nNOS has also been implicated in NO production. In our laboratory, we demonstrated that IMA, despite the absence of endothelial function, exhibited NO release via nNOS in vascular smooth muscle cells (VSMC) and that hypertension impaired this NO production (14). These findings are in agreement with those reported by Webb et al. in IMA and VS grafts (15) and by Buchwalow et al., who showed nNOS of VSMC from human arteries modulates arterial function independently of NO released from endothelial cells (16).

Based on the hypothesis that NO release from extraendothelial nNOS plays a role in the reactivity of IMA and that the hypertensive state increases oxidative stress, the goal of the present study was to determine, in a selected group of hypertensive patients without associated risk factors, if there is a counterregulatory effect of NO on the contractility response to Ang II in isolated IMA with endothelial dysfunction.

MATERIALS AND METHODS

Segments of IMA that would otherwise have been discarded were obtained from patients undergoing coronary artery bypass graft surgery (CABG) at the Centro Modelo de Cardiología (Tucumán, Argentina). In order to establish the impact of hypertension on a counterregulatory effect of NO during the Ang II response, strict inclusion criteria in relation to the risk factors were taken into account: Patients with diabetes, renal failure, pulmonary disease, peripheral vascular disease in clinical report, or uncontrolled dyslipemia and active smoking at the moment of surgery were not included. To test the influence of hypertension in vascular graft reactivity, patients were divided into two groups: hypertensive (HT) and normotensive (NT). The clinical characteristics of each are shown in Table 1. No significant differences were observed between NT vs HT with regard to pharmacologic treatments for coronary disease (aspirin: $p = 0.33$; hypolipemiants: $p = 0.92$; and clopidogrel: $p = 0.34$, NS: test difference between percentages). Informed consent according to institutional guidelines was obtained from each patient before surgery.

After surgery, IMA were immediately placed in Krebs solution maintained at 4°C and brought to the laboratory. The Krebs solution had the following composition (mM): NaCl 118.3; KCl 4.7; $CaCl_2$ 2.5; $MgSO_4$ 1.2; KH_2PO_4 1.2; $NaHCO_3$ 25; Glucose 11.1; Na_2EDTA 0.026. The blood vessels were dissected free of connective tissue and cut into 5 mm ring segments. The number of rings taken from each IMA varied from one to four.

Table 1. Clinical profile of the 21 study patients

	HT (n = 11)	NT (n = 10)
Age, years	66 ± 3	60 ± 4
Sex, male/female	7/4	9/1
Body mass index	26.3 ± 0.8	25.2 ± 1.2
Systolic/diastolic blood pressure at moment of the hospitalization (mmHg)*	123.6 ± 6/77.5 ± 5	121.4 ± 8/74.3 ±4
Antecedents of dyslipidemia, n (%)	4 (37)	3 (30)
Exsmokers, n (%)	6 (54)	5 (50)
Grafts per patient	2.1 ± 0.3	2.2 ± 0.4

*Blood pressure in HT patients was controlled with ACE inhibitors (82% of patients), beta blockers (73%), calcium antagonists 36%, and diuretics (18%).

Isometric Tension Measurement

Intact rings were suspended in organ chambers filled with 6 mL of Krebs solution maintained at 37°C, gassed with a mixture of 95% O_2 and 5% CO_2 (pH 7.4), and mounted between two stainless steel wires. One wire was anchored and the other was connected to an isometric force transducer (Gould UC2, USA) and a recorder (Kipp and Zonnen BD41, Holland). Isometric tension was measured under an initial tension of 2 g, which was found to be the optimal tension at which the depolarizing high-K^+ solution induced contraction. All preparations were allowed to equilibrate for 120 min and were washed with Krebs solution at 15 min intervals (equilibration period).

The function of endothelium was evaluated by a response to acetylcholine (Ach). For this purpose, cumulative dose response curves (CDRC) to Ach (10^{-9}–10^{-4} M) were obtained for precontracted NE (CDRC: 10^{-9}–10^{-4} M). In IMA rings from NT and HT patients, the maximal contractile response (R_{max}) of NE was 1629.6 ± 260.5 mg (n = 13) vs. 846.5 ± 91.6 (n = 11), in NT and HT, respectively ($p < 0.05$). Based on a previous paper in which we found that IMA rings had extraendothelial NO and that they were impaired in hypertension, the present study utilized only IMA rings that did not exhibit a response to Ach in both groups (NT:–2.5 ± 1.2% of NE R_{max} (n = 10) and HT: 1.1 ± 0.6% of NE R_{max} (n = 11), p: NS). Immunohistochemistry (monoclonal CD34 antibodies) after experiments were completed revealed the absence of endothelium in these rings.

Vascular reactivity to Ang II was studied in IMA rings from HT and NT patients. For this purpose,

CDRC to Ang II (10^{-9}–10^{-6} M) were determined. To evaluate whether NO modulates Ang II reactivity, paired IMA rings were pretreated with N□-nitro-L-arginine methyl ester (L-NAME) (inhibitor of NOS) (10^{-4} M). In addition, the effect of antioxidant agents, which enhance NO bioavailability, was evaluated in the contractile response to Ang II in IMA rings from HT patients. For this purpose, CDRC to Ang II (10^{-9}-10^{-6} M) in the presence of diphenylene iodonium (DPI, an inhibitor of flavin-containing enzymes including NADPH oxidase) (10^{-5}M) or tempol (a superoxide dismutase mimetic) (10^{-4} M) were also obtained.

Endothelium-independent relaxation was checked by response to sodium nitroprusside (SNP) in NE-precontracted rings. In all cases, SNP 10^{-5} M induced nearly complete relaxation

At the end of the experiments, maximal contractile force of developed VSMC was checked by the administration of a depolarizing solution of 100 mM KCl.

Calculation of Nitrite Release

Nitrite was measured by the Griess reaction. This technique (17) was previously adapted in our laboratory to measure nitrite release in human vessels. The Griess reaction, in which NO metabolites are transformed in diazoic-colored compounds, is one of the assays most frequently used to indirectly measure NO production. Two sets of standard curves were prepared for each experiment. N-(L-naphthyl) ethylenediamine (50 □ L of a 0.2% solution) and sulfanilamide (450 μL of a 0.1% solution) were added to each tube containing a standard (500 μL of 0, 1, 2.5, 5, 7.5, and 10 μM $NaNO_2$) or an experimental sample. The absorbance was measured at 540 nm with a spectrophotometer (SP 1103, Argentina) that was calibrated to zero with a blank solution. Nitrite absorbance was computed with the use of regression analysis (y = a + bx) and converted to a straight line. Only curves with a correlation coefficient > 0.95 were used.

We previously showed that stretching is an ideal condition for *in-vitro* nitrite dosage in aortic rings isolated from rabbits (18) and IMA grafts. Therefore, nitrite release was performed in this condition. For this purpose, samples (500 □ l) of the bath were extracted during the equilibration period for NT and HT IMA rings.

To evaluate the effect of NOS inhibition, some rings were treated for 30 min with L-NAME (10^{-4} M), L-NAME plus L-arginine (10^{-2} M), S-methyl-L-thiocitrulline (10^{-5} M) (nNOS inhibitor), or aminoguanidine (10^{-4} M) (iNOS inhibitor). Then, IMA rings were incubated with DPI (10^{-5} M) or tempol (10^{-4} M) for the first 30 min of the experiment.

Histologic Studies

To evaluate vascular structures and the presence of VSMC, we subjected samples of IMA rings to histologic and immunohistochemistry examination. For this purpose, after the experiment finished, some IMA segments were immediately fixed in buffer formol 10% (pH 7.4), embedded in paraffin, then cut into 3-□ m thick sections. In order to evaluate wall area, lumen area, and number of nuclei, slides were stained with hematoxylin-eosin and periodic acid Schiff (PAS). Images from transverse sections of the IMA segments were captured using a video camera connected to an optical microscope (×40). To evaluate density of VSMC, specific anti-α-actin antibody (Sigma-Aldrich, USA) was used. Briefly, paraffin sections were deparaffinized in xylol and rehydrated, in a graded alcohol series. Endogenous peroxidase was inhibited with H_2O_2 (3 %) in methanol. Sections were then washed in distilled water and heated in a citrate buffer (10 mM pH 6) for 15 min. Slides were incubated first with normal goat serum for 5 min and then for 30 min (20°C) with antibodies (dilution: 1/160). Following that, slides were incubated in a Link L Label IHC detection system (Bio Genex, USA). Antibody binding was revealed using JHC expressing H_2O_2 as a substrate and diaminobenzidine (DAB) as chromogen (liquid DaB; Bio Genex, USA). Counterstaining was performed with hematoxylin.

Histologic findings and immunohistochemistry-stained areas were measured using image analyzer software (Image J 1.36b).

Electrophysiologic Studies

As NO hyperpolarizes the resting membrane potential in arterial tissues (19) and membrane depolarization induced by Ang II is potentiated in the absence of endothelial function (20), we investigated the effect of Ang II on resting membrane potential. Because the hyperreactivity of the Ang II-contractile response was only observed in HT rings, we used HT rings in this experimental series. Internal mammary artery rings were cut open along the long axis before being pinned, intimal surface upwards, to the silicone rubber base of an organ chamber (volume: 5 ml) at 37°C and gassed with 95% O_2 and 5% CO_2. Internal mammary artery segments were immersed in Krebs solution. Smooth muscle cell impalement was performed from the intimal side of the vessels. The transmembrane potential was recorded with glass electrodes filled with 3 M KCl (tip resistance 50–80 Ω), which were connected to the headstage of a recording amplifier equipped with capacitance neutralization (Intra 767, WPI, USA). An Ag/AgCl pellet, in contact with the bath solution and directly connected to the amplifier, served as the reference electrode. The electrophysiological signal was continuously monitored on an oscilloscope and simultaneously recorded on paper (Gould Chart Recorder, USA). Successful impalements were signaled by a sudden negative drop in potential from the baseline (zero potential reference) followed by a stable negative potential for at least 10 min and were held under

current clamp conditions. The resting membrane potentials were measured after stimulation with L-NAME, or L-NAME plus Ang II. IMA segments were washed with Krebs solution and membrane potential was measured again to examine recovery after treatment with L-NAME.

Drugs

Human angiotensin II, norepinephrine (DL-arterenol), acetylcholine bromide, aminoguanidine, sodium nitroprusside, tempol, diphenylene iodonium, S-methyl-L-thiocitrulline, L-arginine, L-NAME and anti-α-actin antibodies were purchased from Sigma Chemical Company (St. Louis, MO). Stock solutions of the drugs were frozen (–4°C) in aliquots and freshly dissolved in distilled water to the appropriate concentrations, expressed as final molar concentrations in the organ bath.

Data Analysis

Data for contractility measurements are shown as milligrams (mg) of tension. Data for nitrite release are expressed in pmol/milligram of tissue. Detailed results are expressed as mean ± standard error (SE). Maximal contractile response (R_{max}) was only considered in those CDRC that reached a sustained effect or plateau. The pEC_{50} (negative log of molar concentration inducing 50% of the R_{max}) was calculated using a curve-fitting analysis program. For each concentration, the effective curve of the sigmoid equation of the curve fitting program "Graph–Pad" Prism 4.0 was used. Student's *t*-test was used for paired and nonpaired samples. In some cases, the data were analyzed by one-way ANOVA, and a Newman-Keuls test was used when appropriate. A nonparametric test and nonlinear estimation were performed with Statistica 5.0. Results were considered significant when $p < 0.05$.

RESULTS

Ang II Reactivity

Figure 1 shows the effect of Ang II and L-NAME in IMA rings without endothelium. Administration of Ang II (CDRC: 10^{-9}–10^{-5} M) produced a dose-dependent contraction in both NT and HT rings. However, with concentrations higher than 10^{-7}M contractile response to Ang II was higher in HT rings (Figure 1). Incubation with L-NAME (10^{-4}M) produced a significant increase in the response to Ang II only in NT rings that exhibited Ang II-CDRC similar to those of HT rings.

On the other hand, no significant difference in Ang II pEC_{50} between HT (–6.9 ± 0.1, n = 9) and NT rings (–6.5 ± 0.3, n = 8) was observed. Similar Ang II pEC_{50} values were observed in the presence of L-NAME (HT rings: –6.9 ± 0.1 (n = 8) vs. NT rings: –6.8 ± 0.0 (n = 4), p: NS).

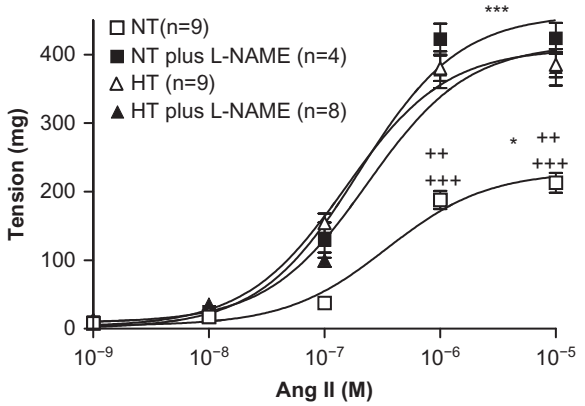

Figure 1. Cumulative dose-response curves (CDRC) to angiotensin II (Ang II) in HT (triangles) and NT (squares) IMA rings in the absence (white) or presence (black) of L-NAME (10^{-4} M). *p < 0.05 Ang II 10^{-6} to 10^{-5} vs. Ang II 10^{-9} to10^{-7} M in NT rings; ***p < 0.001 Ang II 10^{-6} to 10^{-5}M vs. Ang II 10^{-9} to10^{-7} M for the same group of rings in HT, HT plus L-NAME, and NT plus L-NAME; ++p < 0.01 NT vs. HT in absence or presence of same concentration of L-NAME; +++p < 0.001 NT in absence vs. presence of same concentration of L-NAME. ANOVA and Newman-Keuls post-hoc test were used. Data are expressed as mean ± standard error. The number of rings is given in parentheses.

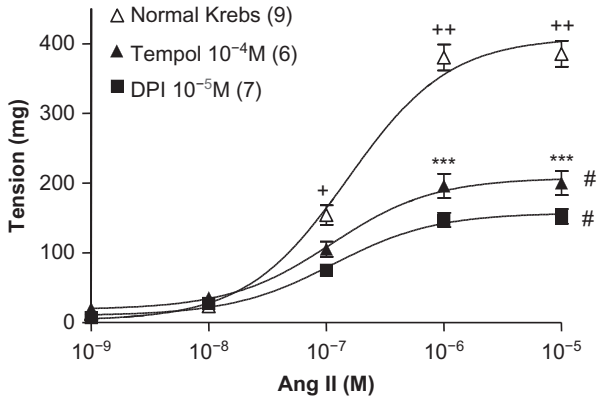

Figure 2. Cumulative dose-response curves (CDRC) to angiotensin II (Ang II) in HT IMA rings incubated with normal Krebs (white triangles), tempol 10^{-4} M (black triangles), or diphenylene iodonium 10^{-5} M (DPI, black squares). +p < 0.05 Ang II 10^{-7} M vs. Ang II 10^{-9} to 10^{-8} M in normal Krebs; ++p < 0.01 Ang II 10^{-6} to 10^{-5}M vs. Ang II 10^{-9} to 10^{-8}M in normal Krebs; ***p < 0.001 tempol and DPI vs. normal Krebs, same Ang II concentration; #p < 0.05 10^{-6} to 10^{-5}M vs. 10^{-9} to 10^{-8} M tempol and DPI. ANOVA and Newman-Keuls post-hoc test were used. Data are expressed as mean ± standard error. The number of rings is given in parentheses.

Figure 2 shows the effect of tempol and DPI in HT rings. Incubation with tempol (10^{-4}M) or DPI (10^{-5} M) decreased the Ang II contractile response, yielding Ang II-CDRC similar to those of NT rings. In NT rings, treatment with tempol or DPI did not affect the Ang II contractile response (data not shown). No difference in pEC_{50} was observed in any case. Aminoguanidine did not modify Ang II reactivity (data not shown).

In all cases, rubbing maneuvers did not modify IMA reactivity.

In contrast to the Ang II response, administration of KCl (100 mM) produced higher contractile responses in NT vs. HT rings (1746.5 ± 149.5 mg; n = 9 vs. 831.1 ± 64.8; n = 10, respectively; $p < 0.001$). In both NT and HT rings, incubation with L-NAME, tempol, or DPI did not modify the KCl contractile response (data not shown).

Nitrite contents

Nitrites were present in IMA rings without endothelium. Figure 3 shows nitrite contents and the effect of L-NAME and tempol in NT and HT rings. Higher values of nitrite contents was observed In NT rings as compared to HT rings ($p < 0.001$). Administration of L-NAME (10^{-4} M) inhibited nitrite levels in both NT and HT rings. However, administration of tempol (10^{-4} M) significantly increased nitrite content in both groups. Similar to tempol, DPI (10^{-5} M) increased nitrite content in both HT (□: 120.3 ± 29.5%; n = 8; $p < 0.001$) and NT rings (□: 141.0 ± 34.3%; n = 6; $p < 0.01$). Treatment with L-arginine blunted the effect of L-NAME on nitrite contents in both NT and HT rings (data not shown).

Similar to L-NAME, incubation with S-methyl-L-thiocitrulline (specific inhibitor of nNOS) significantly decreased nitrite levels (□□59.7 ± 3.0%; n = 8; $p < 0.001$). In contrast, aminoguanidine (10^{-4} M) did not modify nitrite levels in either NT (□□□.7 ± 1.6%; n = 7 and –3.9 ± 15.8%; n = 6, respectively; p: NS) or HT rings (□□1.0 ± 2.1%; n = 7 and 6.2 ± 10.8%; n = 9, respectively; p: NS).

Rubbing maneuvers did not modify nitrite levels in NT (□: 25.0 ± 18.8 pmol/mg tissue; n = 6; p: NS) or HT IMA rings (□: 2.0 ± 8.2 pmol/mg tissue; n = 8; p: NS).

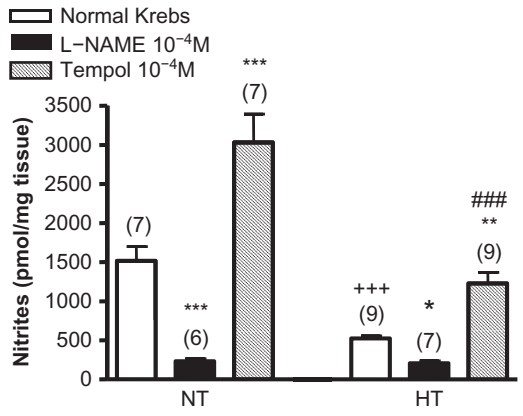

Figure 3. Nitrite levels and effect of L-NAME 10^{-4} M and tempol 10^{-4} M in IMA rings from NT and HT patients. *$p < 0.05$ vs. normal Krebs; **$p < 0.01$ vs. normal Krebs; ***$p < 0.001$ vs. normal Krebs; +++$p < 0.001$ vs. NT normal Krebs; ###$p < 0.001$ vs. NT tempol. ANOVA and Newman-Keuls post-hoc test were used. Data are expressed as mean ± standard error. The number of rings is given in parentheses.

Histologic findings

Figure 4 shows the number of VSMC nuclei, the anti-α-actin stained area and the wall thickness in both HT and NT rings. In HT rings, the number of VSMC was lower than in NT rings (Figure 4A). Similarly, the anti-α-actin stained area was reduced in HT as compared to NT rings (Figure 4B). However, no differences between NT and HT rings in wall thickness (Figure 4C), lumen (NT: 0.6 ± 0.1 mm^2; n = 8 vs. HT: 0.7 ± 0.1 mm^2 n = 7; p: NS), or lumen/wall ratio (NT rings: 0.14 ± 0.04; n = 8 vs. HT rings: 0.2 ± 0.08 n = 6; p: NS) were observed.

Electrophysiologic Studies

In HT rings, resting membrane potential was –21.2 ± 0.6 mV (n = 7). Administration of KCl (□□18.5 ± 3.2 mVolt; $p < 0.0001$; n = 7) and Ang II (10^{-6} M) produced depolarizations of resting membrane potential

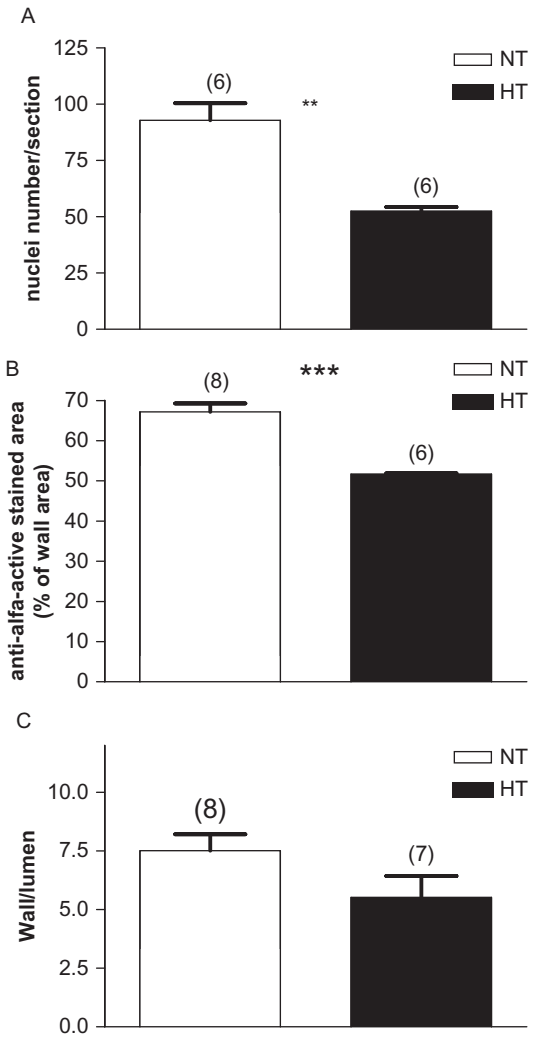

Figure 4. Measurement of (A) nuclei number/section area; (B) anti-α-actin-stained area; and (C) wall/lumen ratio in IMA rings from NT and HT patients. **$p < 0.01$; ***$p < 0.001$ (unpaired student's *t*-test). Data are expressed as mean ± standard error. The number of rings is given in parentheses.

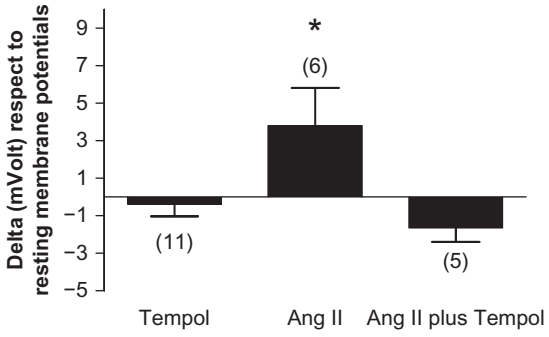

Figure 5. Effects of angiotensin II (Ang II) 10^{-6} M and tempol 10^{-4} M on the resting membrane potentials of unrubbed IMA rings from HT patients. Values are expressed as Delta (mVolt) with respect to resting membrane potentials. *$p < 0.05$ Ang II vs. tempol 10^{-4} M and Ang II plus tempol. ANOVA and Newman-Keuls post-hoc test were used. Data are expressed as mean ± standard error. The number of rings is given in parentheses.

(3.8 ± 2 mVolt; $p < 0.05$, $n = 6$). However, values obtained were lower ($p < 0.001$) than those observed with KCl. Figure 5 shows the effect of tempol on resting membrane potential and on Ang II-depolarization in HT rings. Treatment with tempol (10^{-4} M) did not modify resting membrane potential; however, not modify KCl or Ang II responses.

DISCUSSION

In IMA rings with endothelial dysfunction, we observed hyperreactivity to Ang II in HT patients. This increase in Ang II contractile response was dependent only on hypertension, since the patients did not present other associated risk factors. Similarly, Pompilio et al. reported that hypertension is the major risk factor involved in the hyperreactivity of IMA rings to different agonists (21). Some authors showed a relationship between contractile response to Ang II and other risk factors presented in patients under CABG. Rueda-Clausen et al. found that hyperreactivity to Ang II was associated with the presence of abdominal obesity (22). On the other hand, in animal models, hypertension also induced hyperreactivity to Ang II. In spontaneously hypertensive rats, hydroxyl radical stress induced hyperreactivity to Ang II (23).

Based on the fact that NO contraregulates the Ang II contractile effect and that we observed extraendothelial NO release in IMA rings (14), we hypothesized that hyperreactivity to Ang II in HT rings may be due to decreased extraendothelial NO bioavailability. This hypothesis is supported by our finding that the NOS inhibitor L-NAME increases the contractile response to Ang II only in NT rings. Similar values for the Ang II pEC_{50} between HT and NT rings indicate that modifications in Ang II-receptor agonist affinity are not responsible.

Another finding that supports decreased extraendothelial NO bioavailability in HT rings is the fact that Ang II hyperreactivity is decreased by antioxidant agents (tempol and DPI). In this regard, we postulated that tempol, as well as DPI, principally act through an increase of NO biodisponibilty. However, an additional effect independent of NO could not be ruled out. In this sense, it is known that an increase of superoxide anion not only produced a diminished level of NO bioavailability (24), but also had a direct effect on vascular tone (25). Both mechanisms could be modulated Ang II VSMC reactivity (12, 26). Moreover, the lack of effect of L-NAME in HT rings is in agreement with reports in which it was demonstrated that hypertension decreases NO bioavailability (4,11) through an increase in oxidative stress (27).

We found that extraendothelial NO release was higher in NT than HT rings. This finding is in agreement with the higher reactivity to Ang II in HT rings. The fact that L-NAME and S-methyl-L-thiocitrulline decreased nitrite release in HT and NT rings indicates that nNOS is involved in NO release in both cases. The fact that both tempol and DPI increased nitrite levels in both HT and NT rings suggests that oxidative stress may be implicated in the NO bioavailability of these patients. If well, DPI also might inhibit other flavin-dependent enzymes, like nNOS. The fact that a significative increase in nitrite contents after DPI treatment was obtained suggest that action of this agent in IMA rings with endothelial dysfunction, mainly acts through inhibition of of NADPH oxidase. In addition, other finding that supports an effect of diphenyleneiodonium (DPI) increasing NO is the fact that decreased Ang II reactivity was obtained after treatment with this agent in HT rings (Figure 2). Furthermore, a fact to be considered is that nitrite levels after DPI and tempol treatment were elevated in NT rings. We hypothesized that these antioxidant agents not only prevented NO quenching (28) in NT rings, but also that these patients presented an increased ability to produce NO through nNOS in VSMC. In this sense, our results showed that NT rings presented a higher number of VSMC and higher stained α-actin area density than HT rings. This is in agreement with the fact that maximal contractile response of VSMC induced by KCl (100 mM) was decreased in HT rings, indicating a lower proportion of functional VSMC.

Finally, with respect to the effect of NO on resting membrane potential, the finding that L-NAME did not modify resting membrane potential levels and that tempol reverted Ang II depolarization is in agreement with the fact that NO bioavailability is decreased in HT rings. Since hypertension induces an increased vascular Ang II response and this may be accompanied by impaired regulation of the resting membrane potential in VSMC (29), we suggest that the lack of an NO-hyperpolarizing effect may be implicated in the hyperreactivity to Ang II in HT rings.

The present work demonstrates that extraendothelial NO counterregulates Ang II contractility in IMA rings. However, its action could be altered in hypertensive

situations, although patients did not have associated risk factors. We suggest two mechanisms: 1) increased oxidative stress leading to altered resting membrane potential and vascular reactivity; and 2) a decreased ability of nNOS to produce NO due to a reduced number of VSMC.

ACKNOWLEDGMENTS

This work was supported by grants from the Consejo de Investigaciones de la Universidad Nacional de Tucumán (CIUNT) and the Consejo de Investigaciones Científicas y Técnicas de la República Argentina (INSIBIO-CONICET). The authors thank Residencia of Centro Modelo de Cardiología.

AQ23 **Declaration of interest:**

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