Myocardial Protection by Preconditioning of Heart With Losartan, an Angiotensin II Type 1–Receptor Blocker

Implication of Bradykinin-Dependent and Bradykinin-Independent Mechanisms

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Background—Ischemic preconditioning (PC) represents a state-of-the-art technique for myocardial preservation. Although certain intracellular mediators have been shown to play a role in PC, the exact nature of the trigger for PC is not known. Our previous study demonstrated that intracellular bradykinin released from the heart during ischemia/reperfusion plays a role in myocardial preservation. This study was undertaken to further examine the mechanism of bradykinin-mediated PC.

Methods and Results—Since the bradykinin B₂ receptor is likely to provide cardioprotection by blocking angiotensin II formation, we determined the effects of an angiotensin II type 1 (AT₁) receptor blocker, losartan, and a bradykinin B₂ receptor blocker, HOE 140, on myocardial protection. Isolated rat hearts were perfused initially by the Langendorff mode with Krebs-Henseleit buffer (KHB) for 15 minutes in the absence (control) or presence of losartan (4.5 μmol/L) and/or HOE 140 (10 μmol/L). After conversion to the working mode for 10 minutes (baseline), randomly assigned control and experimental hearts were subjected to 30 minutes of normothermic global ischemia followed by 2 hours of reperfusion. Myocardial function, infarct size, cardiomyocyte apoptosis, and amount of bradykinin/angiotensin released from the hearts were measured at baseline and during reperfusion while in the working mode. Significant postischemic ventricular recovery was demonstrated by improved developed pressure and aortic flow and reduced myocardial infarct size and apoptotic cell death with losartan, whereas the reverse was true for HOE 140. The functional recovery and infarct size–lowering ability of losartan were partially blocked and the antiapoptotic function of losartan was completely blocked by HOE 140.

Conclusions—The results document that losartan reduced whereas HOE 140 increased myocardial ischemia/reperfusion injury by blocking AT₁ and bradykinin B₂ receptors, respectively, suggesting a role of the bradykinin B₂ receptor in PC. Losartan provided cardioprotection through both bradykinin-dependent and bradykinin-independent mechanisms. (Circulation. 2000;102[suppl III]:III-346-III-351.)

Key Words: ischemia ■ reperfusion ■ angiotensin ■ bradykinin ■ receptors

Mammalian hearts have a remarkable ability to adapt themselves to potentially lethal exogenous stresses, which include environmental stresses such as hypoxia, heat shock, and oxidative stress. Increased tolerance to a sustained ischemic insult by hearts previously exposed to cyclic episodes of brief periods of ischemia and reperfusion has been documented in numerous experimental models. This transient adaptive response associated with decreased reperfusion-induced arrhythmias, increased recovery of post-ischemic contractile function, and reduced infarct size is known as ischemic preconditioning (PC). The current hypothesis suggests that the trigger for classic PC is one or more intracellular mediators including catecholamines, adenosine, nitric oxide, bradykinin, and angiotensin. These intracellular mediators are released into the coronary circulation within minutes of ischemia. These mediators either alone or in combination potentiate a cascade of signal transduction involving multiple kinases leading to the induction of the expression of genes.

A large number of studies including our own have implicated a role for bradykinin in PC. We have also demonstrated the cardioprotective abilities of ACE inhibitors. The present study reinforces the hypothesis that ACE inhibition potentiates PC through bradykinin B₂-receptor activation. Isolated rat hearts were perfused in the absence or presence of...
losartan (an angiotensin AT₁ inhibitor), HOE 140 (a bradykinin B₂ blocker) or both losartan and HOE 140 together. The results document that HOE 140 increased myocardial ischemia/reperfusion injury by blocking the bradykinin B₂ receptor and increasing angiotensin II formation. This suggests a role of the bradykinin B₂ receptor in PC. Losartan reduced ischemia-reperfusion injury by modulating the amount of bradykinin release from the heart. The infarct size–lowering ability of losartan was partially blocked and the antiapoptotic role was completely blocked by HOE 140, documenting both the bradykinin-dependent and bradykinin-independent mechanisms of losartan.

Methods

Isolated Perfused Heart Preparation

Sprague-Dawley rats weighing ~300 g were anesthetized with pentobarbital (80 mg/kg IP). After intravenous administration of heparin (500 IU/kg), the chests were opened and the hearts were rapidly excised and mounted on a nonrecirculating Langendorff perfusion apparatus. The perfusion buffer used in this study consisted of a modified Krebs-Henseleit bicarbonate buffer (KHB) (in mmol/L: 118 NaCl, 4.7 KCl, 1.2 MgSO₄, 1.2 KH₂PO₄, 25 NaHCO₃, 10 glucose, and 1.7 CaCl₂, gassed with 95% O₂–5% CO₂, filtered through a 5-μm filter to remove any particulate contaminants, pH 7.4) that was maintained at a constant temperature of 37°C and was gassed continuously for the entire duration of the experiment. Left atrial cannulation was then carried out, and, after allowing for a stabilization period of 10 minutes in the retrograde perfusion mode, the circuit was switched to the antegrade working mode, which allows for the measurement of myocardial contractility as well as aortic and coronary flows, as described in detail in a previous report. Essentially, it is a left heart preparation in which the heart is perfused at a constant preload of 17 cm H₂O (being maintained by means of a Masterflex variable speed modular pump, Cole Palmer Instrument Co) against an afterload of 100 cm H₂O.

At the end of 10 minutes, after the attainment of a steady-state cardiac function, baseline functional parameters were recorded and coronary effluent samples were collected for biochemical assays. The circuit was then switched back to the retrograde mode and hearts were perfused (n=6 per group) with either KHB alone (control) or KHB supplemented with losartan (4.5 μmol/L), HOE 140 (10 μmol/L), or both for 15 minutes. At the end of this period, hearts were subjected to global ischemia for 30 minutes followed by 2 hours of reperfusion. The first 10 minutes of reperfusion was in the retrograde mode to allow for posts ischemic stabilization and thereafter in the antegrade working heart mode to allow for assessment of functional parameters. A schematic of the protocol is shown in Figure 1. Myocardial infarct size and apoptosis were determined in the heart, whereas creatine kinase, bradykinin, and angiotensin release were estimated in the coronary effluent as described below.

Measurement of Ventricular Function

Aortic pressure was measured with a Gould P23XL pressure transducer (Gould Instrument Systems Inc) connected to a side arm of the aortic cannula. The signal was amplified with a Gould 6600 series signal conditioner and monitored on a CORDAT II real-time data acquisition and analysis system (Triton Technologies). Heart rate, developed pressure (defined as the difference of the maximum systolic and diastolic aortic pressures), and the first derivative of developed pressure were all derived or calculated from the continuously obtained pressure signal. Aortic flow was measured with a calibrated flowmeter (Gilmont Instruments Inc), and coronary flow was measured by timed collection of the coronary effluent dripping from the heart.

Figure 1. Schemata of experimental protocol. Isolated perfused rat hearts were stabilized for 15 minutes followed by 30-minute ischemia (30°Isch) and 2-hour reperfusion. Ventricular function (VF) and RIA for bradykinin and angiotensin II were performed at baseline (BL) and during reperfusion. Coronary effluents were taken at baseline and at 30 (R30), 60 (R60) and 120 (R120) minutes of reperfusion. Myocardial infarct size and apoptosis were evaluated at end of 120 minutes of reperfusion.

Evaluation of Myocardial Infarct Size

Hearts to be used for infarct size calculations were taken on termination of the experiment and immersed in 1% triphenyl tetrazolium solution in phosphate buffer (NaH₂PO₄ 88 mmol/L, NaH₂PO₄ 1.8 mmol/L) for 10 minutes at 37°C and then stored at −70°C for later processing. Frozen hearts (including only ventricular tissue) were sliced transversely in a plane perpendicular to the apical-basal axis into ~0.5-mm-thick sections, blotted dry, placed in between microscope slides, and scanned on a Hewlett-Packard Scanjet 5p single-pass flat bed scanner. With the use of NIH 1.61 image processing software, each digitized image was subjected to equivalent degrees of background subtraction, brightness, and contrast enhancement for improved clarity and distinctness. Risk (equivalent to total left ventricular muscle mass) as well as infarct zones of each slice were traced, and the respective areas were calculated in terms of pixels. The weight of each slice was then recorded to facilitate the expression of total and infarct masses of each slice in grams. The risk and infarct volumes (in cm³) of each slice were then calculated on the basis of slice weight to remove the introduction of any errors caused by nonuniformity of heart slice thickness. The risk volumes and infarct volumes of each slice were summed to obtain the risk and infarct volumes for the whole heart. Infarct size was taken to be the percent infarct volume/risk volume for any one heart.

Evaluation of Apoptosis

Immunohistochemical detection of apoptotic cells was carried out with the use of TUNEL, in which residues of digoxigenin-labeled dUTP are catalytically incorporated into the DNA by terminal deoxynucleotidyl transferase II, an enzyme that catalyzes a template-independent addition of nucleotide triphosphate to the 3'-OH ends of double- or single-stranded DNA. The incorporated nucleotide was incubated with a sheep polyclonal antidigoxigenin antibody followed by a FITC-conjugated rabbit anti-sheep IgG as a secondary antibody as described by the manufacturer (Apop Tag Plus, Oncor Inc). The sections (n=3) were washed in PBS 3 times, blocked with normal rabbit serum, and incubated with mouse monoclonal antibody recognizing cardiac myosin heavy chain (Biogenesis Ltd) followed by staining with TRITC-conjugated rabbit anti-mouse IgG (200:1 dilution, Dako Japan). The fluorescence staining was viewed with a confocal laser microscope (Olympus Co). The number of apoptotic cells was counted and expressed as a percentage of total myocyte population.
Measurements of Bradykinin and Angiotensin in Coronary Effluent

Bradykinin and angiotensin II were assayed by radioimmunoassay (RIA) with standard RIA kits obtained from Peninsula Laboratories Inc.

Statistical Analysis

A 2-way analysis of ANOVA followed by Scheffé’s test was first carried out with the Primer Computer Program (McGraw-Hill, 1988) to test for differences between groups. If differences were established, the values were compared by means of a Student’s t test for paired data. The values were expressed as mean ± SEM. The results were considered significant at a value of \( P \leq 0.05 \).

Results

Effects of Losartan and HOE 140 on Postischemic Ventricular Function

There were no differences in baseline function between the control and treatment groups. As expected, on reperfusion, the absolute values of aortic flow, developed pressure, and \( \frac{dP}{dt_{\text{max}}} \) were decreased in all groups as compared with the baseline values, whereas coronary flow did not show a significant change (Figures 2 and 3). Losartan-treated rat hearts displayed significant recovery of postischemic myocardial function. This was evidenced by significantly higher pressure and aortic flow readings throughout the reperfusion period. Significant differences were observed at all time points. In contrast, HOE 140–treated hearts displayed significantly lower postischemic recovery in aortic flow, developed pressure, and \( \frac{dP}{dt_{\text{max}}} \) compared with those for the control group. HOE 140 when combined with losartan significantly attenuated the losartan-mediated improved ventricular function. Heart rates varied from 282 to 302 bpm for all groups and did not vary between groups (results not shown).

Effects of Losartan and HOE 140 on Myocardial Infarction

Thirty minutes of ischemia followed by 2 hours of reperfusion (control) caused a large myocardial infarct (>30% area of risk) (Figure 4). Normalized infarct size in percent (infarct size/area of risk) in the control heart was 34.2 ± 2.0 versus 20.0 ± 1.8 for losartan, 41.3 ± 3.2 for HOE 140, and 25.4 ± 1.9 for losartan + HOE 140–treated hearts. Thus, losartan reduced whereas HOE 140 increased infarct size significantly compared with control. The change in infarct size by losartan was significantly increased by HOE 140.

Figure 2. Effects of losartan, HOE 140, and losartan+HOE 140 on coronary flow (top) and aortic flow (bottom). Results are expressed as mean±SEM of 6 rats per group. * \( P \leq 0.05 \) compared with control; † \( P \leq 0.05 \) compared with losartan. Black bars indicate control; striped bars, losartan; dotted bars, HOE 140; and hatched bars, losartan+HOE 140. R30, R60, and R120 indicate reperfusion at 30, 60, and 120 minutes of reperfusion, respectively.

Figure 3. Effects of losartan, HOE 140, and losartan+HOE 140 on left ventricular developed pressure (LVDP, top) and maximum first derivative of left ventricular developed pressure (LVdp/dt_{\text{max}}, bottom). Results are expressed as mean±SEM of 6 rats per group. * \( P \leq 0.05 \) compared with control; † \( P \leq 0.05 \) compared with losartan. Black bars indicate control; striped bars, losartan; dotted bars, HOE 140; and hatched bars, losartan+HOE 140. R30, R60, and R120 indicate 30, 60, and 120 minutes of reperfusion, respectively.

Figure 4. Effects of losartan, HOE 140, and losartan+HOE 140 on myocardial infarct size. Results are expressed as mean±SEM of 6 rats per group. * \( P \leq 0.05 \) compared with control; † \( P \leq 0.05 \) compared with losartan.
Effects of Losartan and HOE 140 on Cardiomyocyte Apoptosis

We performed double-antibody staining by using antibody in an Apop Tag kit and the monoclonal antibody recognizing cardiac myosin heavy chain to specifically identify cardiomyocyte apoptosis. A significant number of apoptotic cells, which are reduced significantly by losartan (B), were present in hearts treated with HOE 140 (C) or losartan+HOE 140 (D). There were no differences in number of apoptotic cells between losartan and losartan+HOE 140 groups. Bottom, Number of apoptotic cells expressed as percent of cardiomyocyte population. Results are expressed as mean±SEM of 6 rats per group. *P<0.05 compared with control; †P<0.05 compared with losartan.

Effects of Losartan and HOE 140 on Release of Bradykinin and Angiotensin

As expected, an increased amount of bradykinin and angiotensin II was found in the coronary effluent from the postischemic control myocardium (Figure 6). HOE 140 almost completely blocked the release of bradykinin but dramatically increased the angiotensin II content of the effluent. Losartan blocked angiotensin II and dramatically increased bradykinin generation. Combined treatment of the hearts with HOE 140 and losartan reduced both bradykinin and angiotensin II content in the perfusate.

Discussion

The results of our study indicated significant postischemic ventricular recovery with losartan, an AT1 receptor blocker, as demonstrated by improved developed pressure and aortic flow and reduced myocardial infarct size. In contrast, HOE 140 aggravated ischemia-reperfusion injury by reducing the recovery of postischemic contractile function and increasing myocardial infarct size. Additionally, cardiomyocyte apoptosis was reduced with losartan whereas HOE 140 accelerated apoptotic cell death. Ischemia/reperfusion induced the generation of angiotensin II and bradykinin. HOE 140 blocked bradykinin release and augmented the formation of angiotensin II whereas losartan blocked the formation of angiotensin II and augmented bradykinin formation. The results document that losartan reduced whereas HOE 140 increased myocardial ischemia/reperfusion injury by blocking AT1 and bradykinin B2 receptors, respectively, suggesting a role of the bradykinin B2 receptor in PC. Losartan provided cardioprotection in 2 ways: (1) by reducing infarct size and improving ventricular function and (2) by inhibiting cardiomyocyte apoptosis. The antiapoptotic function of losartan was completely blocked and the infarct size–lowering ability was partially blocked by HOE 140, suggesting a bradykinin–dependent and bradykinin–independent function of losartan. This is schematically illustrated in Figure 7, in which blockade of the AT1 receptor by losartan prevented angiotensin II
formation, whereas blockade of the B₂ receptor with HOE 140 both limited bradykinin release and increased angiotensin II formation.

Both bradykinin and angiotensin have been implicated in PC.\textsuperscript{6,11} In the pig heart, the bradykinin level was found to increase within 3 minutes of PC and was blocked by HOE 140.\textsuperscript{14} Perfusion of guinea pig hearts with bradykinin for 10 minutes protected the hearts against free radical injury.\textsuperscript{15} The cardioprotection induced by bradykinin in both the pig and guinea pig was reversed by HOE 140, thus suggesting the role of the bradykinin B₂ receptor in such preservation.\textsuperscript{14,16} In a study with open-chest dogs, the antirrhythmic effects of PC were abolished by blockade of the bradykinin B₂ receptor.\textsuperscript{17} Similar to bradykinin, ACE inhibitors were found to potentiate a preconditioning effect on the myocardium. For example, enalaprilat, an ACE inhibitor, and an angiotensin II receptor antagonist, EXP 3174, reduced infarct size and augmented the PC effect in the pig heart.\textsuperscript{18} Another ACE inhibitor, captopril, also potentiated the myocardial infarct size-limiting effect of PC.\textsuperscript{19}

In the present study, losartan was used to block the AT₁ receptor. There are at least 2 distinct subtypes of angiotensin II receptors, designated as AT₁ and AT₂ receptors.\textsuperscript{20} The best characterized receptor antagonists for AT₁ and AT₂ are losartan and PD123319 (1-{[4-(dimethylamino)-3-methylyphenyl[methyl]-5-(diphenylacetyl)-4,5,6,7tetrahydro-1H-imidazo [4,5-c] pyridine-6-carboxylic acid), respectively.\textsuperscript{20} Although both the AT₁ and AT₂ receptors are known to modulate cardiac function, the AT₁ receptors particularly affect the contractile and mitogenic action of angiotensin II.\textsuperscript{21} The activation of the AT₁ receptor enhances phospholipase C, resulting in the formation of inositol triphosphate (IP₃) and leading to intracellular Ca²⁺ overloading.\textsuperscript{22} Thus, blockade of the AT₁ receptor makes losartan an excellent antihypertensive drug for treatment of hypertension. In addition, losartan has found its use in the treatment of stroke, malignant nephrosclerosis, and myocardial infarction.

Although ACE antagonism or AT₁ receptor blockade has been found to mimic preconditioning, the mechanism(s) of action remains unclear. It has been reported that ACE inhibitors function in part by preserving bradykinin, and captopril, in particular, was found to potentiate the infarct size-limiting effect of PC through the bradykinin B₂ receptor in an isolated rabbit heart model.\textsuperscript{23} Another recent study showed similar results as reported here, namely the bradykinin-dependent cardioprotective effects of losartan against ischemia and reperfusion in rat hearts.\textsuperscript{24} In the present study, both the postischemic ventricular recovery and infarct size–lowering abilities of losartan were partially inhibited by HOE. Interestingly enough, the losartan-mediated reduction of cardiomyocyte apoptosis was completely abolished by HOE 140.

Our own laboratory has supported the notion that cardiomyocyte apoptosis and necrosis are independent contributors to myocardial infarction, and ischemia and reperfusion lead both to apoptotic cell death and cell necrosis whereas PC results in the decrease in both apoptosis and necrosis.\textsuperscript{25} The results of this study also demonstrated increased apoptosis in the ischemic reperfused myocardium, with losartan significantly decreasing apoptotic cell death. This was completely reversed by HOE 140, suggesting that the losartan-mediated decrease in cardiomyocyte apoptosis was due to bradykinin B₂ receptor activation. These results were further supported by the observation that losartan significantly increased bradykinin formation that was blocked by HOE 140.

In conclusion, this study showed that losartan mimicked the preconditioning effects by its ability to reduce infarct size and to improve postischemic ventricular recovery. These cardioprotective properties of losartan were only partially blocked by HOE 140. In contrast, losartan-mediated decrease in cardiomyocyte apoptosis was completely inhibited by HOE 140, suggesting the apoptotic cell death–lowering ability of losartan was due to the bradykinin B₂-receptor activation. This study has documented for the first time that losartan provided cardioprotection by both bradykinin-dependent and bradykinin-independent pathways.

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References

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