Effect of AT\textsubscript{1} receptor blockade on cardiac apoptosis in angiotensin II-induced hypertension

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Angiotensin II (ANG II) via AT\textsubscript{1} receptors induces apoptosis in cardiomyocytes in vitro. We tested the hypothesis that in vivo AT\textsubscript{1} receptor stimulation is accompanied by cardiac apoptosis and attempted to elucidate the molecular mechanisms involved in the death signaling pathway. Male Sprague-Dawley rats received ANG II (120 ng·kg\textsuperscript{-1}·min\textsuperscript{-1}·sc) for 7 days with or without the AT\textsubscript{1} receptor antagonist losartan (10 mg·kg\textsuperscript{-1}·day\textsuperscript{-1} orally). Cardiac function was assessed by echocardiography. Apoptosis in the heart was detected and quantified by in situ TdT-mediated dUTP nick-end labeling (TUNEL) and radiolabeled DNA laddering. Expression of bax, bel-2, caspase 3, and AT\textsubscript{1} and AT\textsubscript{2} receptors was examined by Western blot analysis. Activity of caspase 3 was also measured by a fluorometric immunosorbent enzyme assay. Tail cuff systolic blood pressure was elevated ($P < 0.01, n = 6$) in ANG II-infused rats (173 ± 3 mmHg) versus controls (111 ± 2 mmHg) and reduced by losartan (134 ± 4 mmHg). Cardiac function was essentially unchanged in ANG II-infused rats. Increased internucleosomal DNA cleavage by TUNEL assay and radiolabeled DNA laddering showed results compatible with enhanced cardiomyocyte apoptosis in the hearts of ANG-II infused rats. The bax-to-bcl-2 ratio, expression of the active form of caspase 3 (17 kDa), and activity of caspase 3 in the hearts of the ANG II group increased more than twofold above controls. Protein expression of AT\textsubscript{1} receptors in the heart in vivo is associated with an increased rate of apoptosis without major hemodynamic consequences. Bax and caspase 3 are involved in the apoptotic signaling pathway in this experimental paradigm.

Apoptosis is an active mechanism by which cells respond to some insults by triggering a program of gene-regulated cell death (15, 32, 35). Hallmarks of apoptosis include morphological alterations such as cell shrinkage, membrane blebbing, chromatin condensation, and DNA fragmentation (32). It has been increasingly recognized that apoptosis plays a critical role in normal development and in pathology in a wide variety of tissues (34). Indeed, apoptosis is recognized as one of the mechanisms of myocyte loss in cardiac ischemiareperfusion injury, myocardial infarction, and vascular wall remodeling. Angiotensin II (ANG II) acts as an important mediator in cardiovascular diseases such as hypertension, congestive heart failure, and renal failure. In the heart, ANG II contributes to development of cardiac hypertrophy via its growth factor properties on cardiomyocytes (2, 17) and to cardiac fibrosis via induction of fibroblast proliferation and collagen deposition (24, 28). Furthermore, in vitro studies have demonstrated the capacity of ANG II to induce apoptosis in both neonatal and adult cardiomyocytes (3, 14, 18).

Cardiomyocyte apoptosis may thus contribute to the remodeling that occurs in the transition from compensated hypertrophy to heart failure in hypertensive heart disease (5, 11). However, the molecular mechanisms resulting in cardiomyocyte apoptosis after exposure to ANG II remain largely unknown.

Although little is known of the exact molecular mechanisms controlling apoptosis in cardiac muscle, it has been suggested that members of the bcl-2 family may be general mediators of apoptosis (8). The bcl-2 gene family consists of more than 15 members. They can be classified as antideath or prodeath. Bcl-2 is a prototype for an antideath or survival factor, whereas bax accelerates the apoptotic process. Overexpressed bax also counters the death repressor activity of bcl-2 (13, 25). These proteins appear to dimerize with themselves or each other through bcl-homology domains and thereby determine the susceptibility of the cell to induction of apoptosis. Thus the ratio of bax to bcl-2 determines death or survival after an apoptotic stimulus. Additional factors involved in apoptotic regulation include cysteine proteases belonging to the ICE/CPP32 family, now called caspase 3, also referred to as CPP32, YAMA, or apopain (23). Caspase 3 is expressed in cells as an inactive 32-kDa precursor. During apoptosis, the 32-kDa caspase 3 proenzyme is first cleaved to release a 12-kDa fragment and an inactive, intermediate 20-kDa cleavage product consisting of a 3-kDa fragment, cysteine proteases belonging to the ICE/CPP32 family, now called caspase 3, also referred to as CPP32, YAMA, or apopain (23). Caspase 3 is expressed in cells as an inactive 32-kDa precursor. During apoptosis, the 32-kDa caspase 3 proenzyme is first cleaved to release a 12-kDa fragment and an inactive, intermediate 20-kDa cleavage product consisting of a 3-kDa fragment.

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prodomain plus a 17-kDa subunit. Removal of the 3-kDa propeptide from the 20-kDa peptide generates the 17-kDa mature, active form associated with caspase 3 activity (12, 22).

In the present study, we tested the hypothesis that stimulation of the rat heart in vivo by ANG II is accompanied by apoptosis. We delineated the role of AT1 angiotensin receptors and changes in bax, bcl-2, and caspase 3 activation, critical steps involved in death signaling pathways, in ANG II-associated apoptosis.

METHODS

Animal experiments. The study was approved by the Animal Care Committee of the Clinical Research Institute of Montreal and was performed according to the guidelines of the Canadian Council for Animal Care. As previously described (4), male Sprague-Dawley rats of 7 wk of age (200 g) were infused subcutaneously using Alzet osmotic minipumps (Alza; Palo Alto, CA) with Ile5-ANG II (Peninsula; Palo Alto, CA) at a dose of 120 ng·kg⁻¹·min⁻¹. Losartan (AT1 receptor antagonist) was administered in the drinking water at a dose of 10 mg·kg⁻¹·day⁻¹. After 7 days of treatment, systolic blood pressure (SBP) was taken by the tail cuff method. Rats were killed by decapitation. The heart was removed and weighed. A portion of the ventricle was fixed in 10% buffered formalin solution and embedded in paraffin. Coronal heart sections (5 μm thick) obtained from the equator of the heart were prepared for immunohistochemical analysis. Another portion of the ventricle was immediately frozen in liquid nitrogen and kept at −80°C for DNA and protein extraction.

Echocardiography. Echocardiography was performed 24 h before the rats were killed. The animals were anesthetized with ketamine HCl (15 mg/kg) and xylazine (2 mg/kg). Standard echocardiograms were performed using a 15-MHz linear-array transducer (Hewlett-Packard Sonos 5500). Images were obtained in the parasternal long axis and short axis. M-mode recordings were obtained through the septal and posterior walls. Left ventricular outflow tract velocity was measured with pulsed-wave Doppler on the parasternal long-axis view. Left ventricular chamber dimensions and wall thicknesses were measured according to the American Society of Echocardiography-recommended guidelines (29). Left ventricular ejection fraction (LVEF), left ventricular fractional shortening (LVFS), and cardiac output were calculated with established equations.

In situ detection of apoptosis in the heart. Apoptosis/DNA fragmentation was detected on deparaffinized and rehydrated tissue sections using the Apoptag kit (Intergen; Purchase, NY). The procedure was performed according to the manufacturer’s instructions. This method is based on the binding of fluoresceinlabeled dUTP to 3′-OH ends of fragmented DNA by TdT (TdT-mediated dUTP nick-end labeling (TUNEL)). The number of apoptotic cells shown by nuclei with fluorescence after TUNEL assay was quantified over an image analyzer. Apoptotic density was calculated as the number of positive TUNEL-stained nuclei per 100 nuclei. A positive control and a negative control for the TUNEL assay were carried out. Tissue sections were treated with DNase (positive control) and vehicle (negative control) for 1 h at 37°C before using the Apoptag kit.

Radiolabeled DNA laddering. DNA was extracted from the heart and nick-end radiolabeled as previously described (4). Briefly, a small piece of left ventricular tissue was incubated in DNA lysis buffer [20 mmol/l EDTA, 50 mmol/l Tris-HCl (pH 8.0), 0.5% SDS, and 100 μg/l proteinase K] overnight at 50°C, followed by treatment with RNase A (150 μg/l) for 1 h at 37°C. The DNA was then extracted with phenol-chloroform, precipitated with 100% ethanol, washed with 70% ethanol, dried, and dissolved in sterile water. The DNA concentration was measured by spectrophotometry. Extracted DNA (1 μg) was radiolabeled on free 3′-OH ends using 16 units TdT and 10 μCi [³²P]dCTP (3,000 Ci/mmol) and incubated for 1 h at 37°C. After labeling, one-tenth of the total reaction volume (0.1 μg DNA) was loaded on a 1.5% agarose gel, run for 3.5 h at 90 V, and transferred onto a Hybond nylon membrane (Amersham Canada; Oakville, Ontario, Canada). The membrane was exposed to a 32P-sensitive screen overnight, and the DNA ladders were detected using a PhosphorImager (Molecular Dynamics; Sunnyvale, CA).

Western blot analysis of bax, bcl-2, caspase 3, and AT1 and AT2 receptors. Protein was extracted from frozen tissue as previously described (4). Protein concentration was determined using the Bio-Rad protein assay (Bio-Rad Laboratories; Mississauga, Ontario, Canada). Total protein (30 μg) was separated by electrophoresis on a 15% polyacrylamide gel at 100 V for 1 h and transferred onto a polyvinylidene fluoride membrane in a cooling system at 100 V for 1 h. Membranes were incubated with specific antibodies to bax, bcl-2, and AT1 and AT2 receptors (Santa Cruz Biotechnology; Santa Cruz, CA) at dilutions of 1:2,000, 1:1,600, 1:1,000, and 1:500, respectively, for 1 h at room temperature. To evaluate expression of inactive (32 kDa), intermediate (20 kDa), and active (17 kDa) caspase 3, membranes were incubated overnight with rabbit antiserum to human CPP32 (antibody against caspase 3) at a generous gift from Dr. Raffick Pierre Sekaly, Clinical Research Institute of Montreal, Montreal, Quebec, Canada) at a dilution of 1:1,000 (1). Signals were revealed with chemiluminescence and visualized by autoradiography.

Determination of caspase 3 activity. Frozen hearts were homogenized in lysis buffer [20 mmol/l Tris (pH 7.5), 137 mmol/l NaCl, 1% Nonidet NP-40, 5% glycerol, 2 mmol/l di-
thiothreitol, 5 mmol/l EDTA, 1 mmol/l sodium orthovanadate, 1 μg/ml aprotinin, 0.7 μg/ml pepstatin, and 1 mmol/l phenylmethylsulfonyl fluoride. Homogenates were centrifuged at 15,000 rpm (30 min at 4°C). Caspase 3 activity was measured with a fluorometric immunosorbent enzyme assay (Roche Molecular Biochemicals; Laval, Quebec, Canada), and the methodology was based on the manufacturer’s instructions. The principle of the assay is that activated caspase-3, captured by a monoclonal antibody, cleaves the substrate acetyl-Asp-Glu-Val-Asp-7-amido-4-trifluoromethyl-coumarin (Ac-DEVD-AFC) to generate AFC. Free AFC is quantified fluorometrically at excitation and emission wavelengths of 400 and 505 nm, respectively. Recombinant human caspase 3 (26) was a generous gift from Dr. Rafick Pierre Sekaly, from our institution, and used for evaluation of in vitro effects of losartan on caspase 3 activity.

Statistical analysis. Results are presented as means ± SE. Data were analyzed by one-way ANOVA followed by a Student-Newman-Keuls test. P < 0.05 was considered statistically significant.

RESULTS

Body weight, SBP, and cardiac function by echocardiography. After 7 days of treatment, body weight did not differ significantly between the experimental

![Fig. 1](image-url). A: representative photomicrographs depict end-labeling detection of fragmented DNA with dTd-mediated dUTP nick-end labeling (TUNEL) in sections of heart from control rats, ANG II-infused rats (ANG II), ANG II-infused rats treated with losartan (ANG+Los), and positive and negative controls (magnification ×100). B: density of TUNEL-positive nuclei from each group versus that of controls (Ctrl), presented as a percentage of the total number of nuclei. Values are means ± SE of 3 animals in each group (3 tissue slides for each rat and 10 different fields for each slide). *P < 0.05 vs. controls; †P < 0.05 vs. the ANG II group. C: typical radiolabeled DNA laddering from the heart of controls, ANG-II infused rats, and ANG-II infused rats treated with losartan.
SBP was significantly increased in ANG II-infused rats compared with control rats (P < 0.01; Table 1). Treatment of losartan resulted in significantly lower blood pressure in ANG II-infused rats. As shown in Table 1, ANG II induced a slight, albeit not significant, increase in relative heart weight. By echocardiography (Table 1), there was a trend to increase left ventricular end-diastolic dimension (LVEDD), left ventricular posterior wall thickness at end diastole (LVPWd), interventricular septal wall thickness (IVSD), LVEF, and LVFS in ANG II-infused rats versus controls, without achieving statistical significance.

**Apoptosis by TUNEL and DNA laddering.** Apoptotic density, as shown by the number of TUNEL-positive nuclei per 100 nuclei, was significantly increased in ANG II-infused rats compared with control rats (P < 0.01; Fig. 1, A and B). This increase was reversed by losartan in treated ANG II-infused rats. With the use of the 3′OH-end DNA radiolabeling method, which is much more sensitive than ethidium bromide DNA staining, we detected the DNA laddering typical of apoptosis even in tissue from untreated rats, albeit at much lower level in the latter, as shown in Fig. 1C. Similarly to the TUNEL assay, DNA laddering demonstrated an increase in apoptosis in the ANG II-infused group. This effect was abrogated by losartan (Fig. 1C).

**Expression of pro- and antiapoptotic proteins.** Western blotting was performed to quantify expression of the proapoptotic protein bax and the antiapoptotic protein bcl-2. As shown in Fig. 2A, top, the bax signal was greater in hearts from ANG II-infused rats than control rats. However, no significant changes were found in expression of bcl-2 between both groups (Fig. 2A, bottom), resulting in a significant increase in the bax-to-bcl-2 ratio (P < 0.01) in ANG II-infused rats (0.25 ± 0.02) compared with normotensive rats (0.12 ± 0.03; Fig. 2B). There were no significant differences in the ratio of bax to bcl-2 between controls and ANG II-infused rats treated with losartan (Fig. 2B).

**Effect of ANG II and losartan on activity of caspase 3.** Expression of the active form of caspase 3 (17-kDa peptide) was increased significantly in ANG II-infused rats compared with control levels (Fig. 3A) and significantly diminished in ANG-II infused rats treated with losartan. The activity of caspase 3 in the hearts of ANG...
II-infused rats was elevated 2.3-fold ($P < 0.01$; Fig. 3B), similar to the increase in expression of the active forms of the enzyme (Fig. 3A). Losartan treatment of rats resulted in return of caspase 3 activity to control levels. In vitro, losartan, at concentrations found in vivo (1–100 μg/ml), had no effect on the activity of recombinant human caspase 3 (Table 2). This demonstrated that results found in the ANG II-infused losartan-treated rats could not be attributed to a direct action of losartan on caspase 3 but were the result of an indirect effect via blockade of action of ANG II through AT$_1$ receptors in vivo.

**Protein expression of AT$_1$ and AT$_2$ receptors.** Figure 4, A and B, shows that the protein expression of AT$_1$ and AT$_2$ receptors was significantly increased in ANG II-infused rats compared with controls ($P < 0.05$). Treatment with losartan normalized the protein expression of AT$_1$ receptors in the hearts of ANG II-infused rats. However, losartan slightly increased the protein expression of AT$_2$ receptors in ANG II-infused rats.

**DISCUSSION**

The results of this study support the hypothesis that ANG II infusion into rats with stimulation of AT$_1$ receptors is associated with in vivo apoptosis in the heart. The process of apoptosis involves increased bax expression and caspase 3 activation, although a causal relationship with the apoptotic process triggered by ANG II cannot be established from our results. These findings extend our understanding of the important role of ANG II and its receptor subtypes, particularly AT$_1$ receptors, in cardiac remodeling in hypertensive heart disease.

It is well recognized that cell hypertrophy is mediated by AT$_1$ receptors, whereas stimulation of AT$_2$ receptors may lead to apoptosis (6, 36). However, AT$_1$ receptors have been reported to play an important proapoptotic role in cardiomyocytes of both neonatal and adult rats (3, 14, 18), whereas AT$_2$ receptors seem not to be involved in cardiac apoptosis (33). In addition, angiotensin-converting enzyme inhibitors have been shown to inhibit apoptosis, presumably by preventing the formation of ANG II (11). The observations of the present study extend the previous in vitro studies (3, 14, 18) and show that the reported ability of ANG II to induce cardiomyocyte apoptosis associated with stimulation of AT$_1$ receptors holds true in the in vivo situation.

The selective AT$_1$ receptor antagonist losartan inhibited the upregulation induced by ANG II of one of its targets, the AT$_1$ receptor. In agreement with our study, losartan can downregulate myocardial AT$_1$ receptor mRNA levels induced by aldosterone (27), ischemia, and reperfusion in the isolated rat heart (37) and in spontaneously hypertensive rats (19). In contrast to many in vivo studies, losartan upregulates AT$_1$ receptor mRNA levels in both cardiomyocytes and fibroblasts in vitro (7). ANG II infusion in vivo may upregulate ANG II receptor binding in blood vessels (30, 31) or ANG II receptor mRNA levels (4). ANG II infusion increases aldosterone. We previously demonstrated that aldosterone in vivo and in vitro upregulates ANG II receptor binding (31). Similar effects were more recently shown in vivo for AT$_1$ receptor mRNA in the heart by Robert et al. (27). Downregulation of myocardial AT$_1$ receptor level by losartan in vivo is not necessarily a direct effect but may rather result from inhibition of aldosterone production by the AT$_1$ receptor antagonist. This may explain the contrast between in vivo and in vitro results. Prevention by losartan of

**Table 2. In vitro effect of losartan on recombinant human caspase 3**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>1 μg/ml</th>
<th>10 μg/ml</th>
<th>100 μg/ml</th>
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<tr>
<td>AFC, μmol/l</td>
<td>67.7 ± 3.1</td>
<td>80.0 ± 9.5</td>
<td>71.4 ± 3.1</td>
<td>71.5 ± 7.3</td>
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Values are means ± SE. AFC, acetyl-Asp-Glu-Val-Asp-7-amido-4-trifluoromethyl-coumarin.
upregulation of AT₁ receptor expression indicates that AT₁ expression may be positively regulated by ANG II under some experimental conditions. In contrast to the blockade by losartan of increase of AT₁ receptor protein expression after ANG II infusion, the increase in protein expression of AT₂ receptors in ANG II-infused rats was enhanced after treatment with losartan. This is associated with a decrease in cardiac apoptosis. Whether AT₂ receptors have an antia apoptotic effect or not, particularly in the heart, remains to be clarified. In fact, AT₂ receptors may not be involved in cardiac apoptosis, at least as shown in transgenic mice overexpressing AT₂ receptors (33). In contrast, ANG II induces apoptosis in neonatal cardiomyocytes via both AT₁ and AT₂ receptors (10). The discrepancy between in vivo and in vitro results may be explained by differences in experimental paradigm, including the fact that expression of AT₁ and AT₂ receptors differs between neonatal and adult heart.

Echocardiographic data showed very slight increases in LVEDD, LVSP, IVSD, LVEF, and LVFS in ANG II-infused rats, albeit without achieving statistical significance. This may represent slight left ventricular dilatation (LVEDD) secondary to blood pressure elevation and hypertrophy (LVPWd and IVSD) and enhanced contractility (LVEF and LVFS) responding to ANG II hypertrophic and inotropic action. Apoptosis was not associated with deterioration of cardiac function. Alternatively, increased apoptosis may partly contribute to the left ventricular dilatation. It may also participate in mechanisms leading to remodeling of the left ventricle, which may have functional consequences on a longer term than examined in the present experimental paradigm.

Increased expression of active caspase 3 in the heart of ANG II-infused rats was accompanied by a similarly increased enzyme activity, which returned to control values under treatment with losartan. The inhibition of ANG II-induced apoptosis and caspase 3 activation by the AT₁ receptor antagonist losartan in this and previous reports (4) could potentially be attributed to a putative direct inhibitory effect on caspase 3 of the AT₁ antagonist in the in vivo experiments. However, losartan has no direct effect on the activity of caspase 3 in vitro.

Cell death is controlled in part by a complex interplay between regulatory proteins such as proapoptotic and antiapoptotic mediators, bax and bcl-2, respectively. In cardiomyocytes, bax protein has been shown to act as an accelerator of apoptosis (20), whereas bcl-2 prevents apoptosis (16, 20). Therefore, the ratio of bax to bcl-2 may be considered as a determinant for survival or death of cardiomyocytes after apoptotic stimuli. The main finding of the present study is that compared with controls, expression of bax was increased in the hearts of ANG II-infused rats, whereas expression of bcl-2 remained unchanged, resulting in an abnormal increase in the bax-to-bcl-2 ratio in ANG II-infused rats. Leri et al. (18) showed that stretch-mediated release of ANG II in adult rat ventricular cardiomyocytes in vitro was coupled with stimulation of AT₁ receptors and an increase in p53, bax, and apoptosis. The authors suggested that overexpression of the bax gene is downstream from activation of p53, thereby stimulating cardiomyocytes to undergo apoptosis (18). In agreement, our results support a role of bax in association with cardiomyocyte apoptosis in this in vivo model of ANG II-induced hypertension. However, the study of Fortuno et al. (9) showing that apoptosis in spontaneously hypertensive rats is associated with increased Bax-α and normal levels of p53 does not support a role of p53, which is a transcriptional regulator of bax and bcl-2 (18) in cardiomyocyte apoptosis in vivo, at least in genetic hypertension. A role of p53 in cardiac apoptosis in the ANG II-induced hypertensive model remains to be investigated.

In conclusion, the present results offer insights into the essential role of AT₁ receptors in the control of cardiac apoptosis, which may play an important role in cardiac remodeling associated in hypertension. Cardiac apoptosis induced by ANG II is associated with increased bax expression and caspase 3 activation.

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