Biomedical Research 14 (1) 27-36, 1993

ANALYSIS OF CHANGE WITH TIME IN INTRACELLULAR FREE CALCIUM CONCENTRATION DURING HYPOXIA-REOXYGENATION OF INTACT RAT HEART CONTAINING INDO 1

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ABSTRACT

The effects of hypoxia for 20 min followed by reoxygenation on the intracellular free calcium concentration ($[Ca^{2+}]_i$) were studied on a real-time basis in potassium-arrested perfused rat heart loaded with the fluorescent calcium indicator indo 1. Indo 1-loaded heart was calibrated *in situ* and the concentration of intracellular calcium was determined. When the heart was arrested by a high-potassium medium, the calcium transients remained at the diastolic level. The intracellular calcium concentration did not change during the first 10 min of hypoxia, but increased gradually during the next 10 min to above the normal systolic level. During reoxygenation, it decreased rapidly to the control level and the calcium transients and heartbeat were restored simultaneously by switching to normal medium upon reoxygenation. The exposure to bradykinin, which increases endothelial, but not myocardial $[Ca^{2+}]_i$, did not affect the fluorescence of indo 1-loaded heart, indicating that the contribution of endothelial $[Ca^{2+}]_i$ was negligible. This model seems to be useful in investigating real-time changes in $[Ca^{2+}]_i$ during hypoxia-reoxygenation of perfused rat heart.

Calcium is important in many biological systems, controlling a wide range of subcellular processes. Increase in the intracellular calcium ion concentration ($[Ca^{2+}]_i$) has been proposed to be one cause of the pathological changes occurring during acute ischemia and reperfusion (3, 20). Recently, a striking rise in $[Ca^{2+}]_i$ during acute ischemia of intact perfused heart has been detected by several different methods, including fluorine-19 nuclear magnetic resonance (NMR) spectroscopy with 5F-BAPTA [the 5,5′-difluoro derivative of 1,2-bis (*o*-aminophenoxy) ethane-*N,N,N′,N′*-tetraacetic acid] (10, 18, 24, 25), the measurements of fluorescent Ca^{2+} indicators (11, 12, 19), and bioluminescent protein (10). However, each method has methodological limitations (14, 15, 17, 22). In studies with indo 1 and aequorin in intact perfused heart, the most serious problem relates to non-myocardial cells, especially in that endothelial cells may be disproportionately loaded with the indicator (14). Therefore, with these two methods, the effects of non-myocardial cells must be taken into consideration. On the other hand, the major problem in ^{19}F-NMR spectroscopy with 5F-BAPTA is the extent of time resolution. This method cannot demonstrate beat-to-beat calcium transients or rapid changes that occur within a few minutes, but only time-averaged $[Ca^{2+}]_i$ values for about 5 min periods, and therefore appears to be inappropriate for analysis of rapid changes in $[Ca^{2+}]_i$ during ischemia-reperfusion.

In the present study we used a modified procedure for loading perfused heart with the fluorescent Ca^{2+} indicator indo 1. This procedure enabled us to record the changes in $[Ca^{2+}]_i$ of intact perfused heart on beat-to-beat and real-time bases with little disturbance from the Ca^{2+}-sensitive fluorescence of endothelial cells. Using this method, we investigat-

ed the effect of hypoxia for 20 min followed by reoxygenation on $[Ca^{2+}]_i$ in coronary-perfused, intact rat heart. The results showed that $[Ca^{2+}]_i$ started to increase 10 min after the onset of hypoxia and rapidly returned to normal on reoxygenation.

MATERIALS AND METHODS

Preparation of Isolated Hearts

Adult male Sprague-Dawley rats weighing 200–300 g were starved for 24 h and then anesthetized by intraperitoneal injection of pentobarbital (50 mg/kg body weight). The heart was excised and the aorta was cannulated promptly. Retrograde perfusion was started at a constant pressure of 80 cm H_2O using an overflow chamber. The perfusate, consisting of (in mM) NaCl 120, KCl 4.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $CaCl_2$ 2.5, and $NaHCO_3$ 25, was equilibrated with 95% O_2-5% CO_2 at 30°C.

The left ventricular pressure (LVP) was monitored continuously with a thin latex balloon, which was inserted into the left ventricle (LV) through the mitral valve and connected to a pressure transducer. The balloon was filled with water to an end-diastolic pressure of 8–10 mmHg, which was kept constant throughout the experiment.

Hypoxic perfusion was performed with perfusate equilibrated with 95% N_2-5% CO_2, in which the P_{O_2} was below 15 mmHg as measured with a Clark-type electrode. Oxygen deprivation and delivery to the arrested heart were confirmed by biospectometry as reported previously (26).

The heart was arrested by perfusion with high-potassium medium (high K^+ medium), in which the potassium concentration was raised to 25.7 mM and isotonic osmolarity was maintained by reducing the concentration of NaCl. On switching from perfusion with high K^+ medium to that with the usual low potassium medium, spontaneous beating of the heart was promptly resumed. During hypoxic perfusion, high K^+ medium was used to minimize the leakage of fluorescent calcium indicator because hypoxia has been known to cause the fragility of sarcolemma, which is further damaged by the mechanical stress of heartbeat (26), and results in a release of the calcium indicator as well as cytosolic components. Glucose was omitted from perfusate to restrict energy supply from anaerobic glycolysis because pilot studies in our laboratory have shown that myocardial ATP levels do not reduce even after 60 min of hypoxic perfusion with glucose.

Loading of Indo 1 and Fluorescence Recordings

After an initial 15-min equilibration period, the coronary perfusate was replaced by perfusate containing the acetoxymethyl ester of indo 1 (indo 1/AM) (Dotite, Kumamoto, Japan). Indo 1/AM was solubilized in dimethyl sulfoxide containing Cremophor EL (25% v/v) (Nacalai Tesque, Kyoto, Japan), diluted to a final concentration of 4 μM (Cremophor EL, 0.025% v/v), and sonicated for 30 min unless otherwise stated. The perfusate containing indo 1/AM was recirculated for 15 min at 25°C. Then the heart was returned to a non-recirculating mode and washed with normal perfusate for 15 min to remove indo 1/AM from the extracellular space. During this period, the perfusate was slowly warmed to 33°C and all experiments with indo 1-loaded heart were performed at 33°C. For fluorescence recordings, illumination from a xenon lamp was filtered at 340 nm and directed through a fiber-optic cable, the end of which was attached to the anterior epicardial surface of the left ventricle (Fig. 1). Fluorescence emissions were collected with another fiber optics system and directed through a beam splitter into two photomultipliers fitted with optical band-pass filters centered at 405 and 500 nm (CAF-102, Japan Spectroscopic, Hamamatsu, Japan). The signals of the photomultipliers and the pressure transducer were transferred to a PC-9801 RX personal computer (NEC, Tokyo, Japan) and fluorescence intensity at individual wavelengths (F405 or F500), the ratio of F405 to F500 (F405/F500), and LV isovolumic pressure were recorded.

Correction of F405/F500 with Autofluorescence

To correct for changes in autofluorescence during hypoxia and reoxygenation, we subtracted autofluorescences from the values at F405 and F500. The corrected values of F405 and F500 (cF405 and cF500) and the corrected ratio (cR) were calculated from the following relationship:

$$cF405 = F405 - F_{auto}405$$
$$cF500 = F500 - F_{auto}500$$
$$cR = cF405/cF500$$

where $F_{auto}405$ and $F_{auto}500$ are the autofluorescences at the respective wavelengths. The values of the autofluorescence were obtained in parallel experiments on hypoxia-reoxygenation without indo 1 ($n = 3$), and cF405 and cF500 at certain times were calculated using the mean values of

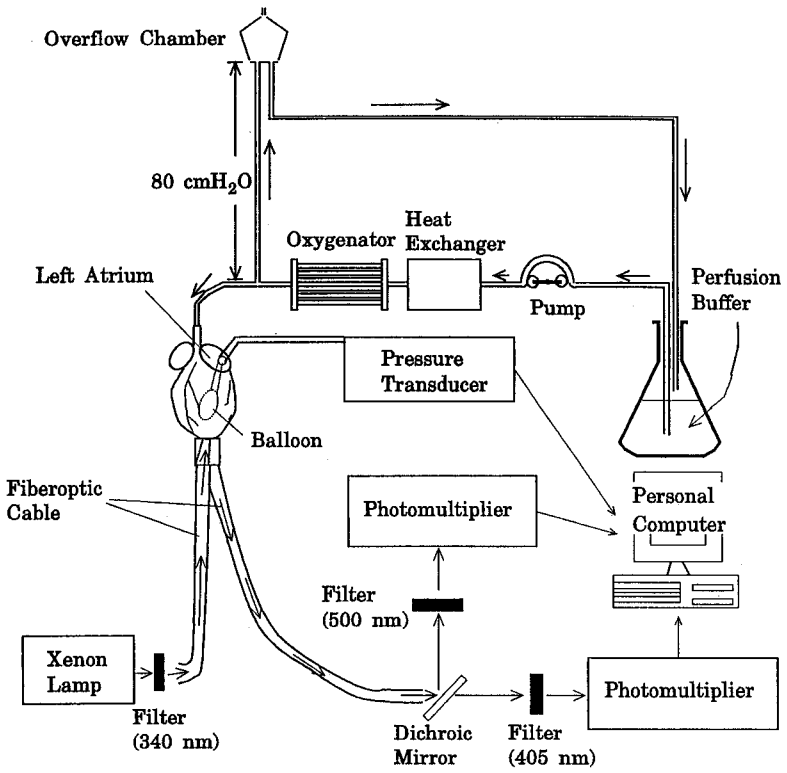

Fig. 1 Measurements of $[Ca^{2+}]_i$ and left ventricular pressure in the isolated and perfused rat heart

autofluorescence at the same time points.

Calibration of Indo-1 Loaded Heart

To determine the maximum and minimum values of F405/F500, we perfused indo 1-loaded heart with a calcium ionophore Br-A23187 and 2,3-butanedione monoxime (BDM). BDM was added to reduce the contracture of myocardium and vascular smooth muscle because it is reported to decrease the sensitivity of the myofibrils to Ca^{2+} (2) and to inhibit cross-bridge formation (13, 21). Heart was arrested with high K^+ medium containing 8 mM EGTA [ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid] and no $CaCl_2$ (calcium-free medium) in the presence of 10 μM Br-A23187 and 10 mM BDM, and after 4 min the minimum value of F405 and F405/F500 (F405min and Rmin) were obtained. Then calcium-free medium was replaced with normal high K^+ medium, which contained 2.5 mM calcium, and the maximum value of F405 and F405/F500 (F405max and Rmax) were obtained. The corrected values of Rmax and Rmin (cRmax and cRmin) and of the minimum to maximum fluorescence intensity ratio at 405 nm (cF405min/cF405max) were calculated by the equations described above. In these calculations, the autofluorescences in the prehypoxic state were used as the values for $F_{auto}405$ and $F_{auto}500$, because autofluorescence showed scarcely any change in normoxic conditions (data not shown).

From these values, the relative value of $[Ca^{2+}]_i$ was calculated from the following equation (22).

$[Ca^{2+}]_i/Kd = (cF405min/cF405max)(1/cR\text{-}1/cRmin)/(1/cRmax\text{-}1/cR)$ where Kd is the dissociation constant of indo 1. The Kd value of indo 1 in the intracellular milieu is reported to be different from that obtained *in vitro* mainly because indo 1 in cells is bound to myoplasmic protein (9). To access the contribution of protein, we estimated the Kd value of indo 1 at 33°C in 50 mM KCl, 10 mM potassium HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid] (pH 7.2), 1 mM $MgCl_2$, 10% bovine serum albumin, 1 μM indo 1, and 10 mM EGTA with variable amounts of $CaCl_2$ added to yield the desired $[Ca^{2+}]$. $[Ca^{2+}]$ of each solution was calculated by use of a computer pro-

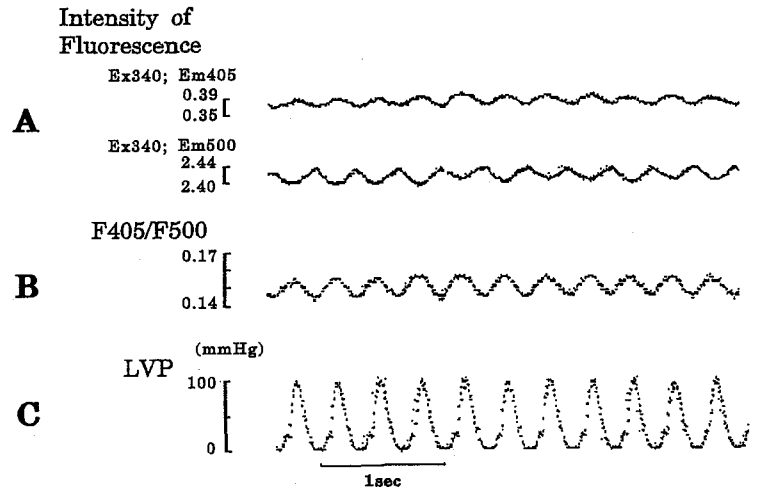

Fig. 2 Simultaneous recordings of $[Ca^{2+}]_i$-dependent fluorescence and ventricular pressure in a rat heart containing indo 1. Isovolumic left ventricular pressure was measured with an intracavitary latex balloon (Panel C). Each beat was accompanied by phasic $[Ca^{2+}]_i$ transients, in which fluorescence increased at 405 nm (Panel A, top) and decreased at 500 nm (Panel A, bottom). Panel B, ratio of intensities at the two wavelengths, F405/F500, calculated with a computer. Fluorescence intensities are expressed in an arbitrary unit.

gram analogous to that described by Fabiato and Fabiato (4), and we could obtain the Kd of 610 ± 64 nM (mean ± SD).

Measurement of LDH Activity

LDH activity was determined spectrophotometrically as reported (26).

Statistical Analysis

Data are presented as means ± SEM. Statistical analysis was performed by the paired or unpaired *t*-test, or by repeated measures analysis of variance with the Dunnett's test where indicated. A *P* value of <0.05 was regarded as significant.

RESULTS

$[Ca^{2+}]_i$-Dependent Fluorescence Transients Under Physiological Conditions

The heart loaded with indo 1 exhibited phasic changes in fluorescence, which were not observed before loading, and which coincided with ventricular contractions (Fig. 2). The fluorescence signals at the two emission wavelengths showed reciprocal changes: a phasic increase in fluorescence at 405 nm (Fig. 2A, top) and a phasic decrease in fluorescence at 500 nm (Fig. 2A, bottom) were observed in systole, while the reversed changes were observed in diastole. These changes in fluorescence were recognized to reflect changes in $[Ca^{2+}]_i$ by Lee *et al.* (11, 12). The autofluorescence signal, which was measured before loading the heart with indo 1, represented 18% and 12% of the total fluorescence at 405 and 500 nm, respectively. The systolic pressure was 96 ± 7 mmHg before loading and 106 ± 13 mmHg after loading (n=10).

Changes in $[Ca^{2+}]_i$-Dependent Fluorescence During Hypoxia-Reoxygenation

Fig. 3 shows a representative result of change in F405/F500 of a heart subjected to 20 min of hypoxia followed by reoxygenation. On switching to high K^+ perfusate, the heart was arrested as shown in the trace of left ventricular pressure, and concomitantly the phasic change in F405/F500 ceased at the diastolic level. No change in F405/F500 was observed during the first 10 min of hypoxia, but during the next 10 min, the ratio increased gradually to above its systolic level before hypoxia.

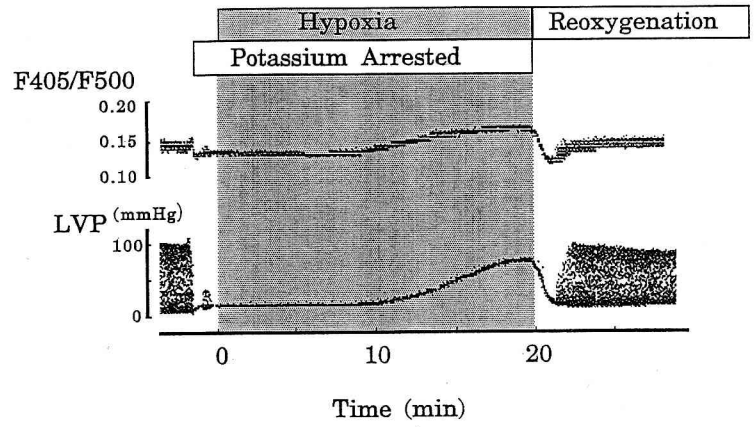

Fig. 3 Effects of hypoxia on the ratio of $[Ca^{2+}]_i$-dependent fluorescence at the two wavelengths (F405/F500) and left ventricular pressure in a heart containing indo 1. On switching to high-potassium perfusate, the transients of F405/F500 were arrested at the diastolic level (top) and, concomitantly, the heart stopped beating, as shown by the left ventricular pressure (bottom). LVP, left ventricular pressure

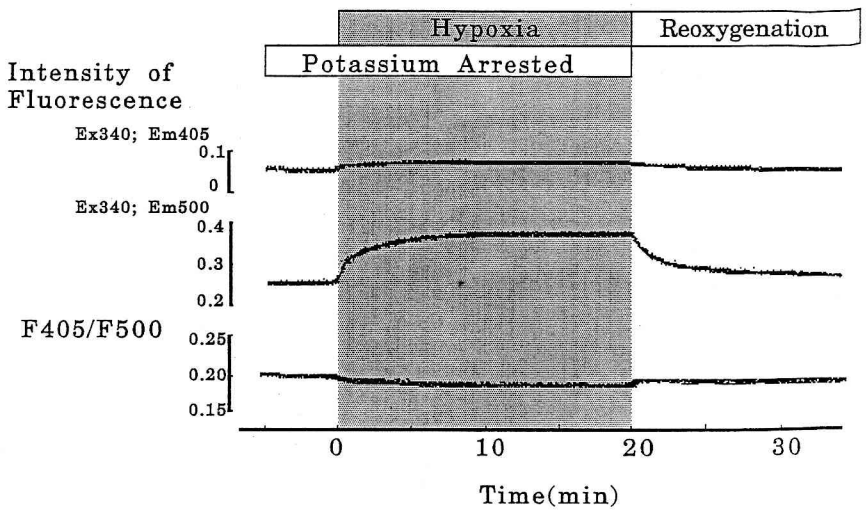

Fig. 4 Changes in autofluorescence and F405/F500 during 20 min of hypoxic perfusion followed by reoxygenation. Fluorescence intensities are expressed in an arbitrary unit.

During reoxygenation, this ratio decreased rapidly to the control value and both the phasic changes in F405/F500 and the heartbeat were restored by switching to normal medium upon reoxygenation. F405/F500 in Fig. 3 was not corrected by the autofluorescence, but the ratio could reflect the changes in $[Ca^{2+}]_i$ because the F405/F500 of heart without indo 1 was scarcely affected during hypoxia-reoxygenation (Fig. 4). Changes in autofluorescence were fairly remarkable at onset of both hypoxia and reoxygenation, probably due to reduction and oxidation of NAD, but they had little contribution to F405/F500 ratio. Fig. 5 shows pooled data on changes in cR, calculated as described in Materials and Methods. The cR value 20 min after the onset of hypoxia was significantly higher than

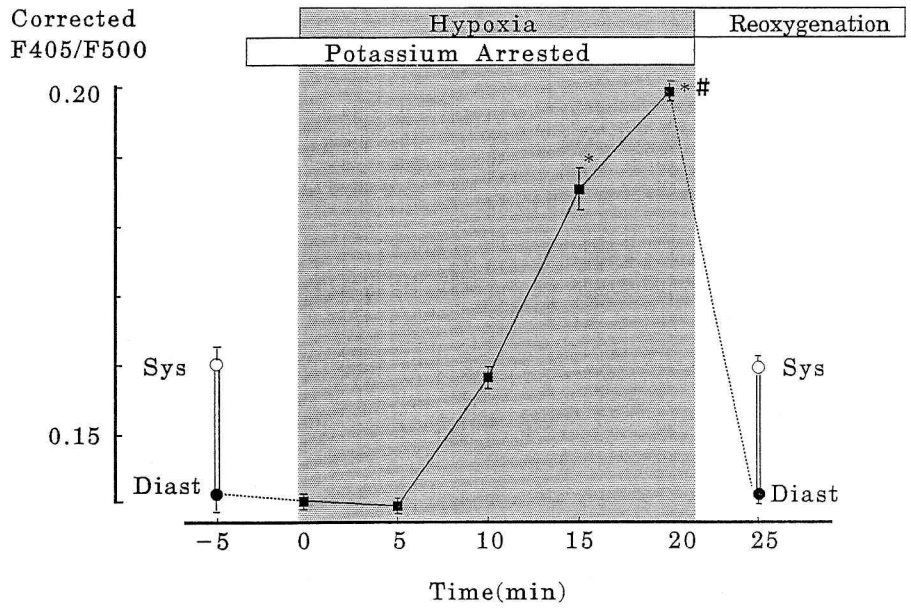

Fig. 5 Pooled data on F405/F500 corrected for changes in autofluorescence (see text) during hypoxia and reoxygenation. $^{*}P<0.05$ vs value at onset of hypoxia; $^{\#}P<0.05$ vs systolic value before hypoxia. Results are expressed as means ± SEM (n=5).

that at the onset of hypoxia ($P<0.05$) and also higher than the systolic value of control ($P<0.05$).

Contribution of Endothelial Cells to the F405/F500 Ratio

To test whether the changes in $[Ca^{2+}]_i$ of endothelial cells affected the changes in F405/F500 of heart loaded with indo 1, we exposed indo 1-loaded heart to bradykinin (10 μM), which is reported to increase the endothelial, but not myocardial, $[Ca^{2+}]_i$ (14). As shown in Fig. 6, the ratio in a potassium-arrested heart did not change significantly by exposure to bradykinin. This result also showed that the ratio was not affected by perfusion with high K^+ medium for more than 20 min. In contrast, when the heart was loaded with lower concentration of indo 1/AM (2.5 μM) (lighter-loaded heart), the ratio rose by exposure to 3 μM bradykinin (Fig. 7). These results were summarized in Table 1. In lighter-loaded model, the F405/F500 ratio was significantly increased by exposure to both 3 μM and 10 μM bradykinin. Because the increase in the F405/F500 ratio of the lighter-loaded heart was almost the same either by 3 μM or 10 μM bradykinin, maximal effect on endothelial $[Ca^{2+}]_i$ could be obtained by 3 μM bradykinin. Nevertheless, the F405/F500 ratio of normally-loaded heart (4 μM) did not change significantly by exposure to 3 μM and even to 10 μM bradykinin.

Estimation of $[Ca^{2+}]_i$

Intracellular indo 1 loaded as its AM form is reported to have greatly different fluorescent characteristics from indo 1 or indo 1/AM *in vitro*, because of its incomplete de-esterification (22). Therefore, *in situ* calibration was necessary to determine $[Ca^{2+}]_i$ (9). This should be measured using intact perfused heart loaded with indo 1, because other procedures such as homogenization of the heart probably change the de-esterificated state of the dye, resulting in different fluorescent characteristics from those of indo 1 in intact heart. Fig. 8 shows a representative trace of changes in F405/F500 obtained by calibration of a perfused heart loaded with indo 1. The pooled values for cRmax, cRmin and cF405min/cF405max were 0.22 ± 0.04, 0.10 ± 0.02 and 0.45 ± 0.07, respectively (n=3). LDH leakage during this calibration procedure was measured in one heart to determine the degree of sarcolemmal injury due to this procedure. Leakage amounted to only 1.8% of the enzyme in the whole heart, which was about the same as that in our

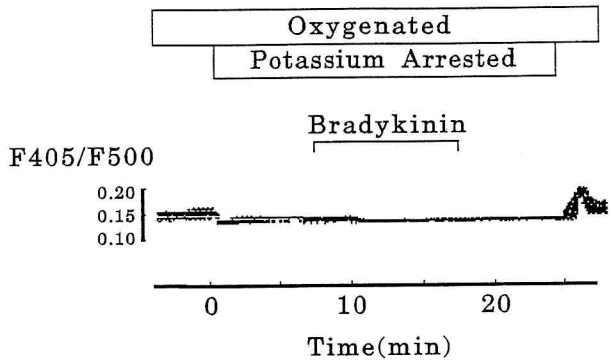

Fig. 6 Effect of 10 μM bradykinin on F405/F500 of indo 1-loaded heart

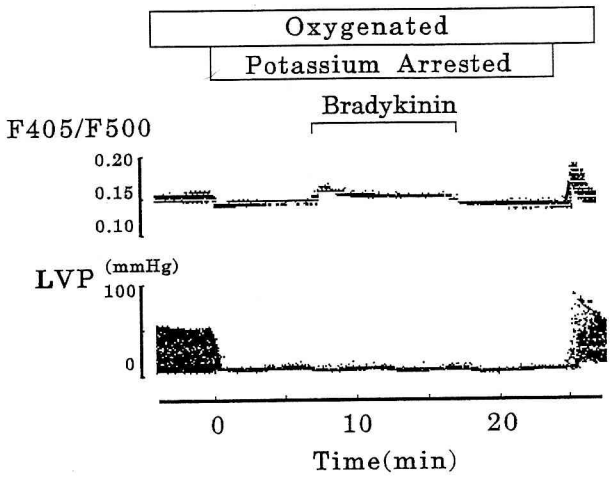

Fig. 7 Effect of 3 μM bradykinin on F405/F500 and LVP of lighter-loaded heart (2.5 μM). LVP, left ventricular pressure

Table 1 *Effect of Bradykinin on F405/F500 of Indo 1-Loaded Heart*

Bradykinin	Normally-loaded hearts (4 μM indo 1/AM)		Lighter-loaded hearts (2.5 μM indo 1/AM)	
	Before treatment	After treatment	Before treatment	After treatment
3 μM	0.143 ± 0.002	0.143 ± 0.001	0.145 ± 0.002	0.173 ± 0.003*
10 μM	0.140 ± 0.003	0.140 ± 0.002	0.147 ± 0.003	0.176 ± 0.005*

Data shows values of F405/F500 in hearts treated with different concentrations of indo 1/AM, and bradykinin. Results are means ± SEM. *$P<0.05$ vs values before treatment

previous model after 20-min hypoxia followed by reoxygenation (26). Therefore, damage of the cell membrane, or leakage of indo 1 into the extracellular space during this procedure was probably negligible.

From these values, the relative value of $[Ca^{2+}]_i$ ($[Ca^{2+}]_i/Kd$) was calculated. As shown in Fig. 9, the $[Ca^{2+}]_i/Kd$ under physiological conditions was 0.99 ± 0.10 in systole and 0.50 ± 0.04 in diastole. After 20 min of hypoxic perfusion, $[Ca^{2+}]_i/Kd$ increased to 3.58 ± 0.65, which was significantly higher than the value at the onset of hypoxia ($P<0.05$) and also the systolic value of control ($P<0.05$). Adapting the Kd of 610 nM, the value obtained in the albumin-containing solution (see Materials and Methods), $[Ca^{2+}]_i$ under physiological conditions was estimated to be 600 ± 60 nM in systole and 300 ± 20 nM in diastole and the estimated $[Ca^{2+}]_i$ rose to 2,200 ± 200 nM after 20 min of hypoxic perfusion.

DISCUSSION

Measuring $[Ca^{2+}]_i$ of Perfused Rat Heart

Recently, monitoring changes in $[Ca^{2+}]_i$ of perfused rat heart has become possible by use of the new fluorescent Ca^{2+} indicators, such as indo 1 (11, 12, 19), ^{19}F-NMR spectroscopy with 5F-BAPTA (10, 16–18) and bioluminescent protein (8). Fluorescent Ca^{2+} indicators are considered to be superior to NMR spectroscopy with 5F-BAPTA in view of time resolution. Also, when compared with bioluminescent protein, these indicators can be introduced more easily to myocardial cells with their cell-permeable acetoxymethyl ester form. However, there are limitations in use of the fluorescent indicators in perfused heart. The major limitation is the presence of the dye incompletely de-estrified

because the non-de-estrified dye exhibits Ca^{2+}-insensitive fluorescence, which is clearly different from that of either indo 1 or indo 1/AM (15, 22). Thus, calibration must be done *in situ* because other procedures such as homogenization of the heart probably influence the de-estrificated state of the cell-associated dye. In fact, homogenate of indo 1-loaded heart had different fluorescence characteristics from those of an indo 1-loaded intact heart (data not shown). Mohabir *et al.* (19) reported a method of *in situ* calibration using one wavelength and they successfully estimated $[Ca^{2+}]_i$ of indo 1-loaded heart in physiological condition. Our method of calibration was essentially the same as theirs, but, because we used the F405/F500 ratio in calibration, we could overcome the leakage or quenching of indo 1 within the cells and could estimate $[Ca^{2+}]_i$ even after hypoxic perfusion.

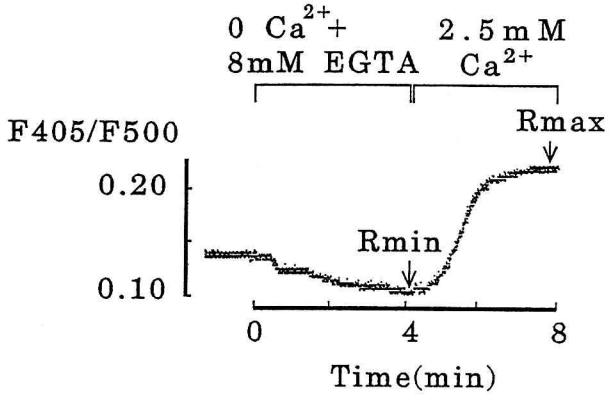

Fig. 8 Changes in F405/F500 during *in situ* calibration. An indo 1-loaded heart was arrested by high-potassium medium. Then the perfusate was replaced by calcium-free medium with 8 mM EGTA and, then, by medium containing calcium in the presence of the calcium ionophore Br-A23187 (10 μM). Minimum and maximum values of F405/F500 were obtained by this procedure (see text). Rmin, minimum value of F405/F500; Rmax, maximum value of F405/F500

Another limitation is the Kd value of indo 1 in cells because fluorescent calcium indicators are known to bind to cytoplasmic proteins and the binding alters many properties of the indicators (9). The indicators in the intracellular environment are considered to have a somewhat higher Kd value than those in the solutions without proteins. Actually, the Kd value of 610 nM, which we obtained in an albumin-containing solution, was rather higher than the reported value, 250 nM, which was obtained in pure salt solution (6). As the exact Kd of indo 1 in cells is unknown, the absolute value of $[Ca^{2+}]_i$ cannot be easily estimated. Therefore, we evaluated the changes in $[Ca^{2+}]_i$ by

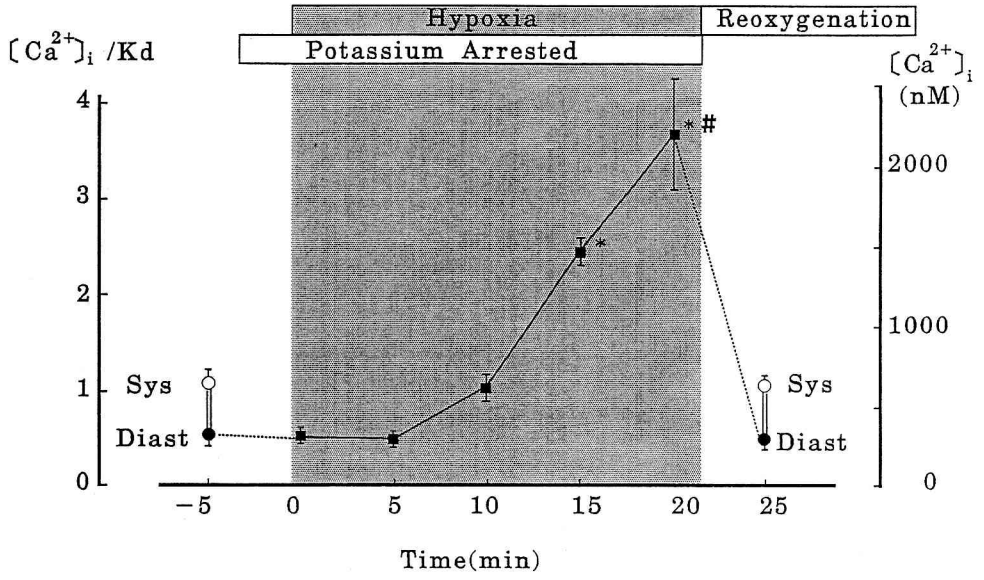

Fig. 9 Changes in relative $[Ca^{2+}]_i$ ($[Ca^{2+}]_i$/Kd) during hypoxia and reoxygenation. $[Ca^{2+}]_i$ was estimated by adapting the Kd of 610 nM, the value obtained in an albumin-containing solution. $^*P<0.05$ vs value at the onset of hypoxia; $^\#P<0.05$ vs systolic value before hypoxia. Results are expressed as means ± SEM (n=5).

$[Ca^{2+}]_i$/Kd, which may be a relative but much more reliable parameter than the simple ratio of the fluorescences at two wavelengths.

Effect of High-Potassium Medium on Myocardial $[Ca^{2+}]_i$

In the present study, we used potassium-arrested heart to minimize the leakage of indo 1 during hypoxia. We found no change in $[Ca^{2+}]_i$ by perfusion with high K^+ medium under aerobic condition for 20 min. However, there are some reports that high K^+ medium causes a small increase in $[Ca^{2+}]_i$ of isolated myocardial cells (5, 23). On the other hand, in perfused rat heart (27) and in ferret papillary muscles (1), $[Ca^{2+}]_i$ was not increased by high K^+ medium. There seems to be two possible reasons for this discrepancy. One is the difference in the experimental model. Isolated cardiomyocytes are reported to be slightly lower in their ATP content and membrane polarization (7), and these can result in increased $[Ca^{2+}]_i$ during depolarized state. The other reason is the concentration of extracellular potassium ($[K^+]_o$). Those reported the elevation of $[Ca^{2+}]_i$ used 50 mM $[K^+]_o$, and others including ourselves used 30 mM or less. $[K^+]_o$ could affect the depolarized state of cells, which would change the state of voltage-dependent Ca^{2+} channel and electrogenic Na^+-Ca^{2+} exchange system and, subsequently, the regulation of $[Ca^{2+}]_i$.

Effect of Endothelial Cells on Ca^{2+}-Dependent Fluorescence

Another problem in investigating $[Ca^{2+}]_i$ in heart loaded with indo 1 is the possible effect of changes in $[Ca^{2+}]_i$ due to endothelial cells. Lorell *et al.* (14) demonstrated that bradykinin caused a pronounced increase in Ca^{2+}-dependent fluorescence in heart loaded with indo 1 and concluded that endothelial cells could accumulate indo 1. In contrast, by our loading procedure, bradykinin did not cause any changes in $[Ca^{2+}]_i$. There are two possible reasons for the small contribution of endothelial $[Ca^{2+}]_i$ in our experiment.

First, our loading solution had a higher concentration of indo 1/AM than that reported previously and did not contain albumin, which binds to the dye and reduces its actual concentration. Also, the longer sonication period in our experiment possibly resulted in smaller micelles of indo 1/AM. If indo 1/AM enters cells only passively, the concentrations of the dye in myocardial and endothelial cells should be the same, and the influence of endothelial cells on Ca^{2+}-dependent fluorescence should be negligible in the context of the populations of the two kinds of cells. Thus, some kind of facilitated transport, such as pinocytosis may be related with the accumulation of the dye in endothelial cells. We think that the higher concentration of indo 1/AM and the longer time of sonication may increase the myocardial fluorescence by enhancing its passive diffusion, and reduce the relative contribution of endothelial cells. Actually, when the concentration of indo 1/AM in the loading solution was reduced to 2.5 μM, the F405/F500 ratio showed a significant change by exposure to only 3 μM bradykinin. Second, Lorell *et al.* (14) used rabbits as the experimental animal. Even in our lighter-loaded model (2.5 μM indo 1/AM), the F405/F500 increase by bradykinin was not so prominent as that of Lorell *et al.* (14). Thus, the endothelial accumulation of indo 1 in rat heart could be smaller than that in rabbit heart.

In conclusion, our model of potassium-arrested perfused heart with indo 1 will be useful in investigating real-time changes in $[Ca^{2+}]_i$ during hypoxia-reoxygenation, or ischemia-reperfusion, especially in the settings of cardiac surgery or heart preservation.

We thank Mrs K. Chihara for technical assistance and Miss T. Ogawa, Miss M. Inoue and Miss M. Hattori for secretarial assistance. This work was supported in part by grants-in-aid for Scientific Research from the Ministry of Education, Science and Culture of Japan.

Received 6 July 1992; and accepted 10 December 1992

REFERENCES

1. ALLEN D. G., EISNER D. A. and ORCHARD C. H. (1984) Factors influencing free intracellular calcium concentration in quiescent ferret ventricular muscle. *J. Physiol.* (London), **350**, 615-630
2. BLANCHARD E. M., MULIERI L. A. and ALPERT N. R. (1984) The effect of 2,3-butanedione monoxime (BDM) on the relation between initial heat and mechanical output of rabbit papillary muscle (Abstract). *Biophys. J.* **45**, 48
3. DHALLA N. S., PIERCE G. N., PANAGIA V., SINGAL P. K. and BEAMISH R. E. (1982) Calcium movements in relation to heart function. *Basic Res. Cardiol.* **77**, 117-139
4. FABIATO A. and FABIATO F. (1979) Calculator programs for computing the composition of the solutions containing multiple metals and ligands used for experiments in skinned muscle cells. *J. Physiol.* (Paris), **75**, 463-505
5. FOSTER K. A. and ROBISHAW J. D. (1991) Effect of calcium and cAMP on $G_{0\alpha}$ expression in neonatal rat cardiac myocytes. *Amer. J. Physiol.* **261**, Suppl. (Oct), 15-20
6. GRYNKIEWICZ G., POENIE M. and TSIEN R. Y. (1985) A new

generation of Ca^{2+} indicatbrs with greatly improved fluorescence properties. *J. Biol. Chem.* **260**, 3440-3450
7. Jacobson S. L. and Piper H. M. (1986) Cell cultures of adult cardiomyocytes as models of the myocardium. *J. Mol. Cell. Cardiol.* **18**, 661-678
8. Kihara Y., Grossman W. and Morgan J. P. (1989) Direct measurement of changes in intracellular calcium transients during hypoxia, ischemia, and reperfusion of the intact mammalian heart. *Circ. Res.* **65**, 1029-1044
9. Konishi M., Olson A., Hollingworth S. and Baylor S. M. (1988) Myoplasmic binding of fura-2 investigated by steady-state fluorescence and absorbance measurements. *Biophys. J.* **54**, 1089-1104
10. Koretsune Y. and Marban E. (1990) Mechanism of ischemic contracture in ferret hearts: relative roles of $[Ca^{2+}]_i$ elevation and ATP depletion. *Amer. J. Physiol.* **258**, H9-H16
11. Lee H.-C., Mohabir R., Smith N., Franz M. R. and Clusin W. T. (1988) Effect of ischemia on calcium-dependent fluorescence transients in rabbit hearts containing indo 1. Correlation with monophasic action potentials and contraction. *Circulation* **78**, 1047-1059
12. Lee H.-C., Smith N., Mohabir R. and Clusin W. T. (1987) Cytosolic calcium transients from the beating mammalian heat. *Proc. Natl. Acad. Sci. USA* **84**, 7793-7797
13. Li T., Sperelakis N., Teneick R. E. and Solaro R. J. (1985) Effects of diacetyl monoxime on cardiac excitation-contraction coupling. *J. Pharmacol. Exp. Ther.* **232**, 688-695
14. Lorell B. H., Apstein C. S., Cunningham M. J., Schoen F. J., Weinberg E. O., Peeters G. A. and Barry W. H. (1990) Contribution of endothelial cells to calcium-dependent fluorescence transients in rabbit hearts loaded with indo 1. *Circ. Res.* **67**, 415-425
15. Lückhoff A. (1986) Measuring cytosolic free calcium concentration in endothelial cells with indo-1: The pitfall of using the ratio of two fluorescence intensities recorded at different wavelengths. *Cell Calcium* **7**, 233-248
16. Marban E., Kitakaze M., Chacko V. P. and Pike M. M. (1988) Ca^{2+} transients in perfused hearts revealed by gated ^{19}F NMR spectroscopy. *Circ. Res.* **63**, 673-678
17. Marban E., Kitakaze M., Koretsune Y., Yue D. T., Chacko V. P. and Pike M. M. (1990) Quantification of $[Ca^{2+}]_i$ in perfused hearts. Critical evaluation of the 5F-BAPTA and nuclear magnetic resonance method as applied to the study of ischemia and reperfusion. *Circ. Res.* **66**, 1255-1267
18. Marban E., Kitakaze M., Kusuoka H., Porterfield J. K., Yue D. T. and Chacko V. P. (1987) Intracellular free calcium concentration measured with ^{19}F NMR spectroscopy in intact ferret hearts. *Proc. Natl. Acad. Sci. USA* **84**, 6005-6009
19. Mohabir R., Lee H.-C., Kurz R. W. and Clusin W. T. (1991) Effects of ischemia and hypercarbic acidosis on myocyte calcium transients, contraction, and pH_i in perfused rabbit hearts. *Circ. Res.* **69**, 1525-1537
20. Murphy J. G., Smith T. W. and Marsh J. D. (1987) Calcium flux measurements during hypoxia in cultured heart cells. *J. Mol. Cell. Cardiol.* **19**, 271-279
21. Nayler W. G., Elz J. S. and Buckley D. J. (1988) The stunned myocardium: effect of electrical and mechanical arrest and osmolarity. *Amer. J. Physiol.* **254**, H60-H69
22. Scanlon M., Williams D. A. and Fay F. S. (1987) A Ca^{2+}-insensitive form of fura-2 associated with polymorphonuclear leukocytes. Assessment and accurate Ca^{2+} measurement. *J. Biol. Chem.* **262**, 6308-6312
23. Sheu S.-S., Sharma V. K. and Uglesity A. (1986) Na^+-Ca^{2+} exchange contributes to increase of cytosolic Ca^{2+} concentration during depolarization in heart muscle. *Amer. J. Physiol.* **250**, C651-C656
24. Steenbergen C., Murphy E., Levy L. and London R. E. (1987) Elevation in cytosolic free calcium concentration early in myocardial ischemia in perfused rat heart. *Circ. Res.* **60**, 700-707
25. Steenbergen C., Murphy E., Watts J. A. and London R. E. (1990) Correlation between cytosolic free calcium, contracture, ATP, and irreversible ischemic injury in perfused rat heart. *Circ. Res.* **66**, 135-146
26. Takami H., Matsuda H., Kuki S., Nishimura M., Kawashima Y., Watari H., Furuya E. and Tagawa K. (1990) Leakage of cytoplasmic enzymes from rat heart by the stress of cardiac beating after increase in cell membrane fragility by anoxia. *Pflügers Arch.* **416**, 144-150
27. Tani M. and Neely J. R. (1990) Mechanism of reduced reperfusion injury by low Ca^{2+} and/or high K^+. *Amer. J. Physiol.* **258**, H1025-H1031

Biochimica et Biophysica Acta, 1098 (1991) 41–48

BBABIO 43513

Protection of cellular and mitochondrial functions against anoxic damage by fructose in perfused liver

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(Received 23 April 1991)
(Revised manuscript received 2 August 1991)

Key words: Anoxia; Fructose; Mitochondrion; ATP; (Perfused liver)

In anoxic perfused liver, conversion of fructose to lactate was greatly increased to about 3 μmol/min per g liver. This increase in lactate implied that the same amount of ATP was also produced. The rate of metabolism of glucose was less than 10% of that of fructose, as judged by rate of production of lactate. In anoxic liver perfused with fructose, the ATP levels of both the tissue and mitochondria remained high, despite lack of oxygen, thus preventing enzyme leakage and preserving processes requiring ATP, such as bile excretion and urea formation. The mitochondrial oxidative phosphorylation capacity of anoxic liver perfused with fructose was also unimpaired. Spectral analysis of light transmitted through the liver revealed that the mitochondrial electron transfer system was in the completely reduced state during anoxia, indicating that the mitochondria were incapable of synthesizing ATP. These results suggest that fructose metabolism during anoxia resulted in sufficient production of ATP for maintaining the physiological functions of the cells and the oxidative phosphorylation capacity of their mitochondria.

Introduction

In the last 10 years, investigations on the mechanism of anoxic injury have revealed that decrease in the ATP level and consequent cellular dysfunction are the primary causes of anoxic or ischemic cell injury [1–7]. Prolonged anoxia causes irreversible damage and cell death, which has been generally believed to be caused by loss of the oxidative phosphorylation capacity of the mitochondria [1,3,8,9]. In anoxic conditions, mitochondria cease to produce ATP, but the cells, as a whole, continue to consume ATP to maintain their integrity, thus leading to depletion of intracellular ATP. ATP is not only a source of energy, but also important in protecting mitochondria from anoxic damage [3,4]. So theoretically, during anoxia, if the ATP level is maintained by more efficient metabolic pathways, or by depressing ATP turnover, mitochondrial function and the integrity of cells should be well preserved [5].

In perfused liver, anoxic damage is easily evaluated as suppression of bile flow, leakage of cytosolic enzymes, or cessation of ATP-requiring reactions such as gluconeogenesis and urea formation [10–16]. Anundi et al. reported that in perfused liver, utilization of fructose increases dramatically during anoxia, and that ATP produced by anaerobic decomposition of fructose is sufficient to prevent enzyme leakage. They did not observe this protective effect using glucose instead of fructose [17]. Similar observations were made by an other group [18]. These findings are reasonable considering the metabolic pathways of glucose and fructose; the metabolism of glucose by liver is hormonally controlled by regulatory enzymes such as glucokinase and phosphofructokinase I, both of which are almost completely inactivated in starved liver [19,20], whereas fructose is first converted to fructose 1-phosphate by ketohexokinase, and then cleaved to dihydroxyacetone

Abbreviations; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; LDH, lactate dehydrogenase; HPLC, high-performance liquid chromatography; PFK, phosphofructokinase; F1*P*, fructose 1-phosphate; F26*P*, fructose 2,6-bisphosphate.

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phosphate and glyceraldehyde, and so escapes the limiting steps catalyzed by these two key glycolytic enzymes [19,21,22].

In the present study, prompted by the work of these earlier investigators, we studied the effect of fructose in preventing anoxic damage of liver and mitochondrial functions. We first confirmed that in anoxic liver perfused with fructose, the ATP content remained high owing to the increased rate of fructose metabolism. In these conditions, despite lack of oxygen, bile flow was maintained at a constant rate, and no enzyme leakage occurred. Moreover, though the electron transfer system of mitochondria was in a completely reduced state, mitochondrial functions were well preserved due to ample ATP supplied by anaerobic decomposition of fructose in the cytosol.

Materials and Methods

Materials. Hepes, glutamate dehydrogenase and urease were purchased from Sigma (St. Louis, U.S.A.). All other reagents were of analytical grade and were obtained from local sources.

Liver perfusion. Male Sprague-Dawley rats, weighing about 230 g, were starved overnight before experiments. The animals were anesthetized by intraperitoneal injection of sodium pentobarbital at 50 mg/kg body weight. The procedure and conditions of liver perfusion were essentially as described by Sugano et al. [23]. Livers were isolated and perfused with hemoglobin-free Krebs-Henseleit bicarbonate buffer (119 mM NaCl, 4.5 mM KCl, 2.5 mM $MgSO_4$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, 2.5 mM $CaCl_2$), or the buffer plus 30 μM taurocholate when bile flow was measured (Fig. 2). The perfusion medium was continuously gassed and equilibrated with either 95% O_2–5% CO_2, or 95%N_2–5%CO_2 at 32 °C. The perfusate was pumped through the liver via a cannula in the portal vein at a constant flow rate of 3.0 ml/min per g liver in a flow-through mode. Samples of efferent perfusate were collected for measurements of lactate, glucose, urea and lactate dehydrogenase (LDH) activity. Livers were first pre-perfused with oxygenated (O_2:CO_2 = 95%:5%) plain perfusate for 20 min, and then the experiment was started by adding substrates, or by switching the perfusate to nitrogen-equilibrated (N_2:CO_2 = 95%:5%) medium, as indicated in the legends to figures. Substrates were infused into the afferent perfusate at appropriate rates to give the indicated final concentrations in the perfusate. In some experiments, the bile duct was cannulated with polyethylene tubing, and bile was collected in pre-weighed Eppendorf microcenrifuge tubes.

Preparation of liver and mitochondrial extracts. Liver extracts were prepared as described by Kamiike et al. [2]. Briefly, the excised samples of the perfused liver were quickly freeze-clamped with bronze tongs pre-cooled in liquid nitrogen, and lyophilized. The lyophilized tissue was weighed and homogenized in 50 vol. of 0.5 N perchloric acid in a Biotron homogenizer. The homogenate was centrifuged and the supernatant was neutralized with potassium hydroxide. The resultant precipitate was removed by centrifugation and the supernatant was used for assay of adenine nucleotides.

Mitochondria were isolated from the perfused liver by a conventional procedure [24], and suspended in 0.25 M sucrose containing 10 mM potassium Hepes and 0.2 mM EDTA (pH 7.4). A portion of the mitochondrial suspension was used for measurements of the respiration rate and protein content. For determination of mitochondrial adenine nucleotides, 0.1 vol. of 5.5 N perchloric acid was added to the suspension, and the mixture was neutralized with potassium hydroxide as described above. The extract thus obtained was used for measurement of adenine nucleotides of mitochondria.

Analytical methods. Adenine nucleotides were determined by high-performance liquid chromatography (HPLC), as described elsewhere [2,25]. Liver and mitochondrial extracts were loaded on a reverse-phase column (Shimpack CLC-ODS, Shimadzu, Kyoto) equilibrated with 100 mM sodium phosphate (pH 6.0) containing 3% methanol, and the absorbance of the eluate at 260 nm was monitored with a Shimadzu SPD-6AV spectrophotometric detector. Activity of lactate dehydrogenase (LDH) was assayed as described [26]. Urea was measured as described by Kerscher and Ziegenhorn [27]. Glucose and lactate were measured with an amperometric detector of hydrogen peroxide (E502 with a platinum electrode, IRICA, Kyoto, Japan) combined with an immobilized enzyme column [28]. The enzyme column packed with immobilized glucose oxidase or lactate oxidase was connected to the amperometric detector, and the content of glucose or lactate in the sample was determined after its enzymic conversion to hydrogen peroxide. Protein was measured by the method of Lowry et al. [29] with bovine serum albumin as a standard.

Spectrophotometry of perfused liver. Changes in the reduction-oxidation state of cytochrome oxidase in the perfused liver were monitored with a Biospectrometer MS-401 (Unisoku, Osaka, Japan) as described previously [28]. Incandescent light from a halogen lamp was introduced through a quartz fiber to the upper surface of the liver, and transmitted light from the opposite side of the liver was directed through another fiber to the photomultiplier. The absorbance of the transmitted light at between 500 nm and 700 nm was scanned for 10 s.

Results

Formation of lactate from fructose in anoxic perfused liver

To estimate glycolytic flux under anoxia, we measured the amount of lactate released from perfused liver with glucose or fructose as substrate. A preliminary experiment revealed that rat liver perfused with glucose or fructose at concentrations of more than 4 mM produced a saturation level of lactate from either glucose or fructose. So we used the concentrations of 10 mM, which were sufficient to generate the maximum velocity of lactate formation. We confirmed the report of Anundi et al. [17] that perfusion of anoxic liver with fructose caused a tremendous increase in lactate formation (Fig. 1). We also observed a remarkable difference between the rates of lactate production during perfusion with fructose and glucose. This difference can be ascribed to the different metabolic pathways of the two substrates (see Discussion). Liver perfused with fructose produced lactate at a constant rate of about 3 μmol/min per g liver. This finding indicated that the sequential process of lactate formation, which consisted of cellular uptake of fructose, its enzymic degradation in the cytosol, and excretion of lactate from the cell, proceeded as a steady state reaction. One molecule of fructose gives rise to two molecules of ATP during its conversion to two molecules of lactate, so we estimated that approx. 3 μmol/min of ATP per g liver was formed by fructose degradation in anoxic perfused liver. This rate may be lower than that of oxidative phosphorylation under

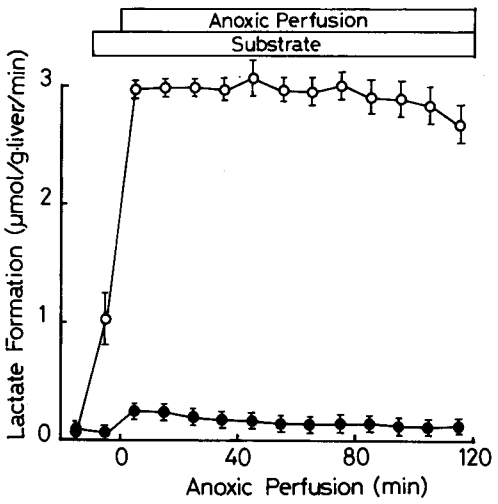

Fig. 1. Lactate formation in anoxic perfused liver. Liver perfusion was carried out as described in Materials and Methods. As substrate, fructose (○) or glucose (●) was added to the perfusion medium at a concentration of 10 mM, and the amounts of lactate released were measured in the efferent perfusate. 10 min after the start of substrate addition, oxygenated medium (O_2:CO_2 = 95:5) was replaced by medium equilibrated with nitrogen (N_2:CO_2 = 95:5). Values are means ± S.D. for four livers.

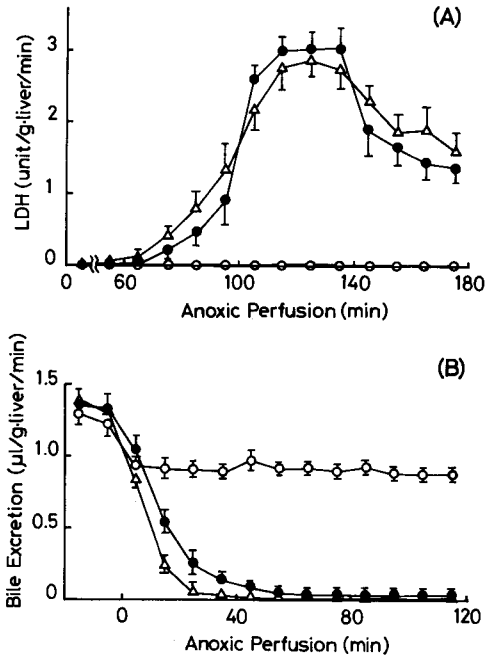

Fig. 2. Time-courses of enzyme leakage (A) and bile excretion (B) in anoxic perfused liver. Liver perfusion was carried out as described in the Materials and Methods. After 20 min of preperfusion, oxygenated medium was switched to medium equilibrated with nitrogen (N_2:CO_2 = 95:5). Simultaneously with the onset of anoxic perfusion, infusion of 10 mM fructose (○), 10 mM glucose (●), or neither (△) was started. (A) Lactate dehydrogenase (LDH) released into the efferent perfusate. (B) Weight of excreted bile (1 μl = 1 mg). Values are means ± S.D. for three livers.

aerobic conditions, but seems to be compensated for by decrease in ATP consumption due to significant decrease in the metabolic rate in anoxic liver [30]. As ATP depletion is considered to be the primary cause of ischemic or anoxic damage of living cells, this finding led us to expect that, even on oxygen deprivation, liver function would be well preserved during perfusion with fructose as substrate.

Effect of fructose on enzyme leakage and bile excretion in anoxic perfused liver

Leakage of cytosolic enzymes is a typical anoxic injury, and has been widely observed in such organs as liver and heart [13–16,28,31–33]. Fig. 2A shows the leakage of a cytosolic enzyme, lactate dehydrogenase (LDH), from anoxic liver. LDH release from liver perfused with substrate-free medium started gradually within 60 min after the onset of anoxic perfusion, and increased enormously during the subsequent 60 min, reaching a plateau and then decreasing. A similar pattern of LDH leakage was observed in anoxic liver perfused with glucose. In contrast, on perfusion with fructose, no enzyme leakage was observed for at least three hours during anoxic perfusion.

Results on bile formation during anoxia are shown in Fig. 2B. The excretion of bile is known to require

energy, and decrease in the intracellular ATP level is thought to be accompanied by decrease in the rate of bile excretion in anoxic liver or liver treated with reagents that reduce the ATP level such as ethionine or potassium cyanide [10–12]. Fig. 2B shows that, upon anoxic perfusion, bile excretion from livers perfused with glucose or substrate-free medium decreased gradually to an almost undetectable level. Use of glucose slightly delayed the cessation of bile excretion, but there seemed virtually no significant difference in the results with and without glucose. In contrast, anoxic liver perfused with fructose continued to excrete bile at a constant flow rate of approx. 0.9 μl/min per g liver. These results may be explained as due to sufficient supply of ATP by anaerobic decomposition of fructose, as predicted from Fig. 1, so we next measured the contents of adenine nucleotides in perfused liver and in mitochondria from the perfused liver.

Changes in adenine nucleotide levels in the liver and mitochondria during anoxic perfusion

Fig. 3 shows the changes in adenine nucleotides levels in anoxic livers perfused with fructose or glucose. On perfusion with glucose (Fig. 3B), the ATP level decreased with concomitant increase in the AMP level,

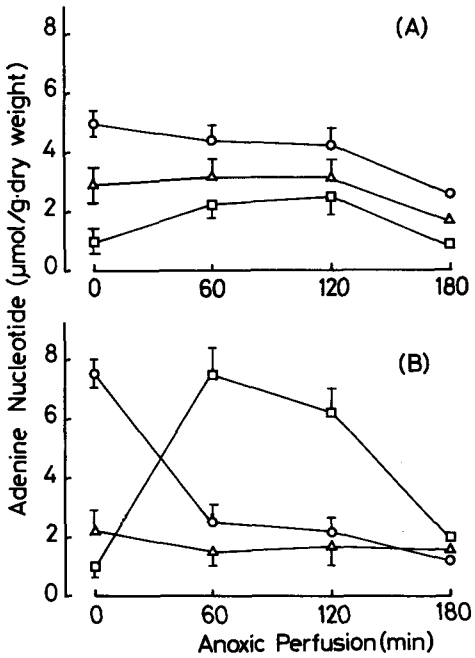

Fig. 3. Changes in adenine nucleotide levels in anoxic perfused liver. Liver perfusion was carried out as described in Materials and Methods, using fructose (A) or glucose (B) as substrate. After preperfusion for 20 min, infusion of substrate at a concentration of 10 mM was started, and 10 min later oxygenated perfusion medium was replaced by medium equilibrated with nitrogen (N_2:CO_2 = 95:5). At the times indicated, liver samples were quickly freeze-clamped for measurement of the contents of ATP (○), ADP(△) and AMP (□), as described in the Materials and Methods. Values are means ± S.D. for three livers or means for two livers.

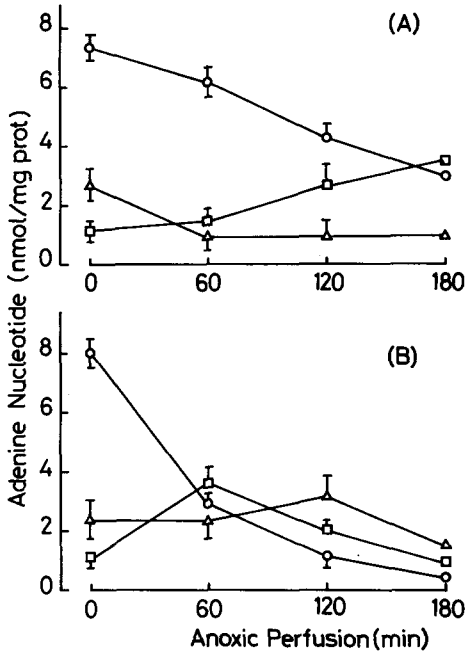

Fig. 4. Changes in adenine nucleotide levels in mitochondria in anoxic perfused liver. Liver perfusion was performed under the same conditions as for Fig. 3 using fructose (A) or glucose (B) as substrate. At the indicated times the liver was quickly removed for preparation of mitochondria, as described in Materials and Methods. The contents of ATP (○), ADP (△), and AMP (□) in the mitochondria were measured.

and then the total adenine nucleotide level gradually decreased, the degraded adenine nucleotide being recovered as purine compounds in the perfusate (data not shown) [25]. This result is consistent with reported findings in many ischemic tissues [2,17]. Perfusion with fructose, however, resulted in different patterns of change in adenine nucleotide contents (Fig. 3A): the initial ATP level was about 25% less in liver perfused with fructose than in that perfused with glucose, but this level was invariably maintained during subsequent anoxic perfusion.

The adenine nucleotides contents of mitochondria from anoxic perfused liver were also examined (Fig. 4). The level of mitochondrial ATP also decreased during anoxic perfusion, but its rate of decrease differed considerably depending on the substrate used. As observed in whole liver tissue, decrease of mitochondrial ATP was significantly slower with fructose (Fig. 4A). These results suggest that ATP produced by glycolytic decomposition of fructose compensates partially, if not completely, for the ATP consumed for basal metabolism, and preserves the liver functions.

Urea synthesis in anoxic liver

We examined whether the ATP in mitochondria of anoxic liver is available for physiological metabolism by measuring urea synthesis, as this is one of the most active reactions for ATP consumption in mitochondria.

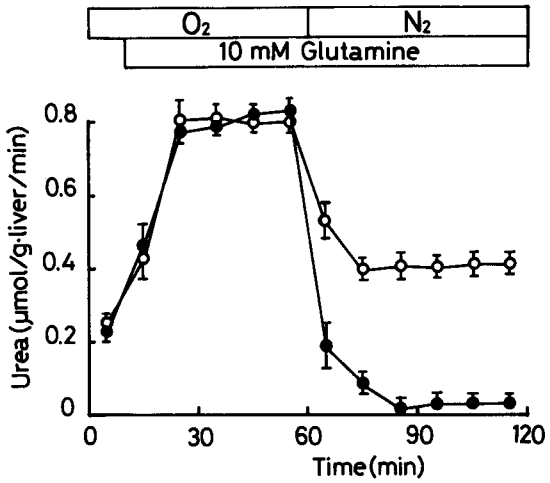

Fig. 5. Urea synthesis in anoxic perfused liver. Liver perfusion was carried out as described in the Materials and Methods with fructose (○), or glucose (●) as substrate. Glutamine at a concentration of 10 mM was infused, and the efferent medium was collected for determination of urea. Livers were first perfused with oxygenated medium and then with medium equilibrated with nitrogen (N_2:CO_2 = 95:5) as indicated in the figure. Values are means ± S.D. for three livers.

Glutamine is known to activate urea synthesis in the liver, so we used it as a nitrogen source in measurement of the output of urea in the efferent perfusate. We used 10 mM glutamine for perfusion because this concentration generates a saturated level of urea output [34]. Addition of glutamine to an oxygenated perfusate of the liver resulted in formation of about 0.8 μmol of urea/min per g liver (Fig. 5). When glucose-perfused liver was set in an anoxic state, urea output rapidly decreased to less than 5% of that initially seen under oxygenated conditions. In contrast, when the liver was supplied with fructose, switching to anoxic perfusate decreased the rate of urea formation only 50%. If NH_4Cl was used in the presence of fructose in anoxic liver, urea was also generated at the same rate as that from glutamine (data not shown). This phenomenon was not observed if glucose was employed. These results indicate that, even when mitochondria cannot synthesize ATP due to lack of oxygen, the ATP formed in the cytosol is transported into the mitochondria via ADP/ATP translocase and utilized for urea synthesis.

Preservation of mitochondrial integrity in anoxic perfused liver

It was of interest to examine whether mitochondria in anoxic liver endowed with sufficient ATP preserved their capacity for oxidative phosphorylation, since ATP is known to protect mitochondria in vitro against anoxic damage [3,4]. Table I shows the oxidative phosphorylation capacity, expressed as the respiratory control ratio (RCR), of mitochondria from anoxic perfused liver. In our experiment the control value of RCR, which was

TABLE I

Respiratory Control Ratio of mitochondria in anoxic perfused liver

Liver perfusion was carried out under the same conditions as for Fig. 3, using glucose, fructose or no added substrate. At the times indicated in the table, samples were taken and quickly homogenized in ice-cold buffer for preparation of mitochondria. The rate of oxygen consumption by isolated mitochondria was measured polarographically with a Clark-type electrode at 25 °C with succinate as substrate. RCR was determined as the ratio of state 3 respiration to the second break of state 4 respiration. Values are means ± S.D. for three determinations, or means for two determinations.

Anoxic perfusion (min)	Substrate		
	none	glucose	fructose
60	2.32 ± 0.31	2.65 ± 0.27	4.21 ± 0.41
120	1	1.66 ± 0.30	3.92 ± 0.38
180	1	1	4.17

that of mitochondria taken directly from liver in vivo, was 4.66 ± 0.38. As shown in Table I, when the liver was perfused with glucose or substrate-free medium, the RCR decreased gradually during anoxia. This decrease of the RCR was attributable mainly to an increase in the rate of state 4 respiration. The respiration rate of state 3 and that released by the uncoupler CCCP remained unchanged (data not shown), implying that the electron transfer system of mitochondria was unimpaired at least during 3 h of anoxia. When anoxic perfused liver was provided with fructose, the RCR of mitochondria in the liver remained as high as that of control mitochondria, indicating that ample ATP production in the cytosol protected the mitochondria from anoxic injury.

Finally, we examined the change of perfused liver during anoxia by spectrophotometric measurements of

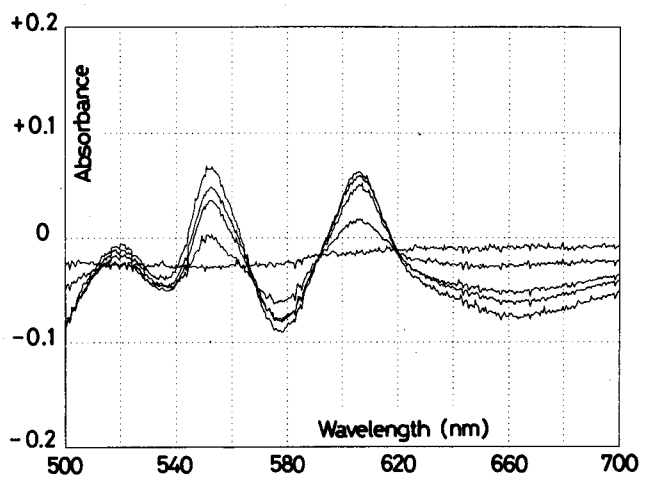

Fig. 6. Spectral change of light transmitted through the liver during anoxia. Liver perfusion was carried out as described in the Materials and Methods with fructose as substrate. Difference spectra between that initially and those after anoxic perfusion are shown. The upward peaks at 605 nm are those before and 1, 3, 5 and 10 min, respectively, after the start of anoxic perfusion.

transmitted light. Since livers were isolated and perfused with hemoglobin-free medium containing fructose, the absorbance of light transmitted through the organ at wavelengths of between 500 nm and 700 nm provided information on the oxidation-reduction state of the mitochondria. Fig. 6 shows the difference spectra between that initially and those after the onset of anoxic perfusion. The upward peaks seen at 550 nm and 605 nm, and the shoulder at about 560 nm are those of cytochrome $c + c_1$, cytochrome oxidase, and cytochrome b, respectively. The isobestic points were at 592 nm and 618 nm, indicating that the upward peak of 605 nm is composed solely of the peak of the reduced form of cytochrome oxidase. Upward change of the peak due to cytochrome oxidase was saturated 10 min after the start of anoxic perfusion. This implies that the electron transport system in mitochondria was kept in the fully reduced state thereafter; namely, the results confirmed that mitochondria were actually incapable of synthesizing ATP during anoxic perfusion.

Discussion

In the present paper we described the effect of fructose in protecting the liver function against anoxic damage. Upon addition of fructose to the perfusion fluid of anoxic liver, lactate formation increased markedly to about 3.0 μmol/min per g liver (Fig. 1), indicating that ATP was also produced at the same rate in the liver. Under normal conditions, the rate of oxygen consumption in the perfused liver is reported to be about 1.5 μmol/min per g liver [35]. Thus the physiological rate of ATP production, which is equal to the ATP consumption rate, was calculated to be 9.0 μmol/min per g liver assuming that the P/O ratio was 3. Although the rate of ATP production during anoxia was one third of its rate of consumption in aerobic conditions, the amount of ATP produced by the accelerated degradation of fructose seemed to be sufficient to maintain cytosolic and mitochondrial functions in anoxic liver, where total metabolism is decreased [30]. In anoxic liver perfused with fructose, we observed continuous excretion of bile (Fig. 2B) which is dependent on the energy of ATP in the cytosol, and persistent biosynthesis of urea (Fig. 5) which consumes ATP in the matrix space, as well as in the cytosol. Moreover, under these conditions the mitochondria in the liver contained a large amount of ATP, although they were incapable of synthesizing ATP, judging from the fact that their electron transfer system was in the completely reduced state (Fig. 6). So, it is reasonable to consider that ATP formed in the cytosol was transported into mitochondria via ATP/ADP translocase. However, physiologically, translocation of external ATP into the mitochondria in exchange for internal ADP is an unfavorable flow, as the inside negative, outside positive electrical gradient across the inner membrane is favorable for extrusion of ATP^{4-} with simultaneous uptake of ADP^{3-}. This situation may be changed under anoxia, since interruption of electron transport by anoxia diminishes the membrane potential, and the decomposition of ATP in the matrix space, if it occurs, generates P_i^{2-} and H^+, the latter presumably contributing to decrease of the proton gradient, or even formation of the reverse proton gradient. Therefore, it is considered that the motive force of ATP transfer in exchange for ADP operates in the reverse direction in anoxia. The urea synthesis observed during anoxia may reflect this situation. As seen in Fig. 5, in perfused liver supplied with fructose, switching from oxygenated to anoxic conditions decreased urea synthesis about 50%. As the cell integrity was well preserved by maintenance of a high level of ATP, the enzymic reactions related to urea synthesis are unlikely to be affected simply by deprivation of oxygen. Thus, the most probable rate-limiting step in these conditions is the transport of ATP from the cytosol into the matrix space.

Protection of mitochondrial functions against anoxic damage has been widely reported, though the precise mechanism is still controversial. Previously we reported that the oxidative phosphorylation capacity of anoxic mitochondria could be preserved not only by ATP but also by its non-metabolizable analogs [4]. This indicates that for protection of mitochondria against anoxic damage, the structure, not the energy of ATP is required. In the present work we demonstrated that if a sufficient ATP-generating system other than that of mitochondria was functioning in the cells, the oxidative phosphorylation capacity of the mitochondria was maintained, even if their electron transfer system was kept in a completely reduced state.

Fructose metabolism requires consideration as an ATP-generating system in the cytosol of the liver. In animals, the liver is the principal organ for fructose metabolism, and the metabolic pathway of fructose was elucidated in the early 1970's [19,21,22,36,37]. Fructose trapped by hepatocyte is first phosphorylated to form fructose 1-phosphate (F1P), and then cleaved to dihydroxyacetone phosphate and glyceraldehyde. The maximal rates of the enzymes synthesizing and splitting F1P, ketohexokinase and ketose 1-phosphate aldolase, respectively, are about equal, being approx. 3.1 μmol/min per g liver tissue [21]. The activities of the enzymes involved in the subsequent degradation steps are much higher than this [21]. So if the step catalyzed by ketohexokinase or aldolase is rate-limiting in fructose metabolism, the possible rate of lactate formation is at most 6.2 μmol/min per g liver. Considering that the velocity of cellular uptake of fructose may be smaller than the activity of ketohexokinase or aldolase [21], our value (Fig. 1) is reasonable compared with this theoretical value. It should be noted, however, that

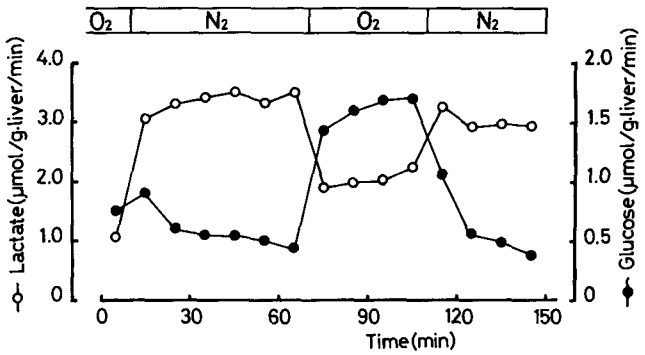

Fig. 7. Conversion of fructose to glucose and lactate during oxygenated/anoxic perfusion of liver. Liver was perfused with oxygenated or anoxic medium containing 10 mM fructose as described in the Materials and Methods. The efferent perfusate was collected for measurement of lactate (○) and glucose (●).

conversion of fructose to lactate is significantly accelerated in the liver in anoxic conditions (Fig. 7). In oxygenated conditions fructose trapped by the cell is more likely to be directed to gluconeogenesis than to lactate production. One possible explanation for this phenomenon is that decrease in the ATP/ADP ratio in anoxia mobilizes ATP formation at the step of phosphoglycerate kinase, leading to increase in lactate formation.

In contrast to fructose, glucose metabolism in the liver is hormonally restricted by glucokinase and phosphofructokinase I (PFK I). Glucokinase is an inducible enzyme under the control of insulin and a high concentration of glucose, and its content in the liver is drastically decreased by starvation [19]. Regulation of PFK I in response to variation in fructose 2,6-bisphosphate (F26*P*) has been studied extensively during the 1980's [20,38–40]. The maximal rate of PFK I is reported to be 2–3 μmol/min per g liver [19,41]. However, in starved rats, the content of F26*P* is as low as 3 nmol per g liver, and in this condition PFK I exhibits less than 5% of its V_{max} activity (our unpublished data). These enzymic analyses explain why fructose is utilized more efficiently than glucose, both in anaerobic and aerobic livers. Acceleration of fructose metabolism in anoxic liver produces a large amount of lactate, as well as ATP. Physiologically, this is not altogether a favorable process, because the liver is the principal organ that traps and metabolizes lactate in the blood vessels.

From a view point of clinical use of fructose, it is interesting to look over effect of fructose on the aerobic liver. In the normal aerobic liver, administration of fructose leads to the rapid accumulation of F1*P* with simultaneous decreases in ATP and inorganic phosphate (P_i) [21,37]. The depletion of hepatic ATP results in the inhibition of protein synthesis and a rapid increase in the serum level of uric acid [37]. These adverse effects of fructose have been considered to be dangerous for critically ill patients. However, these effects are observed only when massive fructose is loaded. Previously we reported that in the liver perfused with fructose at concentrations of 2 mM or less, no decrease in ATP content was observed [42]. Transient decrease in P_i content in liver was caused by fructose, but suppression of respiration due to P_i depletion was significantly observed at fructose concentrations of more than 5 mM. The blood fructose concentration in human subjects given fructose for total parenteral nutrition is much lower than this [43]. Although care must be taken in employing fructose clinically, we believe that low concentrations of fructose do not have unfavorable effects, but that it is useful for supplying the liver with energy.

References

1 Mittnacht, Jr., S. and Farber, J.L. (1981) J. Biol. Chem. 256, 3199–3206.
2 Kamiike, W., Watanabe, F., Hashimoto, T., Tagawa, K., Ikeda, Y., Nakao, K. and Kawashima, Y. (1982) J. Biochem. 91, 1349–1356.
3 Watanabe, F., Kamiike, W., Nishimura, T., Hashimoto, T. and Tagawa, K. (1983) J. Biochem. 94, 493–499.
4 Watanabe, F., Hashimoto, T. and Tagawa, K. (1985) J. Biochem 97 1229–1234.
5 Hochachka, P.W. (1986) Science 231, 234–241.
6 Hochachka, P.W. (1985) Mol. Physiol. 8, 331–350.
7 Jones, D.P. (1986) Annu. Rev. Physiol. 48, 33–50.
8 Mittnacht, Jr., S., Sherman, S.C. and Farber, J.L. (1979) J. Biol. Chem. 254, 9871–9878.
9 Shug, A.L., Shrago, E., Bittar, N., Folts, J.D. and Koke, J.R. (1975) Am. J. Physiol. 228, 689–692.
10 Slater, T.F. and Delaney, V.B. (1971) Toxicol. Appl. Pharmacol. 20, 157–174.
11 Slater, T.F. and Delaney, V.B. (1970) Biochem. J. 116, 303–308.
12 Kamiike, W., Nakahara, M., Nakao, K., Koseki, M., Nishida, T., Kawashima, Y., Watanabe, F. and Tagawa, K. (1985) Transplantation, 39, 50–55.
13 Koseki, M., Nishida, T., Kamiike, W., Nakao, K., Kawashima, Y., Dan, C., Wake, K., Yoshida, Y. and Tagawa, K. (1988) Biomed. Res. 9, 47–57.
14 Lemasters, J.J., Stemkowski, C.J., Ji, S. and Thurman, R.G. (1983) J. Cell. Biol. 97, 778–786.
15 Nishimura, T., Yoshida, Y., Watanabe, F., Koseki, M., Nishida, T., Tagawa, K. and Kawashima, Y. (1986) Hepatology 6, 701–707.
16 Jaeschke, H., Smith, C.V. and Mitchell, J.R. (1988) Biochem. Biophys. Res. Commun. 150, 568–574.
17 Anundi, I., King, J., Owen, D.A., Schneider, H., Lemasters, J.J. and Thurman, R.G. (1987) Am. J. Physiol. 253, G390–G396.
18 Jaeschke, H. and Mitchell, J.R. (1989) Biochem. Biophys. Res. Commun. 160, 140–147.
19 Newsholm, E.A. and Start, C. (1973) Regulation in Metabolism, John Wiley, New York.
20 Pilkis, S.J., El-Maghrabi, M.R. and Claus, T.H. (1988) Ann. Rev. Biochem. 57, 755–783.
21 Woods, H.F., Eggleston, L.V. and Krebs, H.A. (1970) Biochem. J. 119, 501–510.
22 Sillero, M.A.G., Sillero, A. and Sols, A. (1969) Eur. J. Biochem. 10, 345–350.
23 Sugano, T., Suda, K., Shimada, M. and Oshino, N. (1978) J. Biochem. 83, 995–1007.
24 Johnson, D. and Lardy, H. (1967) Methods Enzymol. 10, 94–96.

25 Nishida, T., Koseki, M., Kamiike, W., Nakahara, M., Nakao, K., Kawashima, W., Hashimoto, T. and Tagawa, K. (1987) Transplantation 44, 16–21.
26 Reeves, Jr., W.J. and Fimognari, G.M. (1966) Methods Enzymol. 9, 288–294.
27 Kerscher, L. and Ziegenhorn, J. (1985) Methods of Enzymatic Analysis 3rd edn., Vol. 8, pp. 444–453.
28 Takami, H., Matsuda, H., Kuki, S., Nishimura, M., Kawashima, Y., Furuya, E. and Tagawa, K. (1990) Pflügers Archv. 416, 144–150.
29 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
30 Storey, K.B. (1985) Mol. Physiol 8, 439–461.
31 Hearse, D.J., Garlick, P.B. and Humphrey, S.M. (1977) Am. J. Cardiol. 39, 986–993.
32 Hearse, D.J. and Humphrey, S.M. (1975) J. Mol. Cell. Cardiol. 7, 463–482.
33 Van der Heide, R.S. and Ganote, C.E. (1987) J. Mol. Cell. Cardiol. 19, 1085–1103.
34 Inoue, T., Yamada, T., Furuya, E. and Tagawa, K. (1989) Biochem. J. 262, 965–970.
35 Yoshihara, H. and Thurman, R.C. (1987) Am. J. Physiol. 253, G383–G389.
36 Ravio, K.O., Kekomäki, M.P. and Mäenpää, P.H. (1969) Biochem. Pharmacol. 18, 2615–2624.
37 Mäenpää, P.H., Ravio, K.O. and Kekomäki, M.P. (1968) Science 161, 1253–1254.
38 Furuya, E., Yokoyama, M. and Uyeda, K. (1982) Proc. Natl. Acad. Sci. USA 79, 325–329.
39 Uyeda, K., Furuya, E., Richards, C.S. and Yokoyama, M. (1982) Mol. Cell. Biochem 48, 97–120.
40 Van Schaftingen, E. (1987) Adv. Enzymol. 59, 315–395.
41 Reinhart, G.D. and Lardy, H.A. (1980) Biochemistry 19, 1477–1484.
42 Nishi, T., Kido, Y., Furuya, E., Tagawa, K. and Mori, T. (1989) Jpn. J. Surg. 19, 351–357.
43 Leutenegger, A.F., Cäschke, H., Stutz, K., Mannhart, H., Werdenberg, D., Wolff, G. and Allgower, M. (1977) Am. J. Surg. 133, 199–205.

Pflügers Arch (1990) 416:144–150

参考論文

Pflügers Archiv
European Journal of Physiology

Leakage of cytoplasmic enzymes from rat heart by the stress of cardiac beating after increase in cell membrane fragility by anoxia

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Received March 29/Received after revision June 1, October 2, November 13/Accepted November 20, 1989

Abstract. The effects of spontaneous beating after anoxia and the pumping stress induced by a left ventricular balloon on the leakage of myocardial enzymes from the isolated perfused rat heart were investigated. Beating of the heart was arrested by perfusion with high-K^+ medium. When the beating was arrested during reoxygenation after anoxia, the leakage of lactate dehydrogenase (LDH) was significantly lower than during reoxygenation with spontaneous cardiac beating. After changing from K^+ arrest to spontaneous beating by perfusion with low-K^+ medium during reoxygenation, the leakage of LDH increased markedly. Imposition of left ventricular wall stress on the K^+-arrested heart by repetitive passive distension during aerobic perfusion and after 20 min and 60 min of anoxia caused LDH leakages of 1.0, 4.6 and 21.0 units/g in 30 min, respectively. Under this mechanical stress, the release of LDH as a percentage of its total myocardial activity coincided well with that of cytoplasmic aspartate aminotransferase (AST), while the percentage release of mitochondrial AST was much less. These results appeared to indicate that the leakage of cytoplasmic enzymes during reoxygenation is accelerated by cardiac beating because of fragility of the cell membranes developing during the preceding anoxia.

Key words: Enzyme release – Heart – Anoxia – Reoxygenation – Heart arrest – Adenosine triphosphate

Introduction

The leakage of myocardial enzymes is used as an indicator of ischemic damage of the heart (Hearse 1977). The cause of enzyme leakage is probably due to many factors, including anoxic injury (Hearse and Humphrey 1975), the oxygen paradox (Hearse et al. 1973), mechanical stress (Vander Heide and Ganote 1987) and activated leukocytes (Romson et al. 1983). However, the mechanism by which leakage is induced has not yet been fully elucidated. Recently, anoxic injury has been recognized to cause destruction of the cytoskeleton and bleb formation of the cell membrane (Lemasters et al. 1983; Ganote and Vander Heide 1987), which is likely to lead to membrane fragility. This fragility of the cell membrane may become apparent when the mechanical stress of heartbeat is imposed during reperfusion.

We have reported that in ischemic liver, blebs of cell membrane protruding into the sinusoidal space are formed, and that during reperfusion the shear stress of the bloodstream disrupts these blebs, resulting in the release of cytoplasmic enzymes (Koseki et al. 1988). A similar formation of blebs and, consequently, fragility of the cell membrane occurs in ischemic or anoxic myocardium (Vander Heide and Ganote 1987; Steenbergen et al. 1987). However, the factors that accelerate enzyme leakage through fragile cell membranes in the heart may be different from those in the liver because of structural differences in the cytoskeleton and microvasculature in these organs. We supposed that the beating of the heart after anoxia is a possible trigger of enzyme leakage, since it enhances mechanical stress.

In this study, utilizing elective cardiac arrest induced by perfusion with high-K^+ medium (Melrose et al. 1955), we investigated the influence of cardiac beating after anoxia on myocardial enzyme leakage. We compared the leakages of cytoplasmic and mitochondrial enzymes, and also examined the release of lactate, which is considered to be easily released from myocardial cells by carrier-mediated facilitated diffusion. The vulnerability of the myocardium, caused by anoxic injury, towards mechanical stress was assessed by examining the effect on enzyme leakage of repetitive distension of the left ventricular (LV) wall via an intraventricular balloon. The results showed that cardiac beating plays a significant role in inducing cytoplasmic enzyme leakage after anoxia.

Materials and methods

Preparation of isolated perfused heart. Male Sprague-Dawley rats, weighing about 240 g, were used after fasting for 24 h. They were

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anesthetized by intraperitoneal injection of pentobarbital (50 mg/kg body weight), and their hearts were rapidly removed. The aorta was promptly cannulated and perfusion was started by the Langendorff technique at a perfusion pressure of 90 cm H_2O. The perfusate, consisting of (in mM) NaCl 120, KCl 4.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25 and $CaCl_2$ 2.5, was equilibrated with 95% O_2/5% CO_2 at 33°C. Glucose was omitted from the perfusate to restrict the energy supply from anaerobic glycolysis except in an experiment on lactate release. Anoxic perfusion was performed with perfusate equilibrated with 95% N_2/5% CO_2, in which the P_{O_2} was below 15 mm Hg (2 kPa) as measured with a Clark-type P_{O_2} electrode. Ischemia was induced by clamping the perfusion line, while maintaining the heart at 33°C. Reoxygenation or reperfusion was performed with the oxygenated perfusate.

Cardiac beating was arrested by perfusion with high-K^+ medium, in which the K^+ concentration was raised to 25.7 mM and isotonic osmolarity was maintained by reducing the concentration of NaCl. On switching from perfusion with high-K^+ medium to that with the usual low-K^+ medium, spontaneous beating of the heart was resumed within 3 min. The pressure in the left ventricle was monitored with a loosely fitting LV balloon, which was filled with water and connected to a Statham P-23 DI transducer. During anoxic perfusion, high-K^+ medium was used to restrict the burst of cardiac beating in the transient hypoxic condition.

To induce mechanical stress in hearts arrested by high-K^+ perfusate, LV balloon distension via an intraventricular balloon or a high coronary flow rate was imposed intermittently during aerobic and anoxic perfusion. For creating wall stress, the LV balloon, made of latex rubber, was inflated to 0.15 ml volume and deflated, 70 times/min for 10 min, with a pump normally used for high performance liquid chromatography (HPLC). The peak pressure in the left ventricle, attained at the maximal balloon volume of 1.5 ml, was measured in a separate experiment with perfused hearts of the same size. The LV pressure during balloon inflation was 27 mm Hg at aerobic perfusion, 35 mm Hg after anoxic perfusion for 20 min, and 60 mm Hg after anoxic perfusion for 60 min. These levels of peak LV pressure were lower than those attained during spontaneous cardiac beating. A high coronary flow rate was induced at a constant perfusion rate, and the stress of a high flow rate (15 ml/min) was imposed intermittently for 10 min on the heart, which was otherwise perfused at a low flow rate (3 ml/min) under anoxia.

Biospectrometry of heart. Changes in the oxygenation state of myoglobin and the oxidation-reduction state of cytochrome oxidase in the perfused heart under K^+ arrest were measured with a biospectrometer MS-401 (Unisoku Co.) (Tamura et al. 1978). The absorbance of light transmitted through the heart at wavelengths of between 500 nm and 900 nm was scanned for 10 s. Spectra were recorded once a minute at the start of anoxic perfusion and of reoxygenation.

Measurement of enzyme activity. The total coronary effluent was collected every 2, 5, or 10 min and the volume of the effluent was measured. The activities of lactate dehydrogenase (LDH, EC 1.1.1.27) and cytoplasmic aspartate aminotransferase (cAST, EC 2.6.1.1) in the effluent were measured to assess cytoplasmic enzyme leakage. Mitochondrial AST (mAST) was also measured to assess mitochondrial enzyme leakage. At the end of perfusion, the heart was homogenized and the enzyme activities retained in the myocardium were measured. Enzyme leakage was expressed in the figures as the rate of release [units min^{-1} (g wet weight of heart)$^{-1}$] or as a percentage of the cumulative release of the total myocardial enzyme activity.

The activity of LDH was determined by the spectrophotometric procedure reported by the German Society for Clinical Chemistry (GSCC 1972). The activities of AST were determined by a modification of the GSCC procedure described previously (Nishimura et al. 1986). The mAST and cAST isozymes were separated immunochemically (Morino et al. 1964). Briefly, cAST in the specimen was adsorbed by sheep red blood cells sensitized with antibody against pig cAST, and the remaining unadsorbed AST activity was determined as the activity of mAST (Teranishi et al. 1988). The assay kit was kindly provided by Eiken Co. (Tokyo, Japan).

For the measurement of myocardial enzyme activities, the hearts were minced in cold sodium phosphate buffer and homogenized gently in a polytron. The homogenate was centrifuged at 5 000 *g* for 10 min, the supernatant was diluted and triton X-100 was added to disrupt mitochondria. By this procedure the activities of LDH, cAST, and mAST in the myocardium were found to be 410 ± 75, 63 ± 9, and 84 ± 12 units/g wet weight (means $\pm$ SD, $n = 7$), respectively.

Measurement of lactate. The concentration of lactate was determined electrochemically using a column of immobilized enzyme. The principle of this method was the same as that of the method for measuring glucose described previously (Furuya et al. 1986). Lactate oxidase (EC 1.1.3.2) was immobilized in controlled-pore glass and packed in polyethylene tubing. The flow rate of the elution buffer, consisting of 50 mM sodium phosphate, pH 6.8, 150 mM NaCl, 1 mM NaN_3, and 0.1% triton X-100, was 1 ml/min maintained with an HPLC pump. The H_2O_2 formed by the immobilized lactate oxidase was detected with an electrochemical H_2O_2 detector and the current was amplified and recorded. With this system, levels of lactate of above 10 pmol could be determined precisely.

The concentration of lactate in the coronary effluent was determined directly with this system, while that in the myocardium was measured after extraction of the freeze-clamped and lyophilized myocardium with perchloric acid as in the chemical measurement of ATP described below.

Myocardial ATP level. For estimation of the energy depletion of myocardial cells under anoxia, the myocardial adenosine 5′-triphosphate (ATP) level was measured by both chemical analysis and ^{31}P nuclear magnetic resonance (NMR) analysis. For chemical analysis of ATP, the myocardium was freeze-clamped and lyophilized. The preparation of perchloric acid extracts and determination of ATP by HPLC were carried out as reported previously (Kamiike et al. 1982). Myocardial ATP was measured chemically during aerobic perfusion, after anoxic perfusion for 20 min, 40 min and 60 min. In separate experiments under the same conditions, the myocardial ATP level was measured by ^{31}P-NMR analysis, using a WM-360 wb NMR spectrometer with Fourier transformation. The frequency employed was 145.8 MHz and radiofrequency pulses of 10 μs were used as 15° pulses. Free induction decays of 400 data points were accumulated for about 150 s. The area of the β-phosphate peak of ATP at each point of anoxic perfusion was compared with that of the control and the ATP level was expressed as a percentage of the control.

Results

Spectral change of the heart during anoxia and reoxygenation

The spectrum of a perfused heart under K^+ arrest changed markedly after transition to anoxia and on reoxygenation because of changes in the oxygenation state of myoglobin and in the redox state of cytochrome oxidase (Fig. 1). The spectra in Fig. 1 are difference spectra between that initially and those at the indicated times after the start of anoxic perfusion. The downward peaks at 540 nm and 580 nm are those of deoxymyoglobin and the upward peak at 605 nm is mainly that of the reduced form of cytochrome oxidase (Tamura et al. 1978). This spectral change of the heart due to myoglobin and cytochrome oxidase reached a plateau about 10 min after the start of anoxic perfusion. On reoxygenation after anoxic perfusion for 60 min, the spectrum reverted com-

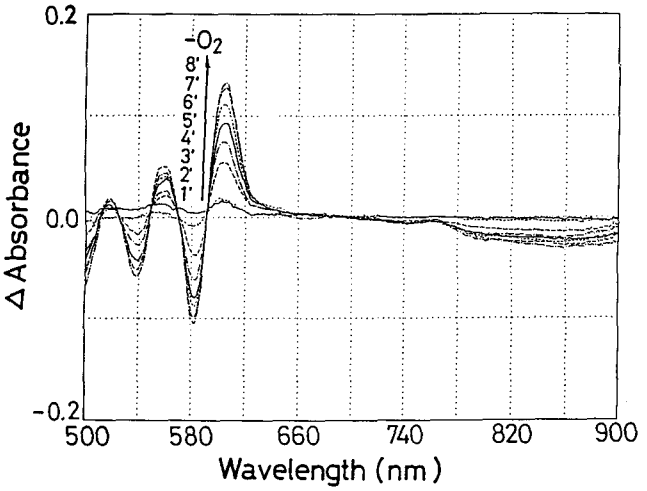

Fig. 1. Spectral change of heart. Typical spectra of five experiments are shown. Spectra were determined by biospectrometry once a minute after the transition from aerobic to anoxic perfusion. The downward peaks at 540 nm and 580 nm are due to the absorbance of deoxymyoglobin, and the upward peak at 605 nm is mainly due to the absorbance of the reduced form of cytochrome oxidase. These spectral changes reached a plateau about 10 min after the start of anoxic perfusion. On reoxygenation after 60 min of anoxic perfusion, the spectrum reverted to that of the previous aerobic state

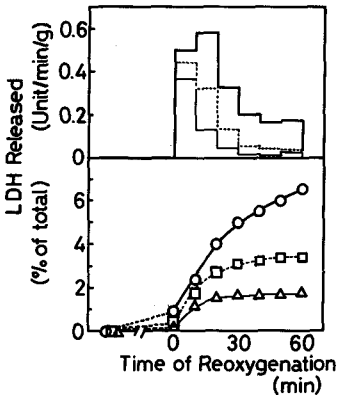

Fig. 2. Lactate dehydrogenase (LDH) release during reoxygenation of hearts beating after anoxia. Rates of LDH release (units min^{-1} g^{-1}) during reoxygenation after 20 min (——), 40 min (-----), and 60 min (|——|) are shown as columns in the *upper panel* ($n = 1$ in respective anoxic period). Cumulative LDH releases, expressed as percentages of total myocardial activities, after 20 min (△——△), 40 min (□——□), and 60 min (○——○) of anoxia are plotted in the *lower panel*

pletely to that of the oxy state. These spectral changes reflected the deprivation and delivery of oxygen in the arrested heart during anoxic perfusion and reoxygenation, respectively.

Enzyme leakage during reoxygenation and spontaneous beating

In this model of the perfused heart there was no significant leakage of LDH or AST during aerobic perfusion for about 150 min with spontaneous cardiac beating; the rate of LDH release was less than 0.05 unit min^{-1} (g wet weight of heart)$^{-1}$. During anoxic perfusion, however, the enzymes started to leak out after about 30 min.

When hearts were perfused with oxygenated low-K^+ medium after 20, 40 and 60 min of anoxic perfusion, they resumed beating and the release of LDH increased markedly and in proportion to the preceding time of anoxia (Fig. 2). Total LDH releases following 20, 40 and 60 min of anoxic perfusion and reoxygenation with beating were 6.6, 11.3 and 22.4 units/g, respectively, which corresponded to 1.8%, 3.3% and 6.5% of the total myocardial LDH activity of these hearts.

The influence of spontaneous cardiac beating on enzyme leakage during reoxygenation was evaluated by controlling the resumption of beating by perfusion with high- or low-K^+ medium. When the heartbeat was arrested by perfusion with high-K^+ medium during reoxygenation after 60 min of anoxia, the rate of LDH leakage doubled during reoxygenation and the cumulative LDH release during 60 min of reoxygenation was 8.4 units/g (Fig. 3, top). When the heart was perfused with low-K^+

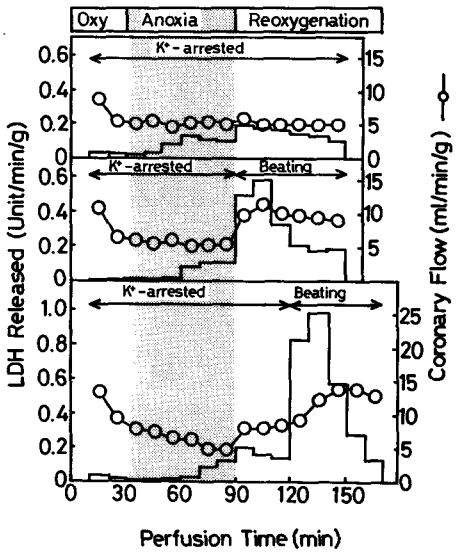

Fig. 3. LDH release from perfused hearts during anoxia, reoxygenation, and spontaneous cardiac beating. *Columns* show the rates of LDH release (units min^{-1} g^{-1}); ○, coronary flow rate (ml min^{-1} g^{-1}). *Top*, K^+-arrested perfusion was continued throughout reoxygenation; *middle*, K^+-arrested perfusion was changed to spontaneous beating by perfusion with low-K^+ medium at the time of reoxygenation; *bottom*, K^+ arrest was changed to spontaneous beating after 30 min of reoxygenation ($n = 1$ in each experiment)

medium and beat spontaneously during reoxygenation, the rate of LDH leakage on reoxygenation increased about fourfold and the cumulative LDH release during 60 min of reoxygenation with beating was 19.3 units/g (Fig. 3, middle). Furthermore, when the heart was perfused with high-K^+ medium for 30 min during reoxygenation and then beating was restored by low-K^+ medium,

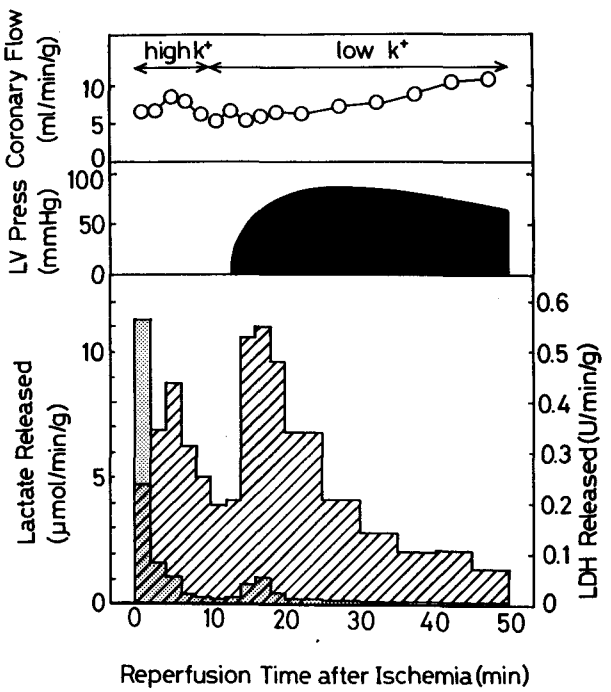

Fig. 4. Release of lactate and LDH from an ischemic heart upon reperfusion and the onset of beating. The heart was reperfused after ischemia for 30 min at 33° C. Reperfusion was started with high-K^+ medium for 10 min, and then the perfusate was switched to low-K^+ medium to reinitiate spontaneous beating. The coronary flow rate (ml min^{-1} g^{-1}) and left ventricular (*LV*) pressure (mm Hg) are shown in the *top* and *middle panels*, respectively. Rates of lactate and LDH releases are shown in the *bottom panel* as *stippled columns* (▩, μmol min^{-1} g^{-1}) and *hatched columns* (▨, units min^{-1} g^{-1}), respectively ($n = 1$)

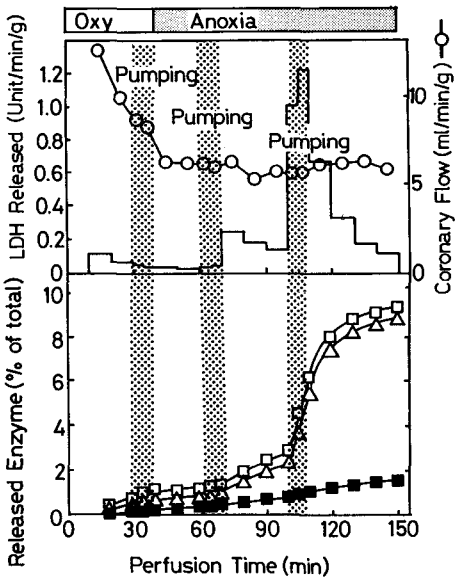

Fig. 5. Influence of mechanical wall stress on enzyme leakage from the heart arrested with high-K^+ medium. Left ventricular wall stress due to repetitive filling of an intraventricular balloon (pumping) was imposed for 10 min during aerobic perfusion, and after anoxic perfusion for 20 min and 60 min. Rates of LDH release (*open columns*, units $min^{-1}g^{-1}$) and coronary flow rates (○, ml min^{-1} g^{-1}) are shown in the *upper panel*. Cumulative releases of LDH (△), cytoplasmic aspartate aminotransferase (□), and mitochondrial aspartate aminotransferase (■), expressed as percentages of their total myocardial activities, are shown in the *lower panel* ($n = 1$)

a marked increase in the rate of LDH leakage occurred on resumption of beating (Fig. 3, bottom). The cumulative LDH release during 30 min of reoxygenation under K^+ arrest was 4.9 units/g, and that during 50 min of subsequent perfusion with beating was 27.8 units/g.

The changes in release of cAST activity coincided well with those of release of LDH described above. However, the release of mAST activity was small, less than 10% of the total release of AST activity.

Releases of lactate and LDH during reperfusion after ischemia

Next we compared the leakage of LDH with the release of lactate during reperfusion after ischemia. For this, the heart was perfused with oxygenated medium containing 20 mM glucose and then subjected to ischemia for 30 min at 33° C. After ischemia, the heart was perfused with oxygenated high-K^+ medium without glucose for 10 min, and then with low-K^+ medium to reinitiate spontaneous beating.

The rate of release of lactate formed during ischemia was greatest during the first 2 min of reperfusion, and the amount released during the first 10 min of reperfusion (K^+-arrested period) was 28.1 μmol/g wet weight of heart. This accounted for 83.4% of the total lactate release during 50 min of reperfusion (Fig. 4). In a parallel experiment, the myocardial lactate contents at the end of ischemia and after reperfusion with high-K^+ medium for 10 min were found to be 29.1 μmol/g and 2.7 μmol/g wet weight of heart, respectively. The amount of lactate released into the effluent in this interval corresponded well with the amount of myocardial lactate lost. In contrast of lactate release in this experiment, the leakage of LDH during reperfusion was biphasic, with one peak after reperfusion for about 5 min under K^+ arrest and another larger peak during the subsequent stage of spontaneous beating. Cumulative LDH release during the first 10 min of reperfusion (K^+-arrested period) was 3.1 units/g, and that during the following 40 min of perfusion with beating was 8.6 units/g.

Enzyme leakage during mechanical stress

To evaluate the vulnerability of the heart to mechanical stress, we distended the LV of the arrested heart passively (repetitive pumping of a balloon) during aerobic perfusion, and after 20 min and 60 min of anoxic perfusion. Mechanical wall stress during aerobic perfusion caused little LDH leakage (1.0 unit/g in 30 min), the stress after 20 min of anoxia caused moderate leakage (4.6 units/g in 30 min), and marked leakage (21.0 units/g in 30 min) was

Table 1. Changes in ATP level during anoxic perfusion measured by chemical and NMR analyses[a]

Anoxic perfusion time (min)	Analysis of ATP level	
	chemical $n = 5$ (μmol/g dw.)	NMR $n = 9$ (%)
0	19.3 ± 2.4	100
20	8.2 ± 2.2	42 ± 8.6
40	3.5 ± 1.3	0 ± 0
60	2.8 ± 1.2	0 ± 0

[a] In the chemical analysis, the myocardium was freeze-clamped, lyophilized, and extracted with perchloric acid. The ATP content was then measured by HPLC. In separate experiments under the same conditions, the myocardial ATP level was measured by ^{31}P-NMR as described in Materials and methods and the level was expressed as a percentage of the control. Data are presented as means ± SD

induced by the stress after 60 min of anoxia (Fig. 5). These LDH releases following mechanical stress after 20 min and 60 min of anoxia were similar to those shown in Fig. 2 following reoxygenation and spontaneous beating after 20 min and 60 min of anoxia, respectively. The cumulative releases of cAST and mAST as percentages of their total myocardial activities are plotted with that of LDH in the lower panel of Fig. 5. As in the case of LDH, mechanical wall stress under anoxia accelerated the leakage of cAST, and its percentage release was the same as that of LDH. In contrast, the leakage of mAST occurred irrespective of mechanical stresses and its percentage was much smaller than those of the two cytoplasmic enzymes LDH and cAST. At the end of this experiment, cumulative LDH, cAST and mAST releases were 8.6%, 9.4% and 1.6% of their respective total myocardial activities.

The influence of a high coronary flow rate on enzyme leakage was also examined. As in the experiment on distention-induced stress, the stress of high flow was imposed during aerobic perfusion, and after 20 min and 60 min of anoxic perfusion. Increase in leakage of LDH, cAST or mAST caused by the stress of high flow in anoxia was much smaller, less than 5% of that elicited by distention.

Myocardial ATP level during anoxic perfusion

The myocardial ATP content measured by chemical analysis during aerobic perfusion under K^+ arrest was 19.3 ± 2.4 μmol/g dry weight of heart (mean ± SD, $n = 5$) (Table 1). It decreased during anoxic perfusion, being 3.5 ± 1.3 μmol/g dry weight (18% of control) after 40 min of anoxia. The ATP level measured by ^{31}P-NMR decreased more rapidly in anoxia, reaching zero after 40 min ($n = 9$). Therefore, the ATP detectable by ^{31}P-NMR was considered to be depleted at about this time.

Discussion

This study showed that during reoxygenation after anoxia and during reperfusion after ischemia, the leakages of LDH and cAST from the heart were small during arrest of spontaneous beating by high-K^+ medium but increased markedly when spontaneous beating was resumed. During this K^+-arrested period, full delivery of oxygen to the myocardium was confirmed biospectrometrically, and most of the lactate in the myocardium was released. These findings appear to indicate that the tissue was thoroughly perfused, and that the influence of the no-reflow phenomenon (Humphrey et al. 1984) was small during the period of cardiac arrest. In the beating state, release of enzymes might be facilitated by the enhanced washout from the interstitial space, and the sudden increase of LDH release with the onset of beating may be partly due to this. However, the large quantities of LDH released indicate that cardiac beating caused extra injury to the myocardium. The release of lactate from cells is thought to be due to carrier-mediated facilitated diffusion, as is the case with purine compounds (Nishida et al. 1987), but the release mechanism of enzymes from cells seems different. Accelerated enzyme leakage after resumption of cardiac beating in this study appears to indicate that the mechanical stress of beating induces leakage of cytoplasmic enzyme from cells. Spontaneous beating of the heart, however, did not provoke enzyme leakage during aerobic perfusion, and the leakage of LDH during reoxygenation after anoxia increased in proportion to the period of anoxia. Therefore, the vulnerability of the myocardium to the stress of cardiac beating appears to develop latently during anoxia. This concept was supported by our finding of marked enzyme leakage in response to mechanical wall stress, induced with a repetitively inflated LV balloon, only after anoxia, but not during aerobic perfusion.

The mechanical stress of spontaneous cardiac beating and that induced by the intraventricular balloon may be different in nature, but in comparison to the state of cardiac arrest they seem to have considerable similarity, because both stresses exert rapid periodic changes in the pressure in myocytes and their shapes. It is likely that cell membranes undergo some stress during these changes. After anoxia, the stresses of spontaneous cardiac beating and balloon distention accelerated the leakage of the cytoplasmic enzymes LDH and cAST, but not of the mitochondrial enzyme mAST. Therefore, these mechanical stresses seem to disrupt the cell membrane, but not necessarily to disrupt the mitochondrial membrane (Lemasters et al. 1983). This may be consistent with the clinical observation of the delayed appearance of mitochondrial enzymes in the circulation compared with cytoplasmic enzymes during mycocardial infarction (Smith et al. 1977).

There are several possible mechanisms by which anoxia may cause fragility of the cell membrane. Ischemic contracture may have contributed to rigidity of myocardial cells and their vulnerability to mechanical stress (Hearse et al. 1977). Another possibility is that disruption of the cytoskeleton anchoring the cell membrane and

formation of blebs caused fragility (Vander Heide and Ganote 1987; Steenbergen et al. 1987). In anoxic liver, saccular blebs protruding into sinusoidal spaces are formed on disruption of the cytoskeleton (Lemasters et al. 1983; Koseki et al. 1988). In the heart, the anchoring of the cytoskeleton to the cell membrane arises mainly from the Z-line of the myofibrils, and consequently flat subsarcolemmal blebs are formed on disruption of the cytoskeleton (Ganote and Vander Heide 1987; Steenbergen et al. 1987). Such blebs do not appear to be disrupted by the shearing stress of the bloodstream, unlike blebs in the liver, but they seem to be vulnerable to the mechanical stress of cardiac beating. Fragile cell membranes of the heart are also reported to be vulnerable to intracellular hyperosmotic stress, which develops during ischemia and reperfusion (Steenbergen et al. 1985).

Explanations for the destruction of the cytoskeleton during anoxia could be an activation of proteases due to increased intracellular calcium (Toyo-oka et al. 1979), and dissociation of cytoskeletal proteins following energy depletion (Lemasters et al. 1987). In any case, the process is probably associated with a change in the energy level of myocardial cells. As we have reported, the ^{31}P-NMR method probably detects only free ATP in the cytoplasm and this ATP decreases rapidly in ischemia (Takami et al. 1988). In this study, we found that during anoxia free ATP detectable by NMR was depleted and the vulnerability of the myocardium to mechanical stress increased. Thus, depletion of free ATP could contribute to disruption of the cytoskeleton and membrane fragility in anoxia.

In myocardial injury on reperfusion, the oxygen paradox due to abrupt reoxygenation has been proposed to be one of the most important mechanisms of injury (Braunwald and Kloner 1985). In this paper we have pointed out additional mechanisms of injury: increase in cell membrane fragility by anoxia, and the mechanical stress of cardiac beating on reperfusion. In this connotation, we do not underestimate the importance of the oxygen paradox, but we think that the contributions of the two mechanisms described to myocardial injury should be evaluated (Piper et al. 1984).

We conclude from this study that the leakage of cytoplasmic enzymes from myocardial cells after anoxia is accelerated by the mechanical stress of heartbeat on reperfusion, and that the vulnerability of myocardial cells to this stress develops during the preceding period of anoxia.

Acknowledgements. This work was supported in part by grants-in-aid for scientific research from the Ministry of Education, Science and Culture of Japan. We thank Ms. K. Chihara for technical assistance.

References

Braunwald E, Kloner RA (1985) Myocardial reperfusion: a double-edged sword? J Clin Invest 76:1713–1719

Furuya E, Hotta K, Tagawa K (1986) Anomer specificity of glucose-6-phosphatase and glucokinase. Biochem Biophys Res Commun 141:931–936

Ganote CE, Vander Heide RS (1987) Cytoskeletal lesions in anoxic myocardial injury. A conventional and high-voltage electron-microscopic and immunofluorescence study. Am J Pathol 129:327–344

GSCC (German Society for Clinical Chemistry) (1972) Recommendations of the German Society for Clinical Chemistry. Standardisation of methods for the estimation of enzyme activities in biological fluids. Experimental basis for the optimized standard conditions. Z Klin Chem Klin Biochem 10:281–291

Hearse DJ (1977) Myocardial enzyme leakage. J Mol Med 2:185–200

Hearse DJ, Humphrey SM (1975) Enzyme release during myocardial anoxia: a study of metabolic protection. J Mol Cell Cardiol 7:463–482

Hearse DJ, Humphrey SM, Chain EB (1973) Abrupt reoxygenation of the anoxic potassium-arrested perfused rat heart: a study of myocardial enzyme release. J Mol Cell Cardiol 5:395–407

Hearse DJ, Garlick PB, Humphrey SM (1977) Ischemic contracture of the myocardium: mechanisms and prevention. Am J Cardiol 39:986–993

Humphrey SM, Thomson RW, Gavin JB (1984) The influence of the no-reflow phenomenon on reperfusion and reoxygenation damage and enzyme release from anoxic and ischaemic isolated rat hearts. J Mol Cell Cardiol 16:915–930

Kamiike W, Watanabe F, Hashimoto T, Tagawa K, Ikeda Y, Nakao K, Kawashima Y (1982) Changes in cellular levels of ATP and its catabolites in ischemic rat liver. J Biochem (Tokyo) 91:1349–1356

Koseki M, Nishida T, Kamiike W, Nakao K, Kawashima Y, Dan C, Wake K, Yoshida Y, Tagawa K (1988) Mechanism of leakage of cytosolic enzymes from anoxic rat liver cells: disruption of blebs by blood flow. Biomed Res 9:47–57

Lemasters JJ, Stemkowski CJ, Ji S, Thurman RG (1983) Cell surface changes and enzyme release during hypoxia and reoxygenation in the isolated, perfused rat liver. J Cell Biol 97:778–786

Lemasters JJ, DiGuiseppi J, Nieminen AL, Herman B (1987) Blebbing, free Ca^{2+} and mitochondrial membrane potential preceding cell death in hepatocytes. Nature 325:78–81

Melrose DG, Dreyer B, Bentall HH, Baker JBE (1955) Elective cardiac arrest. Lancet II:21–22

Morino Y, Kagamiyama H, Wada H (1964) Immunochemical distinction between glutamic-oxaloacetic transaminases from the soluble and mitochondrial fractions of mammalian tissues. J Biol Chem 239:943–944

Nishida T, Koseki M, Kamiike W, Nakahara M, Nakao K, Kawashima Y, Hashimoto T, Tagawa K (1987) Levels of purine compounds in a perfusate as a biochemical marker of ischemic injury of cold-preserved liver. Transplantation 44:16–21

Nishimura T, Yoshida Y, Watanabe F, Koseki M, Nishida T, Tagawa K, Kawashima Y (1986) Blood level of mitochondrial aspartate aminotransferase as an indicator of the extent of ischemic necrosis of the rat liver. Hepatology 6:701–707

Piper HM, Schwartz P, Spahr R, Hütter JF, Spieckermann PG (1984) Absence of reoxygenation damage in isolated heart cells after anoxic injury. Pflügers Arch 401:71–76

Romson JL, Hook BG, Kunkel SL, Abrams GD, Schork MA, Lucchesi BR (1983) Reduction of the extent of ischemic myocardial injury by neutrophil depletion in the dog. Circulation 67:1016–1023

Smith AF, Wong PCP, Oliver MF (1977) Release of mitochondrial enzymes in acute myocardial infarction. J Mol Med 2:265–269

Steenbergen C, Hill ML, Jennings RB (1985) Volume regulation and plasma membrane injury in aerobic, anaerobic, and ischemic myocardium in vitro. Effects of osmotic cell swelling on plasma membrane integrity. Circ Res 57:864–875

Steenbergen C, Hill ML, Jennings RB (1987) Cytoskeletal damage during myocardial ischemia: changes in vinculin immunofluorescence staining during total in vitro ischemia in canine heart. Circ Res 60:478–486

Takami H, Furuya E, Tagawa K, Seo Y, Murakami M, Watari H, Matsuda H, Hirose H, Kawashima Y (1988) NMR-invisible ATP in rat heart and its change in ischemia. J Biochem (Tokyo) 104:35–39

Tamura M, Oshino N, Chance B, Silver IA (1978) Optical measurements of intracellular oxygen concentration of rat heart in vitro. Arch Biochem Biophys 191:8–22

Teranishi H, Morino Y, Nishino T, Murao S, Yagi T, Kagamiyama H, Harada N (1988) Determination of human aspartate aminotransferase isoenzymes by their differential sensitivity to proteases. Clin Biochem 21:347–352

Toyo-oka T, Masaki T, Okamoto J, Tanaka T (1979) Calcium-activated neutral protease from bovine ventricular muscle: isolation and some of its properties. J Mol Cell Cardiol 11:769–786

Vander Heide RS, Ganote CE (1987) Increased myocyte fragility following anoxic injury. J Mol Cell Cardiol 19:1085–1103