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Authors

Ramasamy, Ravichandran

Liu, Hong

Oates, Peter J

et al.

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Attenuation of ischemia induced increases in sodium and calcium by the aldose reductase inhibitor Zopolrestat

Ravichandran Ramasamy[a,*], Hong Liu[b], Peter J. Oates[e], Saul Schaefer[c,d]

[a]*Division of Cardiology, PH 3-Cen-342, College of Physicians and Surgeons, Columbia University, 630 West, 168th Street, New York, NY 10032 USA*

[b]*Department of Human Physiology, University of California, Davis, CA 95616, USA*

[c]*Division of Cardiovascular Medicine, University of California, Davis CA 95616, USA*

[d]*Cardiology Section, VA Northern California Health Care System, Martinez, CA 94553, USA*

[e]*Central Research Division, Department of Metabolic Diseases, Pfizer, Inc., Groton, CT 06340, USA*

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Abstract

Objective: We have previously demonstrated that zopolrestat, an inhibitor of the enzyme aldose reductase, reduces ischemic injury in hearts from diabetic and non-diabetic rats. To further explore potential cardioprotective mechanisms of zopolrestat, we measured changes in intracellular sodium, calcium, and Na^+,K^+-ATPase activity in zopolrestat treated hearts during ischemia and reperfusion. **Methods:** Hearts from acute diabetic (Type I) and age-matched control rats were isolated and retrogradely perfused. Hearts had either control perfusion or exposure to 1 μM zopolrestat for 10 min, followed by 20 min of global ischemia and 60 min of reperfusion. Changes in intracellular sodium and calcium were measured using ^{23}Na and ^{19}F magnetic resonance spectroscopy, respectively, while the activity of Na^+,K^+-ATPase was measured using biochemical assays. **Results:** Zopolrestat blunted the rise in $[Na]_i$ during ischemia in both diabetic hearts and non-diabetic hearts. The end-ischemic $[Na]_i$ was 21.3±2.6 mM in the zopolrestat treated diabetics and 25.9±2.3 in zopolrestat treated non-diabetics, versus 31.6±2.6 mM and 32.9±2.8 mM in the untreated diabetics and untreated non-diabetics, respectively. ($P=0.002$). Similarly, the rise in $[Ca]_i$ at the end of ischemia was significantly reduced in zopolrestat treated diabetic and non-diabetic hearts ($P=0.005$). Zopolrestat increased the activity of Na^+,K^+-ATPase in diabetic hearts under baseline conditions (11.70±0.95 versus 7.28±0.98 μmol/h/mg protein, $P=0.005$) as well as during ischemia and reperfusion. Similar changes in Na^+,K^+-ATPase activity were also observed in non-diabetic hearts. **Conclusions:** The data provide additional support to the protective effects of zopolrestat and suggest that a possible mechanism of action may be associated with the attenuation of the rise in $[Na]_i$ and $[Ca]_i$ during ischemia and reperfusion.

Keywords: Diabetes; Aldose reductase; Na^+,K^+-ATPase; Ischemia; NMR

1. Introduction

Myocardial ischemia impairs energy production and alters pH and ionic homeostasis, which along with other factors, result in myocardial injury. Interventions that limit metabolic and ionic derangements have been demonstrated to protect hearts from ischemic injury. Specifically, studies have demonstrated that limiting the rise in intracellular sodium during ischemia limits the rise in intracellular calcium, thereby reducing ischemic injury [1–4].

Recently, we demonstrated that zopolrestat, an aldose reductase inhibitor, improved functional recovery and reduced ischemic injury in both diabetic and non-diabetic rat hearts [5,6]. Further, it was shown that the cardioprotection afforded by zopolrestat was associated with lowering of cytosolic redox state, increases in glycolysis, and conservation of ATP in diabetic and non-diabetic hearts [5–7]. Since there is evidence that aldose reductase inhibition can increase Na^+,K^+-ATPase activity in some tissues [8–11], we examined if zopolrestat would limit the rise in intracellular sodium and calcium during ischemia

*Corresponding author. Tel.: +1-212-305-2267; fax: +1-212-305-4648.

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via its influence on Na^+,K^+-ATPase activity. To test if the cardioprotection was associated with limitation of sodium and calcium increases during ischemia, we measured Na^+,K^+-ATPase activity, intracellular sodium, and intracellular calcium in isolated hearts from Bio-Bred (BB/W) insulin-dependent acute diabetic and non-diabetic rats perfused with and without the aldose reductase inhibitor zopolrestat. The data show that zopolrestat protects ischemic myocardium and that this protection is associated with decreases in intracellular sodium and calcium during ischemia.

2. Methods

2.1. General methods

All experiments were performed with the approval of the University of California Davis and Columbia University Animal Use and Care Committee. This investigation conforms with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH publication No. 85-23, 1996).

2.2. Diabetic rats

Spontaneously acute diabetic Bio-Bred (BB/W) rats from the colony maintained at the University of Massachusetts Medical Center, Worcester, were used in this study. We chose BB/W rats for our study because the BB/W rats are considered to be a useful model of autoimmune human insulin-dependent diabetes (IDDM) [12]. The BB/W rats used in this study were 3–4 months old with the duration of diabetes being 12 ± 3 days, weighed between 300–350 g, and were receiving daily insulin therapy. Insulin therapy was discontinued 24 h prior to performing the isolated heart perfusion studies, resulting in significant increases in serum glucose levels (386 ± 54 mg/dl) [13].

2.3. Littermate control rats

Age-matched non-diabetic littermates, also from the colony maintained at the University of Massachusetts Medical Center, Worcester, were used in this study. The mean blood glucose levels in the littermate controls were 102 ± 12 mg/dl.

2.4. Isolated perfused heart model

Experiments were performed using an isovolumic isolated rat heart preparation. Acutely diabetic male BB/W rats and age-matched littermate controls were pretreated with heparin (1000 U, I.P.), followed by sodium pentobarbital (65 mg/kg, I.P.). After deep anesthesia was achieved, the hearts were rapidly excised and placed into iced saline. The arrested hearts were retrogradely perfused (in a non-recirculating mode) through the aorta within 2 min. Left ventricular developed pressure (LVDP), left ventricular end diastolic pressure (LVEDP), and heart rates were determined using a latex balloon in the left ventricle with high pressure tubing connected to a pressure transducer. Perfusion pressure was monitored using high pressure tubing off the perfusion line. Hemodynamic measurements were recorded on a 4-channel Gould recorder. The system had two parallel perfusion lines, with separate oxygenators, pumps and bubble traps, allowing rapid change of perfusion media. The hearts were perfused with phosphate-free Krebs–Henseleit buffer consisting of (in mM) NaCl 118, KCl 4.7, $CaCl_2$ 1.2, $MgCl_2$ 1.2, $NaHCO_3$ 25, with the substrate being 11 mM glucose. The perfusion apparatus was tightly temperature controlled, with heated baths used for the perfusate and for the water jacketing around the perfusion tubing to maintain heart temperature at 37°C under all conditions. The oxygenated perfusate in the room temperature reservoir was passed through 25 feet of thin-walled silicone tubing surrounded by distilled water at 37°C saturated with O_2–CO_2 (95:5). The perfusate then entered the water jacketed tubing leading to the heart through a water jacketed bubble trap.

2.5. Protocols

All protocols were performed in hearts from diabetic and non-diabetic rats in the absence and presence of 1 μM zopolrestat. In the experiments without zopolrestat, baseline perfusion was continued for 20 min before institution of global ischemia. In the experiments employing zopolrestat, the inhibitor was added 10 min before global ischemia was instituted. Intracellular sodium $[Na]_i$ or calcium $[Ca]_i$ were measured continuously during baseline perfusion, during ischemia and upon reperfusion for 30 min with concurrent hemodynamic measurements. Na^+,K^+-ATPase activity measurements ($n=9$ in each group) were performed in hearts freeze-clamped after baseline perfusion, after 5 and 20 min of ischemia, and after 5 and 30 min of reperfusion. Homogenates from each of these hearts were prepared and assayed for Na^+,K^+-ATPase activity.

2.6. ^{23}Na NMR spectroscopy

Intracellular sodium concentration $[Na]_i$ was determined using the shift reagent thulium-$DOTP^{5-}$ [14], supplied by Magnetic Resonance Solutions (Dallas, TX, USA). Since the shift reagent contains sodium, the amount sodium added to the buffer was lowered such that the total sodium concentration did not exceed 143 mM. The total calcium in the shift reagent containing buffer was adjusted such that the free calcium concentrations were approximately 1.25

mM. Hearts were placed in a 20 mm NMR tube and were bathed by the perfusate (mean volume of 4.96±0.09 ml). Coronary effluent was removed from the NMR tube approximately 9 mm above the heart. Sodium spectra were acquired on a Bruker AMX 400 MHz spectrometer using the broad band 20 mm probe tuned to 105.85 MHz. One thousand free induction decays were signal averaged over 5 min using 90 degree pulses with a ±4000 Hz sweep width. Intracellular sodium concentration was calculated using the formula:

$$[Na]_i = ([ANa_i]/[ANa_o])((V_o/V_i)(f_o/f_i))[Na]_o$$

where ANa_i and ANa_o are the intracellular and extracellular areas of the sodium resonances, V_o and V_i are the extracellular and intracellular volumes (V_o/V_i ratio is assumed to be 1 based on Ref. [15]), and f_o and f_i are the fractional visibilities of extra- and intra-cellular sodium (assumed as 1.0 and 0.4, respectively) [15]. It was assumed that the intracellular sodium visibility did not change during the experimental protocols.

2.7. Analysis of sodium in freeze-clamped hearts

Intracellular sodium content under baseline conditions in diabetic and non-diabetic freeze-clamped hearts ($n=6$ in each group) were also determined by atomic absorption techniques, using a Varian Spectra AA 10/20 system, as published earlier [16,17].

2.8. ^{19}F NMR spectroscopy

Intracellular calcium concentrations $[Ca]_i$ were measured after loading the hearts with 5F-BAPTA, a compound that provides a signal proportional to $[Ca]_i$ [18,19]. Hearts were perfused in the standard manner and then perfused with the acetoxymethyl ester of 5F-BAPTA (2.5 μM in normal perfusate) over 1 h. The hearts were then perfused for 15 min with 5F-BAPTA-free perfusate to wash the 5F-BAPTA out of the extracellular space. Total perfusate calcium concentration was increased to 2.5 mM to maintain free ionized intracellular calcium concentrations. The probe was tuned to 376.5 MHz (on the Bruker AMX-400 MHz spectrometer) and 1500 free induction decays were acquired in 5 min intervals using 45 degree pulses and ±5000 Hz sweep width. Intracellular calcium concentration in nM was calculated using the equation:

$$[Ca]_i = K_d([Ca-5F-BAPTA]/[5F-BAPTA])$$

where $K_d=308$ nM and the ratio of calcium-bound to free [5F-BAPTA] was equal to the ratio of the corresponding peak areas of the two well defined peaks in the ^{19}F spectrum [18,19].

2.9. Measurements of Na^+,K^+-ATPase activity

Ouabain sensitive Na^+,K^+-ATPase activities were measured in the microsomes of diabetic, zopolrestat perfused diabetic, non-diabetic, and zopolrestat perfused non-diabetic hearts by a previously described spectrophotometric method [20]. Heart tissue was homogenized at 4°C in 2 ml of 0.2 M sucrose–0.02 M Tris–HCl buffer (pH 7.5) containing a protease inhibitor (100 μl of 57 mM phenylmethylsulfonyl fluoride+10 μl of 1 mg/ml leupeptin), with a polytron homogenizer (for four periods, with each period not exceeding 15 s) and then centrifuged at 100 *g* for 10 min at 4°C. The microsomes were prepared from the homogenates using published procedures [21]. Briefly, the microsomes rich in Na^+,K^+-ATPase activity were prepared by subjecting homogenates from the above step to 15 freeze–thaw cycles and then centrifuging at 31 000 *g* for 30 min and suspending the pellet in imidazole buffer until measuring the activity. Earlier investigations from our laboratory had demonstrated that the Na^+,K^+-ATPase activity obtained by this preparation was not different from those obtained using sarcolemma permeabilizers such as alamethycin or valinomycin. Therefore, the Na^+,K^+-ATPase activity measurements reported in this study are from microsomal preparations containing vesicles of all orientations.

The assay was started by the addition of 20 μl of microsomal aliquot to a 1-ml×1-cm cuvette containing 100 mM NaCl, 10 mM KCl, 2.5 mM $MgCl_2$, 2 mM EGTA, 1 mM Tris–ATP, 1 mM phosphoenolpyruvate, 30 mM imidazole–HCl buffer at pH 7.4, 0.15 mM NADH, 50 μg lactate dehydrogenase, 30 μg pyruvate kinase with or without 1 mM ouabain. After a 30-min stabilization period, the linear rate of oxidation of NADH was monitored at 340 nm. ATPase activity was calculated from the linear portion of the curve using extinction coefficient of NADH, volume of the reaction mixture, and the amount of heart homogenate. The Na^+,K^+-ATPase activity was obtained by subtracting the ATPase activity obtained in the presence of ouabain from that obtained without ouabain. In order to determine whether zopolrestat had direct in vitro effects on Na^+,K^+-ATPase activity, homogenates from hearts were added to the above medium containing various concentrations of zopolrestat (0.2 to 10 μM).

2.10. Statistical methods

Data were analyzed using INSTAT (GraphPad, San Diego, CA, USA) software operating on an IBM compatible personal computer. Differences between groups was assessed using ANOVA, with subsequent Student–Newman–Keuls multiple comparisons post-tests if the *P* value for ANOVA was significant. A *P* value of less than 0.05 was used to reject the null hypothesis. All data are expressed as mean±S.E.M.

3. Results

3.1. Functional data

Zopolrestat did not alter LVDP or heart rate under baseline conditions in any group (Table 1). Cessation of cardiac function occurred in all groups shortly after the onset of global ischemia. Reperfusion of diabetic and non-diabetic hearts resulted in poor LVDP and rhythmic activity compared to zopolrestat treated diabetic and non-diabetic hearts. All the zopolrestat perfused diabetic and non-diabetic hearts exhibited recovery of LVDP that was significantly greater than untreated hearts.

LVEDP was set to 10–12 cm H_2O pressure at the beginning of the perfusion period and increased in all groups to a maximum after 15 min of ischemia. The difference in LVEDP among groups at the end of ischemia was not significant. On reperfusion, the zopolrestat treated diabetic and non-diabetic hearts had significantly lower LVEDP than untreated diabetic and non-diabetic hearts. Also, the LVEDP in diabetic hearts was significantly lower than in non-diabetic hearts at the end of reperfusion. Comparing the present functional data obtained in the presence of $[Tm(DOTP)]^{5-}$ with that published in our earlier study [5], it is evident that shift reagent did not influence the functional parameters in diabetic and non-diabetic hearts.

3.2. Effect of zopolrestat on intracellular sodium

3.2.1. Baseline measurements of sodium

The baseline intracellular sodium concentrations measured by atomic absorption were significantly higher in diabetic than non-diabetic hearts (36.8±3.2 mM in diabetic versus 26.9±2.7 mM in non-diabetic, $P=0.003$). Intracellular sodium concentration measured by NMR also exhibited a similar trend, with higher sodium concentrations in diabetic than non-diabetic hearts (15.1±1.4 mM in diabetic versus 9.1±1.1 mM in non-diabetic, $P<0.01$). The differences in intracellular sodium concentrations obtained using NMR and atomic absorption spectroscopy can be explained based on the fact that NMR visibility factor is applied to the free cytoplasmic sodium while the rest is bound Na^+, which is NMR invisible. Nevertheless, both independent techniques demonstrated significantly higher intracellular sodium concentrations under baseline conditions in diabetic compared to non-diabetic hearts.

3.2.2. Time dependent sodium measurements (diabetic hearts)

Since NMR provides a non-destructive means of measuring time dependent changes in intracellular sodium, we used NMR to measure intracellular sodium during ischemia and reperfusion. Fig. 1 displays the ^{23}Na NMR spectra of intracellular and extracellular sodium in diabetic hearts perfused with 4 mM $[Tm(DOTP)]^{5-}$. The areas under the intracellular Na^+ resonances were converted into concentrations as detailed in Section 2. As observed in Fig. 2, ischemia increased intracellular $[Na]_i$ from 15.6±1.1 to 31.6±2.2 mM in diabetic hearts. Perfusing diabetic hearts with 1 μM zopolrestat resulted in a significant reduction in the rise of intracellular $[Na]_i$ during ischemia, resulting in end-ischemic intracellular $[Na]_i$ of 21.3±2.6 mM ($P<0.005$ vs. untreated diabetic control). At the end of reperfusion, the intracellular $[Na]_i$ was significantly lower in zopolrestat treated diabetic hearts than in untreated diabetic control hearts ($P<0.005$).

3.2.3. Time dependent sodium measurements (non-diabetic hearts)

The end-ischemic intracellular sodium concentration was not significantly different between diabetic and non-diabetic hearts ($[Na]_i$ 32.9±2.8 mM in non-diabetic and 31.6±2.2 mM in diabetic hearts). Our observations on the

Table 1
Function data on perfused rat hearts under baseline, ischemic, and reperfusion conditions

Function	C	Diabetic	Diabetic + Zopolrestat	Z
LVDP (cm H_2O)				
Baseline	106±12	96±11	109±12	112±14
End of reperfusion	18±6	9±7	58±12[a]	48±9[b]
LVEDP (cm H_2O)				
Baseline	12±2	11±3	12±1	10±3
End of ischemia	68±9	59±11	52±14	61±12
End of reperfusion	71±12	33±6[c]	16±5[a]	11±5[b]
Heart rate				
Baseline	262±39	198±27	221±31	251±42
End of reperfusion	24±12	12±9	121±22[a]	101±31[b]

Data are presented as means±standard error. Six hearts were used per group.
C, non-diabetic controls; Z, zopolrestat perfused non-diabetics. The presence of $[Tm(DOTP)]^{5-}$ did not alter any of the functional parameters.
[a] $P<0.05$ in diabetic zopolrestat vs. diabetic group.
[b] $P<0.03$ in Z vs. non-diabetic group.
[c] $P<0.05$ in diabetic vs. non-diabetic group.

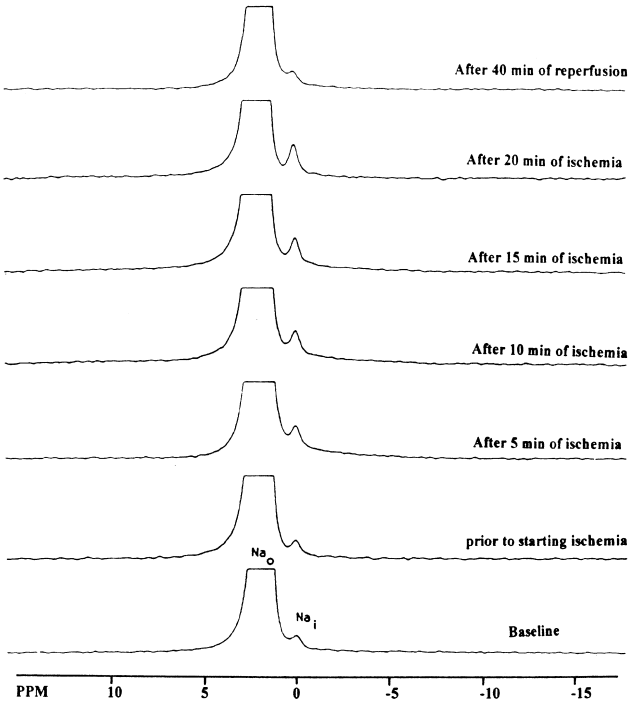

Fig. 1. Representative ^{23}Na NMR spectra from diabetic hearts during baseline, ischemic and reperfusion conditions. The shift reagent $[Tm(DOTP)]^{5-}$ (4 mM) was employed to distinguish between the intra- and extra-cellular sodium resonances.

changes in intracellular sodium at the end of ischemia in diabetic hearts are in agreement with those of Tosaki et al. [22]. As in the diabetic hearts, zopolrestat significantly limited the rise in intracellular sodium in non-diabetic hearts ($[Na]_i$ being 25.9±2.3 mM in zopolrestat treated non-diabetics versus 32.9±2.8 mM in non-diabetic hearts, $P<0.03$).

While zopolrestat attenuated the rise in intracellular sodium in both diabetic and non-diabetic hearts, there was a greater magnitude of reduction in the rise of $[Na]_i$ in zopolrestat treated diabetics compared to zopolrestat treated non-diabetics (11 versus 7 mM, $P=0.03$). In both diabetic and non-diabetic hearts, zopolrestat significantly attenuated the rise in intracellular sodium during ischemia compared to their respective untreated controls.

3.3. Effect of zopolrestat on intracellular calcium

Intracellular calcium measurements were performed using 5F-BAPTA and ^{19}F NMR spectroscopy in perfused hearts during global ischemia and during reperfusion. Loading of the hearts with 5F-BAPTA resulted in a significant reduction in LVDP in both diabetic and non-diabetic hearts. The reduction in baseline LVDP were similar in all groups (LVDP values in cm water pressure were 62±9 in non-diabetic, 56±7 in non-diabetic zopolrestat, 64±11 in diabetic and 52±8 in diabetic zopolrestat hearts). Our observations of reduction in LVDP are consistent with those reported in the literature [15,18,19]. A typical ^{19}F NMR spectrum of a 5F-BAPTA perfused diabetic heart under baseline conditions is illustrated in Fig. 3. The changes in intracellular calcium observed during 20 min of global ischemia and 60 min of reperfusion are illustrated in Fig. 4. The data presented in Fig. 4A demonstrate that zopolrestat abolished the rise in intracellular free Ca^{2+} during ischemia in diabetic hearts. Intracellular calcium after 20 min of global ischemia was 693.3±49.9 nM in diabetic and 286.6±39.8 nM in diabetic zopolrestat hearts. While calcium continued to increase in diabetic hearts during reperfusion, intracellular calcium in zopolrestat perfused diabetic hearts remained at pre-ischemic values. There was no increase in intracellular calcium concentration during ischemia or reperfusion in the diabetic zopolrestat hearts. In non-diabetic hearts (Fig. 4B), zopolrestat limited the rise in intracellular calcium to a lesser extent than in zopolrestat treated diabetic hearts. These observations are consistent with the hypothesis that

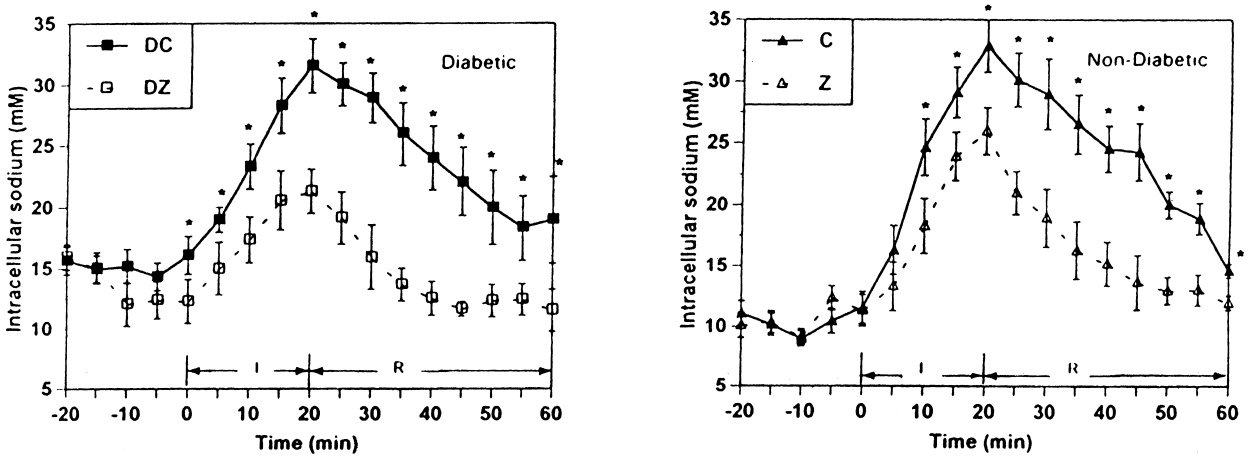

Fig. 2. Changes in intracellular sodium concentration during 20 min of global ischemia and 40 min of reperfusion in diabetic and non-diabetic group of hearts. In each group, data is presented from six hearts perfused with and without zopolrestat. I and R represent ischemic and reperfusion periods, respectively. Each point represents the data obtained every 5 min. *, significantly greater than the zopolrestat treated group ($P<0.03$).

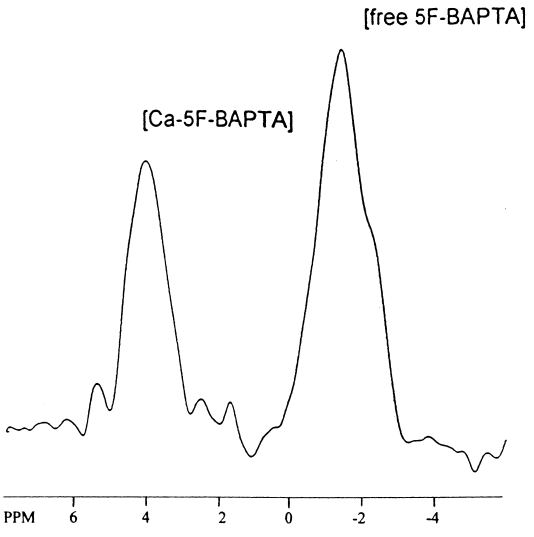

Fig. 3. Typical ^{19}F NMR spectrum from a diabetic heart, loaded with 5F-BAPTA, obtained under baseline conditions. The calcium bound and unbound free 5F-BAPTA resonances are shown in the spectrum.

the protection against ischemia afforded by zopolrestat is mediated, in part, by preventing calcium overload during ischemia and reperfusion.

3.4. Zopolrestat and Na^+,K^+-ATPase activity

Table 2 displays Na^+,K^+-ATPase activity in the four groups under baseline, ischemic, and reperfusion conditions. The microsomal Na^+,K^+-ATPase activity values obtained for non-diabetic hearts are similar to those obtained by Lee et al. [21]. Diabetic hearts had a 44% reduction in Na^+,K^+-ATPase activity compared to non-diabetic hearts ($P<0.05$). Zopolrestat increased Na^+,K^+-ATPase activity in diabetic hearts, restoring the Na^+,K^+,ATPase activity to values observed in non-diabetic hearts. In non-diabetic hearts, zopolrestat also resulted in a significant increase in Na^+,K^+-ATPase activity. The zopolrestat treated hearts (both diabetic and non-diabetic) had significantly greater activity during ischemia and reperfusion compared to the untreated hearts.

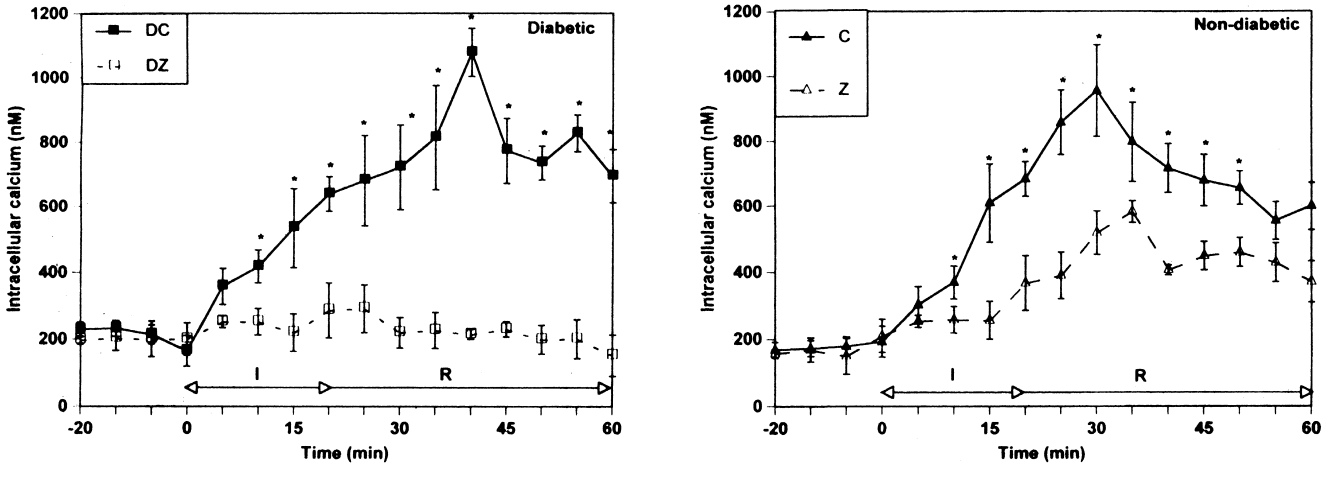

Fig. 4. Changes in intracellular calcium concentration during 20 min of global ischemia and 40 min of reperfusion in diabetic and non-diabetic group of hearts. In each group, data is presented from six hearts perfused with and without zopolrestat. I and R represent ischemic and reperfusion periods, respectively. Each point represents the data obtained every 5 min. *, significantly greater than the zopolrestat treated group ($P<0.05$).

Table 2
Ouabain-sensitive Na^+,K^+-ATPase activity in microsomes from hearts perfused with and without 1 μM zopolrestat

Hearts	Na^+,K^+-ATPase activity (μmol ADP/h/mg total protein)				
	Baseline	Ischemia		Reperfusion	
		5 min	20 min	5 min	30 min
Diabetic	7.29±0.98[a]	6.96±1.08[a]	5.56±1.62[a]	6.92±0.81[a]	6.18±1.18[a]
Diabetic+ Zopolrestat	11.70±0.95	12.12±1.66	11.89±1.06	12.36±1.54	14.01±3.32
C	12.96±1.13	9.91±1.02	10.32±0.81	9.03±1.88	10.08±1.68
Z	16.21±1.68[b]	15.29±1.69[b]	14.67±0.79[b]	13.57±1.13[b]	13.81±0.54[b]

Data are presented as means±standard error. Six hearts were used per group.
C, non-diabetic controls; Z, zopolrestat perfused non-diabetics.
[a] Significantly lower than in diabetic zopolrestat and non-diabetic hearts ($P=0.03$).
[b] Significantly greater than in non-diabetic hearts ($P=0.05$). Six hearts were used per group for baseline, ischemia, and reperfusion.

To determine if zopolrestat may directly influence Na^+,K^+-ATPase activity in vitro, the Na^+,K^+-ATPase assay was performed in the presence and absence of zopolrestat in microsomes from diabetic and non-diabetic hearts. In microsomes obtained from diabetic hearts, zopolrestat had minimal, non-significant direct effects on the microsomal Na^+,K^+-ATPase activity in diabetic hearts. The activity changes due to the addition of zopolrestat in homogenates from diabetic hearts (expressed as μmol ADP/h/mg total protein) were from (a) 7.29 ± 0.98 to 8.11 ± 0.62 after baseline perfusion, (b) 5.56 ± 1.62 to 5.11 ± 0.91 at the end of 20 min of ischemia, and (c) 6.18 ± 1.18 to 8.02 ± 1.77 after 30 min of reperfusion ($n=5$ in each group). In microsomes from non-diabetic hearts, zopolrestat resulted in a marginal, non-significant increase in Na^+,K^+-ATPase activity from 12.96 ± 1.13 to 13.88 ± 0.91 ($n=5$ in each group, $P=0.12$). Measurements of microsomal Na^+,K^+-ATPase activities from non-diabetic hearts after ischemia and reperfusion also demonstrated that the addition of zopolrestat did not result in a significant change in activity. In both diabetic and non-diabetic hearts, the increases in Na^+,K^+-ATPase activity were much greater when hearts were perfused with zopolrestat (see Table 2) than in the in vitro microsomal experiments. These data suggest that increases in Na^+,K^+-ATPase activity observed in zopolrestat treated hearts were unlikely due to a direct effect of zopolrestat on the enzyme.

4. Discussion

This study demonstrated that zopolrestat, an aldose reductase inhibitor, limited the increase in both intracellular sodium and calcium during ischemia and reperfusion. In addition, zopolrestat increased the activity of Na^+,K^+-ATPase under baseline, ischemic and reperfusion conditions. In concert with our previous data showing a reduction in infarct size in both diabetic and non-diabetic rat hearts [5], these findings suggest a mechanism for the protective effect of aldose reductase inhibition during ischemia.

4.1. Inhibition of aldose reductase and intracellular sodium

The steady state intracellular sodium concentration is maintained by a balance between sodium influx down and sodium efflux against the electrochemical gradient. Under baseline conditions, sodium influx occurs via the fast sodium channels, the Na^+–H^+ exchanger, the Na^+–K^+–$2Cl^-$ cotransporter, and the Na^+–Ca^{2+} exchanger, while sodium efflux occurs mainly through the Na^+,K^+-ATPase [23]. Consequently, inhibition of myocardial Na^+,K^+-ATPase, as observed in diabetics, results in increased baseline sodium concentration [9,10].

Several studies have demonstrated that ischemia leads to intracellular acidosis and increases in intracellular sodium and calcium in isolated perfused hearts, and that these increases are related to the severity of ischemic injury [1,2,14,15,24,25]. Studies have demonstrated that interventions which limit sodium or calcium accumulation during ischemia reduce ischemic injury [1,3,4,26]. The data presented here are consistent with the postulate that the cardioprotection afforded by zopolrestat may be due to attenuation of the rise in intracellular sodium and calcium during ischemia.

One mechanism to limit the rise in intracellular sodium during ischemia and reperfusion is to increase sodium efflux via the Na^+,K^+-ATPase. Concurrent with the limitation in the rise in intracellular sodium in these experiments, zopolrestat increased Na^+,K^+-ATPase activity in diabetic hearts under baseline, ischemic, and reperfusion conditions. These observations are consistent with normalization of Na^+,K^+-ATPase activity by zopolrestat, thereby resulting in greater sodium efflux during ischemia and reperfusion. While normalization of Na^+,K^+-ATPase may be an important mechanism of protection in hearts from diabetic animals, other mechanisms apart from effects of zopolrestat on Na^+,K^+-ATPase activity are likely to be involved. This conclusion is suggested by comparing the lower $[Na]_i$ and $[Ca]_i$ values during ischemia in the zopolrestat treated diabetic hearts with the untreated non-diabetic groups with similar Na^+,K^+-ATPase activities.

Treatment of diabetic hearts with zopolrestat resulted in Na^+,K^+-ATPase activity similar to that of non-diabetic hearts, yet had lower sodium and calcium accumulation, to the point of abolition of the rise in $[Ca^{2+}]$ during ischemia. There are several possible explanations for the lower intracellular sodium. First, we have previously shown that ATP levels are higher in the zopolrestat treated hearts during ischemia [5]. Since Na^+,K^+-ATPase activity in vivo may be ATP limited, these higher levels of ATP may afford greater sodium efflux despite similar activity measured using biochemical assays, which are not ATP limited. Calculations of ATP to ADP ratio and the free energy of ATP hydrolysis determined using creatine kinase equilibrium [16], suggests that ATP is not the limiting factor for the observed changes in Na^+,K^+-ATPase activity in this study. Secondly, other sodium regulatory mechanisms are altered in diabetic hearts. These include reduced Na^+–H^+ exchanger activity in diabetic hearts [27,28]. Since the Na^+–H^+ exchanger has a critical role in regulating intracellular sodium during ischemia, reduced Na^+–H^+ exchanger activity, coupled with greater Na^+,K^+-ATPase activity, would result in a lower increase in intracellular sodium diabetic hearts. In addition to the Na^+–H^+exchanger, the Na^+–K^+–$2Cl^-$ cotransporter [25,29,30] and the sodium channels [24] can contribute to the rise in intracellular sodium. We have recently demonstrated that the activity of the Na^+–K^+–$2Cl^-$ cotrans-

porter is increased in diabetic hearts [31], potentially as a consequence of impaired Na^+,K^+-ATPase activity. Increased $Na^+–K^+–2Cl^-$ cotransporter activity would contribute to sodium accumulation during ischemia [30], and, consequently, normalization of Na^+,K^+-ATPase activity could have secondary beneficial effects by reducing cotransporter activity. Hence, the consequence of normalization of Na^+,K^+-ATPase activity, in the face of other perturbations of sodium regulating mechanisms in diabetics, may be greater than in non-diabetic hearts and explain the lower rise in intracellular sodium.

4.2. Inhibition of aldose reductase and intracellular calcium

As noted above, the rise in intracellular calcium during ischemia and reperfusion may result from sodium-dependent calcium influx via the $Na^+–Ca^{2+}$ exchanger [1,19]. While calcium entry via voltage dependent calcium channels is another mechanism of calcium overload, studies have demonstrated that activation of calcium channels cannot be the sole mechanism by which the rise in intracellular calcium can occur during ischemia [32]. In support of the critical role of $Na^+–Ca^{2+}$ exchange in calcium overload with ischemia and reperfusion, numerous experiments have shown that limiting the rise in intracellular sodium using inhibitors of the $Na^+–H^+$ exchanger significantly reduces the rise in intracellular calcium [1,2,26]. The reductions in both intracellular sodium and calcium in the current study supports these previous studies and supports the hypothesis that calcium influx via the $Na^+–Ca^{2+}$ exchanger plays an important role in the rise in intracellular calcium during ischemia.

In parallel with similar increases in intracellular sodium in untreated diabetic and non-diabetic hearts, the increase in intracellular calcium was similar in both untreated groups during ischemia ($\approx$650 nM) as well upon reperfusion ($\approx$1000 nM). These similar increases occurred despite the reported lower activity of the $Na^+–Ca^{2+}$ exchanger in diabetic hearts [9,33] and suggest that the $Na^+–Ca^{2+}$exchanger has sufficient activity to result in large increases in calcium. The rise in calcium during ischemia was limited in both zopolrestat treated diabetic and non-diabetic hearts, reductions that paralleled the reductions in intracellular sodium. Diabetic zopolrestat-treated hearts had no significant increases in intracellular calcium during either ischemia or reperfusion, while non-diabetic zopolrestat hearts had a significant increase in intracellular calcium on reperfusion. These changes likely reflect the 3:1 stoichiometry of the $Na^+–Ca^{2+}$ exchanger and the large effect of relatively small reductions in intracellular sodium on intracellular calcium [1,15,34], as well as a possible threshold value of $[Na]_i$ required for reversal of the $Na^+–Ca^{2+}$ exchanger. Alternatively, either a direct effect on calcium flux via calcium channels or direct inhibition of the $Na^+–Ca^{2+}$ exchanger is possible in the zopolrestat treated diabetic hearts.

4.3. Aldose reductase and Na^+,K^+-ATPase activity

Studies have shown that aldose reductase inhibitors normalize Na^+,K^+-ATPase activity in most tissues of diabetic animals [10,11,35,36], although no studies on perfused hearts have been performed. Consistent with previous studies, we have shown that the Na^+,K^+-ATPase activity is reduced in hearts from spontaneously diabetic (BB) rats, and that treatment with the aldose reductase inhibitor zopolrestat increased Na^+,K^+-ATPase activity.

There are several mechanisms by which zopolrestat could influence the Na^+,K^+-ATPase activity. First, as observed in studies of diabetic kidney [11], zopolrestat may directly stimulate the Na^+,K^+-ATPase by interacting with the low affinity ATP binding site. Second, inhibition of aldose reductase reduces the ratio of $NADH/NAD^+$ [5,8]; this in turn may influence protein kinase C activity, resulting in higher Na^+,K^+-ATPase activity [8,37]. Third, aldose reductase inhibition may result in restoration of myoinositol levels, which may then enhance Na^+,K^+-ATPase activity [9]. The current in vitro experiments using zopolrestat suggest that the direct effect of zopolrestat alone does not account for the significant increases in Na^+,K^+-ATPase activity seen in perfused hearts.

4.4. Limitations

While the current findings show that zopolrestat reduced the rise in sodium and calcium during ischemia, these findings must be interpreted within the limitations of the experimental design. The use of 5F-BAPTA has been shown to influence intracellular buffering and left ventricular pressure development [2,38], this effect can be partially offset by using higher concentrations of calcium in the perfusion medium [18]. Nevertheless, it has been demonstrated that this method is quite useful in comparing relative changes in intracellular calcium during ischemia and reperfusion [15,18,19].

The structural characteristics of zopolrestat may suggest that the cardioprotection could be independent of aldose reductase inhibition. However, preliminary results using a structurally different aldose reductase inhibitor, sorbinil, demonstrated cardioprotection and was also associated with reductions in sodium and calcium during ischemia.

5. Conclusions

The data presented here demonstrate that zopolrestat, an aldose reductase inhibitor, limited the rise in intracellular sodium and, hence, calcium during ischemia and reperfusion. The observed changes in intracellular sodium were partly attributable to increases in Na^+,K^+-ATPase activity.

However, the complex interplay between the Na^+,K^+-ATPase and other ion transport and metabolic systems in diabetic and non-diabetic hearts suggest that the measured changes in Na^+,K^+-ATPase activity are not the sole effectors of reduced intracellular sodium and calcium in zopolrestat treated hearts. Furthermore, the effects of zopolrestat on substrate metabolism and, potentially, ion transport pathways may contribute to the limitation in intracellular sodium and calcium observed in these experiments. These findings support earlier studies that attenuation of the rise in sodium and calcium during ischemia and reperfusion protect hearts from ischemic damage. Further understanding of the mechanism(s) by which aldose reductase inhibitors such as zopolrestat protect ischemic myocardium may lead to novel therapeutic interventions for the treatment of myocardial ischemia.

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