Aldose reductase (AR) is a member of the aldo-keto reductase superfamily. Although this enzyme is expressed in most eukaryotic cells and is known to catalyze the reduction of several aldehydes including aldo-sugars, its physiological role remains unclear. Recent investigations have shown that AR exhibits high affinity for hydrophobic aldehydes, such as those generated during lipid peroxidation. The most abundant among the lipid-derived aldehydes, 4-hydroxy-trans-2-nonenal (HNE) and its glutathione conjugate, are excellent substrates of AR. Because lipid-derived aldehydes are cytotoxic, the ability of AR to metabolize them suggests that this enzyme may be involved in protection against oxidative injury. This function of AR could be important in myocardial ischemia/reperfusion, which is associated with increased generation of reactive oxygen species. Indeed, mounting evidence indicates that HNE is a major product of lipid peroxidation during myocardial ischemia/reperfusion and that the formation of HNE and the accumulation of HNE-modified proteins are related to the extent of tissue damage.

The late phase of ischemic preconditioning (PC) is a long-lasting adaptive response of the myocardium to a mild ischemic stress that confers relative resistance against both mild, reversible ischemia/reperfusion injury (myocardial stunning) and severe, irreversible injury (myocardial infarction). The protection afforded by late PC appears 12 to 24 hours after the initial ischemic challenge, lasts 3 to 4 days, and is mediated by the synthesis of cardioprotective proteins. Among these, recent pharmacological and/or genetic studies have identified inducible NO synthase (iNOS) and cyclooxygenase-2 (COX-2) as essential mediators of the infarct-sparing effects of late PC. However, in view of the evidence that late PC is a complex polygenic adaptation, it seems plausible to postulate that other protein(s) may also be involved. Although AR is known to be a stress-responsive enzyme and could be potentially cardioprotective by virtue of its antioxidant activity, virtually nothing is known regarding its involvement in late PC and its role in myocardial protection remains obscure.

**Abstract**—Aldose reductase (AR), a member of the aldo-keto reductase superfamily, has been shown to metabolize toxic aldehydes generated by lipid peroxidation, suggesting that it may serve as an antioxidant defense. To investigate its role in the late phase of ischemic preconditioning (PC), conscious rabbits underwent 6 cycles of 4-minute coronary occlusion/4-minute reperfusion. Twenty-four hours later, there was a marked increase in AR protein and activity and in the myocardial content of sorbitol, a unique product of AR catalysis. Pretreatment with Nω-nitro-L-arginine, a nitric oxide synthase inhibitor, or chelerythrine, a protein kinase C inhibitor (both given at doses that block late PC in this model), prevented the increase in AR protein 24 hours later, demonstrating that ischemic PC upregulates AR via nitric oxide– and protein kinase C–dependent signaling pathways. The AR-selective inhibitors tolrestat and sorbinil prevented AR-mediated accumulation of sorbitol and abrogated the infarct-sparing effect of late PC, demonstrating that enhanced AR activity is necessary for this cardioprotective phenomenon to occur. Inhibition of AR did not affect infarct size or the myocardial accumulation of the lipid peroxidation product 4-hydroxy-trans-2-nonenal (HNE) in nonpreconditioned rabbits. The accumulation of HNE was inhibited by late PC, and this effect was abrogated by sorbinil. Taken together, these results establish AR as an essential mediator of late PC. Furthermore, the data suggest that myocardial ischemia/reperfusion injury is due in part to the generation of lipid peroxidation products and that late PC diminishes this source of injury by upregulating AR. (Circ Res. 2002;91:240-246.)

**Key Words:** myocardial ischemia/reperfusion injury  ■  aldose reductase  ■  myocardial infarction  ■  4-hydroxy-trans-2-nonenal  ■  ischemic preconditioning
In the present study, we tested the hypothesis that AR plays an obligatory role in mediating the protective effects of the late phase of ischemic PC. Using a well-characterized rabbit model, we first sought to determine whether AR is upregulated 24 hours after a PC protocol known to elicit delayed protection against myocardial infarction.\textsuperscript{12,14,16} We next examined whether upregulation of AR is mediated by NO and/or protein kinase C (PKC), two early elements of the signaling pathways of late PC.\textsuperscript{11,13–15,17,21,22} Finally, we determined whether blocking AR activity with AR selective inhibitors interferes with the delayed protective effects elicited by ischemic PC and prevents the detoxification of HNE generated during ischemia/reperfusion. These studies were conducted in conscious animals to obviate the potentially confounding influence of conditions associated with open-chest animal preparations,\textsuperscript{11,12,16} particularly in view of the fact that ROS generation is markedly exaggerated in open-chest models.\textsuperscript{23} The results demonstrate that ischemic PC up regulates AR in the heart and that AR activity is essential for the infarct-sparing actions of the late phase of PC, thereby identifying, for the first time, AR as a cardioprotective protein.

**Materials and Methods**

The conscious rabbit model of myocardial ischemia has been described in detail previously.\textsuperscript{11–14,16} Briefly, New Zealand White male rabbits (obtained from Myrtle’s Rabbitry, Thompson Station, Tenn.; weight, 2.3±0.1 kg) were instrumented under sterile conditions with a balloon occluder around a major branch of the left coronary artery, a 10-MHz pulsed Doppler ultrasonic crystal in the center of the region to be rendered ischemic, and bipolar ECG leads on the chest wall. Rabbits were allowed to recover for a minimum of 14 days after surgery. The present study was performed in accordance with the guidelines of the Animal Care and Use Committee of the University of Louisville (Ky) School of Medicine and with the Guide for the Care and Use of Laboratory Animals (Department of Health and Human Services, publication No. [NIH] 86–23).

**Phases of Study**

The study consisted of 3 consecutive phases (Phases I through III).

**Phase I: Studies of AR Expression and AR and Sorbitol Dehydrogenase (SDH) Activity**

Rabbits were assigned to 5 groups (online Figure 1 found in the online data supplement available at http://www.circresaha.org). Rabbits in group I (control) did not receive any treatment and did not undergo coronary occlusion. They were euthanized and samples of the anterior and posterior left ventricular (LV) wall were rapidly removed and stored at −140°C until use. Rabbits in groups II (PC) underwent a sequence of six 4-minute coronary occlusion/4-minute reperfusion cycles and were euthanized 24 hours later. Myocardial samples were removed from the ischemic/reperfused and the nonischemic region, and stored at −140°C until use. Rabbits in groups III to V underwent the same sequence of occlusion/reperfusion cycles. Group III (L-NA+PC) received N\textsuperscript{4}-nitro-L-arginine (L-NA, IV, 13 mg/kg) for 10 minutes, starting 20 minutes before and ending 10 minutes before coronary occlusion. Group IV (CHE+PC) received chelerythrine (IV, 5 mg/kg) 5 minutes before the first coronary occlusion. These doses of L-NA and chelerythrine block the cardiovascular effects of late PC in this model.\textsuperscript{11–14} L-NA (Sigma) was dissolved in normal saline. Chelerythrine chloride (Research Bio-medicals International) was dissolved in dimethyl sulfoxide (DMSO) and then diluted with normal saline to a final concentration of 50% DMSO in saline (vol/vol). Rabbits in group V (PC+tolrestat) underwent a sequence of 6 occlusion/reperfusion cycles without any treatment on day 1. Twenty-four hours later, they received tolrestat (10 mg/kg; IV) and were euthanized 30 minutes after the injection. Tolrestat (Wyeth-Ayerst) was dissolved in 25 mmol/L bicarbonate buffer, pH 8.5.

The frozen samples were powdered in liquid nitrogen and suspended in 50 mmol/L potassium phosphate buffer, pH 7.4, containing 5 mmol/L dithiothreitol (DTT) and 1 mmol/L PMSF, 25 μg/mL leupeptin, 25 mmol/L NaF, and 1 mmol/L NaN\textsubscript{3}O. The extract was centrifuged at 13 000 g for 30 minutes at 4°C, and the supernatant was used for the measurements of AR and sorbitol dehydrogenase (SDH) activity.

**Phase II: Studies of Myocardial Infarction**

All rabbits were subjected to a 30-minute coronary artery occlusion followed by 3 days of reperfusion. Rabbits were assigned to eight groups (online Figure 2). Rabbits in group VI (control) underwent the 30-minute occlusion without PC or drug pretreatment. Rabbits in group VII (PC) were preconditioned with a sequence of six 4-minute coronary occlusion/4-minute reperfusion cycles 24 hours before the 30-minute coronary occlusion. Rabbits in groups IX, X, XII, and XIII were also preconditioned with the sequence of 6 occlusion/reperfusion cycles on day 1. Twenty-four hours later, they received either an IV injection of tolrestat (Wyeth-Ayerst; 10 mg/kg; group IX: PC+tolrestat)) or an IP injection of sorbinil (Pfizer; 40 mg/kg [group XII: PC+sorbitol]), 30 minutes before the coronary occlusion. Sorbinil was dissolved in DMSO and then diluted with normal saline to a final concentration of 50% DMSO in saline (vol/vol). Rabbits in group X (PC+vehicle I) and XIII (PC+vehicle II) received only the vehicle used for dissolving tolrestat (intravenous) or sorbinil (intraperitoneal). Rabbits in groups VIII (tolrestat) and XI (sorbitil) received the same doses of tolrestat or sorbinil without a PC protocol 24 hours earlier.

**Postmortem Tissue Analysis**

At the conclusion of the study, the occluded/reperfused vascular bed and the infarct were identified by postmortem perfusion of the heart with Phthalo blue dye and triphenyltetrazolium.\textsuperscript{14,16} Infarct size was calculated by using computerized video planimetry.\textsuperscript{14,16}

**Phase III: Measurements of HNE**

To measure ischemia-induced changes in HNE, rabbits in groups XIV through XVII underwent 30 minutes of coronary occlusion and were euthanized after 60 minutes of reperfusion (online Figure 3). Group XIV (I/R) was subjected to a 30-minute occlusion followed by 1 hour of reperfusion. Group XV received sorbinil (40 mg/kg, IP) 30 minutes before occlusion. Groups XVI (PC+I/R) and XVII (PC+I/R+sorbitil) were preconditioned with 6 occlusion/reperfusion cycles 24 hours before the 30-minute occlusion; additionally, rabbits in group XVII received sorbinil (40 mg/kg, IP) 30 minutes before occlusion on day 2. The concentration of free HNE was measured according to the method of Luo et al\textsuperscript{25} (see online data supplement).

**Statistical Analysis**

Data are reported as mean±SEM. Data were analyzed with either a 1-way or a 2-way repeated-measures ANOVA, as appropriate, followed by paired or unpaired Student’s t test with the Bonferroni correction.

**Results**

A total of 121 rabbits were instrumented for this study.

**Phase I: Studies of AR Expression and Activity**

Exclusions

Of the 26 rabbits instrumented for phase I, 6 were assigned to group I (control), 6 to group II (PC), 5 to group III (L-NA+PC), 5 to group IV (CHE+PC), and 4 to group V...
All rabbits completed the protocol successfully.

Expression of AR Protein

A representative Western blot of AR in membranous fractions prepared from groups I through III is illustrated in Figure 1. In control rabbits (group I), the AR protein was detected in both the cytosolic and the membranous fractions, although more than 85% of the protein was associated with the cytosolic fraction (estimated from the relative band intensities on Western blots). Isolated rabbit myocytes displayed immunoreactivity to anti-AR antibodies, indicating that AR is expressed in cardiac myocytes (online Figure 4). Our previous studies show that myocytes, isolated from rabbit or guinea pig hearts, metabolize aldehydes via AR. When rabbits were preconditioned with six 4-minute occlusion/4-minute reperfusion cycles 24 hours earlier (group II), the expression of AR in the membranous fractions increased markedly in both the ischemic/reperfused and nonischemic region 24 hours after ischemic PC (A), whereas no change was observed in AR protein in the cytosolic fractions (B). Pretreatment before the occlusion/reperfusion cycles with L-NA (C) or chelerythrine (D) prevented the increase in AR protein expression in the membranous fractions 24 hours later.

Myocardial AR Activity and Sorbitol Levels

The increase in AR protein was accompanied by an increase in sorbinil-sensitive AR activity (Figure 3A) and an increase in the myocardial levels of sorbitol (Figure 3B). Administration of the selective AR inhibitor, tolrestat, completely abrogated the increase in myocardial sorbitol levels induced by ischemic PC (Figure 3B). Thus, the dose of tolrestat used in the present study was effective in blocking the increase in AR activity associated with ischemic PC in vivo. In contrast to AR, we found no detectable expression of SDH in the rabbit heart, and SDH was not induced by ischemic PC (online Figure 6).

Phase II: Studies of Myocardial Infarction

Exclusions

Of the 74 rabbits instrumented for the studies of myocardial infarction, 10, 10, 9, 9, 9, 9, 9, and 9 were assigned to groups...
Figure 4. Effect of AR inhibitors on infarct size. Myocardial infarct size in groups VI (control, n=8), VII (PC, n=8), VIII (tolrestat, n=7), IX (PC + tolrestat, n=9), X (PC + tolrestat, n=9), XI (sorbinil, n=8), XII (PC + sorbinil, n=8), and XIII (PC + vehicle, n=7). Infarct size is expressed as a percentage of the region at risk of infarction. Open circles represent individual rabbits, whereas filled circles represent mean ± SEM.

VI, VII, VIII, IX, X, XI, XII, and XIII, respectively. Seven rabbits died of ventricular fibrillation during the 30-minute coronary occlusion (2 in group VI, 2 in group VII, 1 in group XII, and 2 in group XIII). Two rabbits were excluded because of a small region at risk (<10% of the left ventricle) (one each in group VIII and XI). One rabbit in group VIII was excluded because of postoperative problems (pericarditis).

Hemodynamic Parameters
There were no appreciable differences in heart rate throughout the experimental protocol among groups V through XII (data from groups IX, XII, and XIII are shown in online Table 1, which can be found in the online data supplement available at http://www.circresaha.org). In addition, there were no differences in thickness fraction among the eight groups at baseline (before administration of vehicle, tolrestat, or sorbinil) and just before coronary occlusion (data not shown). In four rabbits in each of groups IX, XII, and XIII, arterial blood pressure was measured using a 22-gauge angiocatheter inserted into ear dorsal artery as previously described.11 There was no difference in mean arterial pressure at baseline or before coronary occlusion (online Table 1).

Region at Risk and Infarct Size
The size of the LV and of the region at risk did not differ among the 8 groups (online Table 2). The average infarct size was 47% smaller in group VII (PC) compared with group VI (control), indicating late PC against myocardial infarction (Figure 4). In contrast, in preconditioned rabbits treated with either tolrestat (group IX) or sorbinil (group XII), infarct size was similar to that measured in controls (Figure 4), indicating that both drugs abrogated the protective effect of late PC. Administration of vehicle (groups X and XIII) did not interfere with late PC. In groups VIII and XI, infarct size did not differ from that observed in controls, indicating that tolrestat and sorbinil did not affect the extent of cell death in nonpreconditioned myocardium. For any given size of the region at risk, the resulting infarction was greater in preconditioned rabbits treated with either tolrestat or sorbinil than in untreated preconditioned rabbits (online Figure 7).

Phase III: Measurements of HNE
Exclusions
Of the 21 rabbits instrumented for phase III, 6, 5, 5, and 5 were assigned to groups XIV, XV, XVI, and XVII, respectively. One rabbit was excluded in group XIV due to ventricular fibrillation. All other rabbits completed the protocol successfully.

As shown in Figure 5, 30 minutes of occlusion followed by 60 minutes of reperfusion (group XIV) led to a 5-fold increase in the content of free HNE in the ischemic/reperfused (anterior wall) and nonischemic (posterior wall) regions of the hearts of rabbits that underwent 30 minutes of occlusion and 1 hour of reperfusion without (group XIV) or with sorbinil (group XV) administered 30 minutes before occlusion. Group XVI underwent ischemic PC 24 hours before occlusion. Rabbits in group XVII were preconditioned 24 hours before occlusion, but were treated with sorbinil 30 minutes before occlusion.

Discussion
Although AR and its homologs are widely distributed aldoketo reductases, the physiological role of these enzymes remains unclear. In vitro, AR catalyzes the reduction of a large array of aldehydes including aldosugars.2 Our recent observations showing that the enzyme displays $10^3$- to $10^4$-
fold lower $K_m$ for lipid-derived aldehydes as compared with glucose.\textsuperscript{3,30} suggest that AR may be involved in the metabolism of toxic aldehydes such as those generated during lipid peroxidation. To test whether AR provides antioxidant protection by diminishing tissue injury caused by lipid peroxidation, we examined its regulation by ischemia and its involvement in ischemia/reperfusion injury, which is accompanied by enhanced generation of oxygen-derived free radicals.

Our results show that brief episodes of myocardial ischemia (ischemic PC) upregulate AR. The elevated levels of AR were associated with a parallel increase in the myocardial content of sorbitol and in the sorbinil-sensitive myocardial AR activity, indicating that the newly-synthesized protein is catalytically active. Inhibition of AR with two unrelated compounds completely abrogated the infarct-sparing effects of late PC (Figure 4), demonstrating that AR plays an essential role in the cardioprotection afforded by the late phase of ischemic PC. Inhibition of AR also abrogated the protective effects of late PC against the accumulation of the cytotoxic lipid aldehyde HNE (Figure 5), demonstrating that AR activity is important for the antioxidant actions of late PC. Taken together, these results expand our understanding of late PC by identifying a new molecular effector of this cardioprotective phenomenon and by demonstrating that AR serves an important antioxidant function.

The increase in the expression of AR after ischemia is consistent with its role as a stress-responsive protein. The expression of AR is known to be upregulated by oxidative, osmotic, or chemical stress.\textsuperscript{2,19,20,24} Moreover, AR was reported to be one of the few genes whose expression was consistently enhanced in failing human hearts,\textsuperscript{31} suggesting that upregulation of AR may be a general protective response of the stressed heart. However, the mechanism whereby ischemia upregulates AR is unknown. We tested the role of NO and PKC because previous studies have demonstrated that ischemic PC activates PKC via formation of NO\textsuperscript{14} and that the generation of NO and the activation of PKC are essential for the development of late PC.\textsuperscript{1,13,14} Our results demonstrate that the induction of AR was prevented by the same doses of L-Na and cherytherine that block the activation of NOS and PKC, respectively, in this model,\textsuperscript{13,14} demonstrating that the generation of NO and the subsequent activation of PKC is essential for the induction of AR by ischemic PC. These results suggest that upregulation of AR is an important component of the molecular changes that underlie the development of late PC, thereby prompting us to examine the function of AR in this phenomenon.

To test the role of AR, we used two structurally unrelated inhibitors, so as to minimize the possibility that the results could be due to nonspecific actions of either drug. We found that the infarct-sparing effects of late PC were completely abrogated by both tolrestat and sorbinil. Our measurements of myocardial sorbitol and HNE levels provide direct evidence that tolrestat and sorbinil effectively blocked the increase in AR activity associated with PC (Figures 3B and 5). However, neither drug decreased infarct size in non preconditioned rabbits, indicating that AR does not modulate ischemia/reperfusion injury in the absence of ischemic PC. The reason for this selective role of AR in protecting the preconditioned heart is presently unclear, but may relate to the low abundance of AR in the rabbit heart. Although AR protein, activity, and sorbinil contents were detectable in the rabbit heart, the AR activity was only one-tenth of that detected in the rat heart (unpublished observations, 2002). Another possibility is that the ischemia-induced upregulation of AR in the membranous fraction (Figures 1 and 2) may be essential for this enzyme to reduce lipid-derived aldehydes that are generated and retained in the membrane due to their high hydrophobicity. A third possibility, supported by the greater accumulation of HNE in sorbinil-treated PC hearts (Figure 5), is that late PC induces a prooxidant state, which is counteracted by the upregulation of AR but is unmasked by AR inhibitors. This would be in agreement with previous reports showing that the late preconditioned phenotype is established by an increase in the levels of oxidant-generating enzymes such as COX-2 and iNOS.\textsuperscript{11,12,14,17} According to this hypothesis, the role of AR may be more important in preconditioned hearts because they generate higher levels of lipid peroxidation products compared with non preconditioned hearts. That inhibition of AR did not increase HNE levels in the non preconditioned myocardium (Figure 5, group XV) rules out the possibility that HNE generation is less in preconditioned vis-à-vis non preconditioned hearts and that AR is unable to effectively remove all the HNE that is produced in the non preconditioned hearts. Rather, our results are consistent with the view that the non preconditioned rabbit heart does not contain enough AR in the appropriate compartment (membrane) to prevent HNE accumulation.

In apparent contradiction to our findings, two recent studies\textsuperscript{25,26} have concluded that pharmacological inhibition of AR reduces ischemia/reperfusion injury in isolated perfused hearts. It was speculated that AR inhibitors exert their protective effects by limiting the increase in the NADH/NAD$^+$ ratio as a result of inhibition of the polyol pathway, thereby maintaining glycolysis and limiting ATP depletion during ischemia.\textsuperscript{23} However, these studies\textsuperscript{5,26} examined only one AR inhibitor and it is unclear whether the effects were specific to this drug. One investigation\textsuperscript{23} was conducted in diabetic rats, and therefore comparison with our results is not possible. In the study that examined nondiabetic rabbit hearts,\textsuperscript{26} the infarct size measured after treatment with the AR inhibitor zoprolrestat ranged widely (from $\approx$8% to 70% of the risk region), making conclusions difficult. Importantly, we found no detectable expression or activity of SDH in the heart either in control conditions or after ischemia (online Figure 6), implying that in the rabbit heart sorbitol is not further metabolized to fructose. Consequently, in this species inhibition of AR cannot protect the heart by increasing the NADH/NAD$^+$ ratio.

The finding that infarct size limitation by ischemic PC is mediated by AR, which is primarily an aldehyde-metabolizing enzyme, suggests that ischemia/reperfusion causes lipid peroxidation and that the toxicity of lipid-derived products is a significant component of tissue injury. Although the occurrence of oxidative stress during ischemia/reperfusion is well documented, conflicting results have been reported regarding the ability of antioxidants to limit infarct
size. To date, almost all antioxidant interventions have been targeted at oxygen-free radicals such as superoxide anion or hydroxyl radical. However, these species are highly reactive and short-lived. Consequently, they would be expected to cause damage at or near the site of their formation. In contrast, the toxic aldehydes generated by lipid peroxidation have a considerably longer half-life, and thus can mediate or amplify the cellular effects of their radical precursors. Because in vitro HNE causes metabolic inhibition and death of cardiac myocytes, it is likely that accumulation of HNE is toxic to the ischemic heart. Unsaturated aldehydes have been shown to be generated during myocardial ischemia/reperfusion. Recently, Eaton et al. have reported that proteins modified by HNE increase in ischemic rat hearts with increasing durations of ischemia. Nevertheless, the functional role of unsaturated aldehydes in ischemia/reperfusion injury remains virtually unknown.

Our observations provide important new insights into this issue. Consistent with previous reports, we found that ischemia/reperfusion leads to a marked accumulation of free HNE in the heart (Figure 5), indicating increased lipid peroxidation. The accumulation of HNE was significantly reduced by ischemic PC (Figure 5). To our knowledge, this is the first demonstration that late PC is associated with mitigation of oxidant stress. Sorbinil, given at doses that abrogated protection against infarction, completely blocked the antioxidant actions of late PC, resulting in a marked increase in free HNE formation (Figure 5). Taken together, these data provide the first evidence to suggest that formation of lipid-derived electrophiles is a significant cause of ischemia/reperfusion injury and that the mechanism whereby late PC confers cytoprotection involves, at least in part, AR-dependent attenuation of aldehyde accumulation. Nevertheless, our data suggest that HNE accumulation, at the levels noted in our model, is not sufficient to precipitate cell death in the absence of ischemia/reperfusion injury because the HNE content of the nonischemic region of preconditioned hearts treated with sorbinil (group XVII) was comparable to that of the ischemic/reperfused zone of non preconditioned hearts (group XIV), yet no infarction was present in the nonischemic region of sorbinil-treated preconditioned hearts (group XII). The observation that the HNE content increased in the nonischemic zone of preconditioned hearts treated with sorbinil (group XVII) is somewhat surprising, and suggests that PC is associated with a global increase in oxidative stress. We propose that this increased prooxidant state does not result in increased HNE levels as long as AR activity is upregulated; however, when AR is inhibited, HNE levels increase (Figure 5). The mechanism for the increased prooxidant state in the nonischemic preconditioned myocardium remains speculative at present. Even in non preconditioned hearts, regional ischemia leads to a global increase in lipid peroxidation products. Our data indicate that the accumulation of these products per se does not lead to tissue injury, and that high levels of these oxidants can be tolerated by nonischemic myocardium, whereas ischemic/reperfused myocardium is more sensitive to their toxic actions.

In summary, the present study identifies AR as an essential mediator responsible for the beneficial effects of the late phase of ischemic PC. Our results support the concept that cytotoxic aldehydes are a significant cause of myocardial ischemia/reperfusion injury and that AR is an antioxidant enzyme that detoxifies aldehydes generated by lipid peroxidation. This study also suggests that late PC exerts its cytoprotective effects by a hitherto unrecognized mechanism, namely, decreased accumulation of lipid peroxidation products. Although further investigation will be required to elucidate the relationship between aldehyde detoxification and the other known cardioprotective mechanisms recruited by ischemic PC, the findings reported herein bring a new focus on AR and lipid peroxidation and should stimulate further research into their role in myocardial ischemia/reperfusion. Moreover, the finding that AR mediates the protective effects of ischemic preconditioning has implications for the clinical use of AR inhibitors for treating diabetic complications.

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References


Aldose Reductase Is an Obligatory Mediator of the Late Phase of Ischemic Preconditioning
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SUPPLEMENTAL INFORMATION

**Measurement of AR and SDH:** The frozen samples were pulverized in liquid nitrogen. The powder was suspended in 50 mM potassium phosphate buffer, pH 7.4, containing 5 mM dithiothreitol (DTT) and 1 mM PMSF, 25 µg/ml leupeptin, 25 mM NaF, and 1 mM Na₃VO₄. The tissue was homogenized and sonicated 3 times for 20 s each. The extract was centrifuged at 13,000 x g for 30 min at 4 °C, and the supernatant was used for the measurements of AR and SDH activity. AR activity was measured in a 1 ml system containing 0.4 M Li₂SO₄, 0.1 mM NADPH and 10 mM DL-glyceraldehyde in 0.05 mM potassium phosphate, pH 6.0, and the change in absorbance was measured at room temperature at 340 nm. The SDH activity was determined in a reaction mixture containing 0.24 M Tris-HCl, pH 9.5, 0.1 mM NAD, and 0.3 M sorbitol by monitoring the increase in absorbance at 340 nm². The fructose oxidase activity of SDH was measured in 0.25 M potassium phosphate, pH 7.4 containing 0.2 mM NADH, and 0.2 M fructose. The enzyme activities were defined as the μmoles of NAD(P)(H) oxidized or reduced/ mg protein/min.

For Western analysis, tissue samples were homogenized in buffer A (25 mM Tris-HCl; pH 7.4, 0.5 mM EDTA, 0.5 mM EGTA, 1 mM PMSF, 25 µg/ml leupeptin, 1 mM DTT, 25 mM NaF, and 1 mM Na₃VO₄) and centrifuged at 14,000 g for 12 min at 4°C, and the resulting supernatants were collected as cytosolic fractions². The pellets were incubated in a lysis buffer (buffer A + 1% Triton X-100) for 2 h and centrifuged, and the resulting supernatants were collected as the membranous fractions. The expression of AR was assessed by standard SDS-PAGE Western immunoblotting techniques using anti-AR antibodies. Gel transfer efficiency was recorded carefully by making photocopies of membranes dyed with reversible Ponceau staining; gel retention was determined by Coomassie blue staining. The AR signals and the corresponding records of Ponceau stains of nitrocellulose membranes were quantitated by an image scanning densitometer²,³, and each AR
signal was normalized to the corresponding Ponceau stain signal. The polyclonal anti-sorbitol dehydrogenase (SDH) antibodies were a gift from Dr. S. S. Chung. In all samples, the content of AR protein was expressed as a percentage of the AR protein in the anterior LV wall of group I (control group).

**Measurement of HNE:** Tissue samples (~50 mg) were homogenized in 1.0 ml water containing EDTA (400 µM), butylated hydroxy toluene (20 µM), desferral (20 µM), and 50 pmoles benzaldehyde-ring-D₅ (internal standard) at 0 °C. Four hundred microliters of a 0.05 M solution of O-(2,3,4,5,6-pentafluorobenzyl) hydroxylamine hydrochloride (PFBHA.HCl)⁴ was added to the samples and the mixtures were vortexed for 1.0 min and incubated for 30 min at room temperature. After incubation, the samples were deprotenized by the addition of 1.0 ml methanol, and 4.0 ml hexane was added. The samples were vortexed for one min and 12 drops of concentrated sulfuric acid were added and the samples were vortexed for an additional minute. The mixtures were centrifuged at 5000 x g and the upper hexane layer was aspirated, dried over sodium sulfate and evaporated to dryness, under a stream of nitrogen. Fifty microliter aliquots of n-O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) reagent were added to each vial and the samples were incubated at 80 °C for 5.0 min, and 1.0 µl aliquots were used for GC-MS analysis.

Derivatized extracts were analyzed with a HHP5890/HP5973 GC/MS system (Hewlett-Packard, Palo Alto, CA). The extracts were separated on a bonded-phase capillary column (DB-5MS, 15 m, 0.25 mm I.D., 0.25 mm film thickness) from J & W Scientific (Folsom, CA). The GC injection port and the interface temperature were set to 280 °C with the helium carrier gas maintained at a flow rate of 1.2 ml/min. Injection were made in the splitless mode with the inlet port purged after 1 min, increased at a rate of 8 °C per min to 200 °C, which was held for 10 min, then increased at a rate of 25 °C per min to 280 °C, and held at this temperature for 5 min. Components eluting from
GC were detected in negative chemical ionization mode with ammonia as the reagent gas. Under these conditions, the retention time of the internal standard (d5-benzaldehyde) was 14.43 min and the retention time of the derivatives of HNE was 18.30 and 18.69 min. The ions monitored for quantification were m/z 286 and 403 for the internal standard and HNE, respectively.
FIGURE LEGENDS

**Supplemental Fig. 1**  Experimental protocol for the studies of AR expression and activity (phase I).

Five groups of rabbits were studied. Rabbits in group I (control) did not receive any treatment and did not undergo coronary occlusion. Rabbits in groups II (PC) underwent a sequence of six 4-min coronary occlusion/4-min reperfusion cycles and were euthanized 24 h later. Rabbits in groups III-V also underwent the same sequence of occlusion/reperfusion cycles. In addition, rabbits in group III (L-NA + PC) received an i.v. infusion of \( N^\omega \)-nitro-L-arginine (L-NA) for 10 min, starting 20 min before and ending 10 min before the first coronary occlusion (total dose; 13 mg/kg, total volume infused 20 ml). Rabbits in group IV (CHE + PC) received an i.v. bolus of chelerythrine (5 mg/kg) 5 min before the first coronary occlusion. Rabbits in group V (PC + tolrestat) underwent a sequence of six occlusion/reperfusion cycles without any treatment on day 1. Twenty-four hours later (day 2), they received an i.v. injection of tolrestat (10 mg/kg) and were euthanized 30 min after the injection.

**Supplemental Fig. 2**  Experimental protocol for the studies of myocardial infarction (phase II).

Rabbits were assigned to 8 groups. Rabbits in group VI (control) underwent the 30-min occlusion without PC protocol or drug pretreatment. Rabbits in group VII (PC) were preconditioned with a sequence of six 4-min coronary occlusion/4-min reperfusion cycles 24 h prior to the 30-min coronary occlusion. Rabbits in groups IX, X, XII and XIII were also preconditioned with the sequence of six occlusion/reperfusion cycles on day 1. Twenty-four hours later, rabbits received an i.v. injection of tolrestat (10 mg/kg) 30 min before the coronary occlusion [groups IX (PC + tolrestat)] or an i.p. injection of sorbinil (total dose; 40 mg/kg)[ group XII (PC + sorbinil)], respectively. Rabbits in groups VIII (tolrestat) and XI (sorbinil) received the same dose of tolrestat or sorbinil without the PC protocol 24 h before. Rabbits in
group XII (PC + vehicle) were preconditioned with the sequence of six occlusion/reperfusion cycles on day 1 and received an i.v. infusion of vehicle (used for dissolving sorbinil, total volume infused 5 ml/kg) 30 min before the coronary occlusion on day 2.

**Supplemental Fig. 3** Experimental protocol for measuring HNE (phase III). Rabbits were assigned to 4 groups. Rabbits in group XIV underwent 30 min of occlusion without PC or drug treatment and tissue samples were collected after 1 h of reperfusion. Rabbits in group XV received sorbinil (40 mg/mg, i.p.) 30 min before occlusion. Groups XVI was preconditioned with a sequence of six 4-min coronary occlusion/4 min reperfusion cycles 24 h prior to the 30 min occlusion. The tissue samples were harvested 1 h after reperfusion. Rabbits in group XVII were preconditioned on day 1, and 24 h later, they received an i.p. injection of sorbinil (40 mg/kg), 30 min before coronary occlusion and their hearts were harvested 1 h after reperfusion.

**Supplemental Fig. 4** Expression of AR in isolated rabbit cardiac myocytes. Cardiac myocytes were isolated from rabbit hearts using a standard enzymatic dissociation procedure as described previously⁵. The myocytes were lysed within 3 to 4 h of isolation and the cell extract was separated by SDS-PAGE followed by Western blotting using anti-AR antibodies. Two concurrent lanes (A and B) are shown. The band corresponding to AR is marked with an arrow. The intensity of the AR band normalized to the amount of protein loaded was comparable to that obtained with cardiac homogenates.

**Supplemental Fig. 5** Quantification of AR expression in rabbit hearts. The rabbit hearts were powdered in liquid nitrogen and the extract was separated and immunoblotted as described in the text. Panel A shows a typical Western blot performed to identify the expression of AR protein. Lanes marked “control” were loaded with extract from control hearts, whereas those
from preconditioned rabbits are marked “LPC”. The last lane on the right (marked “Positive Control”) was loaded with AR protein purified from *E. Coli* transfected with human AR cDNA. Panel B shows a Ponceau staining record corresponding to the Western blot in the above panel. The Ponceau staining record was used to correct errors introduced during gel loading and gel transfer processes. Specifically, the predominant protein band with the largest molecular weight (68 KDa for the cytosolic fraction and 43 KDa for the membraneous fraction) was used to correct the measurements of AR protein expression.

**Supplemental Fig. 6**  Measurements of SDH expression and activity. (A) Purified sheep SDH protein (lane 1), rabbit liver cytosol (lane 2), extracts of liver, and non-ischemic (group 1, lane 3) or ischemic (group II, lane 4) cardiac samples were separated on SDS-PAGE, transblotted and stained with anti-SDH antibody. (B) The SDH activity of the rabbit liver (lane 2) is shown (n = 3); no measurable SDH activity was detected in the nonischemic (group I) or ischemic hearts (group II), lanes 3 and 4, respectively.

**Supplemental Fig. 7**  Relationship between size of region at risk and size of myocardial infarction in groups VI (control, n = 8), VII (PC, n = 8), IX (PC + tolrestat, n = 9), and XII (PC + sorbinil n = 7). The figure illustrates both individual values and the regression lines obtained by linear regression analysis for each group. In all groups, infarct size was positively and linearly related to the size of the region at risk. The linear regression equations were as follows: group VI, y = 0.577x (r = 0.92); group VII, y = 0.328x - 0.002 (r = 0.80); group IX, y = 0.689x - 0.031 (r = 0.90); and group XII y = 0.660x - 0.035 (r = 0.91). ANCOVA demonstrated that the regression lines for groups VI, IX, and XII were significantly different from that for group VII (P < 0.05 for each comparison), indicating that for any given risk region size, infarct size was smaller in preconditioned rabbits, compared with controls; in contrast, the lines for groups IX
and XII were similar to that for group VI, indicating that tolrestat and sorbinil abrogated the infarct-sparing effects of late PC.
REFERENCES


Supplemental Figure 1

Phase I

Group I (control)

Day 1
Day 2
Tissue samples

Group II (PC)

Day 1
Day 2
Tissue samples

Group III (L-NA+PC)

Day 1
Day 2
Tissue samples

Group IV (CHE+PC)

Day 1
Day 2
Tissue samples

Group V (PC+tolrestat)

Day 1
Day 2
Tissue samples

L-NA (13 mg/kg i.v. infusion for 10 min)

chelerythrine (5 mg/kg i.v.)

tolrestat (10 mg/kg i.v.)
Phase II

Supplemental Figure 2

<table>
<thead>
<tr>
<th>Group</th>
<th>Day 1</th>
<th>Day 2</th>
</tr>
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<tbody>
<tr>
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<td>(control)</td>
<td></td>
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</tr>
<tr>
<td>Day 1</td>
<td>30 min OCCLUSION</td>
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<tr>
<td>Day 2</td>
<td>1h 3h 5h 24h 48h 72h</td>
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<tr>
<td>Group VII</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(PC)</td>
<td></td>
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</tr>
<tr>
<td>Day 1</td>
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</tr>
<tr>
<td>Day 2</td>
<td>30 min OCCLUSION</td>
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</tr>
<tr>
<td></td>
<td>1h 3h 5h 24h 48h 72h</td>
<td></td>
</tr>
<tr>
<td>Group VIII</td>
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<tr>
<td>Day 1</td>
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</tr>
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<tr>
<td></td>
<td>30 min OCCLUSION</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1h 3h 5h 24h 48h 72h</td>
<td></td>
</tr>
<tr>
<td>Group IX</td>
<td></td>
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<td>(PC+tolrestat)</td>
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<tr>
<td></td>
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<tr>
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</tr>
<tr>
<td></td>
<td>1h 3h 5h 24h 48h 72h</td>
<td></td>
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<tr>
<td>Group XI</td>
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<td>30 min OCCLUSION</td>
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</tr>
<tr>
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<td>1h 3h 5h 24h 48h 72h</td>
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<td>Group XII</td>
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<td>Day 1</td>
<td>sorbinil (40 mg/kg i.p.)</td>
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<td>Day 2</td>
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<tr>
<td></td>
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</tr>
<tr>
<td></td>
<td>1h 3h 5h 24h 48h 72h</td>
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<tr>
<td>Group XIII</td>
<td></td>
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<tr>
<td>(PC+vehicle II)</td>
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<td>DMSO (0.25 ml/kg i.p.)</td>
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<tr>
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<td>1h 3h 5h 24h 48h 72h</td>
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Supplemental Fig. 6

Sorbitol dehydrogenase activity
(µmoles NAD reduced/min/mg protein)

Sheep SDH
Rabbit liver
Rabbit heart (Group 1, Control)
Rabbit heart (Group II, PC)

Supplemental Fig. 11

A

B

Sorbitol dehydrogenase activity
(µmoles NAD reduced/min/mg protein)
Supplemental Table 1. Hemodynamic changes

<table>
<thead>
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<th>Heart rate, bpm</th>
<th>n</th>
<th>Baseline</th>
<th>Pre-occlusion</th>
<th>Occlusion</th>
<th>Reperfusion</th>
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<td>30 min</td>
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<tr>
<td>Group IX (PC+tolrestat)</td>
<td>9</td>
<td>230 ± 10</td>
<td>239 ± 8</td>
<td>256 ± 10</td>
<td>265 ± 10</td>
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<td>9</td>
<td>240 ± 11</td>
<td>241 ± 4</td>
<td>265 ± 4</td>
<td>270 ± 7</td>
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<tr>
<td>Group XII (PC+ sorbinil)</td>
<td>9</td>
<td>253 ± 11</td>
<td>269 ± 14</td>
<td>274 ± 10</td>
<td>284 ± 11</td>
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</table>

Mean arterial pressure, mm Hg

|                                  |    |           |               |           |             |
| Group IX (PC+tolrestat)          | 4  | 81 ± 1    | 78 ± 2        | 75 ± 1    | 79 ± 2      | 80 ± 1      |
| Group X (PC+vehicle I)           | 4  | 81 ± 2    | 77 ± 4        | 79 ± 4    | 83 ± 1      | 83 ± 1      |
| Group XII (PC+ sorbinil)         | 4  | 73 ± 3    | 70 ± 5        | 72 ± 2    | 70 ± 2      | 70 ± 4      |

Values are mean ± SEM
Baseline: before injection of tolrestat, vehicle I, or sorbinil; Pre-occlusion: just before coronary occlusion.
Occlusion: 1 min before reperfusion.
**Supplemental Table 2. Size of LV and Occluded-Reperfused Vascular Bed (Region at Risk)**

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>LV (g)</th>
<th>Region at risk (g)</th>
<th>Region at risk (% of LV)</th>
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<tbody>
<tr>
<td>Group VI (control)</td>
<td>8</td>
<td>4.16±0.18</td>
<td>0.87±0.09</td>
<td>21.1±2.2</td>
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<td>Group VII (PC)</td>
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<td>4.32±0.15</td>
<td>0.87±0.06</td>
<td>20.2±1.0</td>
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<tr>
<td>Group VIII (tolrestst)</td>
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<td>Group IX (PC+tolrestst)</td>
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<td>3.97±0.09</td>
<td>0.94±0.09</td>
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<td>Group X (PC+vehicle I)</td>
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<td>4.15±0.14</td>
<td>1.01±0.09</td>
<td>24.5±2.0</td>
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<tr>
<td>Group XI (sorbinil)</td>
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<td>4.25±0.12</td>
<td>0.98±0.09</td>
<td>23.4±2.3</td>
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<td>Group XII (PC+sorbinil)</td>
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<td>3.87±0.27</td>
<td>0.85±0.13</td>
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<td>Group XIII (PC+vehicle II)</td>
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<td>3.49±0.09</td>
<td>0.76±0.08</td>
<td>21.7±2.0</td>
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</tbody>
</table>

Values are mean ± SEM