Up-Regulation of AT₁ and AT₂ Receptors in Postinfarcted Hypertrophied Myocytes and Stretch-Mediated Apoptotic Cell Death

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To determine whether up-regulation of AT₁ and AT₂ receptors occurred in hypertrophied myocytes after infarction and whether AT₂ played a role in stretch-mediated apoptosis, left ventricular myocytes were dissociated from the surviving portion of the wall 8 days after coronary occlusion and cardiac failure in rats. Control cells were obtained from sham-operated animals. Myocytes were stretched in an equibiaxial stretch apparatus and angiotensin II (Ang II) formation and cell death were measured 3 and 12 hours later. AT₁ and AT₂ proteins were evaluated in freshly isolated myocytes and after stretch. The effects of AT₁ and AT₂ antagonists on stretch-induced Ang II synthesis and apoptosis were also established. Myocardial infarction increased AT₁ and AT₂ in myocytes and stretch further up-regulated these receptors. Ang II levels were higher in postinfarcted myocytes and this peptide increased with the duration of stretch in both groups of cells. Similarly, apoptosis increased with time in control and postinfarcted myocytes. Absolute values of Ang II and apoptosis were greater in myocytes from infarcted hearts at 3 and 12 hours after stretch. Addition of AT₁ blocker to cultures inhibited stretch-activated apoptosis in both myocyte populations as well as the generation of Ang II in postinfarcted myocytes. In contrast, AT₂ antagonists had no impact on these cellular events. In conclusion, Ang II stimulated cell death through AT₁ receptor activation, whereas ligand binding to AT₂ receptor did not alter Ang II concentration and apoptosis in normal and postinfarcted hypertrophied myocytes. (*Am J Pathol* 2000, 156:1663–1672)

The renin-angiotensin system (RAS) is up-regulated after myocardial infarction (MI)¹ and long-term administration of angiotensin-converting enzyme inhibitors improves survival and reduces morbidity and mortality due to cardiovascular events.² Myocyte death affects acutely^{3,4}

and chronically^{5,6} the viable myocardium of the infarcted heart, but different implications have been attributed to the loss of cells in the early and late phases of the ischemic myopathy. The immediate effect of coronary artery occlusion and ventricular dysfunction is a sudden increase in cavitory volume and a decrease in mural thickness, mediated by scattered apoptotic cell death^{3,4} and side-to-side slippage of myocytes within the ventricular wall.^{7,8} Myocyte apoptosis continues during the progression of the disease and may condition the onset of terminal failure.^{5,6,9} Chronic loss of myocytes may impact predominantly on the amount of functioning tissue and pump performance. The initiation and evolution of ischemic cardiomyopathy are characterized hemodynamically by an increase in left ventricular end-diastolic pressure and diastolic wall stress which result in sustained sarcomere stretching.^{10,11} Load-dependent sarcomere elongation *in vivo* up-regulates the cellular RAS leading to an increase in surface angiotensin II (Ang II) receptors on myocytes and local synthesis of Ang II.^{12–14} Similar results have been obtained *in vitro* in stretched myocytes,^{15–18} in which ligand binding to AT₁ receptor has been linked to the activation of the endogenous cell death pathway.^{17,18} Two questions remain to be answered: 1) are AT₁ receptors only present in adult myocytes under normal and pathological states;^{12,14,19–21} and 2) is myocyte apoptosis triggered by Ang II stimulation of AT₁ receptors,^{17,18,22,23} AT₂ receptors,^{21,24} or the consequence of a balance between the response of these receptor subtypes to Ang II?²¹ For this purpose, control myocytes and hypertrophied myocytes, surviving an 8-day myocardial infarct, were studied. This was done because Ang II binding sites are doubled in postinfarcted myocytes at this interval.¹² Myocytes were dissociated and stretched in an equibiaxial stretch apparatus for 3 and 12 hours. The expression of AT₁ and AT₂ receptors was determined at baseline and after stretch. Similarly, Ang II formation, extent of apoptosis, and influence of AT₁ and AT₂ receptor antagonists, alone and in combination, on Ang II formation and cell death with stretch were established.

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Figure 1. Culture of nonstretched (**A, C**) and stretched (**B, D**) left ventricular myocytes obtained from an infarcted heart at 8 days. Nuclei are stained by propidium iodide (yellow) and the cytoplasm by α -sarcomeric actin (red). **Arrows** in **A** and **C** indicate myocytes which are shown at higher magnification in **B** and **D**. **Arrowheads** in **B** and **D** define groups of 10 sarcomeres each. Confocal microscopy: **A** and **B**, $\times 100$; **C** and **D**, $\times 800$.

Materials and Methods

Myocardial Infarction and Cardiac Function

Under ether anesthesia, MI was induced in 84 male Sprague-Dawley rats (Charles River Breeding Labs, North Wilmington, MA), weighing approximately 250 g.^{12,13,25} To reduce pain, buprenorphine hydrochloride was administered (0.65 mg/kg body weight, intramuscularly; Brufenex, Reckitt and Colman Pharmaceuticals, Richmond, VA). Thirty-seven infarcted rats died shortly after the operation; 47 infarcted rats were used. Thirty-one sham-operated (SO) rats were used as controls. Animals were killed 8 days after coronary occlusion or sham-operation. Before sacrifice, animals were anesthetized with chloral hydrate (300 mg/kg body weight, intraperitoneally), and measurements of left ventricular and right ventricular pressures and rate of pressure rise, $+dP/dt$, and decay, $-dP/dt$, were obtained.^{12-14,25} Protocols were approved by the New York Medical College.

Myocyte Isolation and Culture

Hearts were placed on a stainless steel cannula for retrograde perfusion through the aorta and left ventricular myocytes were enzymatically dissociated. The solutions were supplements of modified commercial minimum essential medium (MEM) Joklik (Sigma Chemical Co., St. Louis, MO).^{12-14,17,18,22,23,25} The cell isolation procedure, used for SO and MI hearts, consisted of three main steps: 1) calcium-free perfusion: blood wash-out and collagenase (selected type II, Worthington Biochemical Corp., Freehold, NJ) perfusion of the heart were carried out at 37°C with HEPES/MEM gassed with 85% O₂ and 15% N₂. 2) Mechanical tissue dissociation: after the heart was removed from the cannula, the left ventricle, inclusive of the septum, was separated from the right ventricular free wall and minced. Pieces were subsequently shaken in resuspension medium containing collagenase. 3) Separation of intact cells: intact cells were enriched by

centrifugation at $30 \times g$ for 3 minutes. Supernatant was discarded. This procedure was repeated four times to remove nonmyocytes, cellular debris, and residual collagenase. Myocytes were resuspended in Percoll solution (final concentration 41%) and centrifuged for 10 minutes at $34 \times g$. Intact cells were recovered and washed. Smears were made to control the purity of the preparation. Nonmyocytes constituted 1 to 2% of the cells.^{12–14,25} The average number of myocytes obtained from the left ventricle was 6 to 7×10^6 and 3.3×10^6 in SO and MI rats, respectively. Myocytes were plated on a silicon rubber substrate (Figure 1) and stretched in an equibiaxial strain device.^{17,18,26} Stretching effects on cell injury were established by exposing cultures to ethidium monoazide bromide (EMB; Molecular Probes, Eugene, CA). EMB binds to nucleic acids only in cells with membrane breakage.²⁷ Nonstretched and stretched myocytes from noninfarcted and infarcted hearts were evaluated at 3 and 12 hours. In some cultures, the AT₁ antagonist, losartan (Merck, Rahway, NJ), and/or the AT₂ antagonist, PD123319 (Parke-Davis, Ann Arbor, MI), were added at 10^{-8} mol/L 30 minutes before stretch, and kept for the duration of the experiment. Cells were collected for histochemical and molecular assays.^{17,18}

Myocyte Volume

Myocyte dimensions were measured in freshly isolated binucleated myocytes with a computer system: 200 cells from each control and infarcted ventricle were analyzed to collect length, width, and area. Isolated myocytes flatten and the cross-sectional area resembles an ellipse. The minor-to-major axis ratio of the ellipse was obtained by examining 40 cells in each case by confocal microscopy. These values were used to compute myocyte volume.²⁵

Terminal Deoxynucleotidyl Transferase and Taq Polymerase In Situ Ligation Assays

Cultures were incubated with 5 units of 2.5 mmol/L CoCl₂, 0.2 mol/L potassium cacodylate, 25 mmol/L Tris-HCl, 0.25% bovine serum albumin, and 0.5 nmol/L biotin-16-deoxyuridine triphosphate (dUTP). After exposure to fluorescein-labeled Extravidin (Sigma), cytoplasm was stained by α -sarcomeric actin and nuclei by propidium iodide. Double-strand DNA fragments for *in situ* ligation to 3' overhangs were prepared by polymerase chain reaction using primers 5'-GTGGCCTGCCAAGCTC-TACCT-3' and 5'-GGCTGGTCTGCCGTTTTCGAC-CCTG-3' complementary to pBluescript-bSD11 plasmid.^{28,29} Digoxigenin-dUTP fragments were ligated to DNA using T4 ligase.^{28,29} The number of myocytes labeled by terminal deoxynucleotidyl transferase or Taq was determined by examining 2000 to 3000 cells in each condition by confocal microscopy.^{6,17,18,29}

Western Blot

Myocytes were incubated with lysis buffer (50 mmol/L Tris/HCl, pH 7.5, 5 mmol/L EDTA, 250 mmol/L NaCl, 0.1%

Figure 2. Stretched ventricular myocytes (**A** and **B**) obtained from two infarcted hearts at 8 days. Myocyte nuclei labeled by EMB (yellow; **arrows**) are apparent; myocyte cytoplasm is stained by α -sarcomeric actin (red). EMB-negative myocytes are also shown. Confocal microscopy, $\times 300$.

Triton X-100) containing protease inhibitors (0.2 mmol/L phenylmethylsulfonyl fluoride, 1 μ g/ml aprotinin, 5 mmol/L dithiothreitol, 1 mmol/L Na₃VO₄) on ice for 30 minutes. Equivalents of 50 μ g of myocyte proteins were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to nitrocellulose and exposed to rabbit anti-human AT₁ (306, Santa Cruz, Santa Cruz, CA) and goat anti-human AT₂ (C-18, Santa Cruz) at a concentration of 1 μ g/ml in Tris-buffered saline/Tween 20 (TBST). Bound antibodies were detected by peroxidase-conjugated anti-rabbit and anti-goat IgG. AT₁ was detected as a 41-kd band and AT₂ as a 44-kd band.^{17,18,30} Human cloned AT₁ receptor (BioSignal, Montreal, Canada) was used as positive control.

Ang II

Ang II in conditioned medium was measured by enzyme-linked immunosorbent assay (Peninsula Laboratories, San Carlos, CA). Conditioned medium was treated with

Figure 3. Western blot of AT₁ (A) and AT₂ (B) receptors in freshly (F) isolated, nonstretched (NS), and stretched (S) myocytes for 3 (3) and 12 (12) hours, obtained from SO and MI hearts. Loading of proteins is illustrated by Coomassie blue staining. AT1R, human cloned AT₁ receptor protein. Optical density values for AT₁ and AT₂ are shown in C; *n* = 6 in each determination. *, difference, *P* < 0.05, from myocytes obtained from SO animals; †, difference, *P* < 0.05, from NS myocytes.

10% trifluoroacetic acid and centrifuged. Supernatant was dried and dissolved in 0.1% trifluoroacetic acid. Ang II was purified using a C18 Sep-Pak column (Waters Associates, Milford, MA). Ang II fraction was eluted from the column with 30% acetonitrile in 5 ml of 0.1% trifluoroacetic acid, dried, and dissolved in 0.25 ml of TBST. Samples were analyzed in a microtiter plate using Ang II antibody (1:32,000) and a tracer, biotinylated Ang II. The microtiter plate was treated with streptavidin/horseradish peroxidase. Color reaction was developed with tetramethyl-benzidine substrate and terminated by 2 N HCl. Concentration was calculated from standard curves.¹⁸

Data Analysis

Results are presented as means ± SD. Autoradiograms were analyzed densitometrically by an image analyzer (Gel Doc 1000, Bio-Rad, Hercules, CA). Significance between two values, *P* < 0.05, was determined by Student's *t*-test and among multiple preparations by the Bonferroni method.³¹ In the text and in figure legends *n* values for each determination are listed.

Results

Cardiac Function and Myocyte Volume

MI at 8 days was associated with a 6% and 7% reduction in body weight (SO: 305 ± 30 g, *n* = 10; MI: 286 ± 22 g, *n* = 25; NS) and heart weight which were not significant (SO: 914 ± 83 mg, *n* = 10; MI: 974 ± 90 mg, *n* = 25; NS). Infarcted hearts showed a threefold increase in left-ventricular end-diastolic pressure (SO: 7 ± 2.4 mmHg, *n* = 10; MI: 22 ± 4 mmHg, *n* = 25; *P* < 0.001) and an 18% decrease in left-ventricular systolic pressure (SO: 105 ± 8 mmHg, *n* = 10; MI: 86 ± 15 mmHg, *n* = 25; *P* < 0.001). Left ventricular +dP/dt was reduced 33% (SO: 9,976 ± 928 mmHg/second, *n* = 10; MI: 6,726 ± 902 mmHg/second, *n* = 25; *P* < 0.001) and -dP/dt 35% (SO: 8,840 ± 803 mmHg/second, *n* = 10; MI: 5,747 ± 934 mmHg/second, *n* = 25; *P* < 0.001). After infarction, right-ventricular end-diastolic pressure increased 2.4-fold (SO: 2.7 ± 1.8 mmHg, *n* = 10; MI: 6.5 ± 2.6 mmHg, *n* = 25; *P* < 0.001) and right ventricular systolic pressure 11% (SO: 27 ± 2.2 mmHg, *n* = 10; MI: 30 ± 3 mmHg, *n* = 25; *P* < 0.05). right ventricular +dP/dt (SO: 2,923 ± 399 mmHg/second, *n* = 10; MI: 2,675 ± 450 mmHg/second,

Figure 4. Effects of 3 (3h) and 12 (12h) hours of stretch (S) on the generation of Ang II from myocytes obtained from SO and MI hearts (A). B and C illustrate the impact of losartan (Los) and PD12319 (PD) on the formation of Ang II in myocytes from MI hearts stretched for 3 (B) and 12 (C) hours. NS, nonstretched myocytes. Results are means $\pm$ SD; $n = 5$ in each determination with two exceptions: $n = 6$ in nontreated S-myocytes from MI hearts in B and C. *, difference from NS-myocytes. In A, † indicates a difference from myocytes from SO hearts. In B and C, † indicates a difference from nontreated S-myocytes from MI hearts.

$n = 25$; NS) and right ventricular $-dP/dt$ (SO: $2,508 \pm 258$ mmHg/second, $n = 10$; MI: $2,330 \pm 458$ mmHg/second, $n = 25$; NS) did not change. Moreover, the volume of binucleated myocytes increased 45% ($P < 0.001$) with cardiac failure, from $25,300 \pm 2,200 \mu m^3$ ($n = 6$) in controls to $36,800 \pm 4,900 \mu m^3$ ($n = 6$) in infarcted hearts. These parameters, obtained by confocal microscopy, were the product of cross-sectional area (controls: $234 \pm 19 \mu m^2$, $n = 6$; infarcts: $283 \pm 37 \mu m^2$, $n = 6$; $P < 0.05$) and length (controls: $108 \pm 6 \mu m$, $n = 6$; infarcts: $130 \pm 13 \mu m$, $n = 6$; $P < 0.005$) measurements, evaluated separately in each animal.

Myocyte Stretch

A strain of 20% produced a 12% ($P < 0.001$) increase in sarcomere length in myocytes from SO animals, from $1.820 \pm 0.022 \mu m$ to $2.037 \pm 0.030 \mu m$ ($n = 61$). Corresponding values in MI rats were $1.821 \pm 0.024 \mu m$ and $2.040 \pm 0.032 \mu m$ ($n = 61$; $P < 0.001$). Sarcomere length changes were constant in different preparations, but reflected 60% the extent of strain. Stretch did not increase the fraction of myocytes with plasma membrane damage identified by EMB labeling of nuclei (Figure 2, A and B). At baseline, ie, 24 hours after plating, and 3 and 12 hours later in the absence of stretch, $0.58 \pm 0.24\%$ ($n = 5$), $0.68 \pm 0.35\%$ ($n = 5$), and $0.73 \pm 0.28\%$ ($n = 5$) control myocytes were EMB-positive. Corresponding values in control stretched myocytes were $0.74 \pm 0.41\%$ ($n = 5$), $0.49 \pm 0.33\%$ ($n = 5$), and $0.64 \pm 0.39\%$ ($n = 5$). In postinfarcted nonstretched myocytes, $0.62 \pm 0.32\%$ ($n = 5$), $0.55 \pm 0.37\%$ ($n = 5$), and $0.40 \pm 0.22\%$ ($n = 5$) of cells were stained by EMB. Percentages of EMB-labeled cells in stretched postinfarcted myocytes were $0.56 \pm 0.31\%$ ($n = 5$), $0.66 \pm 0.41\%$ ($n = 5$), and $0.71 \pm 0.29\%$ ($n = 5$).

Myocyte Stretch and AT₁ and AT₂

Figure 3, A and B, illustrates the detection of AT₁ and AT₂ receptor in myocytes before and after stretching. The densitometric analysis of the changes in protein quantity is shown in Figure 3C. AT₁ protein was 1.8-fold ($P < 0.001$) higher in freshly isolated myocytes from infarcted hearts than in cells from control animals. Three hours of stretch had no effect on the quantity of AT₁ in myocytes from SO and MI rats but, at 12 hours, a 2.0-fold ($P < 0.001$) and 2.4-fold ($P < 0.001$) increase in AT₁ expression was detected in the two groups of cells, respectively. Up-regulation of AT₁ in myocytes, induced *in vitro* by the mechanical stimulus, left essentially intact the difference in AT₁ between SO and MI animals. Similarly, AT₂ increased 1.7-fold ($P < 0.001$) in myocytes dissociated from infarcted rats and stretch at 3 hours enhanced AT₂ quantity by 1.9-fold ($P < 0.001$ - $P < 0.002$) in control and stressed cells; 12 hours of stretch resulted in a 2.2-fold ($P < 0.001$) and 2.3-fold ($P < 0.001$) accumulation of AT₂ in myocytes from SO and MI rats, respectively.

Figure 5. (Starts on facing page) Apoptosis in two binucleated (**A–C**; **G–I**) and one mononucleated (**D–F**) myocyte from post-MI cells stretched for 3 (**A–C**; **G–I**) and 12 (**D–F**) hours. Apoptosis, characterized by nuclear fragmentation, cellular shrinkage, and partial loss of contractile material, was apparent in the two cells shown in panels **A–F**, exhibiting advanced phases of cell death. Cellular morphology was much more preserved in the early stages of apoptosis depicted in panels **G–I**. Confocal microscopy: **A–F**, $\times 1,200$; **G–I**, $\times 1,000$.

Myocyte Stretch and Ang II Formation

Figure 4A illustrates that the concentration of Ang II in conditioned medium was 42% ($P < 0.05$) and 52% ($P < 0.05$) higher at 3 and 12 hours in cultures of nonstretched myocytes from MI animals than in culture of nonstretched

Figure 6. Effects of 3 (**A**) and 12 (**B**) hours of stretch (**S**) on *Taq*-labeled myocytes obtained from SO and MI hearts in the presence of losartan (**Los**), PD123319 (**PD**), and losartan and PD123319 (**L+P**). **NS**, nonstretched myocytes. Results are means $\pm$ SD; $n = 8$ in each group. *, difference from **NS**-myocytes; †, difference from **S**-myocytes kept in **SFM**; ‡, difference from **S**-myocytes from **SO** hearts.

myocytes from **SO** rats. Stretch of control cells for 3 and 12 hours increased Ang II formation 66% ($P < 0.005$) and 91% ($P < 0.001$), respectively. Corresponding increases associated with stretch of postinfarcted myocytes were 62% ($P < 0.001$) and 97% ($P < 0.001$). The concentration of Ang II in conditioned medium of stretched **MI** myocytes at 3 and 12 hours had values 39% ($P < 0.001$) and 57% ($P < 0.001$) greater than those in conditioned medium of stretched control myocytes. Stretching of postinfarcted myocytes in the presence of 10^{-8} mol/L losartan, 10^{-8} mol/L PD123319 and losartan, or PD123319, had different consequences on Ang II generation (Figure 4, **B** and **C**). Losartan inhibited the synthesis of Ang II produced by 3 and 12 hours of stretch of **MI** myocytes. PD123319 did not interfere with Ang II secretion or enhance the impact of losartan on stretch-mediated release of Ang II.

Myocyte Stretch and Apoptosis

Apoptosis was measured by confocal microscopy. Nuclei were identified by the red fluorescence of propidium iodide, and double-strand DNA cleavage with single-

base 3' overhang was recognized by the green fluorescence of *Taq* labeling. Myocytes were distinguished by the red fluorescence of α -sarcomeric actin staining. Yellow fluorescence of nuclei reflected the combination of propidium iodide and *Taq* labeling (Figure 5, A–I). Alterations in chromatin structure are apparent in the apoptotic myocytes shown in Figure 5, A–F, whereas nuclear morphology was preserved in the dying cell illustrated in Figure 5, G–I. The shape of the two first dying myocytes was altered by cellular shrinkage (Figure 5, C and F) and partial loss of myofibrillar structures (Figure 5C). In contrast, cell morphology was primarily preserved in the myocyte shown in Figure 5, G and I. Similar observations were made when terminal deoxynucleotidyl transferase was used.

Sarcomere elongation for 3 and 12 hours increased apoptosis sixfold ($P < 0.001$) and 5.9-fold ($P < 0.001$) in myocytes from SO hearts, and fivefold ($P < 0.001$) and 6.6-fold ($P < 0.001$) in myocytes from MI hearts (Figure 6, A and B). However, values in MI myocytes were 1.39-fold ($P < 0.001$) and 1.44-fold ($P < 0.001$) greater than in control cells at 3 and 12 hours after stretch. In the absence of stretch, baseline apoptosis was consistently higher in myocytes from MI rats. Losartan prevented stretch-induced apoptosis in both groups of myocytes. PD123319 had no effect on stretch-activated cell death (Figures 6, A and B). Because PD123319 had no impact on Ang II formation and apoptosis, Ang II-mediated cell death most likely occurred through ligand binding to the AT_1 receptor subtype. This is consistent with results obtained by the addition of nanomolar concentrations of Ang II to myocyte cultures pretreated with losartan.²³ Under this setting, apoptosis was inhibited despite the presence of Ang II in the medium.

Discussion

Results in this study indicate that hypertrophied left ventricular myocytes, surviving for 8 days a myocardial infarct and cardiac failure, were characterized by enhanced expression of AT_1 and AT_2 receptors and increased generation of Ang II *in vitro*. These conditions did not attenuate the response of these cells to mechanical stretch in terms of up-regulation of Ang II receptor subtypes, synthesis and secretion of Ang II, and Ang II-mediated apoptosis. Sarcomere elongation produced qualitatively identical consequences in control myocytes. Inhibition of ligand binding to AT_1 receptors prevented the activation of cell death promoted by stretch and Ang II formation in both myocyte populations. AT_2 receptor had no effect on the ability of Ang II to initiate apoptosis after stretch in myocytes from noninfarcted and infarcted hearts.

AT₁ and AT₂ Receptors and Cardiac Myocyte Death

The role of AT_1 and AT_2 receptor subtypes in the activation of apoptotic death signals varies in different cell

populations. Ligand binding to AT_2 receptors in PC12W and R3T3 cells, which do not express AT_1 receptors, leads to DNA fragmentation³² possibly through dephosphorylation of the survival factor mitogen-activated protein kinase. Mitogen-activated protein kinase phosphatase-1 dephosphorylates Bcl-2 inhibiting its antiapoptotic effect.²⁴ AT_2 receptor stimulation triggers apoptosis in vascular smooth muscle cells by antagonizing extracellular signal-regulated protein kinase (ERK) function, whereas AT_1 receptor opposes the initiation of the endogenous cell death pathway.^{33–35} The AT_1 receptor seems to prevent the impact of AT_2 on ERK activity.³⁴ Neonatal rat cardiac myocytes have been claimed to behave in a similar manner after exposure to Ang II *in vitro*.^{36,37} These observations are at variance with the results of the current study in which myocyte death mediated by Ang II occurred *via* AT_1 receptor exclusively; AT_2 receptor activation had no influence on cell viability of stretched myocytes.

Primary cultures of adult myocytes from SO and MI hearts showed a qualitatively identical response to stretch-induced synthesis and release of Ang II. Acute MI increased significantly the expression of AT_1 and AT_2 receptors on the remaining hypertrophied cells, but the AT_2 receptor had no effect on the enhanced generation of ligand and level of myocyte apoptosis after the imposition of the mechanical stimulus. Pretreatment of myocytes from infarcted hearts with losartan blocked the formation of Ang II and the increase of cell death associated with 3 and 12 hours of stretch. Conversely, PD123319 alone did not interfere with the consequences of stretch on these cellular parameters. Moreover, the combination of losartan and PD123319 did not modify the results obtained by losartan only. Similar findings on the role of AT_1 and AT_2 in cell survival have previously been obtained in cultures of neonatal³⁸ and adult²⁰ myocytes exposed to Ang II. The discrepancy between our results and those indicated above^{36,37} is difficult to explain. However, our *in vitro* experimentation used Ang II at 10^{-9} mol/L,^{20,38} whereas concentrations 500,000³⁶ and 500³⁷ times higher were used in the other studies. In a comparable manner, doses of 10^{-7} to 10^{-8} mol/L of losartan and PD123319 were used by us which contrast the utilization of concentrations of these Ang II receptor antagonists 10,000,000³⁶ and 1,000³⁷ times greater. Our preparations of cardiac myocytes were all done in serum-free medium, but an initial 5% and a subsequent 0.5% serum, in combination with Ang II, was used in the cited work. Baseline apoptosis was markedly lower in our cultures, because the effects of changes in serum levels were avoided. Although these factors may account for some of the differences in the accumulated data, they still leave unexplained the contrasting results on the regulatory function of AT_1 and AT_2 receptors on myocyte death.

Myocardial Infarction, AT₁ Receptor, Ang II Formation, and Myocyte Death

Myocytes surviving ischemic injury are characterized by hypertrophy^{1,8} and up-regulation of the local RAS which

involves enhanced expression of angiotensinogen,³⁹ renin,¹⁴ angiotensin converting enzyme⁴⁰ and, as shown here in nonstretched cells, an increased formation of Ang II. Ang II binding sites in myocytes are also increased^{12–14} and this change in receptor density comprises both AT₁ and AT₂ subtypes. Additionally, these myocytes are more susceptible to apoptotic death signals; Bcl-2 quantity is reduced and Bax is increased,⁴¹ possibly through activation of p53 function.^{42,43} Forced induction of p53 in adult ventricular myocytes alters the Bcl-2-to-Bax protein ratio, up-regulates the local RAS, and potentiates apoptosis.²² Mechanical stretch mimics the effects of p53 overexpression on these cellular events.¹⁷

Postinfarcted hypertrophied myocytes are subjected to sarcomere elongation *in vivo*^{1,8} and this structural modification may be implicated in the changes in the local RAS detected in cultured myocytes in the absence of stretch. Moreover, these cells responded to stretch by enhancing the synthesis of Ang II and Ang II receptors. Although p53 function was not characterized in the current investigation, inhibition of the AT₁ effector pathway prevented the formation and secretion of Ang II and myocyte apoptosis. This intervention may have interfered with protein kinase C phosphorylation of the C-terminal of p53,⁴⁴ down-regulating the p53-dependent gene, angiotensinogen⁴⁵ and the generation of Ang II. *In vitro* competition with mutated p53 abrogates stretch-mediated up-regulation of angiotensinogen, AT₁ receptor, Ang II synthesis, and cell death despite an increased expression of renin, angiotensin converting enzyme, and AT₂ receptor in myocytes.⁴⁵ These observations are consistent with the notion that p53 is a major regulator of myocyte RAS and ligand-binding to AT₁ receptors triggers apoptosis. Protein kinase C phosphorylation of L-type Ca²⁺ channels, elevation in cytosolic Ca²⁺, activation of Ca²⁺-dependent DNase I and, ultimately, DNA fragmentation have been implemented in the execution of the death process.^{17,20,38} Apoptosis was found here to consist of double-strand cleavage of the DNA with single-base 3' overhang which occurs only through the stimulation of Ca²⁺-dependent DNase I.²⁸

The *in vitro* model of myocyte stretching and apoptosis requires some comments. The equibiaxial stretch apparatus leads to a stable condition of mechanical deformation of myocytes that does not apply to any *in vivo* state. It mimics only in part the diastolic stretch associated with increases in end-diastolic pressure and diastolic wall stress. Some similarities with the model may be found in the surviving myocardium after infarction in which lengthening of sarcomeres and a marked elevation in diastolic loading occur in the absence of alterations in the interstitial compartment two days after coronary artery occlusion.^{8,46} Myocyte apoptosis is the exclusive form of cell death found in this viable portion of the wall.⁴¹ Necrosis does not participate in the remodeling process of the acutely postinfarcted heart.⁴¹ However, the type of myocyte stretching used here cannot be assumed to reflect the increases in preload with anemia or dynamic exercise. Whether stretching of neonatal and adult ventricular myocytes produces comparable results is difficult to es-

tablish. Neonatal myocytes differ significantly from adult cells; a large portion of the cytoplasm is undifferentiated, DNA replication occurs in more than 10% of the cell population, and their volume is only 5% of adult cells.⁴⁷

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