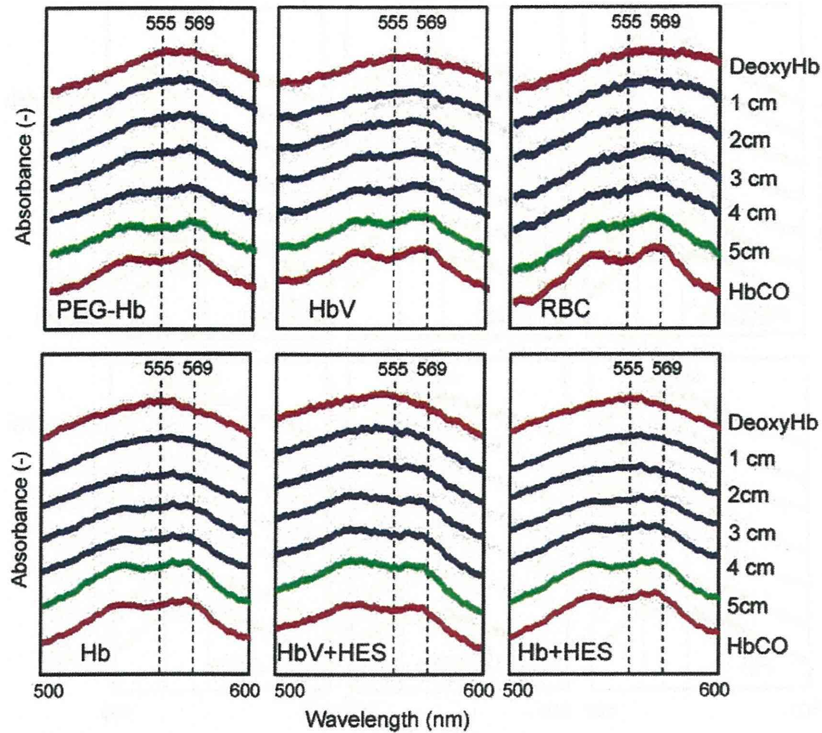

Fig. 6. Spectroscopic changes in Q bands of the Hb-containing fluids with CO-binding perfused through the narrow tube. Two new characteristic peaks attributed to HbCO (λ_{max} = 540 and 569 nm) increased with the traveling distance; deoxyHb (555 nm) decreased.

in Table 1. The NO gas molecules diffused through the tube inner wall bind rapidly to Hb. In contrast, CO gas has more time to diffuse in the inner fluid to bind to Hb. That CO-binding is retarded slightly by encapsulation and PEGylation, but we were unable to produce clear retardation to the level of RBCs merely by the addition of HES to HbV.

One limitation of our experimental setup for evaluating NO reactions is that we cannot measure the reaction of NO and HbO_2 to form metHb and nitrate, even though this reaction is predominant in *in vivo* blood circulation. In our system in Figure 2, the narrow tube is immersed in a solution bubbled with NO/N_2 mixed gas (oxygen tension is zero). Perfusion of HbO_2 in the tube causes not only metHb and nitrate formation but also deoxygenation and NO-binding to deoxygenated Hb. Accordingly we did not have the experiment of the reaction of NO and HbO_2. However, we speculate that such reaction would be more influenced by the increased viscosity, because the reaction rate constant of NO and HbO_2 ($k_{ox}^{(NO)} = 8.7 \times 10^7\ M^{-1}\ s^{-1}$) is essentially larger than that of NO-binding ($k_{on}^{(NO)} = 2.4 \times 10^7\ M^{-1}\ s^{-1}$) and CO-binding ($k_{on}^{(CO)} = 2.1 \times 10^5\ M^{-1}\ s^{-1}$) (Sakai et al., 2008b) in the order, $k_{ox}^{(NO)} > k_{on}^{(NO)} > k_{on}^{(CO)}$, and the gas reaction would be completed at the closer place to the inner wall.

One remaining question is whether the retarded NO-binding and CO-binding rates found in this study are sufficient to explain the absence or presence of vasoconstriction. We infer the presence of a threshold particle diameter not only in terms of diffusiveness in the plasma phase, as discussed herein, but also in terms of penetration across the perforated endothelial cell layer to approach a space (such as the space of Disse near the sinusoidal endothelial layer in a hepatic microcirculation, or the space between the endothelium and the smooth muscle). At that space, CO or NO is produced as a vasorelaxation factor to bind to soluble guanylate cyclase, which catalyzes the conversion of guanosine triphosphate to cyclic guanosine monophosphate (Sakai et al., 2000a; Nakai et al., 1998; Goda et al., 1998; Matheson et al., 2002). At the very least, both HbVs and PEG-Hb are unable to permeate across the endothelial layers in the vascular wall. As summarized by Olson et al. (2004) both the retardation of the NO reaction (reduced NO affinity) and the larger particle diameter are inferred to be keys to suppression of vasoconstriction and hypertension induced by HBOCs because the simple combination of Hb solution and HES would not prevent vasoconstriction even if the mixed fluid were highly viscous.

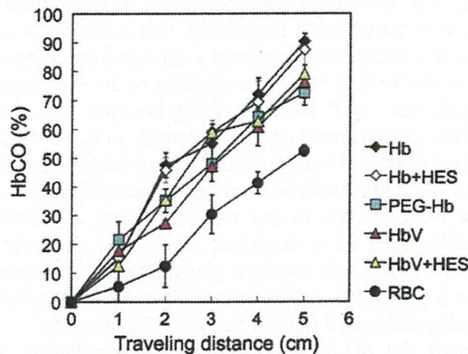

Fig. 7. Change of the level of CO-binding reactions of the Hb-containing fluids, Hb solution (black rhombi), PEG-Hb (blue squares), HbV (pink triangles), Hb + HES (white rhombi), HbV + HES (yellow triangles), and RBC (black circles) with traveling distance.

Conclusion

In summary, results of this study clarified that increased viscosity or increased size can reduce the gas reaction rates for NO-binding, probably because of the lowered lateral diffusivity in the tube. That can partly explain the mechanism of vasoconstriction. Several biological mechanisms of vasoconstriction other than gas reactions are reported, such as induction of hypersensitivity of adrenergic receptors and plasma enthothelin-1 increase (Gulati et al., 1999). Reportedly, increased viscosity of HBOCs is effective to increase the

shear stress on the vascular wall and enhance production of vasorelaxation factor. In our study, we additionally uncovered advantages of increased viscosity, and retardation of HBOCs reaction with NO as vasorelaxation factors. This finding will be important to encourage reconsideration of the design of the optimal HBOC chemical structure.

Conflict of interest

Of the authors, HS, ST, and ET are the inventors of patents related to the production and utilization of Hb-vesicles.

Acknowledgments

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ORIGINAL ARTICLE

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Severe, short-term food restriction improves cardiac function following ischemia/reperfusion in perfused rat hearts

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Abstract The purpose of this study was to clarify the characteristics of improved ischemic tolerance induced by severe, short-term food restriction in isolated, perfused rat hearts. Male Wistar (8 week-old) rats were given a food intake equivalent to a 70% reduction on the food intake of ad-libitum fed rats for 11 days (FR group and AL group, respectively). After this period, hearts were isolated and perfused in the Langendorff mode, and subjected to 20 min of global ischemia followed by 30 min of reperfusion. Although the coronary flow rate in the FR group (63.0 ± 3.1 ml/min/g dry weight) was higher than that in the AL group (47.1 ± 1.3 ml/min/g dry weight) during preischemic perfusion, the lactate release into the coronary effluent and absolute values of +dP/dt and –dP/dt in the FR group (2422 ± 161 and –1282 ± 51) were inversely lower than in the AL group (2971 ± 156 and –1538 ± 74, respectively). An increase in ischemic contracture was suppressed in the FR group. Following reperfusion, cardiac function, high-energy phosphate content, and intracellular pH, as measured by ^{31}P-nuclear magnetic resonance spectroscopy, had recovered to a much greater degree in the FR group than in the AL group. The serum T_3 level was significantly lower in the FR group (2.7 ± 0.1 pg/ml) than in the AL group (3.6 ± 0.1 pg/ml), and the levels of triglycerides, free fatty acids, insulin, and glucose were also significantly lower in the FR group than in the AL group. The protein expressions of myocyte enhancer factor 2A, Na^+,K^+-ATPase, and phospholamban in the cardiac tissue were higher in the FR group than in the AL group. These results suggested that severe, short-term food restriction improves ischemic tolerance in rat hearts via altered expression of functional proteins induced by low serum T3 levels, decreased coronary conductance, and change in metabolic flux.

Key words Caloric restriction · Glycolysis · Lactate · Phosphorus · Reperfusion injury

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Introduction

Calorie restriction (CR) has been shown to extend life span, decrease age-related physiological changes, and delay cancer progress in laboratory animals,[1,2] and has also been reported to have a protective effect with respect to many aspects of cardiac mechanical function via diverse mechanisms.[3–5] Recently, Shinmura et al. noted that short-term CR may be useful in the clinical setting, having found that the ischemic tolerance of hearts was improved after short-term, graded CR (10% food reduction for 2–3 weeks/35% food reduction for 2 weeks) in rats[6] and mice.[7] In these studies, the effects of CR on life span, age-related physiological functions, and cardiac function were investigated under relatively long-term (3 weeks to 8 months or over), mild calorie restriction (30%–40% reduction in calorie intake over ad-libitum fed animals) conditions in senescent rats. Furthermore, the effects of CR on cardiac function appeared to be independent of the period of CR and age of animals.

In contrast to CR, starvation and severe food restriction do not extend the life span since they lead to malnutrition. Although some studies have found that cardiac function was improved under starvation[8,9] or severe food restriction conditions,[9] it has been reported that the functional properties of rat hearts were depressed after 75% food restriction for 6 weeks.[10] Thus, the relationships between cardiac adaptation and severe food restriction (over 70% food restriction) seem to be inconsistent and not necessarily clarified. Therefore, it would be interesting to compare the nature of myocardial ischemic tolerance induced by severe, short-term food restriction with that produced by usual CR and to clarify the mechanism by which severe, short-term food restriction attenuates myocardial ischemia/reperfusion injury.

In this study, we investigated the effects of severe (70% reduction in food intake on that of ad-libitum fed rats), short-term (11 days) food restriction on cardiac functions, high-energy phosphate metabolite contents, and lactate release into coronary effluent during ischemia/reperfusion with Langendorff perfusion in the isolated hearts of young (10-week-old) male Wistar rats. We also estimated the systemic nutritional state and measured tissue glycolytic metabolite contents, glycolytic enzyme activities, adenosine triphosphatase (ATPase) activities, as well as expressions of functional proteins related to glycolysis, fatty acid utilization, and Ca-handling. We found that the improved ischemic tolerance of the hearts due to severe, short-term food restriction had different characteristics from that induced by usual CR.

Materials and methods

All experiments were performed in accordance with the National Defense Medical College Institutional Animal Care and Use Committee Guidelines.

Treatment of animals

Seven-week-old male Wistar rats were maintained under specific pathogen-free conditions on a constant dark/light cycle (12 h each) in our animal facility throughout the study, and given free access to CE-7 laboratory chow (Clea Japan, Tokyo, Japan) and water for a week before the experiments. Since the mean daily food consumption in this period was 33 g/rat, the daily quantity of food for the rats in the food-restricted group was set at 10 g/rat. A total of 35 rats were used in the present study: 11 for cardiac function measurement and ^{31}P-nuclear magnetic resonance (^{31}P-NMR) spectroscopy (ad-libitum fed group [AL; $n = 5$]; food-restricted group [FR; $n = 6$]), and 24 for the measurement of blood serum and cardiac tissue components (AL, $n = 11$ and FR, $n = 13$). On the first day of the two experiments, the rats were weighed and randomly assigned to either the AL group or FR group and the mean body weights of the two groups were made nearly equal. The FR rats were individually housed and received a daily quantity of food equal to 10 ± 1 g (approximately 70% reduction on food intake of AL group) from 09:00 h to 10:00 h for 11 days. The CE-7 laboratory chow contained 63.8% carbohydrate, 17.6% protein, 3.7% fat, and 341 kcal per 100 g of weight. Based on the National Academy of Science's daily minimal requirements for growing male Sprague-Dawley rats, the 10-g ration of CE-7 chow provided the FR rats with approximately 60% of their gross energy requirements, 98% and 49% of their respective protein and fat requirements, and sufficient levels of vitamins and minerals except for vitamin B_{12} and copper whose levels were 48% and 80% of requirements, respectively.[11] The AL rats received food ad libitum. All rats in the two groups had free access to water and were weighed every 24 h at 10:00 h.

Measurement of cardiac function and ^{31}P-NMR spectroscopy

Heart preparation and perfusion method

Measurements were performed for rats in both groups from 1 to 4 h after the last day's feeding for the FR group. Each rat was premedicated with heparin (1000 U, intraperitoneally [i.p.]) and anesthetized with ketamine (90 mg/kg, i.p.) and xylazine (10 mg/kg, i.p.). The hearts were excised, put into ice-cold modified Krebs–Henseleit buffer, quickly trimmed, weighed, and perfused in the Langendorff mode at a constant perfusion pressure of 100 cmH_2O at 37°C with modified Krebs–Henseleit buffer solution consisting of NaCl 116 mM, KCl 4.7 mM, $MgSO_4$ 1.2 mM, $CaCl_2$ 2.5 mM, $NaHCO_3$ 25 mM, and glucose 11 mM. The buffer solution was continuously aerated with 95% O_2 + 5% CO_2, and the pH was adjusted to 7.4. Heart function was monitored and recorded continuously throughout the experiments using a fluid-filled left ventricular balloon in line with a transducer (P-50, Gould, Valley View, OH, USA) and a WS-641G multichannel recorder (Nihon Kohden, Tokyo, Japan). The balloon volume was set to produce a left ventricular end-diastolic pressure (LVEDP) of 5 mmHg. Each heart was perfused for 15–20 min (control perfusion) and then subjected to 20 min of global ischemia by clamping the aortic cannula, followed by 30 min of reperfusion. The coronary effluent was collected at 5-min intervals throughout the experiments. After the volume of the effluent was measured, some of it was frozen and stored at −80°C until analysis of lactate content, and creatine kinase (CK) and lactic dehydrogenase (LDH) activities.

NMR spectroscopy

All ^{31}P-NMR spectra were obtained using a Bruker AM-360 WB spectrometer, operating at 161.9 MHz with a pulse width of 45°, 120 transients, and a recycle time of 1 s, using a Bruker 20-mm broadband probe maintained at 37°C. Three control ^{31}P-NMR spectra were obtained at 3-min intervals during the control perfusion before ischemia. Spectra were then obtained at 0, 5, 10, and 15 min during ischemia, and at 0, 5, 10, 15, 20, and 25 min during reperfusion. Inorganic phosphate, adenosine triphosphate (ATP), and phosphocreatine (PCr) values for the ischemia and reperfusion periods were quantified through comparison with spectra obtained for a standard solution (KH_2PO_4, 40 μM solution) in a heart-sized glass sphere before the experiments. The intracellular pH (pHi) was calculated from the distance of the chemical shifts between the inorganic phosphorus (P_i) and PCr peaks using the equation $pH = 6.9 - \log(\delta - 5.805)/(3.29 - \delta)$.[12] The free induction decay was multiplied by an exponential function corresponding to 20-Hz line broadening preceding Fourier transformation using the Nuts software (Acorn NMR, Livermore, CA, USA).

Measurement of blood serum and cardiac tissue components

Blood and heart tissue sampling

Measurements were performed for rats in both groups according to the same time schedule as described above. After anesthetizing the rats, the abdomen was opened, blood was collected from the abdominal aorta, and then heparin was injected before removing the heart. After removal, hearts were perfused for 2–5 min in the same manner as for cardiac function measurements. This was done to wash out any remaining blood in the heart tissue and bring the condition of hearts into line with that just after the beginning of the perfusion of isolated hearts for the cardiac function measurements. Then, the hearts were freeze-clamped with aluminum tongs precooled in liquid nitrogen, powdered with a mortar and pestle in liquid nitrogen, and stored at –80°C until measurement of tissue components. The supernatant serum of the collected blood was also stored at –80°C until chemical analysis. Some of the serum was deproteinized by treating with perchloric acid and the resulting supernatant was stored at –80°C for the measurement of lactate and pyruvate concentrations.

Subcellular fractionation

Powdered cardiac tissues were homogenized in 10 parts by weight of ice-cold buffer containing 25 mM Hepes, 250 mM sucrose, 4 mM ethylenediamine tetra-acetic acid (EDTA, 25 mM benzamidine, and 0.2 mM phenylmethylsulfonyl fluoride at pH 7.4 supplemented with proteinase inhibitor cocktail (Complete Mini; Roche, Basel, Switzerland).[13] Homogenates were centrifuged at 600×*g* for 10 min at 4°C (supernatant 1 and crude nuclear fraction). The crude nuclear fraction was resuspended in homogenization buffer, layered on an ice-cold buffer containing 0.9 M sucrose, 50 mM Tris–HCl, 1 mM EDTA, and 25 mM KCl at pH 7.5,[14] and then centrifuged at 1800×*g* for 20 min at 4°C. The pelleted nuclei were resuspended in 40% glycerol, 50 mM Tris–HCl, 5 mM $MgCl_2$, and 0.1 mM EDTA at pH 8,[14] and stored at –80°C. Supernatant 1 was then centrifuged at 10,000×*g* for 10 min at 4°C (supernatant 2 and crude mitochondrial fraction). The crude mitochondrial fraction was resuspended in a buffer containing 210 mM mannitol, 70 mM sucrose, 1 mM EDTA, and 10 mM Hepes at pH 7.5, and 1% Triton X-100,[15] and stored at –80°C. Supernatant 2 was centrifuged at 150,000×*g* for 2 h at 4°C. The resulting supernatant was stored at –80°C as a cytosol fraction. The final membrane pellet was resuspended in homogenization buffer by passing repeatedly through a 29-gauge needle, and stored at –80°C as a microsome fraction. The protein concentration in all fractions was determined by the BCA protein assay method.

Western blot analysis of proteins in each subcellular fraction

Five to thirty micrograms of proteins extracted from each subcellular fraction were separated using sodium dodecyl sulfate–polyacrylamide gel electrophoresis and blotted on a polyvinylidene fluoride membrane (Amersham, Bucks, UK). Blots were blocked for 1 h at room temperature with 5% nonfat dry milk in phosphate-buffered saline supplemented with 0.1% Tween-20 (PBS-T). Primary antibodies against myocyte enhancer factor 2A (MEF2A; 1:1000, Santa Cruz Biochemical, Santa Cruz, CA, USA), peroxisome proliferator activated receptor α (PPARα; 1:1000, Santa Cruz), glucose transporter 1 (GLUT1; 1:300, Santa Cruz), glucose transporter 4 (GLUT4; 1:1000, Santa Cruz), sarcoplasmic reticulum Ca-ATPase (SERCA2; 1:1000, Santa Cruz), phospholamban (PLB; 1:1000, Upstate Biochemical, Lake Placid, NY, USA), Na^+,K^+-ATPase (1:1000, RBI, Natick, MA, USA), Na–H exchanger (NHE; 1:1000, Chemicon International, Temecula, CA, USA), and mitochondrial voltage-dependent anion channel 1 (VDAC1; 1:1000, Santa Cruz) were used. Glyceraldehyde-3-phosphate dehydrogenase (1:1000, Santa Cruz) and actin (1:1000, Santa Cruz) were used as internal controls for the cytosol fraction and other fractions (microsome and mitochondrial fractions), respectively. Each blot was incubated with the corresponding primary antibodies overnight in 1% nonfat dry milk in PBS-T and washed twice in PBS-T. Then the blots were incubated for 1 h with secondary antibodies (Santa Cruz) at the manufacturer's recommended dilution in 1% nonfat dry milk in PBS-T. Immunodetection was performed by means of ECL plus (Amersham Bioscience), and signals were quantified using a LAS 3000 image analyzer (Fuji Film, Tokyo, Japan). Each protein signal intensity in the FR group was normalized with the mean value of that in the AL group.

Enzyme activity assay for each subcellular fraction

ATPase activities were measured according to the methods of Nørby (Na^+,K^+-ATPase),[16] Chu et al. (Ca^{2+}-ATPase),[17] and McEnery and Pedersen (F1-ATPase).[18] Glycolytic enzyme activities were measured according to the methods of Easterby and Quadri (hexokinase, HK),[19] Storey (phosphofructokinase, PFK),[20] Cardenas (pyruvate kinase, PK)[21] and Lee and Goldberg (lactate dehydrogenase, LDH).[22] All enzyme activities were expressed as amounts of decrease or increase in NADH or NADPH/min per μg protein extracted.

Chemical analyses

Cardiac tissue glucose and glycogen contents were fluorometrically determined by the method of Keppler and Decker.[23] Serum cholesterol, triglyceride, high-density lipoprotein cholesterol (HDL-C), free fatty acid (FFA), and glucose levels were determined using an enzyme assay kit (Kyowa Medical, Tokyo, Japan). Serum thyroid-stimulating hormone (TSH), free tri-iodothyronine (fT3), and free thyroxine (fT4) levels were determined using a chemiluminescent immunoassay kit (Bayer Healthcare, Leverkusen, Germany). Serum insulin was measured using a rat insulin radioimmunoassay kit (Linco Research, St. Charles, MO, USA). The other serum components were measured using

a Hitachi 7600 autoanalyzer. Lactate and pyruvate in the deproteinized serum and lactate in the coronary effluent were analyzed enzymatically by the method of Lowry and Passonneau.[24]

Statistical analysis

All values are expressed as mean ± SE. Cardiac function and NMR data were analyzed by means of time-series analysis of variance, and then the differences between the mean values in the AL group and FR group at each measurement time were analyzed using the unpaired Student *t*-test. For the other data, differences between the mean values in the two groups were also analyzed using the unpaired Student *t*-test. Software used for statistical analysis was SAS version 8.1 and Stat View for Windows version J-5.0 (SAS, Cary, NC, USA). $P < 0.05$ was considered as significant.

Results

Cardiac function in Langendorff perfused hearts

During the preischemic perfusion, the coronary flow rate (CFR) in the FR group was significantly higher than in the AL group. Absolute values of maximal positive and negative peaks of the first derivative of left ventricular pressure (+dP/dT and −dP/dT) in the FR group (2422 ± 161 and −1282 ± 51) were significantly lower than in the AL group (2971 ± 156 and −1538 ± 74, respectively), but no significant difference was observed in the other heart function parameters between the AL and FR groups (Table 1).

The LVEDP in the FR group was significantly lower than in the AL group at the end of ischemia. During the 30 min of reperfusion, although CFR, +dP/dT, and −dP/dT did not recover in the AL group, the values of these parameters in the FR group recovered to near the preischemic values. After reperfusion, recoveries in the other cardiac function parameters (left ventricular developed pressure [LVDP] and LVEDP) were more remarkable in the FR group than in the AL group.

Energy metabolite contents and intracellular pH

The ATP content in both groups decreased in a similar manner during ischemia but its recovery in the FR group was significantly greater than in the AL group during reperfusion (Fig. 1A). Interestingly, the PCr content in the FR group was significantly higher than in the AL group at the second measurement point during the control perfusion (Fig. 1B). After reperfusion, the PCr content in the FR group had recovered to near the control level, though the recovery in the AL group was limited (only 42% ± 9%, Fig. 1B). The other parameters (P_i content and pHi) recovered to a greater extent in the FR group than in the AL group during reperfusion (Fig. 1C and D).

Lactate, CK, and LDH release into coronary effluent

Lactate release into the coronary effluent over the 5 min of the control perfusion was significantly lower in the FR group than in the AL group (Fig. 2). However, it was inversely higher in the FR group than in the AL group during the first 5 min of reperfusion. Thereafter, it decreased

Table 1. Hemodynamic parameters from pre-ischemia to end of reperfusion in Langendorff perfused hearts

	Preischemia	End of ischemia	Reperfusion, min		
			10	20	30
CFR, ml/min/g dry weight					
AL group	47.1 ± 1.3	0	25.4 ± 1.4	30.3 ± 1.5	31.3 ± 1.4
FR group	63.0 ± 3.1*	0	62.5 ± 4.4*	61.7 ± 2.5*	57.7 ± 3.0*
LVDP, mmHg					
AL group	111.7 ± 3.9	0	21.8 ± 5.6	31.4 ± 8.0	43.0 ± 7.8
FR group	108.4 ± 8.1	0	69.7 ± 17.4*	95.1 ± 19.3*	99.6 ± 18.1*
LVEDP, mmHg					
AL group	4.0 ± 0.4	46.1 ± 2.6	70.9 ± 4.1	63.7 ± 4.2	57.4 ± 4.5
FR group	4.2 ± 0.7	27.7 ± 4.9*	16.6 ± 3.7*	14.0 ± 3.4*	12.2 ± 3.8*
HR, beats/min					
AL group	191 ± 9	0	120 ± 17	171 ± 13	172 ± 8
FR group	174 ± 13	0	226 ± 44	209 ± 50	206 ± 47
+dP/dT					
AL group	2971 ± 156	0	402 ± 106	730 ± 198	1013 ± 214
FR group	2422 ± 161*	0	1567 ± 357*	2081 ± 369*	2250 ± 386*
−dP/dT					
AL group	−1538 ± 74	0	−247 ± 59	−421 ± 105	−562 ± 102
FR group	−1282 ± 51*	0	−884 ± 191*	−1190 ± 183*	−1257 ± 189*

Values are mean ± SE
AL group, ad-libitum fed group; FR group, food restriction group; CFR, coronary flow rate; LVDP, left ventricular developed pressure; LVEDP, left ventricular end-diastolic pressure; HR, heart rate; +dP/dT and −dP/dT, maximal positive and negative peak of first derivative of left ventricular pressure
* $P < 0.05$ vs. data in corresponding AL group

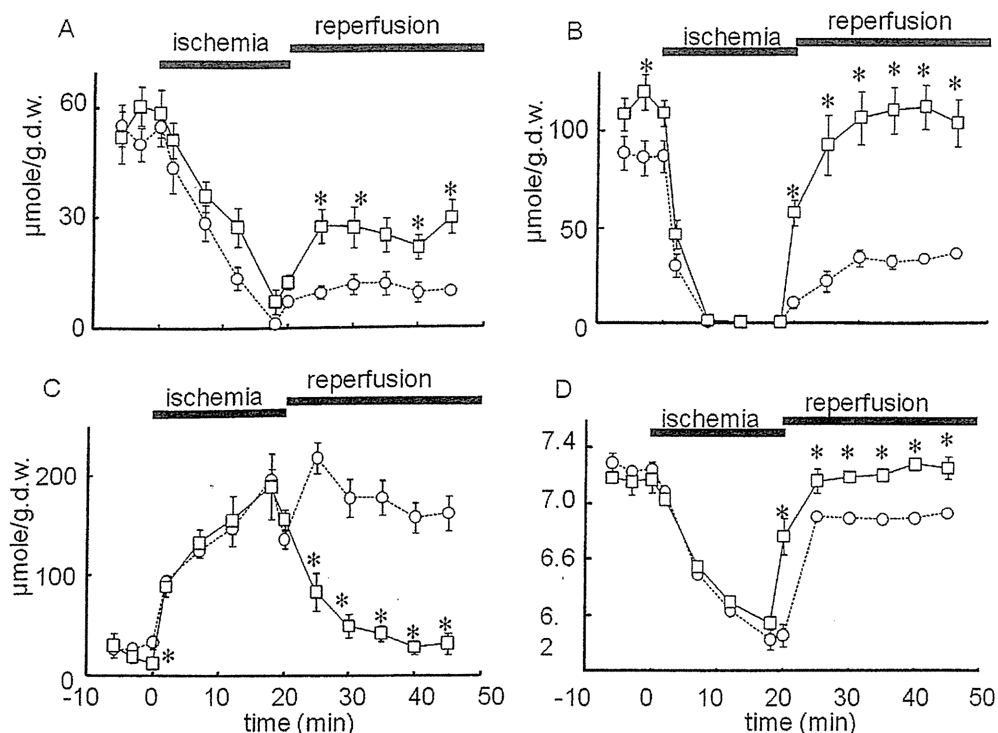

Fig. 1. Changes in adenosine triphosphate (*ATP*) (**A**), phosphocreatine (*PCr*) (**B**), and inorganic phosphorus (P_i) (**C**) contents, and intracellular pH (*pHi*) (**D**) in the hearts of the ad-libitum fed (AL; *circles*) and food restriction (FR; *squares*) groups during ischemia and reperfusion. Data are shown as mean ± SE. *$P < 0.05$ vs the corresponding value in the AL group

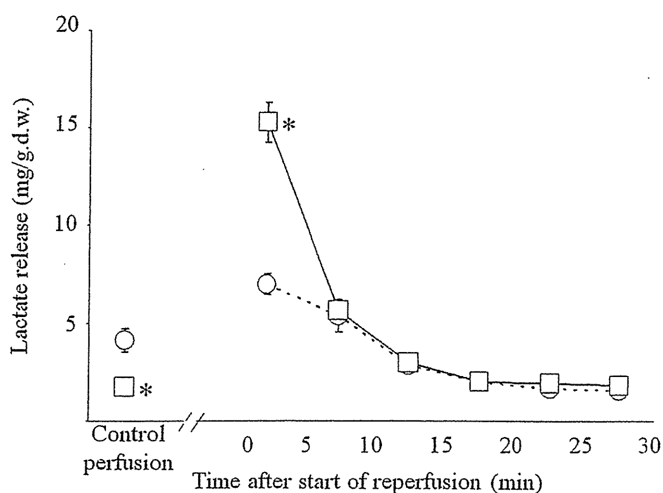

Fig. 2. Release of lactate into the coronary effluent of the hearts in the AL (*circles*) and FR (*squares*) groups during control perfusion and after reperfusion. *$P < 0.05$ vs the value in the AL group

in a similar fashion in the two groups and, after 20 min of reperfusion, had reached a level close to that of the control for the FR group. There was no difference in CK and LDH release during reperfusion between the FR and AL groups (data not shown).

Body weight, heart weight, and food consumption

During the experimental period, the body weight in the AL group increased by 29% (Table 2) while that in the FR group decreased by 21% during the 11 days beginning on the day the amount of feed was restricted to 10 ± 1 g/day.

Table 2. Body weight, heart weight, and average daily food consumption during experimental period

	AL group $n = 16$	FR group $n = 19$
Body weight (g) pre[a]	296 ± 2.2	298 ± 1.7
Body weight (g) post[a]	381 ± 4.3	235 ± 2.3*
Mean food consumption (g/day)	33 ± 1	10 ± 1*
Heart weight (g) wet	1.10 ± 0.03	0.75 ± 0.01*
Heart/body weight ratio (g/kg × 10^3)[b]	2.89 ± 0.05	3.18 ± 0.06

Values are mean ± SE
[a]Pre and post indicate data at the start and end of experiment, respectively
[b]Wet heart weight/body weight post
*$P < 0.05$ vs. data in AL group

The heart weight in the FR group was 68% of that in the AL group ($P < 0.05$). Although the mean heart weight/body weight ratio in the FR group was slightly higher than in the AL group, the difference was not large enough to imply the presence of myocardial hypertrophy or edema.

Serum component levels and heart tissue glucose and glycogen contents

Serum TSH, fT3, fT4, triglyceride, FFA, glucose, and insulin levels were significantly lower in the FR group than in the AL group (Table 3). Serum fT3 and fT4 levels in the FR group decreased by 25% and 19%, respectively, compared with those in the AL group. On the other hand, serum lactate, pyruvate, creatine, Na, and Cl were significantly higher in the FR group than in the AL group. There was no significant difference in serum LDH, CK, total protein, albumin, total cholesterol, and HDL-C concentrations

between the AL group and FR group at the end of the experiments. Serum 3-hydroxybutyrate was significantly higher in the FR than in the AL group, while the acetoacetic acid level was similar in both groups. The tissue glucose content was significantly higher in the FR group than in the AL group.

Table 3. Serum component levels, and cardiac tissue glucose and glycogen contents in AL and FR groups

	AL group $n = 11$	FR group $n = 12$
Serum		
TSH, μIU/ml	2.1 ± 0.3	0.3 ± 0.1*
fT3, pg/ml	3.6 ± 0.1	2.7 ± 0.1*
fT4, ng/dl	2.6 ± 0.1	2.1 ± 0.1*
LDH, IU/l	701 ± 132	668 ± 97
CK, IU/l	540 ± 87	477 ± 46
Total protein, g/dl	5.4 ± 0.1	5.3 ± 0.1
Albumin, g/dl	3.6 ± 0.1	3.6 ± 0.1
Creatinine, mg/dl	0.25 ± 0.01	0.31 ± 0.01*
Na, mEq/l	141 ± 0.5	145 ± 0.5*
K, mEq/l	4.1 ± 0.2	4.3 ± 0.2
Cl, mEq/l	102 ± 0.5	106 ± 0.5*
Lactate, mM	3.12 ± 0.32	5.22 ± 0.86*
Pyruvate, mM	0.22 ± 0.03	0.44 ± 0.07*
Total cholesterol, mg/dl	60 ± 2	60 ± 6
HDL-C, mg/dl	38 ± 1	36 ± 3
Triglyceride, mg/dl	104 ± 8	32 ± 10*
Free fatty acid, mEq/l	0.35 ± 0.03	0.23 ± 0.03*
Acetoacetic acid, μmol/l	88.5 ± 6.9	78.9 ± 8.5
3-Hydroxybutyrate, μmol/l	137.5 ± 8.3	272.5 ± 43.9*
Glucose, mg/dl	292 ± 21	182 ± 22*
Insulin, ng/ml	0.65 ± 0.05	0.21 ± 0.02*
Tissue glucose, μg/mg-tissue wet wt	0.89 ± 0.05	1.12 ± 0.06*
Tissue glycogen, μg/mg-tissue wet wt	3.24 ± 0.31	2.68 ± 0.53

We failed to obtain blood samples for one rat of the FR group, so the number of rats for the analysis of serum components was 12. Values are mean ± SE
TSH, thyroid-stimulating hormone; fT3, free tri-iodothyronine; fT4, free thyroxine; LDH, lactate dehydrogenase; CK, creatine kinase; HDL-C, high-density lipoprotein cholesterol
$*P < 0.05$ vs data in AL group

Glycolytic enzyme and ATPase activities in heart tissues

The amount of protein extracted from each subcellular fraction/g wet weight of heart was nearly the same in the AL and FR groups (data not shown). This result implies that neither the protein content of heart tissue nor that of the subcellular fractions was affected by the 70% food restriction for 11 days. Therefore, despite the difference in mean heart weight between the groups, enzyme activities were expressed as amounts of decrease or increase in NADH or NADPH/min per μg protein extracted. The mean HK activity for the microsome fraction was significantly lower in the FR group (Table 4). There was no significant difference in the activities of other glycolytic enzymes or ATPase activities between the groups for the three subcellular fractions tested.

Western blot analysis of functional proteins

Among the functional proteins examined by Western blot analysis, the expressions of MEF2A in the cytosol fraction, and Na^+,K^+-ATPase and PLB in the microsome fraction were significantly higher in the FR group than in the AL group, but that of VDAC1 in the mitochondrial fraction was significantly lower in the FR group than in the AL group (Fig. 3). There was no difference between the groups in expressions of the other proteins (GLUT1 and GLUT4, SERCA2, and NHE in the microsome fraction, MEF2A in the nuclear fraction, and PPARα in the cytosol fraction).

Discussion

We found that there was a significant recovery in contractile function after reperfusion following global ischemia in the hearts of rats exposed to severe, short-term food restriction. First, among cardiac function parameters we noticed that in the preischemic (control) perfusion, the absolute values of

Table 4. Glycolytic enzyme and ATPase activities in heart tissue subcellular fractions of AL and FR groups

	AL group $n = 11$	FR group $n = 13$
Cytosol fraction		
PK, μg-NADH/min/μg-protein	1.37 ± 0.07	1.38 ± 0.07
PFK, ng-NADH/min/μg-protein	79.8 ± 8.5	78.4 ± 7.1
LDH, μg-NADH/min/μg-protein	5.37 ± 0.11	5.44 ± 0.14
HK, ng-NADPH/min/μg-protein	14.0 ± 0.5	12.3 ± 0.6
Mitochondrial fraction		
HK, ng-NADPH/min/μg-protein	11.2 ± 1.3	8.2 ± 1.2
F_1-ATPase, ng-NADH/min/μg-protein	43.0 ± 3.0	41.6 ± 3.2
Microsomal fraction		
HK, ng-NADPH/min/μg-protein	31.9 ± 2.5	24.6 ± 1.9*
Na^+,K^+-ATPase, ng-NADH/min/μg-protein	127.0 ± 18.3	149.5 ± 24.5
Ca^{2+}-ATPase, ng-NADH/min/μg-protein	109.5 ± 21.8	161.3 ± 22.1

Values are mean ± SE
PK, pyruvate kinase; PFK, phosphofructokinase; LDH, lactate dehydrogenase; HK, hexokinase
$*P < 0.05$ vs. data in AL group

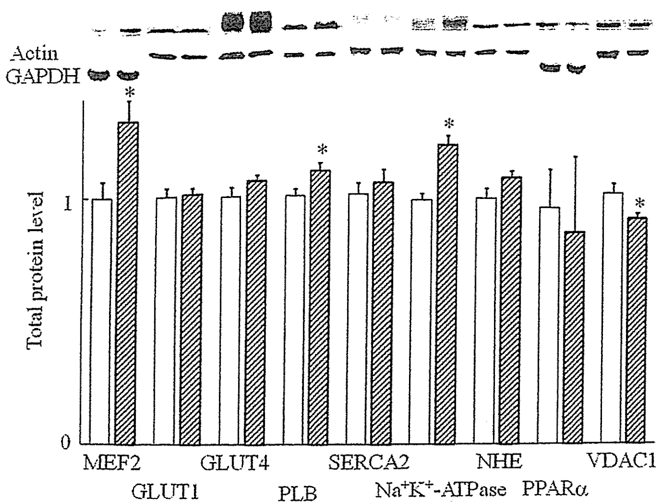

Fig. 3. Western blot analysis of MEF2A and PPARα in the cytosol fraction, GLUT1 and GLUT2, PLB, SERCA2, Na^+,K^+-ATPase, and NHE in the microsome fraction, and VDAC1 in the mitochondrial fraction. *Open bars*, AL group; *hatched bars*, FR group. *$P < 0.05$ vs the value in the AL group. *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; *MEF2*, myocyte enhancer factor 2A; *GLUT1*, glucose transporter 1; *GLUT4*, glucose transporter 4; *PLB*, phospholamban; *SERCA2*, sarcoplasmic reticulum Ca-ATPase; *NHE*, Na–H exchanger; *PPARα*, peroxisome proliferator activated receptor α; *VDAC1*, mitochondrial voltage-dependent anion channel 1

preischemic +dP/dT and −dP/dt in the FR group were 19% and 17% lower, respectively, than the respective values in the AL group. Blood thyroid hormone levels are generally considered to decrease with food restriction[25] and in line with this, we found that serum fT3 and fT4 levels in the FR group had decreased by 25% and 19% on the AL group levels, respectively. Katzeff et al.[26] reported that the serum T_3 level decreased by 82% and preischemic +dP/dT and −dP/dt by 13% and 18%, respectively, in young female SD rats after 28 days of feeding on a 50% calorie-restricted diet. They also found that the expressions of SERCA2 mRNA and α-myosin heavy chain (MHC) mRNA were suppressed in these animals, and concluded that decreases in the serum T_3 level accompanying chronic calorie-restriction were associated with impaired cardiac contractility due, in part, to alterations in cardiac SERCA2 and MHC gene expression. In our experiments, although there was no suppression of SERCA2 protein expression (Fig. 3), there was a significant increase in PLB expression in the FR group. Thus, our experimental results do not seem to be consistent with those of Katzett et al.[26] The inconsistency may be attributable to differences in the experimental conditions (animals, severity and period of food restriction) used. In our study, we assume that both the decrease in the absolute values of ±dP/dt during the control perfusion and adequate recovery of cardiac function after reperfusion in the FR group were induced by the sustained and increased expressions of SERCA2 and PLB, respectively. Since we could not find any studies showing differences in baseline +dP/dt and −dP/dt between ad-libitum fed and food-restricted animals under mild CR conditions,[3,6] these characteristics (decreased serum T_3 levels and absolute values of ±dP/dt) are assumed to be specific to severe food restriction.

Second, we observed that CFR in the FR group was higher than in the AL group during preischemic perfusion. As a similar finding was reported by Broderick et al.[4] and Klevanov et al.[5] under normal mild CR conditions (they did not measure blood T_3 levels), the increase in CFR is thought to be common to both mild and severe food restriction conditions. The relationship between an increase in CFR and the degree of decrease in serum T_3 levels observed under severe food restriction conditions is still unclear. Although the mechanism underlying this phenomenon should be properly clarified, improved coronary circulation during the control perfusion and reperfusion would enhance the ability to remove toxic metabolic intermediates and modulate cardiac ischemic tolerance.

Third, we noted that the PCr content of the heart tissues was higher and there was less lactate release into the coronary effluent in the FR group than in the AL group during the preischemic period. Since lactate release is a marker of oxidative flux from pyruvate to lactate via lactate dehydrogenase, if we postulate that tissue PCr content is a marker of mitochondrial oxidative activity in cardiac tissue, these results suggest that compared with the AL group, mitochondrial oxidation in the FR group was more highly stimulated in the control perfusion, as suggested by Broderick et al.[27] based on their results obtained under mild food restriction. Although it is not clear whether the higher PCr content in this situation is due to low serum T_3 levels,[28] our results could be related to the systemic metabolic changes described below.

We observed that the protein expressions of Na^+,K^+-ATPase in the microsome fraction and MEF2A in the cytosol fraction were greater in the FR group than in the AL group. Since expression of Na^+,K^+-ATPase in the heart is thought to be positively controlled by thyroid hormone similar to the case of SERCA2,[28] this experimental result may present a contradiction. However, the elevated level of this enzyme in the FR group might more effectively maintain ion transport through sarcolemma in cardiac cells. As MEF2A is thought to regulate the activation of glucose transport via the MEF2A–GLUT4 axis,[29] the greater expression of MEF2A could activate glucose transport, resulting in lower serum glucose levels and higher tissue glucose levels in the FR group as compared with the AL group, though these results are not necessarily consistent with lower insulin levels in the FR group.

We also found that an increase in LVEDP during ischemia was significantly suppressed in the FR group as compared to the AL group, and lactate release into the perfusate during the first 5 min of reperfusion (washout lactate produced during ischemic period) was greater in the FR group than the AL group. These results indicate that oxidation of pyruvate to lactate (anaerobic glycolysis) in the heart tissues was more highly activated during ischemia for the FR group. As shown by Kingsley et al.[30] and Vanoverschelde et al.,[31] activation of glycolytic flux in ischemia is fundamental to effective recovery of cardiac function during reperfusion after ischemia. Thus, the metabolic changes mentioned

above and the greater expression of functional proteins in cardiac tissue that we observed in our study play important roles in the recovery of cardiac functions during reperfusion in the hearts of rats subjected to severe, short-term food-restriction.

Finally, we would like to draw attention to the relationship between systemic metabolic state and cardiac function. The fact that serum triglyceride and FFA levels were lower in the FR group than in the AL group suggests that there was possibly a slight increase in the proportion of glucose with respect to fatty acid in the fuel used by cardiac cells in the FR group.[4] Also, the higher serum lactate and pyruvate levels in the FR group as compared with the AL group suggest that metabolic acidosis was induced systemically. As acidic perfusion is thought to reduce ischemia/reperfusion injury in heart tissue,[32] these metabolic changes might alter metabolism in the cardiac tissue and aid recovery of cardiac function after reperfusion. Furthermore, phosphorylation of adenosine monophosphate-activated protein kinase[6] and increases in serum adiponectin levels[7] have been observed to improve ischemic tolerance under mild food restriction, and the hypoxia inducible factor system[33] and osteopontin[34] are known to play a protective role in ischemia/reperfusion injury. These enzyme and/or cytokine systems may be related to the improved recovery of cardiac function under severe food restriction. In conclusion, our results suggested that cardiac function in isolated, perfused rat hearts was protected against ischemia/reperfusion injury by severe, short-term FR loading via altered expression of functional proteins induced by low serum T_3 levels, decreased coronary conductance, and change in metabolic flux.

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Original

Transient increase in contraction observed during early global ischemia in Langendorff perfused rat heart is glycolysis dependent

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Abstract: A transient increase in contraction (TIC) occurred 20 to 40 sec after the start of global ischemia in the Langendorff perfused rat heart. The TIC did not appear when the heart was perfused with Krebs-Henseleit buffer containing 11 mM 2-deoxyglucose or 5 mM 2-deoxyglucose + 6 mM pyruvate for 3 or 10 min before ischemia initiation. The TIC did not appear when the heart was perfused with buffer containing 150 μM iodoacetate, but was partly evident when it was perfused with 2 mM NaCN containing buffer. When the heart was perfused with buffer containing 30 μM ouabain, there was an obvious inotropy prior to the TIC in ischemia. 10 μM of KB-R7943 had no effect on the occurrence of TIC. The NAD/NADH ratio, an aerobic marker, rapidly decreased, registering about one-fourth of the baseline level after 40 sec of ischemia. There was hardly any change in lactate content, an anaerobic marker, up to 40 sec of ischemia but it suddenly increased after 90 sec of ischemia. ^{31}P-NMR spectroscopy analysis revealed a rapid decrease in creatine phosphate and a concomitant increase in Pi up to 40 sec of ischemia, but showed that the total ATP content hardly changed up to 90 sec of ischemia. During the first 40 sec of ischemia, there was a slight drop in intracellular pH. These results suggest that the TIC observed in early global ischemia of the Langendorff perfused rat heart, occurs during a short period preceding metabolic switching from oxidative phosphorylation to anaerobic glycolysis, and that it is closely connected with glycolysis.

Key words: NAD/NADH ratio ／ Lactate ／ Na^+, K^+-ATPase ／ Energy metabolism ／ Metabolic switching

Introduction

When global ischemia is initiated in the Langendorff-perfused heart, heart function rapidly decreases and finally it ceases to beat in response to its oxygen and metabolic substrate being completely cut off. However, on careful observation, a transient increase in contraction (TIC) can be noticed before the heart stops beating completely.

In the normal beating heart, energy (ATP) is supplied by the cooperative system of oxidative phosphorylation and aerobic glycolysis. Cardiac energy metabolism switches from this aerobic cooperative system to anaerobic glycolysis

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when supplies of oxygen (O_2) and/or substrates to heart tissue are cut off by anoxia and/or ischemia[1, 17]. Using surface fluorophotography, it has been detected qualitatively that an increase in intracellular NADH content, a typical ischemic marker, occurs one second after the onset of ischemia[23]. Further, through chemical analysis, intracellular lactate, a marker of anaerobic glycolysis, has been shown to increase more slowly (within the first minute of ischemia or hypoxia)[12, 24]. However, up till now, there have been no studies on the relationship between the increase in lactate content and NADH content or NAD/NADH ratio changes during the early stage of ischemia in Langendorff-perfused rat hearts. Also, it remains to be clarified whether the myocardial dysfunction seen in early ischemia is related to energy metabolite loss, especially that of creatine phosphate[2, 4, 11].

The aim of this study was therefore to clarify the nature of the TIC that occurs in early global ischemia in the Langendorff-perfused rat heart. We first preperfused hearts with metabolic, pump, or exchanger inhibitors before ischemia induction to see if there was any effect on the TIC. Then, when global ischemia had been induced, changes in intracellular pH, and NAD, NADH, lactate, and energy metabolite contents with time were measured chemically or by ^{31}P-NMR spectroscopy. Our results showed that the TIC was closely related to glycolysis and occurred during a short period preceding metabolic switching from the aerobic cooperative system to anaerobic glycolysis in global ischemia.

Methods

Animals and perfusion method

All procedures complied with the *National Defense Medical College Institutional Animal Care and Use Committee Guidelines.*

Male Wistar rats (Charles River Japan Inc.) of 8 to 10 weeks old were used. The rats were given an intraperitoneal injection of heparin (800 U/kg body wt) and anesthetized with xylazine hydrochloride (10 mg/kg) and ketamine hydrochloride (90 mg/kg), also given by intraperitoneal injection. Hearts were excised, quickly arrested in ice-cold Krebs-Henseleit buffer, and then their wet weights were measured. The ascending aortas were cannulated, and the hearts were perfused for 15-20 min (control perfusion) by the Langendorff procedure using Krebs-Henseleit buffer (NaCl 118 mM, KCl 4.7 mM, $CaCl_2$ 2.55 mM, $MgSO_4$ 1.18 mM, KH_2PO_4 1.18 mM, $NaHCO_3$ 24.9 mM, and glucose 11.1 mM; pH 7.3) at a hydrostatic pressure of 100 cmH_2O. The perfusate was equilibrated with a gas mixture of 95% O_2 and 5% CO_2 and maintained at 37℃ throughout the experiment. Pulmonary arteries were carefully incised to prevent build-up of venous pressure. Global ischemia was induced by stopping the perfusion.

Measurement of left ventricular function

Twenty-two isolated hearts were perfused as described above. An air-free latex balloon was inserted into the left ventricle via the left atrial appendage, and the left ventricular pressure and heart rate were recorded continuously with a pressure-transducer (P-50, Gould Inc.) equipped with an amplifier (PMP-6004, Nihon Kohden) connected to a recorder (WS-641G, Nihon Kohden) for the duration of the experiment. In the control perfusion, the left ventricular end-diastolic pressure (EDP) was set at approximately 5 mmHg by infusing bubble-free saline into the balloon. Hearts were excluded from the experiment if either the systolic pressure was below 70 mmHg or the heart rate was under 150 beats/min. Then, the hearts were divided into nine groups of two to three hearts each whose perfusion details were as follows: 1) control (n=3), 2) 2-deoxyglucose (2-DG) 11 mM (n=3), 3) 2-DG 5 mM + Na pyruvate 6 mM (n=3), 4) iodoacetate (IAA) 150

μM (n=2), 5) NaCN 2 mM (n=3), 6) ouabain 30 μM (n=2), 7) ouabain 40 μM (n=2), 8) ouabain 50 μM (n=2) and 9) KB-R7943 10 μM (n=2). KB-R7943 is a selective inhibitor of the reverse mode reaction of Na^+-Ca^{2+} exchanger (NCX)[8]. In the control group, global ischemia was induced immediately after the control perfusion. In the other groups, hearts were perfused with Krebs-Henseleit buffer containing the respective reagents for 3 to 10 min following the control perfusion, and then global ischemia was induced. In the 2-DG 5 mM + Na pyruvate 6 mM group, to adjust the sodium concentration of the perfusion medium, the NaCl concentration was decreased to 110 mM.

Measurement of intracellular pH, and ATP, creatine phosphate and Pi contents by ^{31}P-NMR spectroscopy

^{31}P-NMR spectra were obtained at 109.25 MHz with a JEOL GX 270 spectrometer (JEOL, Tokyo, Japan). The diameter of the probe was 15 mm, and spectra were collected from transients following 45° pulses delivered at 1 sec intervals for 1 min according to the method previously reported[14]. Perfused hearts were put into a NMR tube which was placed inside the probe. Coronary effluent was evacuated from the NMR tube through an overflow outlet placed above the heart. The heart temperature was maintained at 37°C using a variable temperature unit attached to the spectrometer and a water-jacketed perfusion line. One-min spectra were collected during control perfusion, as well as from 10 to 70 sec, 120 to 180 sec, and 270 to 330 sec of ischemia for each of six hearts (Experiment 1). In another 6 hearts, 1-min spectra were collected for the control, as well as from 60 to 120 sec, and 270 to 330 sec of ischemia (Experiment 2). The mean values of the spectra obtained during ischemia were considered to be at the center of each collection period (40, 90, 150, and 300 sec), since creatine phosphate (PCr) and Pi tissue amounts are assumed to change linearly during each collection period of ischemia. At the end of the experiments, all hearts were dried at 80°C for 48 hrs and then their dry weights were measured.

To determine the intracellular pH, the equation below was used[6]:

$$pH = 6.90 - \log[(\delta - 5.805)/(3.290 - \delta)]$$

where δ is the chemical shift (in parts per million) of the resonance of intracellular Pi relative to PCr.

Absolute intracellular concentrations of ATP, PCr, and Pi were determined from the ^{31}P-NMR spectra according to the method of Pike et al.[16] using KH_2PO_4 solution as an external standard.

Chemical analysis of lactate and pyridine dinucleotides

(1) Samples

Thirty-six isolated hearts were perfused as described above for the control perfusion and global ischemia was induced afterwards. Six hearts each were freeze-clamped with aluminum tongs pre-cooled in liquid nitrogen at the following times: during control perfusion; after 20, 40, 90, 150 and 300 sec of ischemia. The freeze-clamped samples were powdered in liquid nitrogen.

(2) Extraction of lactate and NAD

Lactate and NAD were extracted by the method of Katz et al.[10] The powdered heart tissue samples (15-25 mg wet wt) prepared as described above were homogenized with 1 ml of ice-cold 0.6 M perchloric acid solution. The homogenates were centrifuged at 1,970×g for 10 min (0°C) and then the supernatants were mixed with an equal volume of ice-cold 0.6 M $KHCO_3$ solution for neutralization. After $KClO_4$ crystals had sunk, the supernatant was removed and stored at -80°C until the lactate and NAD assays were performed. Precipitates obtained by centrifugation as described above were dissolved in a 0.1 M NaOH solution and then protein contents were measured by the Lowry method[13].

(3) Extraction of NADH

NADH was extracted by the Klingenberg method[3]. Powdered heart tissue samples (15-25 mg wet wt) were homogenized with 1 ml of a 0.5 M KOH/50% alcohol solution. The homogenates were then heated at 90°C for 5 min in a water bath and then cooled at 0°C for 5 min. An amount of 0.1 ml of each of these extracts was set aside for protein measurement and the remaining 0.9 ml was neutralized with 0.5 ml of a mixture of 0.5 M triethanolamine, 0.4 M KH_2PO_4 and 0.1 M K_2HPO_4. The resulting mixture was centrifuged as described above and the supernatant was stored at -80°C until the assay for NADH. The protein content of the alcohol extracts was measured by the Lowry method after dilution with 0.1 M NaOH solution.

(4) Chemical analyses

The lactate contents of the perchloric acid extracts were measured spectrophotometrically by the lactate oxidase-peroxidase method[19]. NAD and NADH contents were measured by the modified Karp method[9] using bacterial luciferase and alcohol dehydrogenase. The NADH assay was performed within 12 h of extraction. The bioluminescence was measured with a 101C luminescence reader (Aloka Co., Ltd.).

Calculation and statistics

From the data obtained by ^{31}P-NMR, the ATP, PCr, and Pi contents were calculated as μmol/g-dry-wt. In Experiment 1 and 2, the mean values of pH, and ATP, PCr, and Pi contents for the control perfusion and 300 sec of ischemia were nearly the same. Therefore, the results used were those from the control perfusion, and 40, 150, and 300 sec of ischemia in the case of Experiment 1 and those for 90 sec of ischemia in the case of Experiment 2.

In the chemical analyses, the lactate and pyridine dinucleotide contents were calculated as nmol/mg protein and NAD/NADH ratios were calculated for each sample. All the results were expressed as mean ± SD for each chemical analysis and ^{31}P-NMR determination time point. The uniformity of variance of the data with time was analyzed by the Bartlett test. Statistical differences between the control data and the ischemia data were checked by one-way analysis of variance and then Dunnett's multiple comparison test using a software package (SAS 6.03, SAS Institute Japan Ltd.). $P<0.05$ was considered significant. Regressions between lactate contents and NADH contents and NAD/NADH ratios were calculated according to the simple regression line calculation method.

Results

Transient increase in contraction (TIC)

In the control perfusion, the left ventricular developed pressure (LVDP) and the heart rate ranged from 80 to 120 mmHg and 160 to 210 beats/min (n=22), respectively. In the control group, there was no remarkable change in EDP during the first 5 min of global ischemia. A typical TIC is shown in Fig. 1A. This TIC occurred 20 to 40 sec after the start of global ischemia and its pressure did not go above the half of the preceding LVDP. The typical TIC was observed in 17 hearts except for 3 hearts in the 2-DG 11 mM group and each one heart in the IAA 150μM and NaCN 2 mM groups. Therefore the appearance rate of typical TIC was 77% (17/22). Under constant-flow perfusion, the TIC was also observed, with electrical pacing and without it (data not shown). Furthermore, when hearts were perfused repetitively in a short-term ischemia (5 min) and reperfusion (10 min) cycle, the TIC was observed at a similar time from the initiation of ischemia (data not shown). When hearts were perfused for 3 min before ischemia initiation with 11 mM 2-DG, which inhibits glycolytic flux, the TIC was completely absent and there was no change in EDP or LVDP during perfusion (Fig. 1B). When the 2-DG concentration was reduced to 5 mM (2-DG 5 mM + pyruvate 6 mM group), these effects of

2-DG were still achieved by increasing the perfusion period to 10 min (data not shown). However, the effect of IAA, another glycolytic inhibitor, was not as constant as that of 2-DG in this respect. The TIC was not diminished when hearts were perfused with 100 μM IAA for 5 min before ischemia induction, but in the case of perfusion with IAA at 150 μM, the TIC was completely absent in one of the two hearts tested (Fig. 1C), but in the other one, the TIC

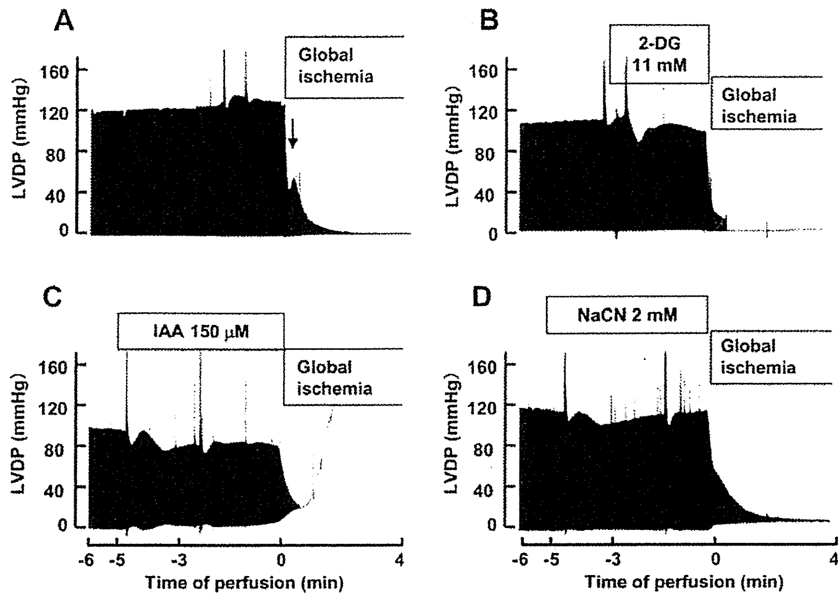

Fig. 1. Continuous recording of heart function during the control perfusion and early stage of ischemia. A: control perfusion and global ischemia - arrow shows a typical TIC, B: 2-DG 11 mM perfusion and global ischemia, C: IAA 150 μM perfusion and global ischemia, D: NaCN 2 mM perfusion and global ischemia.

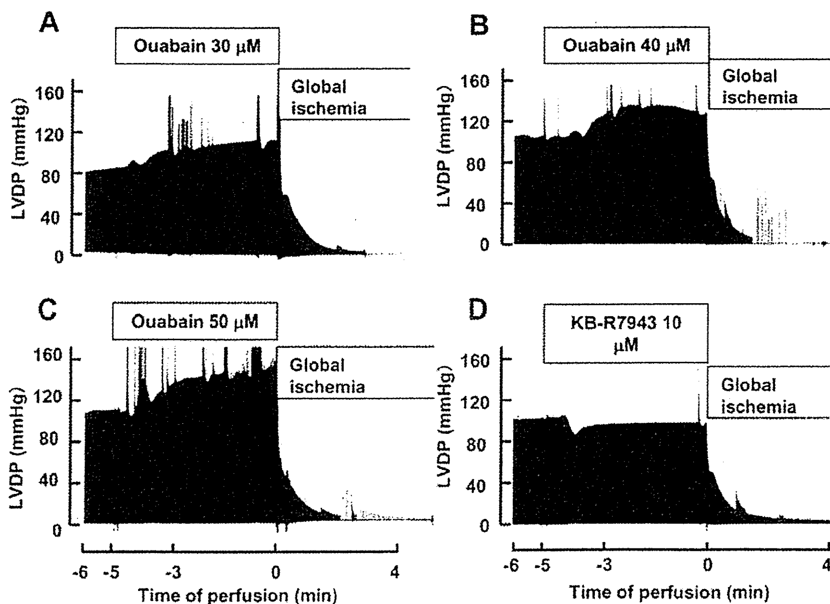

Fig. 2. Continuous recording of heart function during the control perfusion and early stage of ischemia. A: ouabain 30 μM perfusion and global ischemia, B: ouabain 40 μM perfusion and global ischemia, C: ouabain 50 μM perfusion and global ischemia, and D: KB-R7943 10 μM perfusion and global ischemia.

partially remained. With IAA perfusion, there was a more rapid rise in the EDP just after the heart stopped beating in ischemia than there was for 2-DG perfusion (Fig. 1C). Compared with these glycolytic inhibitors, it was more difficult to evaluate the effect of NaCN, a mitochondrial respiratory inhibitor. When hearts were perfused with 2 mM NaCN for 5 min, in many cases, the LVDP rapidly decreased to less than 2/3 of the preceding level and no TIC appeared after ischemia initiation. However, in three other hearts used to evaluate the effect of NaCN under the same perfusion conditions, there was only a slight change in the LVDP or there was no change at all, and the TIC was attenuated, though it did not fully disappear (Fig. 1D). When hearts were perfused with ouabain, a Na^+, K^+-ATPase inhibitor, for 5 min before ischemia initiation, a positive inotropic effect began at a concentration of 30 μM, which progressively increased up to 100 μM. In the case of initiating ischemia after perfusion with 30 μM ouabain, the TIC clearly persisted (Fig. 2A), but when the ouabain concentration was increased to 40 μM, the TIC seemed to be buried in the ischemic function declining curve (Fig. 2B), and then at 50 μM, the TIC was completely absent (Fig. 2C). Thus, the concentration of ouabain that caused the TIC to completely disappear was higher than the concentration that induced a positive inotropic effect. A TIC was clearly evident when hearts were perfused with 10 μM of KB-R7943 for 5 min before ischemia initiation (Fig. 2D). In the substances tested in this study, only 2-DG completely blocked the occurrence of TIC in all hearts used without affecting on the cardiac function.

Changes with time of pyridine dinucleotide contents, NAD/NADH ratio, and lactate content

Changes with time of pyridine dinucleotide contents, NAD/NADH ratio, and lactate content under the experimental conditions of our study are shown in Table 1. For the control perfusion, the NADH and NAD contents were 0.3 ± 0.1 nmol/mg protein and 5.8 ± 0.6 nmol/mg protein, respectively, and the NAD/NADH ratio was 24.6 ± 5.3 (n=6). The mean NADH content rapidly increased initially, being about twice the control value at 20 sec of ischemia and 5 times the control level at 40 sec of ischemia ($p<0.05$ vs control), and continued to increase until 300 sec of ischemia. There was no significant change in the NAD content during ischemia. As a result, the total pyridine dinucleotide content (NAD + NADH) steadily increased in pace with the NADH content increase up to 150 sec of ischemia, and the mean values at 150 and 300 sec of ischemia were significantly higher ($p<0.05$) than that of the control perfusion. Initially the NAD/NADH ratio decreased sharply - to about two-thirds of the control ($p<0.05$) at 20 sec of ischemia and one-fifth of the control after 40 sec of ischemia. It continued

Table 1. NAD, NADH, and Lactate Contents in the Perfused Rat Heart, NAD/NADH Ratios, and Changes in these Parameters after Global Ischemia

	Time (sec) after the onset of global ischemia					
	Control	20 sec	40 sec	90 sec	150 sec	300 sec
NAD	5.8 ± 0.6	6.2 ± 0.8	5.9 ± 0.7	5.6 ± 0.8	5.6 ± 0.7	5.1 ± 0.8
NADH	0.3 ± 0.1	0.5 ± 0.2	1.3 ± 0.4*	1.6 ± 0.2*	1.9 ± 0.2*	2.2 ± 0.1*
NAD+NADH	6.1 ± 0.7	6.6 ± 0.9	7.2 ± 0.8	7.2 ± 0.9	7.5 ± 0.8*	7.4 ± 0.7*
NAD/NADH	24.6 ± 5.3	16.4 ± 9.4*	5.0 ± 2.1*	3.4 ± 0.3*	2.9 ± 0.4*	2.3 ± 0.4*
Lactate	4.6 ± 1.9	5.2 ± 2.8	6.4 ± 2.9	26.6 ± 6.8*	50.8 ± 10.3*	98.8 ± 12.0*

Numerical values are shown as nmol/mg protein (mean ± SD, n=6)
*$p<0.05$ vs control values by Dunnett multiple comparison test

to gradually decrease until 300 sec of ischemia.

The lactate content was 4.6 ± 1.9 nmol/mg protein (n=6) in the control perfusion, and hardly changed up to 40 sec of ischemia. Thereafter, there was a sharp increase and it was about 5 times the control value after 90 sec of ischemia ($p<0.05$). It continued to increase up to 300 sec of ischemia.

Correlations between lactate content and NADH content, and NAD/NADH ratio

The correlations between lactate content and NADH content, and NAD/NADH ratio obtained under these experimental conditions, are shown in Figs. 3A and 3B. From both graphs, it is clear that the regressions between lactate content and NADH content, and NAD/NADH ratio switch in a non-continuous manner during ischemia progression. There was a critical switching point from aerobic oxidation (low NADH content) to anaerobic glycolysis (lactate production) at 40 to 90 sec of ischemia.

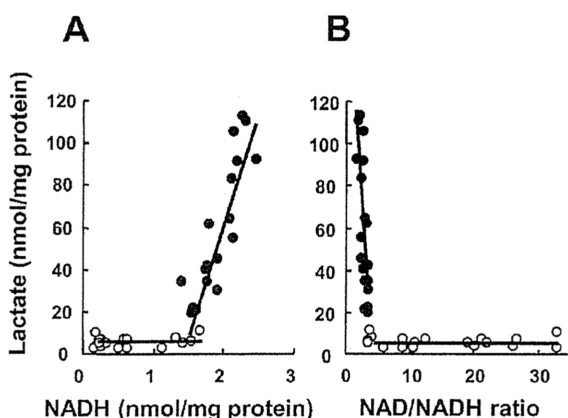

Fig. 3. Regressions between lactate content and NADH content (A) and NAD/NADH ratio (B) in the early stage of ischemia. Data were obtained from six isolated heart tissue samples during control perfusion, and after 20, 40, 90, 150 and 300 sec of ischemia (○ : control perfusion to 40 sec of ischemia, ● : 90 to 300 sec of ischemia). In Fig. 3A, regression line for control perfusion to 40 sec of ischemia: $Y = 1.44X + 4.42$, $r = 0.30$, and regression line for 90 to 300 sec of ischemia: $Y = 93.8X - 122.4$, $r = 0.86$ ($p<0.01$). Regression line for control perfusion to 40 sec of ischemia: $Y = -0.02X + 5.62$, $r = 0.06$, and regression line for 90 to 300 sec of ischemia: $Y = -39.7X + 172.9$, $r = 0.73$ ($p<0.01$) in Fig. 3B.

Changes in intracellular pH and ATP, PCr, and Pi contents with time

The mean intracellular pH was 7.36 ± 0.07 (n=6) during the control perfusion (Fig. 4). After the start of ischemia, the pH dropped to 7.18 ± 0.03 in 40 sec. Thereafter, the pH dropped continuously, registering 6.60 ± 0.05 at 300 sec of ischemia.

The mean PCr, ATP, and Pi contents during the control perfusion were 46.8 ± 8.9, 23.1 ± 3.8, and 16.0 ± 4.4 μmol/g-dry wt, respectively. Following the start of global ischemia, the PCr content rapidly decreased and at 40 and 90 sec of ischemia, the contents were approximately half and one-sixth of the control level, respectively (net decrease at 40 sec of ischemia was about 22 μmol/g-dry wt). On the other hand, there was hardly any change in ATP content up to 300 sec of ischemia. The Pi content rapidly increased with global ischemia, and at 40 sec of ischemia it was approximately 2.5 times the control level (net increase was about 23 μmol/g-dry wt).

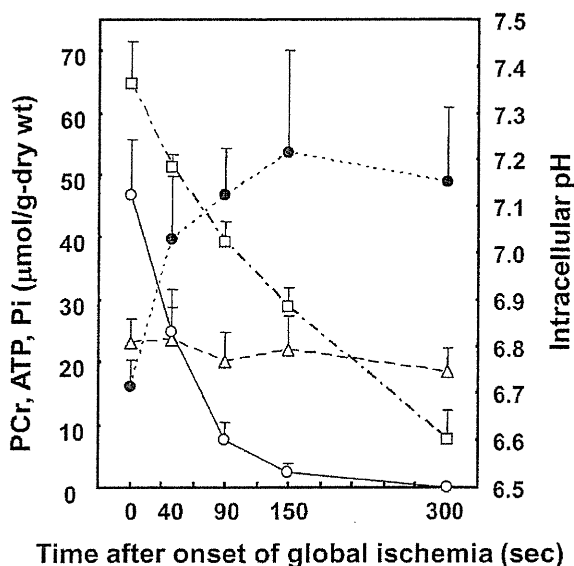

Fig. 4. Changes in intracellular pH, and ATP, creatine phosphate and Pi contents during the early stage of ischemia. □ : Intracellular pH, ○ : creatine phosphate, △ : ATP, ● : Pi

Discussion

A similar TIC has been observed in other studies in which the functions of isolated and perfused rat heart were continuously recorded[15, 26] but little mention was made of it. TIC is different from ischemic contracture and it is interesting to note that it occurs in the early stage of global ischemia when both metabolic and mechanical functions rapidly change.

We first noted that the occurrence of TIC is related to glycolytic activity. As mentioned in the results section, the TIC was completely absent with pre-perfusion of 2-DG, and this result was reproducible in all cases. However, in the case of pre-perfusion with IAA, the TIC did not always disappear and the results were not very reliable. The TIC did not disappear in the case of perfusion with 100 μM IAA, a concentration of IAA widely considered to be effective for inhibiting glycolysis[5]. On increasing the IAA concentration, the TIC was first inhibited at 150 μM (Fig. 1C). The difference in the inhibitory effect of these two reagents on TIC may be attributed to their modes of action and delivery. 2-DG is phosphorylated by hexokinase like glucose but cannot be further metabolized later on and blocks glycolysis, whereas IAA blocks glycolytic flux by inhibiting glyceraldehyde-3-phosphate dehydrogenase[21]. 2-DG is actively taken into the cardiomyocytes through the glucose transport pathway but IAA can only enter cells by passive diffusion.

ATP generated by glycolysis is thought to be intimately connected with the functions of the channel and pumps in the sarcolemma (SL) and sarcoplasmic reticulum (SR) membranes of cardiac myocytes. Glycolytic enzymes are compartmentalized (localized) in or near these membranes: in the SL membrane, the local ATP drives the Na^+, K^+-pump (ATPase)[7] and controls the opening of the K_{ATP}-channel[22], and in the SR membrane, it drives the Ca^{2+}-pump (ATPase)[25]. In global ischemia, the functions of the channel and pumps mentioned above are thought to be maintained until the very last moment when the supply of ATP from the corresponding glycolytic pathway ends. But the fact is that the glycolytic activity or flux localized in the membrane systems may be possibly affected even in the early period of ischemia, since the intracellular NADH and Pi concentrations increase rapidly while the intracellular pH has not yet decreased so much (Fig. 4). However, it is unclear whether these metabolic changes induced by global ischemia equally affect the glycolytic activity or flux localized by the two membrane systems. It is also unclear how the change of local glycolytic activity or flux affects the functions of the channel and pumps under consideration in the early stage of ischemia. However, the fact that TIC could be diminished by inhibiting glycolytic flux, suggests that it might be related to changes in activity in the channel and/or pumps since they are regulated by glycolysis.

Our second important finding was that TIC seemed to be diminished by inhibiting Na^+, K^+-pump activity with ouabain. However, the ouabain concentration that diminished the TIC was different from the concentration that induced a positive inotropic effect, so the results in this case should be carefully considered. It has been shown that when Na^+, K^+-pump activity is inhibited by cardiac glycosides, the intracellular Na^+ concentration ($[Na^+]i$) increases, and positive inotropy and Ca^{2+} overload occur[20]. Another study has reported that the positive inotropic effect is induced by intracellular Ca^{2+} ($[Ca^{2+}]i$) accumulation after the forward mode reaction of NCX, which is linked to Na^+, K^+-pump activity, is inhibited[18]. In this study, there was an obvious inotropic effect after starting the 30 μM ouabain perfusion, though a distinct TIC was evident after the start of global ischemia (Fig. 2A). As the ouabain concentration was increased, the TIC was buried in the ischemic function declining curve (Fig. 2B) to a greater