Symposium

An Angiotensin II Type 1 Receptor Blocker, Candesartan, Increases Myocardial Apoptosis in Rats with Acute Ischemia-Reperfusion

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Angiotensin II (Ang II) and apoptosis contribute significantly to myocardial ischemia-reperfusion (I-R) injury. Evidence indicates that Ang II may activate apoptosis in myocytes. The present study was undertaken to investigate the effects of angiotensin receptor blockers (ARBs), candesartan, on the apoptosis of cardiac myocytes in rats after I-R. Rats were divided into a control group, a candesartan group I (0.015 mg/kg), and a candesartan group II (0.03 mg/kg). Candesartan was intravenously administered 30 min before ischemia. All rats were subjected to 30 min of coronary occlusion followed by 3 h of reperfusion. The data showed that left ventricular (LV) systolic pressure and LV $+dp/dt$ was decreased after administration of candesartan, but increased after reperfusion in the candesartan group II, compared with those in the candesartan group I and control group. LV $-dp/dt$ was decreased after candesartan administration in candesartan group II. The number of apoptotic cells in the candesartan groups (497 ± 204 and 543 ± 254 , respectively) was higher than that in the control group (287 ± 166 ; $p < 0.05$). There was no significant difference in infarct size among the three groups. However, plasma CPK was lower in the candesartan groups than in the control group. Northern blot analysis showed that p53 mRNA was upregulated in the candesartan groups, in association with increased expression of bax mRNA. Immunohistochemical analysis showed that p53 and bax immunoreactivity were increased in both of the candesartan groups. In conclusion, candesartan increased apoptosis in the rat hearts after acute I-R, and this increase was possibly mediated by upregulation of p53 and bax gene expressions. In addition, candesartan was shown to improve LV function, in association with reduction of CPK release. (*Hypertens Res* 2001; 24: 323-329)

Key Words: angiotensin receptor blocker, apoptosis, ischemia-reperfusion, p53, bax

Introduction

Ischemia-reperfusion (I-R) myocardial injury is a key issue in the management of ischemic heart disease. It has been well established that the renin-angiotensin system plays an important role in myocardial I-R injury (1-4). ACE inhibitors and angiotensin II receptor blockers (ARBs) have been shown to be cardioprotective in ex-

perimental animal models with I-R injury, such as by reducing infarct size and reperfusion arrhythmias and improving LV function (5-9). Clinical studies have also demonstrated that use of ACE inhibitors and ARBs in patients with ischemic heart disease improves LV function and prevents geometric remodeling, thereby suppressing post-myocardial-infarction cardiovascular events and ultimately increasing the survival of patients (3, 10-12). However, the precise mechanism of the beneficial effects

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of angiotensin II (Ang II) blockade remains to be fully determined.

Recently, numerous studies have shown that apoptosis of cardiomyocytes might be induced in a variety of insults, including hypoxemia, acute I-R, myocardial infarction, cardiomyopathic end-stage heart failure and myocardial pressure stretch (13–16). Significantly, clear evidence of apoptosis has also been well documented recently in the I-R rat myocardium in a number of injurious settings as well as in myocardial autopsy tissue after death from acute myocardial infarction. It appears likely that apoptosis contributes significantly to myocardial I-R injury (13–16).

Although experimental evidence has suggested that ARBs may provide cardioprotection in various animal models of I-R injury, the effect of ARBs on apoptosis induced by acute I-R injury remains to be shown (17). We hypothesize that the role of Ang II receptor blockade on salvaging the I-R myocardium might involve changes in myocardial apoptosis and/or myocardial necrosis. The present study was undertaken to investigate the effects of a potent and long-lasting angiotensin type 1 receptor blocker, candesartan, on the appearance of apoptosis in cardiac myocytes using an acute I-R Sprague-Dawley (SD) rat model. We also investigated the gene expressions of p53 and bax, which are known to be highly related to cardiac apoptosis.

Materials and Methods

Animal Preparation and Experimental Protocol

Male SD rats weighing 380–420 g were anaesthetized with sodium pentobarbital (50 mg/kg, i.p.). After tracheotomy, the rats were ventilated with room air using a respirator for small rodents (a stroke volume, 15 ml/kg; rate, 80 strokes/min). A polyethylene catheter (PE 50) was inserted into the right carotid artery for the hemodynamic measurement. The chest was then opened and the ribs were gently spread. The heart was well exposed and a 6.0 silk ligature was used to ligate the left anterior descending (LAD) coronary artery. Successful ligation of LAD was verified visually by the color change of the ischemic area and ECG recording. The ligation was removed after 30 min and followed by 180 min of reperfusion. The blood samples were taken for measuring plasma Ang II and creatine phosphokinase (CPK) and the hearts were excised rapidly at the end of the 180-min reperfusion. The hearts were fixed in 4% paraformaldehyde overnight for terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) and immunohistochemistry. The remaining specimens were immediately frozen in liquid nitrogen, stored at -80°C , and later processed for Northern analysis. ECG and tracings from the LV catheter were recorded continuously during the operation and

used to obtain the heart rate, LV systolic pressure, LV end-diastolic pressure, $\text{max} + \text{dp/dt}$ and $\text{max} - \text{dp/dt}$.

Rats ($n=24$) were divided into three groups ($n=8$ each) as follows. The control group received I-R without drugs. Candesartan group I received I-R with pretreatment of 0.015 mg/kg candesartan (provided by Takeda Chemical Industries, Ltd., Osaka, Japan). Candesartan group II received I-R with pretreatment of 0.03 mg/kg candesartan. Candesartan was intravenously administered 30 min before ischemia. The experimental protocol was approved by the Animal Care and Use Committee of Ehime University School of Medicine.

An additional 15 SD rats ($n=5$, each group) were used to investigate infarct size, which was calculated and expressed as a percentage of the LV surface area as described previously (18). After 180 min of reperfusion the loop around the LAD was retightened, and 1 ml of 2% Evans blue was rapidly injected into the LV to distinguish the nonischemic area from the area at risk. The heart was then excised, and the atria, great vessels, and right ventricle were dissected. The LV was cut into 5–6 slices perpendicular to the base-apex axis. The slices were incubated in PBS of triphenyltetrazolium chloride (1% TTC) for 10 min. The noninfarcted region of the stained brick red in the presence of dehydrogenase enzymes, whereas necrotic (infarcted) tissue remained unstained due to a lack of enzymes. Infarct size was expressed as a percentage of the ischemic risk area to the total area of LV.

In Situ TdT Assay

In situ detection of apoptosis was performed using the ApoTag *in situ* apoptosis detection kit (Oncor Inc., Gaithersburg, USA) with some modifications. In brief, the hearts collected from all groups were incubated in 4% paraformaldehyde overnight at 4°C and were then embedded in paraffin. From each heart, 5- μm -thick cross sections, which included the left and right ventricles and septum, were cut by a microtome and mounted on silane-coated slides. The slides were deparaffinized and rehydrated by serial changes of xylene and ethanol. Endogenous peroxidase was blocked by immersing the sections in phosphate buffered saline (PBS) containing 3% H_2O_2 . The sections were then incubated with 20 $\mu\text{g/ml}$ proteinase K (Sigma, Deisenhofen, Germany) for 20 min. After washing in PBS, the sections were covered with equilibration buffer for 5 s, then incubated with a mixture of terminal deoxynucleotidyl transferase (TdT) and reaction buffer containing digoxigenin labeled dUTP at 37°C for 1 h. The sections were washed in diluted working strength stop/wash buffer, incubated with an anti-digoxigenin antibody that had been conjugated with peroxidase for 30 min, washed in PBS for 5 min, and finally stained with 0.05% diaminobenzidine (Wako Pure Chemical Industries, Ltd., Osaka, Japan). The slides were

slightly counterstained with Mayer's hematoxylin to visualize the nuclei. Tissue sections from each specimen were examined microscopically at $\times 400$ magnification. At least 30 views of cardiomyocytes were counted separately in the subepicardial, midmyocardial, and subendocardial regions in the ischemic areas. The percentage of apoptotic cells was determined with an apoptotic index (the number of TUNEL positive cells/the total number of cardiomyocyte nuclei). This evaluation was carried out independently by 2 persons who were unaware of the experimental protocol. We also evaluated the intramyocardial localization of cardiomyocyte apoptosis.

Northern Blot Analysis

Total RNA was extracted from about 100 mg heart tissue from an approximately 100-mg heart-tissue sample taken from the ischemic LV area. RNA was extracted using an ISOGEN kit (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions. The RNA concentration in the myocardium was determined by spectrophotometer, with the absorbance at 260 nm. The ratio of the absorbance at 280 nm was >1.8 in all samples. From each rat, 15 μ g of RNA were fractionated on a 1.0% agarose-formaldehyde gel at 5 V/cm, then transferred onto a nylon membrane (Hybond-N⁺; Amersham Pharmacia Biotech., Inc., Buckinghamshire, UK). Hybridization was performed using a QuickHyb Hybridization Solution kit (Stratagene, La Jolla, USA) according to the manufacturer's instructions. The probes used for Northern blot analysis were rat p53 cDNA and rat bax cDNA. All cDNA probes were synthesized by the reverse transcriptase-polymerase chain reaction method. The oligonucleotides used as primers of p53 and bax were as follows: p53 5'-ATGGAGGATTACAGTCGGA-3' (sense primer) and 5'-TGGGCTGACTCAGACTGA-3' (antisense primer), bax 5'-GACACCTGAGCTGACCTTGG-3' (sense primer) and 5'-GAGGAAGCTCAGTGCCAGC-3' (antisense primer). For the cDNA of GAPDH, RT-PCR control Amplimer Sets were used (Clontech Laboratories, Inc., Palo Alto, USA). The resultant probes were subcloned into pBluescript II vectors (Stratagene), and DNA sequences were determined by the dideoxy-chain termination method. ³²P-labeled cDNA probes specific for rat p53 and bax were prepared by the random primer method using a Prime-It II random primer labeling kit (Stratagene). The radioactive intensity of the resulting bands was quantified with a BAS-1000 (Fuji Film Co., Ltd., Kanagawa, Japan). Results were normalized to hybridization signals of GAPDH mRNA, which encodes a constitutively expressed glycolytic enzyme, as internal control. After washing, autoradiography was performed with HyperfilmTM-MP (Amersham Pharmacia Biotech., Inc.) with an intensifying screen at -80°C .

Immunohistochemistry

The above-described slides were also used for the immunohistochemical analysis. After washing in PBS, inactivation of endogenous peroxidase was performed as described above, followed by washing in PBS. Nonspecific antigens were blocked by incubation of the slides with 10% normal goat serum for 30 min. The sections were then incubated for 1 h with the following primary antibodies: goat polyclonal anti-bax antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, USA) and anti-p53 antibody (Santa Cruz Biotechnology) at a dilution of 20 μ g/ml, 1:100 and 1:200 in PBS, respectively. After washing in PBS, the immunostaining was performed with an avidin-biotin immunoperoxidase using a Vectastain elite ABC kit (Vector Laboratories, Inc., Burlingame, USA) followed by visualization with diaminobenzidine. Tissue sections were lightly counterstained with hematoxylin, dehydrated, and placed under coverslips.

Determination of Plasma CPK and Ang II levels

Plasma was frozen at -80°C and samples were analyzed by double antibody Ang II radioimmunoassay as described previously (19).

Statistical Analysis

Data are expressed as the means $\pm$ SD and were analyzed by analysis of variance for multiple comparison of results. Unpaired Student's *t*-test was used to compare the candesartan groups with the control group under identical conditions. In all cases, probability values less than 0.05 were considered to indicate statistical significance.

Results

Hemodynamic Changes

There were no significant differences in heart rate among the three groups throughout the experiment (Table 1). After administration of candesartan, LVSP was significantly decreased in candesartan group II (0.03 mg/kg candesartan) but not significantly changed in either candesartan group I (0.015 mg/kg) or the control group (Table 1). LVEDP levels were not significantly different among the three groups (Table 1). We also measured $+dp/dt$ and $-dp/dt$, which are sensitive indicators for LV function. The results showed that $+dp/dt$ was decreased after administration of 0.03 mg/kg candesartan. After reperfusion in this group, however, $+dp/dt$ increased to a level higher than those in either candesartan group I or control groups. The $-dp/dt$ decreased obviously after administration of drug in candesartan II group (Table 1). In the case of $-dp/dt$, there were clear decreases in this param-

Table 1. Hemodynamic Changes in Three Groups

	Group	Baseline	Candesartan 30 min after	Ischemia 30 min after	Reperfusion 180 min after
Heart rate (bpm)	Control	421±29	426±25	415±14	364±31
	Candesar-I	436±33	420±24	413±14	358±35
	Candesar-II	429±43	413±35	418±26	366±51
LVSP (mmHg)	Control	131±19	135±12	119±14	115±13
	Candesar-I	130±12	125±14	114±21	116±16
	Candesar-II	134±18	109±16**	110±20	118±10
LVEDP (mmHg)	Control	6.9±3.2	7.3±6.2	10.1±4.4	8.5±3.6
	Candesar-I	7.1±4.7	6.6±4.3	11.4±5.1	8.7±3.1
	Candesar-II	8.6±4.9	7.9±3.9	11.1±3.5	7.2±3.2
+dp/dt (mmHg/s)	Control	4,223±1,091	4,977±1,900	3,335±1,791	2,945±904
	Candesar-I	3,977±789	3,887±1,207	2,687±82	3,275±754
	Candesar-II	4,847±1,125	3,309±1,454**	3,135±681	3,960±814**
-dp/dt (mmHg/s)	Control	3,413±1,880	3,038±784	3,872±1,179	2,462±1,207
	Candesar-I	2,570±675	2,586±603	2,098±977*	1,848±585
	Candesar-II	3,288±1,073	2,041±401**	2,365±942**	2,546±766

Values are expressed as the means±SD. LVSP, left ventricular systolic pressure; LVEDP, left ventricular end-diastolic pressure; dp/dt, maximum rate of change in left ventricular pressure with time. * $p<0.05$ vs. control; ** $p<0.01$ vs. control, $n=8$ for each group.

Fig. 1. Photomicrographs of apoptotic cardiomyocytes in three groups. A: Control group. B: Candesartan group I. C: Candesartan group II. D: Comparison of apoptotic cardiomyocyte cell count among groups. Values are the means±SD; * $p<0.05$; $n=8$ for each group.

eter after ischemia in candesartan groups I and II (Table 1). However, -dp/dt in candesartan groups I and II also

increased to a level in control group after reperfusion.

Apoptotic Myocytes

As shown in Fig. 1, the numbers of TUNEL-positive apoptotic myocytes in candesartan groups I and II (497 ± 204 , 543 ± 254 , respectively) was significantly higher than that in the control group (287 ± 166 ; $p < 0.05$). The apoptotic indexes in candesartan groups I and II were 0.40 and 0.41, respectively, vs. 0.23 for the control group ($p < 0.05$).

Infarct Size and Plasma CPK

The infarct size in candesartan groups I and II and in the controls were 0.38, 0.39, and 0.40, respectively. There were no significant differences among the three groups. However, plasma CPK was much lower in the candesartan groups ($8,668 \pm 3,085$ IU/ml, $6,825 \pm 2,208$ IU/ml, respectively) than in the control group ($14,131 \pm 4,206$ IU/ml; $p < 0.05$).

Northern Blot Analysis

The Northern blot analysis indicated that p53 mRNA was more highly expressed in the candesartan groups than in the control group, and that this increased expression was associated with an increase in the expression of bax mRNA (Fig. 2).

Immunohistochemical Findings of p53 and Bax

As shown in Fig. 3, cardiomyocytes of all three groups showed immunoreactivity for both p53 and bax. However, the numbers of cells positive for p53 immunoreactivity (87 ± 8 and 108 ± 12 , respectively), as well as the numbers positive for bax immunoreactivity (78 ± 12 and 106 ± 9 , respectively), were markedly higher in candesartan groups I and II than in the control group (5 ± 2 for p53, $p < 0.001$; 19 ± 3 for bax, $p < 0.001$).

Discussion

Many studies have demonstrated that candesartan improves LV function in association with its effect of lowering blood pressure (3, 4). Our data showed that candesartan decreased LV systolic pressure rapidly and dose-dependently, in association with a decrease of $+dp/dt$ after the administration. After reperfusion, however, $+dp/dt$ increased to a level higher than that in control group. This effect was much more obvious in the 0.03 mg/kg candesartan group. These results suggest that candesartan may improve LV function in the present rat model after acute I-R.

Recently, Anversa *et al.* reported that apoptosis of myocyte cells was the prevailing form of myocardial damage produced by occlusion of a major epicardial coronary artery, whereas necrotic myocyte cell death followed

Fig. 2. Results of Northern blot analysis. *A*: Representative autoradiograms of Northern blots of GAPDH, bax and p53 in three groups. Total RNA (15 μ g) was subjected to each lane and these experiments were repeated twice with similar results. *B*: Quantitative analysis of Northern blots of bax mRNA. Results were normalized to hybridization signals of GAPDH mRNA. Values are the means $\pm$ SD; * $p < 0.05$; $n = 8$ for each group. *C*: Quantitative analysis of Northern blots of p53 mRNA. Results were normalized to hybridization signals of GAPDH mRNA. Values are the means $\pm$ SD; * $p < 0.05$; $n = 8$ for each group.

apoptosis and contributed minimally to the progressive loss of myocytes after infarction (20). Apoptosis also affected the non-infarcted portions of myocytes, mural thinning and chamber dilation. The positive apoptotic cells in the present study were located in all regions of the myocardium, even in those regions remote from the ischemic area, a finding that was consistent with previous studies (17, 20, 21). To our knowledge, the present study is the first to document the effects of an ARBs on the apoptosis induced by ischemia and reperfusion. The results indicate that myocyte apoptosis may occur early after an acute ischemia and reperfusion injury, as reported

Fig. 3. Results of immunohistochemical analysis for p53 and bax in three groups. A: Representative photomicrograph of bax in the control group. B: Representative photomicrograph of bax in candesartan group I. C: Representative photomicrograph of bax in candesartan group II. D: Representative photomicrograph of p53 in the control group. E: Representative photomicrograph of p53 in candesartan group I. F: Representative photomicrograph of p53 in candesartan group II.

in previous studies (14, 20). Clearly, further studies will be needed to explore the biological and possible clinical implications of this increase in myocardial apoptosis by candesartan.

Studies have shown that Ang II activates apoptosis in a variety of models, including neonatal rat ventricular myocytes and kidney, pheochromocytoma, and granulosa cells. However, the role of different Ang II receptor subtypes in the induction of apoptosis in different tissues remains controversial (17, 22-24). Both AT1 and AT2 receptors are expressed in the rat myocardium. It is very interesting that the AT2 receptor exerts its effects, such as proapoptosis and antiproliferation, as an antagonistic receptor against the AT1 receptor in the myocardium (25-27). Although the AT2 subtype is detected in only a small number of organs and at very low level of expression, both AT1 and AT2 receptors appear to be upregulated after tissue injury (5, 26, 27). Since the ARBs do not block the AT2 receptor, the increased apoptosis of cardiomyocytes in the candesartan groups may have occurred *via* the enhanced binding of AT2 receptors due to the increased plasma Ang II levels after administration of candesartan (17, 25-27).

There is increasing awareness that the p53 tumor suppressor protein may be involved in apoptosis induced by

ischemia and reperfusion in many organs and tissues (17, 28). The molecular mechanisms underlying p53-induced apoptosis in mammalian cells are obscure but may involve the transactivation by p53 of certain cellular genes that are involved in the apoptotic pathway. In this regard, transcription of the death-promoting gene bax has been shown to be upregulated by p53 and to form a potential link between p53 and programmed cell death (17, 28-30). Expressions of p53 and bax have been documented in the cardiac tissue under different pathological conditions (14, 31). Overexpression of bax accelerates apoptotic death. Northern blot analysis in our study showed that p53 mRNA was clearly upregulated more in the candesartan groups than in the control group, and that this upregulation was associated with bax gene expression. These changes were considered responsible for the increased apoptosis in the candesartan groups. Bax gene expression is usually induced by chronic cellular response of the salvaged myocytes against various stimuli, such as chronic ischemia, mechanical stress of myocytes and so on (17, 32). However, our results suggested that the bax gene could be expressed soon after an apoptotic stimulus, such as acute ischemia and reperfusion.

In summary, we conclude that candesartan increased myocardial apoptosis in the rat heart after acute I-R in-

jury, and that this increase was possibly mediated by up-regulation of the p53 and bax gene expressions via AT2 receptors.

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