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Fasting limits the increase in intracellular calcium during ischemia in isolated rat hearts

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Abstract *Introduction* Fasting has been shown to limit ischemic injury and improve functional activity after global ischemia. Because calcium overload is considered a mechanism of ischemic injury, we hypothesized that fasting would limit the accumulation of intracellular calcium $[Ca]_i$ during ischemia, potentially due to reduced accumulation of intracellular sodium $[Na]_i$. *Methods* To address this hypothesis, hearts isolated from rats fed either a normal diet or fasted for 24 hours underwent 20 min of global ischemia at 37°. In addition to functional parameters, $[Na]_i$ and $[Ca]_i$ were measured using ^{23}Na and ^{19}F spectroscopy using thulium-DOTP^{-5} and 5F-BAPTA, respectively. *In vitro* measurement of sarcoplasmic reticulum calcium uptake and release, as well as activity of the sarcolemmal Na-Ca exchanger, was performed in hearts from fed and fasted animals under baseline and ischemic conditions. *Results* Hearts from fasted animals showed greater recovery of developed pressure (37 ± 9 vs. 11 ± 6 cm H_2O, $p < 0.05$) and less contracture (end-diastolic pressure 25 ± 2 vs. 47 ± 2 cm H_2O, $p < 0.05$) by the end of the reperfusion period. $[Na]_i$ was similar in the 2 groups during the first half of the ischemic period, albeit with a higher concentration of $[Na]_i$ in hearts from fed compared to fasted animals at reperfusion. Fasting markedly limited calcium accumulation during ischemia, with end-ischemic calcium being 419 ± 46 nM in the hearts from fasted animals and 858 ± 140 nM in the hearts from fed animals ($p < 0.01$). There was no significant effect of fasting on calcium uptake or release by the SR, nor on sarcolemmal Na-Ca exchange activity. *Conclusions* Fasting for 24 hours improves functional recovery and markedly limits $[Ca]_i$ accumulation during ischemia and early reperfusion. The mechanism for this phenomenon remains to be elucidated.

Key words Sodium – NMR spectroscopy – 5F-BAPTA – calcium

Introduction

Fasting has been shown to protect against myocardial ischemia. In studies from the laboratory of Dr. Taegtmeyer (1–3) and experiments in our laboratory (4), hearts from fasted animals showed greater functional recovery and reduced enzyme release on reperfusion following global ischemia. Experiments to elucidate the mechanism of this protective effect in hearts from fasted animals have shown that these hearts had a) higher baseline glycogen levels and decreased glucose uptake compared to hearts from fed animals (3), b) higher adenine nucleotide content at the end of ischemia, and c) preserved glucose metabolic rate on reperfusion (1). In addition, our earlier studies have shown that hearts from fasted animals exhibit a lower baseline cytosolic redox state ($NADH/NAD^+$) and increased glycogen utilization

BRC 288

during ischemia (4). However, unlike preconditioned hearts (5), hearts from fasted animals were not less acidotic during ischemia.

Reductions in the increase in intracellular sodium $[Na]_i$ and intracellular calcium $[Ca]_i$, both during ischemia and reperfusion, have commonly been cited to be mechanisms limiting ischemia/reperfusion injury (6–8). In one paradigm (6, 8), the increase in intracellular sodium, primarily due to intracellular acidosis and resultant sodium-proton exchange (NHE), is the proximate cause of calcium overload due to the reduction in the membrane sodium gradient and either reduced calcium efflux or frank calcium influx during ischemia or reperfusion due to reversal of the Na-Ca exchanger (6). While this paradigm is supported by beneficial effects seen with NHE inhibition (9), there are experimental models of reduced ischemia/reperfusion injury in which it is difficult to demonstrate lower $[Na]_i$ accumulation (10), in spite of lower $[Ca]_i$ (8). These data suggest that, under some conditions, lower $[Ca]_i$ during ischemia and reperfusion may result from altered calcium transport across the sarcolemmal membrane (such as through calcium channels (11)) or sarcoplasmic reticulum (12, 13), rather than a consequence of lower $[Na]_i$.

In light of the known protective effect of fasting on cardiac ischemia/reperfusion injury, coupled with the possible deleterious role of calcium accumulation, we measured both $[Na]_i$ and $[Ca]_i$ during ischemia and reperfusion in isolated rat hearts. These studies were aimed at testing the hypothesis that fasting limits ischemia/reperfusion injury by reducing the accumulation of $[Ca]_i$ during ischemia and calcium overload on reperfusion.

Methods

Isolated heart preparation

The preparation has been described previously in detail (14). Briefly, it is a retrogradely perfused isovolumic rat heart preparation operating at 37 °C with dual perfusion lines. The buffer was phosphate-free Krebs-Henseleit saline containing (in mM) NaCl 118, KCl 4.7, $CaCl_2$ 1.25, $MgCl_2$ 1.2, $NaHCO_3$ 25. The carbon substrate was glucose 11 mM. Hemodynamic measurements (left ventricular developed pressure [LVDP] and end-diastolic pressure [LVEDP]) were obtained from a latex balloon placed in the left ventricle through the left atrium. LVEDP was set at ~10 cmH_2O at the beginning of the data collection period and was maintained throughout the protocol.

Fasting protocol

Rats were fed *ad libitum* on standard rat chow (Purina) and water. Fed control animals were allowed to eat until the time of the experiment. Fasted animals were allowed only water for 24 hours prior to sacrifice. This period of time was chosen because most of the metabolic changes occur within 24 hours of fasting (15, 16) and this period of fasting was sufficient to show protection during ischemia in our previous experiments as well as those in other laboratories (1, 2).

Sodium-23 spectroscopy

Intracellular sodium concentration $[Na]_i$ was determined using the shift reagent thulium-DOTP^{-5} (4 mM, Magnetic Resonance Solutions, Dallas, TX) added 10 min prior to the ischemic period and continued in a non-recirculating mode throughout reperfusion. Because the shift reagent contains sodium, the amount of sodium added to the buffer was lowered such that the total perfusate sodium concentration did not exceed 143 mM. The total calcium in the shift reagent containing buffer was adjusted such that the free calcium concentration was 1.25 mM. Sodium spectra were acquired on a GE 300 MHz spectrometer using a broad band probe tuned to 79 MHz. 1000 free induction decays were signal averaged over 5 minutes using 90 degree pulses with a ± 4000 Hz sweep width. $[Na]_i$ in mM was calculated from the calibrated area under the unshifted intracellular peak of the sodium spectrum using the equation $[Na]_i = \{ANa_i/ANa_o\} (f_o/f_i) (V_o/V_i) [Na]_o$, where ANa_i and ANa_o are the intracellular and extracellular areas of the sodium resonances, V_i and V_o are intracellular and extracellular volumes (assumed to be 1 according to Steenbergen et al. (8), and f_o and f_i are the fractional visibilities of extra- and intra-cellular sodium (assumed as 1.0 and 0.4, respectively) (8).

Fluorine-19 spectroscopy

These measurements were obtained using a Bruker 400 MHz spectrometer. Intracellular calcium concentrations $[Ca]_i$ were measured after loading the heart with 5F-BAPTA (2.5 μM in normal perfusate) over 1 hour. The hearts were perfused for 15 minutes with 5F-BAPTA-free perfusate to wash the 5F-BAPTA out of the extracellular space. The NMR probe (Doty Scientific, Inc.) was tuned to 376.5 MHz and 1500 free induction decays were acquired in 5 minute intervals using 45 degree pulses and ± 5000 Hz sweep width. Intracellular calcium concentration (mEq/L cell water) was calculated using the equation $[Ca2+]_i = K_d$ [Ca-5F-BAPTA]/[5F-BAPTA], where K_d = 308 nM and the ratio of calcium bound to free 5F-BAPTA

is equal to the ratio of the corresponding peak areas of the two well-defined peaks in the ^{19}F spectrum (17).

Sarcoplasmic reticulum Ca uptake and release

Sarcoplasmic reticulum (SR) were isolated from hearts of both fed and fasted animals after baseline normoxic perfusion, as well as after 15 minutes of global ischemia (n = 5 in each group). This time period was chosen because previous experiments demonstrated significant differences in intracellular calcium in the absence of marked changes in sodium, suggesting the possibility that calcium overload was not secondary to sarcolemmal Na-Ca exchange, but possible re-distribution of intracellular calcium stores. Briefly, SR membrane fractions enriched in terminal cisternae (junctional SR) were isolated from heart muscle homogenates by differential centrifugation as described by Harris and Doroshow (18). Preparations were stored in 0.3 M sucrose, 10 mM imidazole, pH 7.0, at –80 °C until use. Macroscopic Ca^{2+} fluxes were measured spectrophotometrically using the metallochromic calcium dye antipyrylazo III (250 μM) in transport buffer containing 125 μg of cardiac SR protein and (in mM) KCl (92.5), $Na_4P_2O_7$ (7.5), MgATP (1), phosphocreatine (5), creatine kinase (20 μg/ml), MOPS (18.5) at pH 7.0 and 37 °C. Extravesicular calcium concentration changes were detected by a diode array spectrophotometer (Hewlett Packard, model 8452A or 8453) by measuring the difference in absorbance at 710 and 790 nm. SR vesicles were first actively loaded with Ca by several consecutive additions of 6 nmoles of Ca. After reaching a steady-state level, calcium-induced Ca release (CICR) was initiated by rapid injection of bolus Ca (final concentration 50 μM) in the presence of the Ca-pump inhibitor thapsigargin (1 μM). The initial rate of Ca release was calculated by linear regression analysis.

In another set of experiments, Ca loading capacity of SR vesicles (*i.e.* maximal amount of Ca loaded) was determined by measuring total Ca released after addition of Ca ionophore A23187. At the end of each experiment, the absorbance signal was calibrated by addition of a known amount of Ca.

Sarcolemmal Na-Ca exchange

The Na-Ca exchanger, at normal or moderately elevated sodium concentrations (6), operates at 3:1 stoichiometry, and generally results in net calcium efflux (19). Given the increase in $[Ca]_i$ during ischemia, we postulated that relatively lower activity of the exchanger during ischemia would result in a higher $[Ca]_i$ for any given $[Na]_i$. Therefore, intrinsic activity of the Na-Ca exchanger was measured in plasma membrane preparations of hearts of fed and fasted animals at the same time points (baseline and 15 minutes of ischemia, n = 5 in each group). For determination of sarcolemmal Na-Ca exchange activity, highly purified plasma membrane preparations were isolated from left ventricular homogenates by differential and sucrose density centrifugation using a modification of the procedure described by Frank et al. (20) in our modification. Final preparations were frozen and stored at –80 °C. Protein was determined by the method of Hartree (21). Na-Ca exchange was determined as Na_i-dependent Ca uptake (22). A small volume of sarcolemmal vesicle suspension (20 μg of protein) preloaded with 140 mM NaCl by passive diffusion was diluted by 50 volumes of uptake medium maintained at 37 °C. The medium contained either KCl or NaCl (140 mM), 20 mM Tris and 50 μM $CaCl_2$ with ^{45}Ca (0.3 μCi/tube). The uptake was initiated by vortex mixing and quenched at the appropriate time by rapid filtration of vesicle suspension through a 0.45 μm Millipore filter. The filters were washed with cold iso-osmotic buffer containing 1 mM $LaCl_3$. Dried filters were counted in a liquid scintillation spectrometer. Calcium uptake by vesicles diluted into NaCl medium was used as the blank and was subtracted for all time points.

Na,K-ATPase activity measurement

The catalytic activity was determined in left ventricle whole homogenate. Homogenate protein (250 μg) was preincubated at 37 °C in the medium consisting of (in mM) NaCl 100, KCl 10, NaN_3 5, EGTA 1, imidazole 50, pH 7.4, with and without 1 mM ouabain. The reaction was initiated by addition of 5 mM MgATP and stopped 15 min by the addition of trichloroacetic acid. Inorganic phosphate liberated was determined by the method of Taussky and Shorr (23). The Na,K-ATPase activity was the activity inhibited by ouabain.

Statistical analysis

Data were analyzed on a personal computer using GB Stat (Dynamic Microsystems, Silver Spring, MD). Differences between groups were assessed using ANOVA with Student-Neuman-Keuls post-tests employed if $p < 0.05$. Data are presented as mean ± S.E.M.

Results

Functional recovery

Systolic and diastolic pressures for both groups are shown in Table 1. Consistent with previous experiments

Table 1 Hemodynamics on FED and FASTED hearts at three time points in the experimental period (*LVDP* left ventricular developed pressure, *LVEDP* left ventricular end-diastolic pressure, cm H_2O). Values are mean ± SE

	FED		FASTED	
	LVDP	LVEDP	LVDP	LVEDP
Baseline	96 ± 10	12 ± 1	102 ± 10	13 ± 2
End-ischemia	0	81 ± 4	0	69 ± 6
End-reperfusion	11 ± 6	47 ± 2	37 ± 9*	25 ± 2§

* $p < 0.05$ vs. FED, § $p < 0.001$ vs. FED

(4), fasting increased developed pressure and reduced end-diastolic pressure on reperfusion. Of note, these functional measures, obtained in the presence of thulium-DOTP-5, did not differ significantly from previous values found without the shift reagent (4). Thus, there was no apparent effect of the shift reagent on cardiac function.

Intracellular sodium $[Na]_i$

Baseline concentrations of $[Na]_i$ were similar in both groups (Fig. 1). The increase in $[Na]_i$ during ischemia in hearts from fed animals was not significantly greater than that of fasted animals (32.9 ± 2.8 vs. 26.8 ± 1.0 mM, $p = 0.08$). Of note, while sodium accumulation between groups during the first 10 min of ischemia was similar, there was a greater (although not statistically significant) increase in $[Na]_i$ in the fed animals between 10 and 20 min of ischemia than in the hearts of fasted animals (8.4 ± 2.1 vs. 4.8 ± 1.7 mM, respectively). On reperfusion, $[Na]_i$ fell more rapidly in the hearts of fasted animals, resulting in significantly lower concentrations after 10 min of reperfusion.

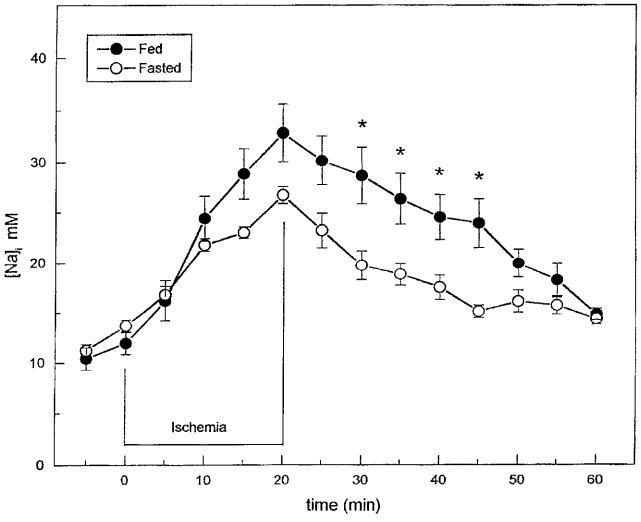

Fig. 1 Intracellular sodium ($[Na]_i$) in mM determined using ^{23}Na spectroscopy in hearts from FED and FASTED animals under baseline, ischemic, and reperfusion conditions. $[Na]_i$ was significantly higher in the hearts from fed animals only during the reperfusion time points noted (*, $p < 0.05$), $n = 6$.

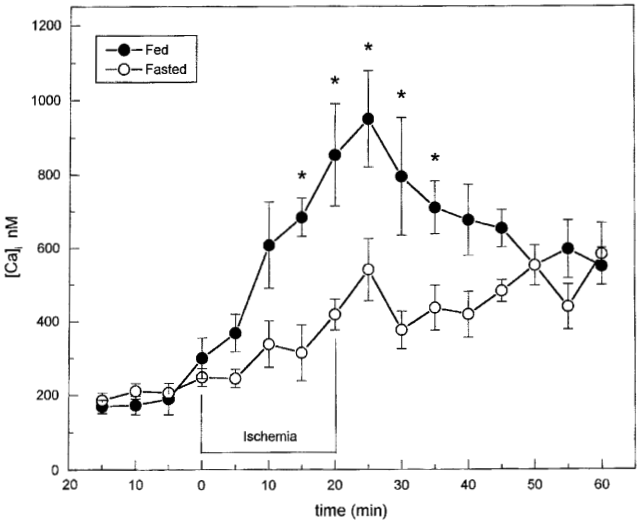

Fig. 2 Intracellular calcium ($[Ca]_i$) in nM determined using ^{19}F spectroscopy in hearts from FED and FASTED animals under baseline, ischemic, and reperfusion conditions. $[Ca]_i$ was significantly higher in the hearts from fed animals during the last two ischemic time points, as well as during the initial reperfusion period (*, $p < 0.05$). Despite the lower $[Ca]_i$ at the end of ischemia (t = 20 min) in the hearts from fasted animals, both groups demonstrated equivalent increases in $[Ca]_i$ upon reperfusion ($n = 6$).

Intracellular calcium $[Ca]_i$

As seen in Fig. 2, baseline $[Ca]_i$ concentrations were similar in the two groups (fed: 182 ± 28 nM, fasted 184 ± 25 nM). $[Ca]_i$ increased more during ischemia in the hearts from fed animals (end-ischemic concentration 858 ± 140 nM), than in the hearts from fasted animals (419 ± 46 nM, $p < 0.05$). In contrast to similar increases in $[Na]_i$ during the first 10 min of ischemia, $[Ca]_i$ increased twofold in the hearts from fed animals, while there was only a modest increase in $[Ca]_i$ in the hearts from fasted animals. In both groups, $[Ca]_i$ increased immediately upon reperfusion to its maximal value. $[Ca]_i$ then fell during the reperfusion period in the hearts from fed animals, while it remained between 400 – 500 nM during the entire reperfusion period in the hearts from fasted animals. $[Ca]_i$ was similar on both groups at the end of the reperfusion period.

Sarcoplasmic reticulum (SR) calcium uptake and release

Global ischemia uniformly decreased maximal calcium capacity of SR isolated from myocardium of fed and fasted animals (Table 2). Fasting itself did not induce any

Table 2 Sarcoplasmic reticulum calcium transport activities and sarcolemmal Na,K-ATPase activity in fed and fasted hearts

		FED	FASTED
SR Ca uptake	Baseline	0.709 ± 0.051	0.664 ± 0.042
	Ischemia	0.342 ± 0.013 *	0.359 ± 0.033[§]
SR Ca release	Baseline	0.804 ± 0.19	0.849 ± 0.17
	Ischemia	0.405 ± 0.02[+]	0.515 ± 0.05[+]
SL Na,K-ATPase	Baseline	0.0183 ± 0.003	0.0166 ± 0.001
	Ischemia	0.0168 ± 0.003	0.0164 ± 0.003

* $p < 0.001$, [§]$p < 0.0005$, [+]$p < 0.05$ ischemia vs. baseline
SR Ca uptake expressed as μmol Ca/mg protein, *SR Ca release* – as nmol Ca/mg/s, *SL Na,K-ATPase* activity – as μmol P_i/mg/min.

changes in SR Ca capacity as compared to fed control. Fasting also did not affect the rate of Ca-induced Ca release, either in control or ischemic groups (Table 2).

Na-Ca exchange

As shown in Fig. 3, no-flow ischemia resulted in a decrease in Na-Ca exchange activity during the initial rapid phase of Ca uptake as compared to the non-ischemic group for both fed and fasted hearts. The difference in steady-state Ca loading was not statistically significant. There was also no difference in exchanger activity found in sarcolemma obtained from fed vs. fasted animals within ischemic and nonischemic groups.

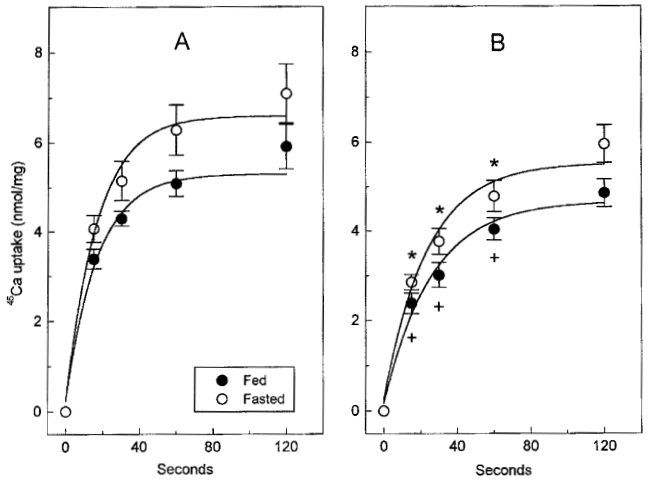

Fig. 3 Time course of Na-Ca exchange in cardiac sarcolemma after baseline perfusion (**A**) and no-flow ischemia (**B**). Sarcolemma was obtained from hearts of FED (●) and FASTED (○) animals. *$p < 0.05$, *$p < 0.02$ vs. comparing to corresponding traces in (A) ($n = 5$).

Na,K-ATPase

No difference in catalytic activity was found within any pair of experimental groups (Table 2).

Discussion

We have presented evidence in rat heart in support of the protective effect of fasting on functional recovery after global ischemia (1, 2, 4). A potential mechanism of this protective effect is a decrease in Ca^{2+} influx during ischemia and reperfusion.

Mechanisms of lower $[Ca]_i$ accumulation

An increase in intracellular calcium during ischemia and reperfusion is considered one of the important mechanisms of ischemic injury (7). An important finding of the current study is that fasting markedly limited the increase in $[Ca]_i$ during ischemia in the absence of significant reductions in $[Na]_i$. Despite this reduction, fasting did not blunt the increase in $[Ca]_i$ immediately upon reperfusion.

Cytosolic calcium may be increased during ischemia by greater net inward transport across the sarcolemma (SL) and/or SR or reduced buffering by intracellular proteins (24). There is no consensus as to the primary mechanism of calcium accumulation during ischemia (12, 25–29), with data supporting both calcium entry from extracellular (Na-Ca exchange (6, 8, 30) or influx via calcium channels (31)) as well as calcium release from intracellular stores. Thermodynamic calculations allow prediction of the change in $[Ca]_i$ for a given change in $[Na]_i$ (assuming equilibrium conditions), an process described by Steenbergen et al. (8). In that study of preconditioned hearts, the authors showed that small changes in $[Na]_i$ (from 25 to 28.5 mM) by the end of ischemia could result in large changes in $[Ca]_i$ (from 2.1 to 3.2 μM). Indeed, although the increases in $[Ca]_i$ during ischemia are less in the current study, the changes in $[Na]_i$ and $[Ca]_i$ in these experiments are qualitatively similar to those seen previously (8). These data suggest, once again, that small changes in $[Na]_i$ can result in large changes in $[Ca]_i$ (Figs. 1 and 2). These findings, however, do not provide a mechanism for a lower increase in $[Na]_i$ in the fasted animals, nor exclude differential effects of fasting on other calcium regulatory mechanisms such as less entry via calcium channels (27, 32), lower calcium release from the sarcoplasmic reticulum calcium release channels (13) or greater SR calcium uptake due to preferential use of glycogen to maintain SR ATP levels (33).

Our experiments addressed several of these mechanisms. Fasting did not result in either increased SR

uptake of calcium, less release of calcium via the calcium release channels, or greater Na-Ca exchange activity. Additionally, the equal calcium overload on reperfusion in the two groups does not support any effect of fasting on the Na-H exchanger. Because calcium overload on reperfusion is postulated to be caused by rapid sodium influx through the Na-H exchanger when the pH gradient across the SL membrane is re-established, resulting in Na-Ca exchange (19), the increase in $[Ca]_i$ in both groups suggests that both the Na-H and Na-Ca exchangers were functionally equivalent in both groups.

Mechanisms of sodium accumulation

One finding of this study was that fasting had no significant effect on sodium accumulation during the ischemic period, although there was a marginal reduction in $[Na]_i$ by the end of 20 min of ischemia. A modest, but not statistically different, reduction in $[Na]_i$ in the protected hearts is similar to the findings of Steenbergen et al. (8) in preconditioned hearts.

Potential mechanisms for sodium accumulation include sodium influx (via the Na-H exchanger or Na channels) (34, 35) or efflux (primarily via the Na,K-ATPase) (36, 37). The Na-H exchanger can profoundly influence the accumulation of sodium as the cell acidifies during ischemia and is primarily driven by the pH gradient across the cell (6). However, in the current setting, it is unlikely that any differences between hearts were due to changes in the Na-H exchanger since a) intracellular pH has been shown to be identical in hearts from both fed and fasted animals (4) and b) the exchanger is inhibited by acidosis, (38) limiting its effect during the latter phases of ischemia when differences in $[Na]_i$ were greatest in these experiments.

Decreased sodium efflux could be postulated by changes in the Na,K-ATPase, either due to changes in the trans-membrane ion gradients or intrinsic activity of the Na,K-ATPase (possible due to phosphorylation) (39). This study does not support a change in the intrinsic activity of the Na,K-ATPase. While, global ATP levels during ischemia are not altered by fasting (4), it is possible that glycolytically produced ATP preferentially supports Na,K-ATPase function (40), resulting in greater sodium efflux and lower $[Na]_i$. Indeed, studies from this laboratory (41) and others (42) support the hypothesis that increased glycogen utilization in fasted animals plays an important role in supporting ion homeostasis during ischemia.

Limitations

This study was performed in hearts isolated from rats fasted for 24 hours. It is unclear whether a lesser duration of fasting would provide equivalent results, or whether a greater duration of fasting would provide even greater benefit. The 24 hour time period was chosen because protection from ischemic injury has been demonstrated with this time period in several studies (1, 3) and because significant metabolic changes occur within 24 hours, with only modest change with fasts up to 72 hours (15).

The use of the isolated heart, while providing the ability to measure $[Na]_i$ and $[Ca]_i$ during ischemia and reperfusion, eliminated the potential effects of other substrates and neuro-humoral influences on ischemic injury and, potentially, $[Na]_i$ and $[Ca]_i$. Thus, further experiments using an in situ model may be required to verify that these changes occur under more physiologic conditions. As well, the use of glucose 11 mM as the sole carbon substrate may not reflect the in situ condition where other substrates, such as free fatty acids and ketone bodies, may affect the influence of fasting on both functional recovery and ion transport. However, previous studies using a variety of substrate conditions (2, 3, 43) or in the in situ fasted rat (44) have not demonstrated a substantial dimunition of the beneficial effect of fasting on either functional recovery or infarct size. Thus, it is likely, although unproven, that the effects of fasting on ion transport are also not altered by substrate conditions.

The measurement of $[Ca]_i$ using 5F-BAPTA introduces the effect of calcium buffering by the compound and may limit the accuracy of the calculated $[Ca]_i$. However, relative changes in $[Ca]_i$ should be valid using this technique, especially if they are of the order of magnitude observed in this study (17).

Lastly, the *in vitro* measurement of Na-Ca exchange activity and SR uptake and release do not consider the effect of changing *in vivo* conditions that could modulate calcium transport. For example, there are data indicating that calcium-induced calcium release is potentially regulated by the cytosolic redox state (45) and prior measurements have shown that fasting significantly lowers the redox state under both baseline and ischemic conditions (41). Thus, it is possible that dynamic regulation of SR and cytosolic calcium occurred that was not measured in the current experiments.

Conclusions

Fasting, which protects the heart from ischemia without affecting intracellular pH, has minimal effects on $[Na]_i$, yet profound effects on $[Ca]_i$ during ischemia. These changes in $[Ca]_i$ are not due to alterations in Na-Ca exchanger, SR Ca-ATPase, or Na,K-ATPase activity. Our

data support an important role for lower sodium accumulation and resultant Na-Ca exchange in limiting calcium overload under these conditions. However, the exact mechanism for this phenomenon remains to be elucidated.

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