

# Metabolic effects of aldose reductase inhibition during low-flow ischemia and reperfusion

RAVICHANDRAN RAMASAMY,<sup>1</sup> NATHAN TRUEBLOOD,<sup>2</sup> AND SAUL SCHAEFER<sup>2,3</sup>

<sup>1</sup>Division of Cardiology, College of Physicians and Surgeons, Columbia University, New York, New York 10032; <sup>2</sup>Division of Cardiovascular Medicine, University of California, Davis, California 95616; and <sup>3</sup>Cardiology Section, Department of Veterans Affairs Northern California Health Care System, Martinez, California 94553

**Ramasamy, Ravichandran, Nathan Trueblood, and Saul Schaefer.** Metabolic effects of aldose reductase inhibition during low-flow ischemia and reperfusion. *Am. J. Physiol.* 275 (*Heart Circ. Physiol.* 44): H195–H203, 1998.—Several studies have shown that maintenance of glycolysis limits the metabolic and functional consequences of low-flow ischemia. Because diabetic animals are known to have impaired glycolytic metabolism coupled with increased flux through the aldose reductase (AR) pathway, we hypothesized that inhibition of AR would enhance glycolysis and thereby improve metabolic and functional recovery during low-flow ischemia. Hearts ( $n = 12$ ) from nondiabetic control and diabetic rats were isolated and retrograde perfused using 11 mM glucose with or without the AR inhibitor zopolrestat (1  $\mu$ M). Hearts were subjected to 30 min of low-flow ischemia (10% of baseline flow) and 30 min of reperfusion. <sup>31</sup>P NMR spectroscopy was used to monitor time-dependent changes in phosphocreatine (PCr), ATP, and intracellular pH. Changes in the cytosolic redox ratio of NADH to NAD<sup>+</sup> were obtained by measuring the ratio of tissue lactate to pyruvate. Effluent lactate concentrations and oxygen consumption were determined from the perfusate. AR inhibition improved functional recovery in both control and diabetic hearts, coupled with a lower cytosolic redox state and greater effluent lactate concentrations during ischemia. ATP levels during ischemia were significantly higher in AR-inhibited hearts, as was recovery of PCr. In diabetic hearts, AR inhibition also limited acidosis during ischemia and normalized pH recovery on reperfusion. These data demonstrate that AR inhibition maintains higher levels of high-energy phosphates and improves functional recovery upon reperfusion in hearts subjected to low-flow ischemia, consistent with an increase in glycolysis. Accordingly, this approach of inhibiting AR offers a novel method for protecting ischemic myocardium.

diabetes; nuclear magnetic resonance; zopolrestat

REDUCED BLOOD FLOW and oxygen delivery to the myocardium result in metabolic and functional changes, characterized by impaired production of high-energy phosphates, intracellular acidosis, reduced developed pressure, onset of contracture, and an increased dependence on anaerobic glycolysis for energy (18, 22, 32, 40). During ischemia, production of ATP ceases because of feedback inhibition of glycolysis by protons, lactate, and fatty acid metabolites (13, 14, 32). Numerous studies have demonstrated that maintenance of glycolytic metabolism can preserve viability in some circumstances and delay contracture, a hallmark of glycolytic failure and irreversible cell damage (24, 32, 40).

As a consequence of this dependence on glycolysis, conditions that impair glycolytic flux can have deleterious effects under low-flow conditions. We have recently

demonstrated that diabetic rat hearts exhibit impaired utilization of glycolytic substrates (24), an effect that is potentially due to increased flux of glucose through the polyol pathway involving the enzymes aldose reductase and sorbitol dehydrogenase (43). This increased flux via the polyol pathway results in a greater cytosolic NADH/NAD<sup>+</sup> (24) and inhibition of glycolytic enzymes such as glyceraldehyde-3-phosphate dehydrogenase (GAPDH). As a consequence of this glycolytic inhibition, we hypothesized 1) that diabetic hearts would have impaired metabolic and functional adaptation to low-flow ischemia and 2) that inhibition of flux through the polyol pathway would thereby improve metabolic and functional adaptation to low-flow ischemia in conjunction with increased glycolysis and preservation of ATP.

These hypotheses were tested by perfusing both nondiabetic and diabetic hearts with 1  $\mu$ M zopolrestat, a potent and specific inhibitor of aldose reductase (17, 34), and by subjecting them to low-flow ischemia and reperfusion. <sup>31</sup>P NMR spectroscopy was used to continuously measure intracellular pH and high-energy phosphates [phosphocreatine (PCr) and ATP] during normal and low-flow conditions. Tissue lactate, pyruvate, and sorbitol concentrations were measured biochemically, as was lactate release in the effluent. The results demonstrate that aldose reductase inhibition protects both control and diabetic hearts during ischemia and reperfusion.

## METHODS

All studies were performed with the approval of the Animal Care Committee at the University of California Davis and Columbia University (New York, NY).

### Control Rats

Normal male nondiabetic rats in this study weighed between 300 and 350 g and had mean blood glucose levels of  $112 \pm 12$  mg/dl.

### Diabetic Rats

The spontaneously acute diabetic Bio-Bred (BB/W) rats were obtained from the University of Massachusetts Medical Center (Worcester, MA). These diabetic rats weighed between 300 and 350 g, with the duration of diabetes being  $12 \pm 2$  days. They were receiving daily insulin therapy, which was discontinued 24 h before the isolated heart perfusion studies were performed. The mean blood glucose levels in these rats were  $398 \pm 54$  mg/dl.

### Isolated Perfused Heart Preparation

Experiments were performed using an isovolumic isolated rat heart preparation as published by us earlier (23, 26, 28, 29). All rats were pretreated with heparin sodium, followed by pentobarbital sodium (65 mg/kg ip). After deep anesthesia was achieved in the rats, the hearts were rapidly excised, placed into iced saline, and retrogradely perfused (in a nonrecirculating mode) through the aorta as previously described (23, 26, 29). Hearts were perfused with modified Krebs-Henseleit buffer containing (in mM) 118 NaCl, 4.7 KCl, 1.2 CaCl<sub>2</sub>, 1.2 MgSO<sub>4</sub>, and 25 NaHCO<sub>3</sub>, with the substrate being 11 mM glucose. Phosphate was omitted in the perfusate to improve the accuracy of intracellular pH measurements. The perfusate was equilibrated with a mixture of 95% O<sub>2</sub>-5% CO<sub>2</sub>, which maintained perfusate P<sub>O<sub>2</sub></sub> >600 mmHg. Left ventricular developed pressure (LVDP) was measured using a latex balloon in the left ventricle. LVDP, heart rate, and coronary perfusion pressure were monitored continuously on a four-channel Gould recorder.

### Protocols

Hearts from control and diabetic rats were perfused as outlined. Diabetic hearts were used because they exhibit reduced glycolysis and have increased flux of glucose via the polyol pathway. Isolated hearts from control and diabetic rats were either perfused with glucose for 20 min before flow reduction (untreated groups) or perfused with glucose for 10 min followed by perfusion with glucose plus the aldose reductase inhibitor zopolrestat (1 μM) for 10 min before low-flow ischemia (zopolrestat-treated groups). The concentration of zopolrestat (1 μM) used in this study was based on our earlier findings that this dose was sufficient to protect hearts from ischemic injury (24). Zopolrestat was a gift from Pfizer Central Research (Groton, CT).

All hearts were perfused at 12.5 ml/min before and after ischemia, without recirculating the buffer. All hearts were subjected to 30 min of low-flow ischemia, with the flow rate being 1.25 ml/min (10% of baseline flow rate).

### Collection and Analysis of Perfusate and Coronary Effluent Samples

Coronary venous effluent was collected through the pulmonary artery and analyzed for P<sub>O<sub>2</sub></sub> and P<sub>CO<sub>2</sub></sub> with an Instrumentation Laboratories IL 213 pH-blood gas analyzer. Myocardial oxygen consumption was calculated by the arteriovenous difference (39):  $P_{O_2} = [(0.003 \times \text{arterial } P_{O_2} - 0.003 \times \text{effluent } P_{O_2}) \times \text{total flow}] / \text{ventricular weight}$ . Lactate production was calculated from the measured perfusate-effluent differences, multiplied by flow, divided by heart weight, and expressed as micromoles per gram dry weight per minute.

### Tissue Assays

To determine changes in the sorbitol levels and cytosolic redox state, parallel experiments were performed using hearts in each of the four groups ( $n = 6$  in each group) and freeze-clamped before ischemia, at the end of low-flow ischemia, and after 30 min of reperfusion. Lactate and pyruvate were extracted from the freeze-clamped tissue using perchloric acid and measured using standard biochemical assays published in the literature (2), whereas sorbitol in the extract was measured using the enzymatic assay of Malone (15) modified by us (24). Glycogen content in freeze-clamped hearts was measured using the procedure as published by us earlier (26, 29), whereas the content of ATP, PCr, ADP, and AMP in the tissue extracts was analyzed using HPLC (39, 40).

P<sub>i</sub> in the tissue extract was measured using a biochemical assay (16).

### <sup>31</sup>P NMR Spectroscopy

Four groups of hearts ( $n = 6$  in each group) were studied using <sup>31</sup>P NMR, with spectra obtained every 5 min during baseline, ischemia, and reperfusion. Simultaneous measurements of heart rate, left ventricular end-diastolic pressure, and LVDP were obtained in conjunction with NMR spectra. To determine changes in high-energy phosphates and intracellular pH, <sup>31</sup>P NMR spectroscopy was performed on a Bruker AMX 400/or GE Omega-300 vertical-bore spectrometer. NMR spectroscopy was performed using 248 acquisitions of a 45° pulse and 1.21-s interpulse delay, with spectra processed using an exponential multiplication of 20 Hz and manual phasing. Intracellular pH was determined from the chemical shift of the P<sub>i</sub> resonance using a titration curve established in this laboratory. Metabolites were referenced to their baseline value determined in duplicate at the start of the experiment and expressed as a fraction of baseline.

### Statistical Methods

Data were analyzed using INSTAT (GraphPad, San Diego, CA) software operating on an IBM-compatible personal computer. Differences between different groups were assessed using ANOVA for repeated measures, with subsequent Student-Newman-Keuls multiple comparisons post tests if the  $P$  value for ANOVA was significant. All data are expressed as means ± SE.

## RESULTS

### Hemodynamics

LVDP and end-diastolic pressure (EDP) were similar in all groups under baseline conditions (Table 1). Reduction of perfusate flow resulted in significant and stable reductions in LVDP in all groups. During ischemia, the rise in EDP was greater in the untreated control and diabetic than in the aldose reductase-inhibited control and diabetic hearts ( $P = 0.01$ ). On reperfusion, the aldose reductase-inhibited hearts exhibited greater LVDP recovery than the untreated hearts. Myocardial oxygen consumption was similar in all groups under baseline, ischemic, and reperfusion conditions and was unaffected by aldose reductase inhibition (Table 1).

### Metabolites

**Biochemical measurements.** The concentrations of ATP, PCr, ADP, and AMP in diabetic and control hearts were measured using HPLC as described above, whereas P<sub>i</sub> was measured using a spectrophotometric method. The concentration of ATP was significantly lower ( $16.11 \pm 1.95$  vs.  $21.91 \pm 1.92$  μmol/g dry wt,  $P = 0.02$ ) and AMP significantly higher ( $3.29 \pm 0.77$  vs.  $1.91 \pm 0.36$  μmol/g dry wt,  $P = 0.01$ ) in the diabetic hearts compared with the control hearts. There were no differences in the levels of PCr ( $36.09 \pm 4.92$  vs.  $32.54 \pm 2.96$  μmol/g dry wt), P<sub>i</sub> ( $2.21 \pm 0.29$  vs.  $1.88 \pm 0.31$  μmol/g dry wt), or ADP ( $4.31 \pm 0.62$  vs.  $4.88 \pm 0.55$  μmol/g dry wt) between diabetic and control hearts. These observations are consistent with the reports in the literature (9, 10).

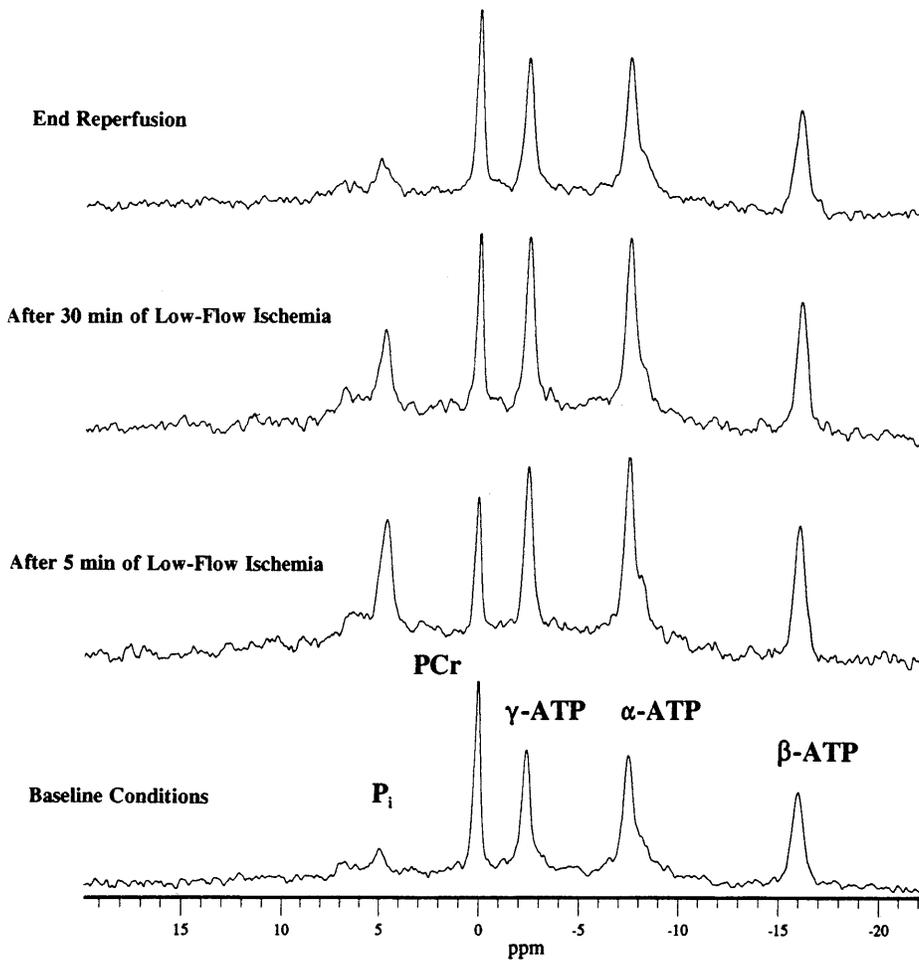


Fig. 1. Representative  $^{31}\text{P}$  NMR spectra from a nondiabetic heart during baseline (bottom spectra), 5 min after low-flow ischemia, at end of low-flow ischemia, and at end of reperfusion.  $\text{P}_i$  denotes intracellular inorganic phosphate resonance, and PCr and PME represent phosphocreatine and phosphomonoester resonances, respectively.  $\gamma$ -ATP,  $\alpha$ -ATP, and  $\beta$ -ATP represent resonances of ATP.

Table 1. Hemodynamic values from control and diabetic hearts perfused with and without the aldose reductase inhibitor zopolrestat

Group	n	Measure	Baseline	Ischemia (30 min)	Reperfusion (30 min)
<i>Control</i>					
Controls	12	LVDP	115 ± 19	6 ± 4	76 ± 19
		EDP	11 ± 4	44 ± 9	25 ± 7
		$\text{M}\dot{\text{V}}\text{O}_2$	29.2 ± 6.7	0.49 ± 0.008	26.9 ± 7.2
Zopolrestat treated	11	LVDP	111 ± 24	11 ± 6	119 ± 23*
		EDP	11 ± 4	6 ± 2*	6 ± 3*
		$\text{M}\dot{\text{V}}\text{O}_2$	31.9 ± 6.8	0.48 ± 0.006	26.7 ± 6.6
<i>Diabetic</i>					
Controls	10	LVDP	108 ± 17	4 ± 5	51 ± 15
		EDP	11 ± 3	59 ± 11	29 ± 6
		$\text{M}\dot{\text{V}}\text{O}_2$	27.6 ± 2.9	0.45 ± 0.006	22.9 ± 5.2
Zopolrestat treated	9	LVDP	122 ± 21	14 ± 6	96 ± 12†
		EDP	9 ± 2	7 ± 3†	8 ± 2†
		$\text{M}\dot{\text{V}}\text{O}_2$	25.9 ± 4.5	0.46 ± 0.009	23.1 ± 2.7

Values are means ± SE; n, no. of hearts. Left ventricular developed pressure (LVDP) and end-diastolic pressure (EDP) are expressed as  $\text{cmH}_2\text{O}$  pressure. Myocardial oxygen consumption ( $\text{M}\dot{\text{V}}\text{O}_2$ ) is expressed as  $\mu\text{mol oxygen}\cdot\text{g}^{-1}\cdot\text{min}^{-1}$ . \*Significantly different from control hearts ( $P < 0.03$ ). †Significantly different from diabetic control hearts ( $P < 0.04$ ).

*NMR measurements.* Representative  $^{31}\text{P}$  NMR spectra acquired at the various time points demonstrating the changes in PCr, ATP, and  $\text{P}_i$  are shown in Fig. 1.

*PCr.* Ischemia reduced PCr to 50% of baseline in all groups (Fig. 2). In diabetic hearts, there was a progressive fall in PCr throughout the ischemic period. In control hearts, the decline in PCr during ischemia was less pronounced than in diabetic hearts. Aldose reductase inhibition resulted in a partial recovery of PCr during ischemia in both control and diabetic hearts. In addition, normalization of PCr on reperfusion was more rapid in aldose reductase-inhibited control and diabetic hearts.

*ATP.* ATP fell progressively in control and diabetic hearts during ischemia, with the decline in ATP being greater in the diabetic hearts (34 vs. 22%, Fig. 3). This is consistent with inhibition of glycolysis in diabetic hearts as noted earlier. In contrast, aldose reductase inhibition significantly preserved ATP during ischemia in diabetic hearts. The decline in ATP during ischemia in the control hearts, although modest in comparison with diabetics, was also attenuated by aldose reductase inhibition. Thus aldose reductase inhibition was beneficial in control and diabetic hearts by preventing the decline in ATP during ischemia.

*Intracellular pH.* Intracellular pH was identical in all groups under baseline conditions before ischemia,

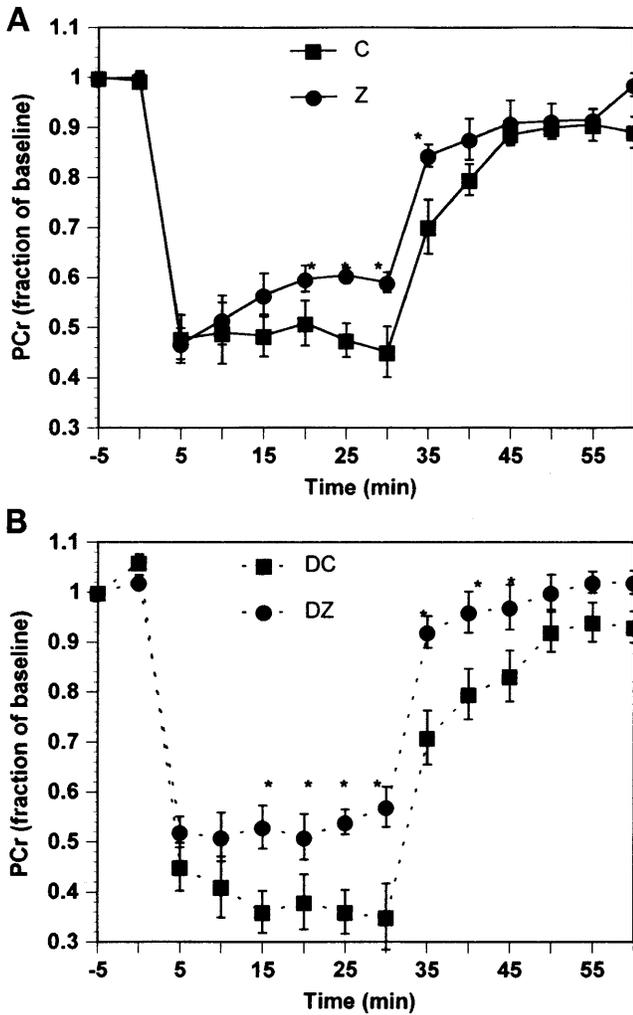


Fig. 2. Changes in PCr, expressed as fraction of baseline, during 30 min of low-flow ischemia and 30 min of reperfusion period in control group (A) and diabetic group (B) of hearts. In each group, data are presented from 6 hearts perfused with (●) and without (■) zopolrestat. C, untreated control hearts; Z, zopolrestat-treated control hearts; DC, untreated diabetic control hearts; DZ, zopolrestat-treated diabetic hearts. Low-flow ischemia was initiated at time 0, whereas reperfusion was started after 30 min of ischemia. Each point represents data obtained every 5 min. \*PCr significantly greater than in DC and C hearts ( $P = 0.001$ ).

with no effect seen with aldose reductase inhibition (Fig. 4). Intracellular pH during ischemia fell similarly in both diabetic and control hearts (to  $\sim 6.8$ ), but recovery of pH on reperfusion in diabetic hearts was, as previously described (24), delayed compared with control hearts. Aldose reductase inhibition resulted in a significantly higher intracellular pH during ischemia in diabetic hearts, with pH at the end of 30 min of low-flow ischemia being  $6.94 \pm 0.02$  in treated and  $6.83 \pm 0.03$  in untreated hearts ( $P = 0.02$ ).

**Tissue sorbitol levels.** Sorbitol levels were significantly higher in diabetic compared with control hearts under baseline ( $86.6 \pm 14.9$  vs.  $12.9 \pm 2.7$  nmol/g tissue), low-flow ischemia ( $69.2 \pm 11.5$  vs.  $8.1 \pm 1.1$  nmol/g tissue), and reperfusion conditions ( $71.8 \pm 16.3$  vs.  $15.1 \pm 3.9$  nmol/g tissue) ( $P = 0.009$ ). Aldose

reductase inhibition with zopolrestat significantly lowered the sorbitol levels in diabetic hearts under all conditions such that the levels of sorbitol in treated diabetic hearts were similar to those of control hearts ( $15.6 \pm 3.6$  nmol/g tissue under baseline,  $17.9 \pm 3.8$  nmol/g tissue under low-flow ischemia conditions, and  $20.8 \pm 2.9$  nmol/g tissue upon reperfusion,  $P = 0.03$  vs. untreated diabetic hearts). There was no significant lowering of sorbitol with aldose reductase inhibition in control hearts.

**Tissue lactate-to-pyruvate ratio.** The tissue concentrations of lactate and pyruvate were measured in freeze-clamped heart tissue extracts. These measurements allowed calculation of the tissue lactate-to-pyruvate ratio (L/P), a measure of the cytosolic redox state ( $NADH/NAD^+$ ). As shown in Table 2, tissue lactate concentrations were markedly elevated in diabetic

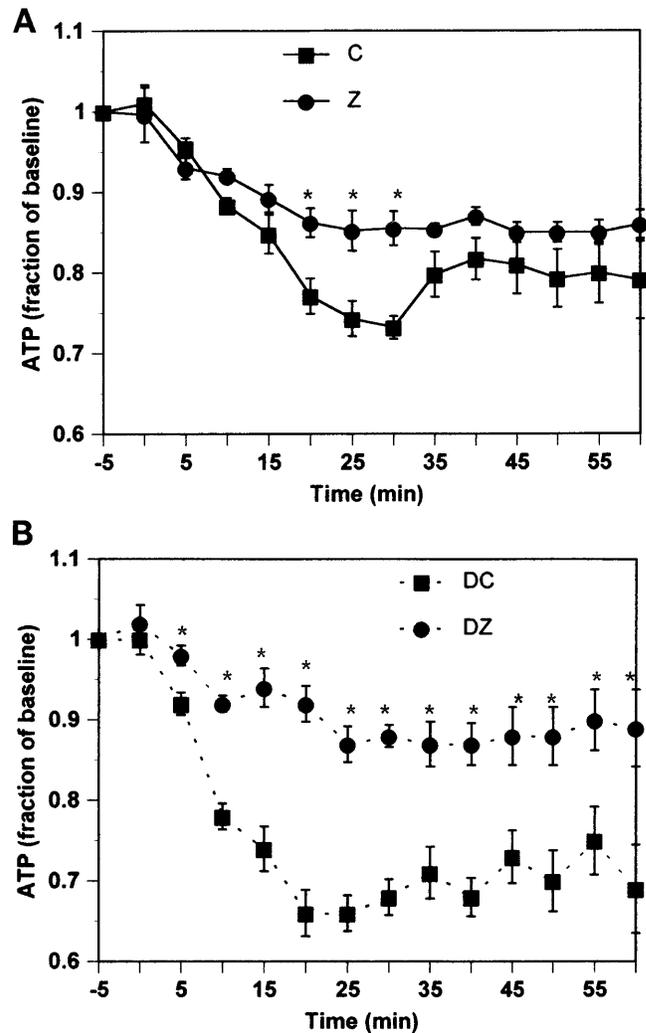


Fig. 3. Changes in ATP, expressed as fraction of baseline, during 30 min of low-flow ischemia and 30 min of reperfusion period in control group (A) and diabetic group (B) of hearts. In each group, data are presented from 6 hearts perfused with (●) and without (■) zopolrestat. Definitions are as in Fig. 2. Low-flow ischemia was initiated at time 0, whereas reperfusion was started after 30 min of ischemia. Each point represents data obtained every 5 min. \*ATP significantly greater than in DC and C hearts ( $P < 0.005$ ).

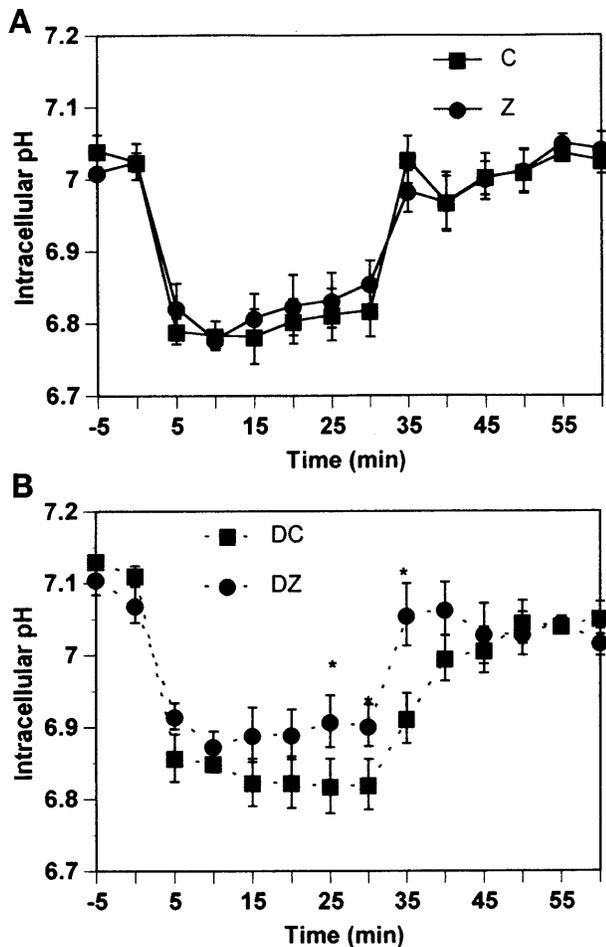


Fig. 4. Intracellular pH during 30 min of low-flow ischemia and during 30 min of reperfusion in control group (A) and diabetic group (B) of hearts. In each group, data are presented from 6 hearts perfused with (●) and without (■) zopolrestat. Definitions are as in Fig. 2. Low-flow ischemia was initiated at time 0, whereas reperfusion was started after 30 min of ischemia. \* Intracellular pH significantly higher than in DC hearts ( $P = 0.002$ ).

hearts under baseline conditions, resulting in a high L/P compared with control hearts. This increase in lactate during ischemia was blunted in the diabetic hearts. However, the decrease in pyruvate during ischemia resulted in a much greater L/P during ischemia in diabetics. Upon reperfusion, metabolite concentrations (lactate and pyruvate) both returned to their respective baseline levels, as did the L/P. Aldose reductase inhibition lowered the L/P in both diabetic and control hearts to similar values under baseline conditions. During ischemia, aldose reductase inhibition with zopolrestat attenuated the rise in lactate in parallel with increased pyruvate concentrations, resulting in significantly lower L/P in both control and diabetic hearts.

*Lactate release in the effluent during low-flow ischemia.* Diabetic hearts had significantly lower levels of lactate release under baseline, ischemic, and reperfusion conditions than control hearts (Fig. 5). The baseline concentrations of lactate in the effluent were increased by aldose reductase inhibition in both diabetic and control hearts compared with the untreated hearts. Similarly, effluent lactate concentrations were significantly greater in aldose reductase-inhibited hearts during low-flow ischemia, as well as during reperfusion. The lower tissue lactate and greater effluent lactate concentrations are consistent with increased production and efflux of lactate during ischemia in aldose reductase-inhibited hearts.

*Glycogen content and utilization.* Compared with control hearts, hearts from diabetic animals had significantly increased glycogen content ( $90.1 \pm 9$  vs.  $38.6 \pm 3$   $\mu\text{mol/g}$  dry wt,  $P = 0.02$ ), an observation consistent with that reported in the literature (9). Perfusion with the aldose reductase inhibitor zopolrestat did not alter the glycogen content in diabetic ( $83.6 \pm 5$   $\mu\text{mol/g}$  dry wt) or control ( $40.4 \pm 6$   $\mu\text{mol/g}$  dry wt) hearts. To assess the utilization of glycogen during ischemia, the glycogen content in all the groups of hearts was measured at the end of 30 min of low-flow ischemia. The glycogen

Table 2. Tissue lactate and pyruvate concentration from untreated and zopolrestat-treated control and diabetic rat hearts

Group	n	Measure	Baseline	Ischemia	Reperfusion
<i>Control</i>					
Untreated	10	Lactate	$5.42 \pm 0.38$	$86.13 \pm 4.98$	$6.37 \pm 1.11$
		Pyruvate	$0.32 \pm 0.07$	$0.38 \pm 0.05$	$0.36 \pm 0.03$
		L/P	$16.96 \pm 2.79$	$226.67 \pm 21.12$	$17.69 \pm 3.29$
Zopolrestat treated	9	Lactate	$3.29 \pm 0.79^*$	$42.60 \pm 2.77^*$	$4.76 \pm 0.52$
		Pyruvate	$0.52 \pm 0.09^*$	$0.58 \pm 0.10^*$	$0.49 \pm 0.04^*$
		L/P	$6.33 \pm 1.88^*$	$73.45 \pm 9.73^*$	$9.71 \pm 1.09^*$
<i>Diabetic</i>					
Untreated	12	Lactate	$18.08 \pm 2.09\ddagger$	$68.4 \pm 3.94\ddagger$	$21.11 \pm 1.93\ddagger$
		Pyruvate	$0.29 \pm 0.05$	$0.24 \pm 0.02$	$0.27 \pm 0.05$
		L/P	$62.34 \pm 4.41\ddagger$	$285.00 \pm 15.61\ddagger$	$78.19 \pm 7.56\ddagger$
Zopolrestat treated	11	Lactate	$3.92 \pm 1.02\ddagger$	$51.11 \pm 5.43\ddagger$	$3.77 \pm 0.89\ddagger$
		Pyruvate	$0.48 \pm 0.04\ddagger$	$0.61 \pm 0.09\ddagger$	$0.52 \pm 0.08\ddagger$
		L/P	$8.17 \pm 1.09\ddagger$	$83.79 \pm 8.81\ddagger$	$7.25 \pm 2.07\ddagger$

Values are means  $\pm$  SE; n, no. of hearts. Lactate and pyruvate concentrations are expressed as  $\mu\text{mol/g}$  dry wt. Lactate-to-pyruvate ratios (L/P) are measured under baseline conditions, at end of 30 min of low-flow ischemia, and after 30 min of reperfusion. L/P is used as a measure of cytosolic redox state (NADH/NAD<sup>+</sup>). \*  $P < 0.05$  zopolrestat vs. control. †  $P < 0.03$  diabetic zopolrestat vs. diabetic control. ‡  $P < 0.05$  diabetic control vs. control.

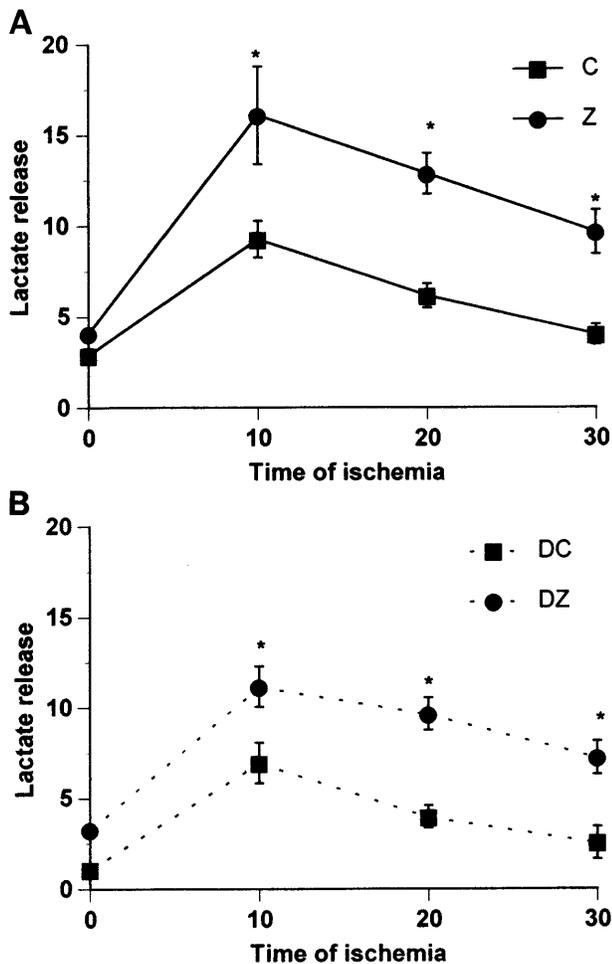


Fig. 5. Lactate release (expressed as  $\mu\text{mol} \cdot \text{g dry wt}^{-1} \cdot \text{min}^{-1}$ ) in effluent during 30 min of low-flow ischemia. Flow was set to 1.25 ml/min during low-flow ischemia. In each group, data are presented from 6 hearts perfused with (●) and without (■) zopolrestat. Definitions are as in Fig. 2. \*Lactate release significantly greater than in C and DC hearts ( $P = 0.002$ ).

content at the end of ischemia was  $17.8 \pm 4 \mu\text{mol/g dry wt}$  in untreated control hearts,  $67.9 \pm 6 \mu\text{mol/g dry wt}$  in untreated diabetic hearts,  $59.1 \pm 7 \mu\text{mol/g dry wt}$  in aldose reductase-inhibited diabetic hearts, and  $24.1 \pm 5 \mu\text{mol/g dry wt}$  in aldose reductase-inhibited control hearts. The differences between preischemic and end-ischemic glycogen content were similar in all groups ( $\sim 20 \mu\text{mol/g dry wt}$ ), suggesting that aldose reductase inhibition did not alter glycogen utilization during ischemia.

## DISCUSSION

These experiments have demonstrated that aldose reductase inhibition maintained higher levels of high-energy phosphates and improved functional recovery on reperfusion in diabetic and control hearts subjected to low-flow ischemia. These beneficial effects were coupled with evidence of increased glycolysis and lactate efflux, providing further evidence that increased glycolysis and lactate efflux can contribute to metabolic adaptation during low-flow ischemia.

## Metabolic Response of Diabetic Hearts to Low-Flow Ischemia

When compared with control hearts, diabetic hearts had higher baseline tissue lactate concentrations and a higher L/P, reflecting a higher NADH/NAD<sup>+</sup> (Table 2). As well, the tissue concentrations of lactate and the L/P remained higher during ischemia and reperfusion in the diabetic hearts, coupled with lower effluent lactate concentrations. These findings are consistent with both reduced glycolysis and lower lactate efflux in the diabetic hearts and agree with previous experimental observations (24). In addition to these changes, the diabetic hearts were characterized by significantly greater reductions in ATP during ischemia, suggesting that glycolytic production of ATP may have been reduced.

The impaired glucose metabolism can also be because of lower uptake of glucose by the diabetic myocardium. Studies have demonstrated that hearts from diabetic rats have lower levels of the glucose transporter GLUT-4 (7, 21, 31), which results in lower uptake of glucose (20, 31, 32). As a consequence of lower glucose transport, glycolysis will likely be lowered in diabetics (3, 24, 37). From the data presented in this study, it is not possible to dissociate if the impaired glycolysis in diabetic hearts is due to lower uptake or to increased flux via the polyol pathway, or both. However, the effluent lactate data suggest that inhibition of polyol pathway increases lactate production (a measure of glycolysis), suggesting that increased flux via polyol pathway is partly responsible for impaired glycolysis. Experiments on the glucose transporters are necessary to precisely determine the extent to which they contribute to impaired glycolysis.

Despite these differences between untreated control and diabetic hearts, aldose reductase inhibition was protective in both groups, resulting in enhancement of glycolysis (as evidenced by significantly higher effluent lactate concentrations), as well as recovery of PCr and maintenance of ATP during ischemia. In the diabetic hearts, aldose reductase inhibition also resulted in higher intracellular pH during ischemia. Although there is only indirect evidence in this study for an increase in glycolysis in zopolrestat-treated hearts, previous studies using <sup>13</sup>C-NMR have shown that aldose reductase inhibition increased glycolysis under similar baseline conditions (37).

## Protective Effect of Glycolysis During Ischemia

The data from this study indicate that metabolic recovery, defined as recovery of high-energy phosphates during low-flow ischemia (27, 32, 39), was observed when hearts were perfused with the aldose reductase inhibitor zopolrestat. In addition, aldose reductase inhibition improved functional recovery on reperfusion in both diabetic and control hearts. The beneficial effect of aldose reductase inhibition on metabolic recovery is consistent with previous models of low-flow ischemia that have demonstrated a benefit of maintaining or enhancing glycolysis (27, 32, 39). For example, it was

demonstrated by Apstein et al. (1) that increasing glycolytic flux using high glucose and insulin prevented contracture, increased high-energy phosphate levels, and improved recovery after 3 h of reduced flow. Similarly, Owen et al. (22) demonstrated that increasing perfusate glucose concentration, in the presence of acetate, prevented contracture during 2 h of low-flow ischemia. The beneficial effects of increasing glycolytic flux by enhancing glucose availability have also been demonstrated in animal and human studies of ischemia (19, 30, 39). In our study, as well as those published by other investigators (24, 25, 27, 32, 39), myocardial lactate was greatest in hearts with poor recovery of function during reperfusion. In addition, we observed that accumulation of lactate in the tissue was accompanied by reduced rates of lactate release in the coronary effluent from hearts that had poor functional recovery on reperfusion (39). These findings are consistent with deleterious effects of inhibition of the lactate- $H^+$  cotransporter (11), particularly in untreated diabetic hearts.

Mechanisms by which glycolysis affords beneficial effects during low-flow ischemia are numerous. First, metabolism of glucose via glycolysis yields ATP (the process does not require oxygen), thus increasing the total amount of ATP produced under conditions of low-flow and oxygen supply. Second, although total production of ATP is clearly important in maintaining cellular function, studies suggest that the source of ATP (glycolytic vs. mitochondrial) is important for specific cellular functions and that inhibition of ATP production via glycolysis impairs the activity of  $Na^+K^+$ -ATPase activity and the ATP-sensitive potassium channels (4, 41, 42). The dependence of  $Na^+K^+$ -ATPase on glycolysis has been previously established under both normoxic and hypoxic conditions. Weiss and Hiltbrand (42) have shown that inhibition of glycolysis under normoxic conditions results in significant impairment of the  $Na^+K^+$ -ATPase. Under low-flow conditions, the studies by Tani and Neely (35, 36) concluded that maintenance of  $Na^+K^+$ -ATPase activity limited reperfusion injury. Similarly, it was demonstrated recently that ischemia (4) in the absence of glucose resulted in more marked abnormalities in high-energy phosphates and intracellular sodium in the perfused heart.

#### *Aldose Reductase Inhibition and Glycolysis*

One possible mechanism for the observed effect of aldose reductase inhibition on glycolysis can be explained on the basis of changes in the cytosolic redox state. It has been suggested that the flux via GAPDH is a rate-limiting step for glycolysis under ischemic conditions because of increases in the cytosolic redox state  $NADH/NAD^+$  (24, 25, 32). Conservation of  $NAD^+$  in any of the cytosolic pathways is likely to attenuate the increases in the cytosolic redox state. In this context, we recently demonstrated that inhibition of aldose reductase conserves  $NAD^+$  (because of lower flux via sorbitol dehydrogenase) and lowers the cytosolic redox state,  $NADH/NAD^+$  (24). The  $NAD^+$  conserved by aldose reductase inhibition is readily available for con-

version of glyceraldehyde-3-phosphate to 1,3-bisphosphoglycerate by GAPDH (24, 43) in the glycolytic pathway. Thus aldose reductase inhibition can increase glycolysis by providing  $NAD^+$  for GAPDH.

#### *Intracellular pH*

*Acidification during ischemia.* Acidification during ischemia results from the balance of proton production (primarily from anaerobic glycolysis and ATP hydrolysis) (5) and proton utilization either by buffering mechanisms (6) or proton efflux pathways (8, 11, 38). The evidence from the current experiments suggests that acidosis during ischemia in diabetic hearts was reduced by aldose reductase inhibition. The higher pH in aldose reductase-inhibited hearts, coupled with preservation of ATP and increased lactate release during ischemia, is consistent with lower ATP hydrolysis and/or greater proton efflux via the lactate- $H^+$  cotransporter. Because reduced lactate- $H^+$  cotransporter activity has previously been reported in diabetics (11), these observations, coupled with greater lactate release, suggest that aldose reductase inhibition likely improves proton efflux via this mechanism.

*Recovery on reperfusion.* The rate of intracellular pH recovery on reperfusion is primarily mediated by lactate and  $CO_2$  efflux, with smaller contributions by  $Na^+/H^+$  exchanger and  $HCO_3^-$  influx via the  $Cl^-/HCO_3^-$  exchanger. Consistent with previous reports (11, 24), we observed that pH recovery in diabetic hearts was slower than in control hearts, presumably because of impaired flux of protons via the lactate-proton cotransporter and  $Na^+/H^+$  exchanger in diabetics (11). As during ischemia, normalization of pH recovery by zopolrestat further supports the postulate that aldose reductase inhibition can positively influence proton efflux mechanisms.

#### *Aldose Reductase and Oxidative Stress*

The cofactor NADPH is required for aldose reductase activity. It has been suggested that an increase in substrate flux via aldose reductase can increase oxidative stress (12, 43) secondary to diminished NADPH-dependent reduction of glutathione (12). In addition to the reduction of glutathione, flux of substrate via aldose reductase can diminish nitric oxide synthase activity because of competition between these enzymes for NADPH (33). Therefore, inhibition of aldose reductase can protect any tissue from oxidative stress. It is possible that the cardioprotection observed in this study may, in part, be because of lowering of oxidative stress.

#### *Limitations*

The data obtained from an isolated rat heart model have to be interpreted within the limits of the experimental setting. This isolated perfused heart model permitted 1) careful control of factors, and thus facilitated comparison between groups in the absence of confounding variables present in vivo, and 2) measurements of numerous metabolites using NMR and bio-

chemical assays, thereby examination of the beneficial effects of aldose reductase inhibition and potential mechanisms.

Despite higher levels of sorbitol (possibly because of higher aldose reductase activity) in diabetic hearts, a similar magnitude of cardioprotection due to zopolrestat was observed in both diabetics and controls. To interpret these findings, it is important to note that in addition to glucose, some of the glycolytic intermediates are also substrates for aldose reductase. Therefore, it is possible that in both diabetic and control hearts, other substrates are being reduced by aldose reductase during ischemia.

The mechanism of cardioprotection afforded by aldose reductase inhibition may be based on the reduction of the cytosolic redox state (L/P) observed in diabetics and controls. In both cases, the reduction in the L/P during ischemia by zopolrestat was similar, suggesting that the beneficial effects of increased glycolysis were secondary to lowering of the cytosolic redox state. To account for the significant reduction in the cytosolic redox state due to aldose reductase inhibition, one has to take into consideration inhibition of enzymes that also use  $\text{NAD}^+$  as a cofactor. For example, the aldehyde reductase that reduces D-glucuronate to L-glucuronate in the uronic acid pathway has been reported to be inhibited by aldose reductase inhibitors. Because the glucuronic acid pathway reduces up to 3  $\text{NAD}^+$  to NADH for each mole of glucose metabolized to L-xylulose, inhibition of this enzyme would similarly alter the redox state as measured in this study. Detailed investigations are necessary to confirm this speculation.

In conclusion, the data presented here demonstrate that aldose reductase inhibition using zopolrestat maintained higher levels of high-energy phosphates and significantly improved functional and metabolic recovery from low-flow ischemia. Measurements of tissue and effluent lactate are consistent with aldose reductase inhibition increasing glycolysis and/or lactate efflux during ischemia and reperfusion. The changes in effluent lactate during ischemia were also associated with a decreased tissue L/P, a measure of the cytosolic redox state. Although a precise mechanism for the beneficial effect of aldose reductase inhibition remains to be elucidated, the increases in effluent lactate and the tissue L/P are consistent with increases in glycolysis by aldose reductase inhibition, possibly because of redox modulation of flux via glycolytic enzymes. These findings suggest a potential role for aldose reductase inhibitors as a novel metabolic adjunct in the treatment of myocardial ischemia.

We thank Dr. Steven R. Bergmann for helpful suggestions and discussions. The expert technical assistance of Li-Feng Wang and the secretarial assistance of Penny Jones is greatly appreciated.

R. Ramasamy was supported by an American Diabetes Association Career Development Award, Juvenile Diabetes Foundation International Grant 196098, National Heart, Lung, and Blood Institute Grant HL-58408, and a grant from Pfizer. S. Schaefer was supported by a grant from the American Heart Association.

Address for reprint requests: R. Ramasamy, Div. of Cardiology, PH 3-342, College of Physicians and Surgeons, Columbia University, 630 West 168th St., New York, NY 10032.

Received 29 December 1997; accepted in final form 30 March 1998.

## REFERENCES

1. **Apstein, C. S., F. N. Gravino, and C. C. Haudenschild.** Determinants of a protective effect of glucose and insulin on the ischemic myocardium. *Circ. Res.* 52: 515-526, 1983.
2. **Bergmeyer, H. U., J. Bergmeyer, and M. Grabl.** Carbohydrates. In: *Methods of Enzymatic Analysis*. Verlag Chemie, Deerfield Beach, FL: 1995, p. 570-587.
3. **Chatham, J. C., and J. R. Forder.** A  $^{13}\text{C}$  NMR study of glucose oxidation in the intact functioning rat heart after diabetes induced cardiomyopathy. *J. Mol. Cell. Cardiol.* 25: 1203-1213, 1993.
4. **Cross, H. R., K. Clarke, and G. K. Radda.** The role of  $\text{Na}^+/\text{K}^+$  ATPase activity during low flow ischemia in preventing myocardial injury: a  $^{31}\text{P}$ ,  $^{23}\text{Na}$ , and  $^{87}\text{Rb}$  NMR spectroscopic study. *Magn. Reson. Med.* 34: 673-685, 1995.
5. **Dennis, S. C., W. Gevers, and L. H. Opie.** Protons in ischemia: where do they come from, where do they go to? *J. Mol. Cell. Cardiol.* 23: 1077-1086, 1991.
6. **Garlick, P. B., G. K. Radda, and P. J. Seeley.** Studies of acidosis in the ischaemic heart by phosphorus nuclear magnetic resonance. *Biochem. J.* 184: 547-554, 1979.
7. **Garvey, W. T., M. Hardin, M. Juhászova, and J. H. Dominguez.** Effects of diabetes on myocardial glucose transport system in rats: implications for diabetic cardiomyopathy. *Am. J. Physiol.* 264 (Heart Circ. Physiol. 33): H837-H844, 1993.
8. **Grace, A. A., H. L. Kirschenlohr, J. C. Metcalfe, G. A. Smith, P. I. Weissberg, E. J. Cragoe, Jr., and J. I. Vandenberg.** Regulation of intracellular pH in the perfused heart by external  $\text{HCO}_3^-$  and  $\text{Na}^+/\text{H}^+$  exchange. *Am. J. Physiol.* 265 (Heart Circ. Physiol. 34): H289-H298, 1993.
9. **Higuchi, M., K. Miyagi, J. Nakasone, and M. Sakanashi.** Role of high glycogen in underperfused diabetic rat hearts with added norepinephrine. *J. Cardiovasc. Pharmacol.* 26: 899-907, 1995.
10. **Jenkins, R. L., H. G. McDaniel, S. Digerness, S. W. Parrish, and R. L. Ong.** Adenine nucleotide metabolism in hearts of diabetic rats. Comparison to diaphragm, liver and kidney. *Diabetes* 37: 629-636, 1988.
11. **Khandoudi, N., M. Bernard, P. Cozzone, and D. Feuvray.** Mechanisms of intracellular pH regulation during posts ischemic reperfusion of diabetic rat hearts. *Diabetes* 44: 196-202, 1995.
12. **Lee, S.-M., S. Z. Schade, and C. C. Doughty.** Aldose reductase, NADPH and NADP $^+$  in normal galactose-fed and diabetic rat lens. *Biochim. Biophys. Acta* 841: 247-253, 1985.
13. **Liedtke, A. J.** Alterations of carbohydrate and lipid metabolism in the acutely ischemic heart. *Prog. Cardiovasc. Dis.* 23: 321-336, 1981.
14. **Liedtke, A. J., H. C. Hughes, and J. R. Neely.** Metabolic responses to varying restrictions of coronary blood flow in swine. *Am. J. Physiol.* 228: 655-662, 1975.
15. **Malone, J.** Red cell sorbitol, an indicator of diabetic control. *Diabetes* 29: 861-864, 1980.
16. **Masuda, T., G. P. Dobson, and R. L. Veech.** The Gibbs-Donnan near equilibrium system of the heart. *J. Biol. Chem.* 265: 20321-20334, 1990.
17. **Mylari, B. L., E. R. Larson, T. A. Beyer, W. J. Zembrowski, C. E. Aldinger, M. F. Dee, T. W. Siegel, and D. H. Singleton.** Novel, potent aldose reductase inhibitors: 3,4-dihydroxy-4-oxo-3-[[trifluoromethyl]-2-benzothiazolyl]methyl]-1-phthalazineacetic acid (zopolrestat) and congeners. *J. Med. Chem.* 34: 108-122, 1991.
18. **Opie, L. H., K. Bruyneel, and P. Owen.** Effects of glucose, insulin and potassium infusion on tissue metabolic changes within the first hour of myocardial infarction in the baboon. *Circulation* 52: 49-57, 1975.
19. **Opie, L. H., and P. Owen.** Effect of glucose-insulin-potassium infusions on arteriovenous differences of glucose and free fatty acids and on tissue metabolic changes in dogs with developing myocardial infarction. *Am. J. Cardiol.* 38: 310-321, 1976.

20. **Opie, L. H., M. J. Tansey, and B. M. Kennelly.** The heart in diabetes mellitus. Part I. Biochemical basis for myocardial dysfunction. *S. Afr. Med. J.* 56: 207–211, 1979.
21. **Osborn, B. A., J. T. Daar, R. A. Laddaga, F. D. Romano, and D. J. Paulson.** Exercise training increases sarcolemmal GLUT-4 protein and mRNA content in diabetic heart. *J. Appl. Physiol.* 82: 828–834, 1997.
22. **Owen, P., S. Dennis, and L. H. Opie.** Glucose flux rate regulates onset of ischemic contracture in globally underperfused rat hearts. *Circ. Res.* 66: 344–354, 1990.
23. **Ramasamy, R., H. Liu, S. Anderson, J. Lundmark, and S. Schaefer.** Ischemic preconditioning stimulates sodium and proton transport in the isolated rat heart. *J. Clin. Invest.* 96: 1464–1472, 1995.
24. **Ramasamy, R., P. J. Oates, and S. Schaefer.** Aldose reductase inhibition protects diabetic and nondiabetic rat hearts from ischemic injury. *Diabetes* 46: 292–300, 1997.
25. **Rovetto, M. J., W. F. Lamberton, and J. R. Neely.** Mechanisms of glycolytic inhibition in ischemic rat hearts. *Circ. Res.* 37: 742–751, 1975.
26. **Schaefer, S., L. J. Carr, E. Prussel, and R. Ramasamy.** Effects of glycogen depletion on ischemic injury in the isolated rat heart: insights into preconditioning. *Am. J. Physiol.* 268 (*Heart Circ. Physiol.* 37): H935–H944, 1995.
27. **Schaefer, S., E. Prussel, and L. J. Carr.** Requirement of glycolytic substrate for metabolic recovery during moderate low flow ischemia. *J. Mol. Cell. Cardiol.* 27: 2167–2176, 1995.
28. **Schaefer, S., and R. Ramasamy.** Transient inhibition of the Na-H exchanger limits acidosis and ischemic injury during global ischemia. *Cardiovasc. Res.* 34: 329–336, 1997.
29. **Schaefer, S., and R. Ramasamy.** Glycogen utilization and ischemic injury in the isolated rat heart. *Cardiovasc. Res.* 35: 90–98, 1997.
30. **Stanley, A. W., R. E. Moraski, R. O. Russell, W. J. Rogers, J. A. Mantle, R. A. Kreisberg, H. G. McDaniel, and C. E. Rackley.** Effects of glucose-insulin-potassium on myocardial substrate availability and utilization in stable coronary artery disease. *Am. J. Cardiol.* 36: 929–937, 1975.
31. **Stanley, W. C., J. R. Hall, K. R. Smith, G. D. Cartee, T. A. Hacker, and J. A. Wisneski.** Myocardial glucose transporters and glycolytic metabolism during ischemia in hyperglycemic diabetic swine. *Metabolism* 43: 61–69, 1994.
32. **Stanley, W. C., G. D. Lopaschuk, J. L. Hall, and J. G. McCormack.** Regulation of myocardial carbohydrate metabolism under normal and ischemic conditions. Potential for pharmacological interventions. *Cardiovasc. Res.* 33: 243–257, 1997.
33. **Stevens, M. J., J. Dananberg, E. L. Feldman, S. A. Lattimer, M. Kamijo, T. P. Thomas, H. Shindo, A. A. F. Sima, and D. A. Greene.** The linked roles of nitric oxide, aldose reductase and (Na<sup>+</sup>,K<sup>+</sup>)-ATPase in the slowing of nerve conduction in the streptozotocin diabetic rat. *J. Clin. Invest.* 94: 853–859, 1994.
34. **Surges, R., and P. J. Oates.** Aldose reductase inhibitors: recent developments. *Prog. Drug Res.* 40: 99–161, 1993.
35. **Tani, M., and J. R. Neely.** Role of intracellular Na<sup>+</sup> and Ca<sup>2+</sup> overload and depressed recovery of ventricular function of reperfused ischemic rat hearts. *Circ. Res.* 65: 1045–1056, 1989.
36. **Tani, M., and J. R. Neely.** Na<sup>+</sup> accumulation increases Ca<sup>2+</sup> overload and impairs function in anoxic rat heart. *J. Mol. Cell. Cardiol.* 22: 57–72, 1990.
37. **Trueblood, N., and R. Ramasamy.** Aldose reductase inhibition improves altered glucose metabolism of isolated diabetic rat hearts. *Am. J. Physiol.* 275 (*Heart Circ. Physiol.* 44): H75–H83, 1998.
38. **Vandenberg, J. I., J. C. Metcalfe, and A. A. Grace.** Mechanisms of pH<sub>i</sub> recovery after global ischemia in the perfused heart. *Circ. Res.* 72: 993–1003, 1993.
39. **Vanoverschelde, J.-L. J., M. F. Janier, J. E. Bakke, D. R. Marshall, and S. R. Bergmann.** Rate of glycolysis during ischemia determines extent of ischemic injury and functional recovery after reperfusion. *Am. J. Physiol.* 267 (*Heart Circ. Physiol.* 36): H1785–H1794, 1994.
40. **Vanoverschelde, J.-L. J., M. F. Janier, and S. R. Bergmann.** The relative importance of myocardial energy metabolism compared with ischemic contracture in the determination of ischemic injury in isolated rabbit hearts. *Circ. Res.* 74: 817–828, 1994.
41. **Venkatesh, N., J. S. Stuart, S. T. Lamp, L. D. Alexander, and J. N. Weiss.** Activation of ATP-sensitive K<sup>+</sup> channels by cromakalim: effects on cellular K<sup>+</sup> loss and cardiac function in ischemic and reperfused mammalian ventricle. *Circ. Res.* 71: 1324–1333, 1992.
42. **Weiss, J., and B. Hiltbrand.** Functional compartmentation of glycolytic versus oxidative metabolism in isolated rabbit heart. *J. Clin. Invest.* 75: 436–447, 1985.
43. **Williamson, J. R., K. Chang, M. Frangos, K. S. Hasan, Y. Ido, T. Kawamura, J. R. Nyengaard, M. Van den Enden, C. Kilo, and R. G. Tilton.** Hyperglycemic pseudohypoxia and diabetic complications. *Diabetes* 42: 801–813, 1993.