Effect of AT₁ receptor blockade on cardiac apoptosis in angiotensin II-induced hypertension

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Diep, Quy N., Mohammed El Mabrouk, Ping Yue, and Ernesto L. Schiffrin. Effect of AT₁ receptor blockade on cardiac apoptosis in angiotensin II-induced hypertension. Am J Physiol Heart Circ Physiol 282: H1635–H1641, 2002. —Angiotensin II (ANG II) induces apoptosis in cardiomyocytes in vitro. We tested the hypothesis that in vivo AT₁ 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⁻¹·min⁻¹) for 7 days with or without the AT₁ receptor antagonist losartan (10 mg·kg⁻¹·day⁻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) assay and radiolabeled DNA laddering. Expression of bax, bel-2, caspase 3, and AT₁ and AT₂ 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 (137 ± 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 cardiac myocyte 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 AT₁ and AT₂ receptors was significantly increased in ANG II-infused rats compared with control rats. Losartan-treated ANG II-infused rats exhibited normalized apoptosis, bax, caspase 3 activity, and AT₁ receptors. ANG II stimulation of AT₁ 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.

caspase; bax; bel-2

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 known as a mechanism of myocardial cell death in cardiac ischemia-reperfusion 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

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AT₁ ANGIOTENSIN RECEPTORS AND APOPTOSIS IN HEART

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 administrated in the drinking water at a concentration of 0.1 mmol/l NaCl, 1% Nonidet NP-40, 5% glycerol, 2 mmol/l di-throsenol, incubated 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.

Determination of caspase 3 activity. 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). Tissue homogenization was performed as previously described (4).

Table 1. Body weight, SBP, and echocardiographic data of ANG II-infused rats ± losartan

<table>
<thead>
<tr>
<th>Groups</th>
<th>Body Weight, g</th>
<th>SBP, mmHg</th>
<th>Heart Weight, mg</th>
<th>Heart Weight/Body Weight, mg/g</th>
<th>HR, beats/min</th>
<th>LVEDD, mm</th>
<th>LVPWd, mm</th>
<th>IVSD, mm</th>
<th>LVEF, %</th>
<th>LVFS, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>242 ± 6</td>
<td>111 ± 2</td>
<td>0.86 ± 0.03</td>
<td>3.57 ± 0.13</td>
<td>366 ± 15</td>
<td>6.24 ± 0.05</td>
<td>2.44 ± 0.04</td>
<td>1.46 ± 0.01</td>
<td>78.8 ± 4.0</td>
<td>43.2 ± 4.0</td>
</tr>
<tr>
<td>ANG II</td>
<td>233 ± 10</td>
<td>173 ± 3***</td>
<td>0.91 ± 0.02</td>
<td>3.88 ± 0.05</td>
<td>363 ± 16</td>
<td>5.88 ± 0.05</td>
<td>2.51 ± 0.04</td>
<td>1.53 ± 0.01</td>
<td>82.1 ± 5.4</td>
<td>47.6 ± 5.8</td>
</tr>
<tr>
<td>ANG II + losartan</td>
<td>234 ± 9</td>
<td>134 ± 4†‡</td>
<td>0.85 ± 0.02</td>
<td>3.66 ± 0.10</td>
<td>384 ± 12</td>
<td>6.62 ± 0.03</td>
<td>2.33 ± 0.01</td>
<td>1.34 ± 0.01</td>
<td>77.9 ± 4.0</td>
<td>42.5 ± 4.3</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 6–8 rats. SBP, systolic blood pressure; HR, heart rate; LVEDD, left ventricular end-diastolic dimension; LVPWd, left ventricular posterior wall thickness at end diastole; IVSD, interventricular septal wall thickness; LVEF, left ventricular ejection fraction; LVFS, left ventricular fractional shortening. *P < 0.01 vs. controls; †P < 0.01 vs. ANG II group.
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

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Fig. 1. A: representative photomicrographs depict end-labeling detection of fragmented DNA with dTdT-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.
groups. SBP was significantly increased in ANG II-infused rats compared with control rats \((P < 0.01; \text{Table 1})\). 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 significantly, 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 \((P < 0.01; \text{Fig. 1, A and B})\). 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 \((0.25 \pm 0.02)\) compared with normotensive rats \((0.12 \pm 0.03; \text{Fig. 2B})\). 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 measured with a fluorometric immunosorbent enzyme assay. Activated caspase 3, captured by a monoclonal antibody, cleaves the substrate acetyl-Asp-Glu-Val-Asp-7-amino-4-trifluoromethyl-coumarin (Ac-DEVD-AFC) to generate AFC. Free AFC was quantified fluorometrically. Error bars indicate SE of results from hearts of 4 rats/group (each in triplicate). *\(P < 0.01\) vs. other groups.
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 demon-
strated that results found in the ANG II-infused losar-
tan-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
AT1 receptors in vivo.

Protein expression of AT1 and AT2 receptors. Figure
4, A and B, shows that the protein expression of AT1
and AT2 receptors was significantly increased in ANG
II-infused rats compared with controls (P < 0.05).
Treatment with losartan normalized the protein ex-
pression of AT1 receptors in the hearts of ANG II-
infused rats. However, losartan slightly increased the
protein expression of AT2 receptors in ANG II-infused
rats.

DISCUSSION
The results of this study support the hypothesis that
ANG II infusion into rats with stimulation of AT1
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
AT1 receptors, in cardiac remodeling in hypertensive
heart disease.

It is well recognized that cell hypertrophy is medi-
ated by AT1 receptors, whereas stimulation of AT2
receptors may lead to apoptosis (6, 36). However, AT1
receptors have been reported to play an important
proapoptotic role in cardiomyocytes of both neonatal
and adult rats (3, 14, 18), whereas AT2 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 stim-
ulation of AT1 receptors holds true in the in vivo situation.

The selective AT1 receptor antagonist losartan inhib-
ited the upregulation induced by ANG II of one of its
targets, the AT1 receptor. In agreement with our study,
losartan can downregulate myocardial AT1 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 AT1 recep-
tor mRNA levels in both cardiomyocytes and fibro-
blasts in vitro (7). ANG II infusion in vivo may upregu-
late 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 AT1 receptor mRNA in the
heart by Robert et al. (27). Downregulation of myocar-
dial AT1 receptor level by losartan in vivo is not nec-
essarily a direct effect but may rather result from
inhibition of aldosterone production by the AT1 recep-
tor 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>
</tr>
</thead>
<tbody>
<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>
</tr>
</tbody>
</table>

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 antiapoptotic 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, LVPWd, 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 stimulus. 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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