The Positive Inotropic Effect of Angiotensin II
Role of Endothelin-1 and Reactive Oxygen Species

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Abstract—Many effects believed to be because of angiotensin II (Ang II) are attributable to the action of endothelin (ET)-1, which is released/produced by Ang II. We investigated whether Ang II elicits its positive inotropic effect (PIE) by the action of endogenous ET-1, in addition to the role played by reactive oxygen species (ROS) in this mechanism. Cat cardiomyocytes were used for: (1) sarcomere shortening measurements; (2) ROS measurements by epifluorescence; (3) immunohistochemical staining for preproET-1, BigET-1, and ET-1; and (4) measurement of preproET-1 mRNA by RT-PCR. Cells were exposed to 1 nmol/L Ang II for 15 minutes. This low concentration of Ang II increases sarcomere shortening by 29.2±3.7% (P<0.05). This PIE was abrogated by Na⁺/H⁺ exchanger or Na⁺/Ca²⁺ exchanger reverse mode inhibition. The production of ROS increased in response to Ang II treatment (ΔROS respect to control: 68±15 fluorescence units; P<0.05). The Ang II–induced PIE and ROS production were blocked by the Ang II type 1 receptor blocker losartan, the nonselective ET-1 receptor blocker TAK044, the selective ETA receptor blocker BQ-123, or the ROS scavenger N-(2-mercapto-propionyl)glycine. Exogenous ET-1 (0.4 nmol/L) induced a similar PIE and increase in ROS production to those caused by Ang II. Immunostaining for preproET-1, BigET-1, and ET-1 was positive in cardiomyocytes. The preproET-1 mRNA abundance increased from 100±4.6% in control to 241.9±39.9% in Ang II–treated cells (P<0.05). We conclude that the PIE after exposure to 1 nmol/L Ang II is due to endogenous ET-1 acting through the ETA receptor and triggering ROS production, Na⁺/H⁺ exchanger stimulation, and Na⁺/Ca²⁺ exchanger reverse mode activation. (Hypertension. 2006;47:727-734.)

Key Words: membranes ■ ion channels ■ oxidative stress ■ receptors, angiotensin

There is a large amount of evidence to demonstrate that several effects believed to be attributed to angiotensin II (Ang II) are caused by the release/production of endothelin (ET) from different cells. Ito et al reported Ang II–induced hypertrophy in neonatal rat cardiomyocytes via the release/production of ET-1. The resulting hypertrophy was prevented by blocking ETA receptors or by using antisense oligonucleotides directed against preproET mRNA. In addition, Liang and Gardner, also working with rat neonatal myocytes, reported that the increase in brain natriuretic peptide (BNP) gene promoter activity induced by Ang II was prevented by blocking ETα receptors. Furthermore, in vivo studies have also shown that several effects of Ang II can be attenuated or abolished by blocking ET receptors. These findings strongly suggest that Ang II induces the release/production of ET-1 from cells which, acting in an autocrine fashion on ET receptors, trigger intracellular signals.

Ang II is a classical stimulus for NADPH oxidase and superoxide production, and a role for this intracellular signaling pathway has been proposed to explain several physiological effects of this peptide. Alternatively, the stimulation of NADPH oxidase by ET-1 and the involvement of this mechanism in determining some of its physiological effects have also been reported. The aim of the present study, which was performed in isolated adult cat ventricular myocytes was to determine: (1) whether the release/production of ET-1 by Ang II could be detected in adult cardiomyocytes, (2) its implications in cardiac contractility, and (3) the role played by reactive oxygen species (ROS) in this mechanism.

Methods

The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (Publication No. 85-23, revised 1996).

Myocytes Isolation

Cat ventricular myocytes were isolated according to the technique described previously.
Sarcomere Shortening

To measure sarcomere length (SL), myocytes were placed in a perfusion chamber on the stage of an inverted microscope (Nikon) and continuously superfused with a solution containing (mM) 5 KCl, 118 NaCl, 1.2 MgSO₄, 0.8 MgCl₂, 1.35 CaCl₂, 10 glucose, and 20 NaHCO₃ (pH 7.4) after continuous bubbling with 5% CO₂ and 95% O₂. The myocytes were stimulated via 2-platinum electrodes on either side of the bath at 0.5 Hz. The SL was recorded by a specific software (Ion Wizard). The myocytes were observed using a video camera connected to the microscope. The SL was measured in a determined region of the myocyte. The software estimated the most frequent SL in that region using fast Fourier transform analysis (Ion Optix). The SL was measured at 30°C (TC2, Cell micro controls).

RNA Measurements by RT-PCR

Total RNA was isolated from the cardiomyocytes suspension using the RNeasy kit (Qiagen) according to the manufacturer’s instructions. RNA (0.8 μg) was reverse-transcribed using the Omniscript RT kit (Qiagen). A dilution of the resulting cDNA was used to quantify the relative content of mRNA by real-time PCR (iCycler IQ Real-Time PCR Detection System, Bio-Rad) using appropriate primers and SYBR Green as fluorescent probe.¹³ The following primers designed using Primer3 software were used: GAPDH: forward primer 5'-GGGTGTGAAACGAGAACAT-3'; reverse primer 5'-CCACAGTCTTCTGAGTGCA-3'; preproET-1: forward primer 5'-CAGACAAAGAACGTCCGAGCC-3'; reverse primer 5'-GGTCTTGATGACTGCTTGCTGA-3'; preproET-3: forward primer 5'-TCTCCACAGACGCTTACG-3'; reverse primer 5'-TGACTTAGCTTGGTCATG-3'; and ET converting enzyme 1 (ECE-1): forward primer 5'-ACAAGCTCTTTTCTGACCA-3'; 3'-GCCAGGTGTTTCTTGT-5'. PCR reactions were performed with TaqDNA polymerase (Invitrogen). Fluorescence data were acquired at the end of extension. A melt analysis was run for all of the products to determine the specificity of the amplification. The cycle threshold values for each gene were measured and calculated by computer software (iCycler IQ OSS, version 3.0a, Bio-Rad). The

Figure 1. Effect of Ang II on SL shortening of cardiac isolated myocytes. (A) Continuous recording of a myocyte SL shortening before and after the addition of 1 nmol/L Ang II, which induced an increase in contractility of ~30%. (B) Individual traces corresponding with “a” and “b” of A. (C) average changes in SL shortening (n=11), expressed as % of initial length (L₀). (D) Dose-response curve for different concentrations of Ang II, from 1 to 500 nmol/L in the absence or presence of TAK044 (1 μmol/L). The maximal PIE was obtained with 100 nmol/L Ang II. TAK044 shifted the dose-response curve to the right and completely blocked the inotropic effect of 1 nmol/L Ang II indicating that this effect was entirely caused by the action of the endogenous ET released/produced by Ang II. However, the data show that concentrations >1 nmol/L are activating other mechanisms than the autocrine signal triggered by Ang II. *P<0.05 vs control.
cycle threshold values for each gene were normalized by those GAPDH in the same sample. The primers for preproET-1 and GAPDH amplification were designed against the feline sequences. With respect to preproET-3 and ECE-1, the primers were designed against the rat sequence for these genes, because the feline sequences have not been published yet. For every experiment of real-time RT-PCR we obtained a melting curve of the amplified gene where it could be appreciated that only a specific product was amplified in each case with no primer-dimer formation.

**Reactive Oxygen Species Measurements**

Intracellular reactive oxygen species (ROS) production was measured by epifluorescence. The myocytes were incubated with 20 μmol/L dichloro-hydrofluorescein diacetate at room temperature for 1 hour. After washing with extracellular solution, the myocytes were placed on a perfusion chamber of an inverted microscope and excited at 480 nm. The emitted light was collected at 530 nm by a photomultiplier connected to a digital converter and stored in a computer (Ionoptix system). Before obtaining the signal, background fluorescence was subtracted.

**Immunohistochemistry**

Myocyte suspensions were fixed with 4% paraformaldehyde in phosphate buffer during 30 minutes and washed in 5% sucrose in phosphate-buffered saline (PBS). They were additionally incubated for 48 hours with antibodies against preproET (preproET-1, Bachem T-4306, rabbit polyclonal against fragment 94-09 or Bachem T-4751, rabbit polyclonal against fragment 110-30), BigET-1 (Bachem T-4572, rabbit polyclonal), and ET-1 (rabbit polyclonal, Bachem T-4050 or mouse monoclonal, clone TR.ET.48.5., Oncogene). Epitopes recognized by the antiserum against preproET-1 are not present in the mature peptides. On the other hand, mature peptides are recognized by the antiserum against preproET-3. Positive controls were made by replacing the primary antibody with its diluent.15

Glycerol mounted specimens were observed with the Laser Scanning System Radiance 2000 (BioRad) using the 488 line of the argon laser followed by the 543 line of a helium-neon laser and the emission filters HQ515/30 and HQ590/70, respectively. Optical sections (1 μm) were performed in the z axis, and images were processed using Lasersharp 2000 (BioRad) and Confocal Assistant Software (BioRad).

**Statistics**

Data are expressed as mean±SEM. Paired t test or repeated-measures 1-way ANOVA followed by Student–Newman–Keuls as post-hoc test were used as appropriate. A P<0.05 was considered to indicate significant differences.

**Drugs**

BQ123 and N-(2-mercapto-propionyl)glycine (MPG) were purchased from Sigma Chemical. TAK044 and HOE642 were kindly donated by Takeda Chemical and Aventis Pharma, respectively. KB-R7943 and Losartan were purchased from Tocris and Merk, Sharp, and Dhome, respectively.

The drugs used in the present study did not significantly affect basal contractility, with the exception of KB-R7943, which slightly but statistically significantly decreased basal contractility (SL shortening relative to initial SL were 5.1±0.4% in control and 4.2±0.5% in KB-R7943; P<0.05). Although the nonselective actions of this compound were mainly reported to be present at higher doses than that used in this study,16,17 we cannot rule out completely an action of this compound mediated through mechanisms other than the inhibition of the Na+/Ca2+ exchanger (NCX) reverse mode.

**Results**

Figure 1A and 1B shows the positive inotropic effect (PIE) induced by 1 nmol/L Ang II on a continuous recording of a myocyte SL. The overall results obtained using 11 myocytes are shown in Figure 1C. Ang II induced an increase in SL shortening of ~30% over basal levels, a PIE similar to that observed previously in cat papillary muscles.18 Figure 1D shows the concentration-effect curve of Ang II before and after the inhibition of the ET receptors by the nonselective blocker (blocks ETα/ETβ) TAK044 (1 μmol/L). These results indicate that Ang II induces a concentration-dependent increase in sarcomere shortening, reaching the maximal effect at concentrations of 100 nmol/L (~70% increase in basal contractility). The PIE induced by 500 nmol/L Ang II was lower than that observed with 100 nmol/L. ET receptor blockade induces a downward shift in the concentration–effect curve. This shift decreased the maximal effect of Ang II by ~30% and cancelled the effect of 1 nmol/L Ang II. Therefore, these findings demonstrate that the PIE induced by 1 nmol/L Ang II is entirely attributable to an autocrine pathway involving an ET isoform. Concentrations >1 nmol/L are, perhaps, also acting through mechanisms other than the autocrine signals triggered by ET and are beyond the scope of the present study. Therefore, based on these results, we decided...
to analyze in detail the PIE induced by 1 nmol/L Ang II, which is mediated by the autocrine effect of ET.

Figure 2 shows that the PIE induced by 1 nmol/L Ang II was totally prevented by pretreatment of the myocytes with losartan (1 μmol/L) indicating that the release/production of ET is entirely attributable to stimulation of the Ang II type 1 (AT1) receptors. The effect of Ang II was also blunted by TAK044 and by the ETA blocker BQ123 (10 μmol/L; Figure 2). Therefore, 1 nmol/L of Ang II induces an increase in inotropism through release/production of ET after stimulation of its AT1 receptor.

We have shown previously that the PIE produced by 1 nmol/L Ang II in cat papillary muscles was totally prevented by the inhibition of the Na+/H+ exchanger (NHE)-1 or of the reverse mode of the NCX with the selective blockers HOE642 and KB-R7943, respectively.18 Results similar to those we reported previously in papillary muscles were obtained in the isolated myocytes (Figure 2). These results indicate that the activation of the NHE-1 and the stimulation of the NCX reverse mode are the only mechanisms by which that concentration of Ang II increases contractility.

To provide additional evidence that Ang II induces the release/production of ET from the myocyte, a number of experiments were performed in which isolated cat myocytes were exposed to 1 nmol/L Ang II, and preproET-1, preproET-3, and ECE-1 mRNAs were measured. After 15 minutes of exposure to Ang II, isolated cat ventricular myocytes showed a significant increase in the expression of preproET-1 mRNA (Figure 3A) but not in preproET-3 or ECE-1 mRNAs (Figure 3B and 3C). The Ang II–induced increase in preproET-1 mRNA was blocked by losartan (Figure 3A).

The concentrated cardiomyocyte suspension used in RT-PCR experiments could possibly be contaminated with non-myocytes (fibroblasts, endothelial cells, etc), leading to a misinterpretation of the above results. Since the presence of the synthetic and processing machinery for ET in adult cardiomyocytes is controversial,19,20 and species differences might exist, we examined the potential presence of preproET-1, BigET-1, and ET-1 in cat cardiomyocytes by immunohistochemistry. The signal for preproET-1, BigET-1, and ET-1 was positive in cardiac myocytes (Figure 4). Positive staining was observed in 5 of 5 preparations. Immunostaining of isolated myocytes was obtained with all of the tested antibodies. No significant differences were observed with monoclonal or polyclonal anti-ET1 antibodies or with antibodies against different fragments of preproET-1. Immunoreactivity was present in the myocyte cytoplasm and absent from myocyte nuclei. The presence of immunoreactivity for preproET-1, BigET1, and ET-1 indicates that myocytes contain the synthetic and processing machinery for ET.

Since the generation of ROS by Ang II and/or ET-1 is well established,5–11 the effect of the ROS scavenger, MPG (1 mmol/L), on the PIE induced by the autocrine pathway of Ang II/ET was examined. This compound has been confirmed previously as a scavenger of the hydroxyl radical.21,22 The Ang II–induced PIE was totally prevented by pretreatment of myocytes with MPG (Figure 2).

To determine whether the ROS generated by Ang II results in the release/production of ET or whether ROS are acting downstream of ET, we examined the effect of the scavenger MPG on the PIE induced by exogenous ET-1 applied at a concentration (0.4 nmol/L) that resulted equipotent to the effect of 1 nmol/L Ang II. The PIE induced by 0.4 nmol/L ET-1 was inhibited by MPG (Figure 5), suggesting that the ROS are acting downstream to the activation of the ETA receptors by ET-1. Therefore, it appears that Ang II–induced ET-1 release stimulates an increase in ROS generation, which, in turn, triggers the increase in contractility. To evaluate this hypothesis, we assessed the role that ET-1 plays in ROS generation using epifluorescence techniques. Figure 6 shows that Ang II increased ROS production and that this increase was blunted by the nonselective blockade of ET.
receptors. A representative time course of these experiments is shown in Figure 6A. Figure 6B depicts the average increase in ROS after 15 minutes of Ang II treatment and the blocking effect of MPG, losartan, TAK044, or BQ123 on ROS production. The suppression of ROS production is consistent with the lack of Ang II–induced PIE in the presence of MPG. The possibility that TAK044 was acting as a scavenger was eliminated, because this compound failed to prevent the H$_2$O$_2$ (5 μmol/L)-induced increase in contractility (H$_2$O$_2$: 112%±9%, n=4; H$_2$O$_2$+TAK044: 112%±20%, n=4) and in ROS production (n=2, data not shown). Finally, we detected that 0.4 nmol/L ET-1 induced a similar increase in ROS production (ΔROS with respect to the control: 101±36 fluorescence units, n=5) to that produced by 1 nmol/L Ang II (Figure 6B). Thus, the data indicate that the production of ROS by Ang II is downstream to the Ang II–mediated release/production of ET-1.

**Discussion**

The present study shows that Ang II induces the release/production of ET, which acts in an autocrine fashion to trigger ROS production and the intracellular signaling that leads to the Ang II–induced PIE. Herein, we detected the presence of proET-1, BigET-1, and ET-1 in cardiomyocytes and found that Ang II was able to increase the preproET-1 mRNA level in the cardiomyocyte suspension. These results, together with the results of contractility and epifluorescence, which were performed in isolated myocytes in a flow-superfusing chamber, suggest that the interaction between Ang II and ET-1 involves an autocrine mechanism. It was reported recently that the hypertrophic action of tri-iodothyronine was completely abolished by cardiomyocyte-specific disruption of the ET-1 gene, which supports the autocrine action of this peptide. However, the potential presence of endothelial cells in the cardiomyocyte suspension used in the present study does not allow us to unequivocally disregard cells other than myocytes as putative sources of this peptide. Therefore, although unlikely, the possibility exists that the increase in ET-1 mRNA detected in response to Ang II could be produced, at least in part, by those nonmyocyte cells.

Although the RT-PCR experiments performed in the present work do not elucidate the mechanism by which Ang II induces the release/production of ET, they suggest that Ang II...
increases the de novo production of ET-1 in the isolated myocytes. It seems unlikely that the increase in preproET-1 mRNA levels in 15 minutes on Ang II exposure could explain the acute PIE induced by this peptide during that time frame, because production of mature ET-1 to be released by the myocyte would require a greater time period. However, it appears valid to assume, if the translation efficiency is not altered, that the Ang II–induced release of preformed ET-1 may be followed by an increase in its mRNA abundance to restore the intracellular pools. On the other hand, we were unable to detect changes in ECE-1 mRNA after 15 minutes of Ang II. This finding in adult cat myocytes is in accordance with the report of Xia and Karmazyn23 in cultured neonatal rat cardiomyocytes treated with Ang II in which an increase in preproET-1 mRNA without any change in ECE mRNA was detected.

In the present study, we have shown that TAK044 was able to completely block the PIE induced by 1 nmol/L Ang II and to partially inhibit the PIE induced by higher concentrations of this peptide. Two alternative explanations can be proposed for the inability of this blocker to suppress the PIE induced by the higher concentrations of Ang II tested: (1) incomplete blockade of ET receptors by TAK044, or (2) an inotropic effect mediated through a different mechanism than that triggered by the autocrine effect of ET-1. The latter possibility appears more likely considering that we12,24 and others25,26 have reported the stimulation of L-type calcium channels by concentrations of Ang II >1 nmol/L. Nevertheless, we would like to emphasize that concentrations of 1 nmol/L are similar to the physiological concentration range of Ang II in the interstitial myocardium27 and are perhaps the most relevant concentrations involved in pathophysiological actions.

It was reported recently that the production of ROS is not involved in the negative inotropic effect of 1 nmol/L Ang II observed in rat cardiomyocytes.28 The discrepancy in the opposite inotropic effects and in the involvement of ROS in these Ang II–induced effects could be attributed to species differences and/or to the significantly higher doses (1000-fold) used in rat myocytes.29 Furthermore, it is important to mention that ET receptors do not appear to play any role in the contractile effects of Ang II on rodent cardiomyocytes.29,30

The experiments in the present study were performed in the presence of extracellular HCO₃⁻. Thus, the activation of the NHE would lead to an increase in intracellular Na⁺ in the absence of changes in intracellular pH,18,31,32 making it unlikely that changes in intracellular pH are the cause of the inotropic changes. The reverse mode of the NCX appears to be the mechanism playing a pivotal role in the Ang II–induced PIE, considering previous data obtained in papillary muscles18 and the data presented herein. In addition, the fact that the Ang II/ET pathway induced similar inotropic responses in multicellular preparations and in isolated myocytes and that, in both cases, these responses were prevented by NHE or NCX reverse-mode inhibition, enable us to suggest that the mechanism involved is of an autocrine nature, with the myocyte being the source and target of endogenous ET-1.

The finding that Ang II increases ROS production was expected according to several reports, which indicate that Ang II stimulates NADPH oxidase.5–7 Surprisingly, this augmented ROS production induced by Ang II was abolished by blocking ETA receptors. Nevertheless, the stimulation of NADPH oxidase by ET-1 has been reported previously9,10 in addition to the role played by ROS generation after ET-1 in determining the increase in contractility in the atria.11 Furthermore, in smooth muscle, vasopressin exerts the increase in superoxide levels through the action of endogenous ET-1.33 It remains currently unknown whether or not every effect of Ang II on ROS level is mediated through ET-1. However, based on the present findings and those of Ortiz et al,34 it appears that at least the effects of suppressor doses of Ang II are mediated through ET-1.

The mechanism by which Ang II releases/produces ET was not investigated in the present study. A recent study performed in smooth muscle myocytes suggested that Ang II...
induces the synthesis of ET-1 through the production of superoxide and the stimulation of extracellular-regulated kinase 1/2. However, in the present study, ROS production and the PIE induced by Ang II were abolished by blocking the ET receptors. However, the possibility that a certain amount of superoxide not detected in our ROS measurements is inducing ET synthesis and that the ET released by this mechanism is additionally increasing the production of the same or another ROS from the same or another source cannot be completely ruled out. In relation to this, Kimura et al. suggested recently that low levels of superoxide produced by Ang II-induced NADPH oxidase activation might be responsible for triggering the production of a greater level of ROS by mitochondria (ROS-induced ROS-release mechanism). Moreover, in smooth muscle, it was reported that Ang II increases ROS production by the stimulation of the NADPH oxidase, whereas ET-1 increases ROS production through mitochondrial-derived pathways. In summary, although we can certainly conclude that the ET released/produced by Ang II is promoting the production of ROS, we cannot disregard the possibility that, in the Ang II/ET autocrine pathway described in the present work, ROS may be involved in multiple steps.

Perspectives

Our results in adult cat ventricular myocytes are, perhaps, relevant to the hypertrophic effect of Ang II. In the present work, we present evidence that Ang II, through its AT1 receptors, increased the release/production of ET-1, which enhances the activity of the NHE-1 through ROS generation. The central role of ET-1 in cardiac hypertrophy is well established. Inactivation of the genes for Go11 and Goq, which transduce the signal from the ET receptors, produces mice resistant to pressure-overload hypertrophy. Furthermore, cardiac hypertrophy induced by tri-iodotironine was mice resistant to pressure-overload hypertrophy. Further-This study was partly supported by grants (PICT 05-08512 to H.E.C. and PICT 12412 to M.C.C.d.H.) of the Agencia Nacional de Promocion Cientifica y Tecnologica de Argentina. HOE642 and TAK044 were kindly provided by Aventis Pharma (Germany) and Takeda Pharmaceuticals (Japan), respectively. H.E.C., I.L.E., M.C.C.d.H., A.M.S., and E.A.A. are Established Investigators of Consejo Nacional de Investigaciones Cientificas y Tecnicas, Argentina. M.C.V.-A., V.T., and C.G. are Fellows of Consejo Nacional de Investigaciones Cientificas y Tecnicas, Argentina. M.V.C. is a Fellow of the Agencia de Promocion Cientifica de Argentina.

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