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Effects of Hypoxia and Reoxygenation on Adult Rat Heart Cell Metabolism (41681)

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Abstract. Isolated adult rat heart cells were used to study the effects of oxygen deprivation followed by reoxygenation upon myocardial metabolism. Calcium-tolerant nonbeating myocytes were incubated for 5, 30, or 60 min under 100% oxygen or 100% nitrogen and then rinsed with oxygenated buffer. Substrate oxidation was studied by incubating the cells with ^{14}C-labeled glucose, pyruvate, or octanoate and determining the rates of $^{14}CO_2$ production from the individual substrates. After 5 min of hypoxia, metabolism of glucose, as assessed by glucose oxidation and lactate production, was significantly depressed. Pyruvate and octanoate oxidation were unaltered. Oxygen consumption was also unchanged by short-term hypoxia and reoxygenation. With reoxygenation after 30 min of oxygen deprivation, more exaggerated changes in glucose metabolism were noted as well as a depression in pyruvate oxidation and unaltered octanoate oxidation. Oxidation of octanoate was slightly depressed after 60 min of hypoxia. Cell viability assessed after reoxygenation was not significantly altered until 60 min of oxygen deprivation. The results indicate that cytosolic changes occur after short periods of hypoxia followed by reoxygenation, whereas mitochondrial function is more resistant to damage inflicted by hypoxia and reoxygenation.

Periodic, recurring, short-lasting oxygen deprivation of the myocardium due to vascular spasm is not uncommon in human patients. Although the metabolic consequences of long-lasting global and regional anoxia or ischemia have been studied extensively in various experimental models, little information is available concerning the metabolic alterations in the myocardium following a short-lasting, transient O_2 deprivation followed by reperfusion. Since contractile activity and metabolism are functionally related (1, 2), alterations in mechanical activity during oxygen deprivation may obscure the effects of such an insult on myocardial metabolism. In order to separate contractile activity from metabolism, the isolated quiescent adult rat heart cells have been selected as an experimental model to investigate the influence of transient oxygen deprivation on myocardial metabolism. Similar experimental models have been employed by other investigators (3–6) for assessing the structural and metabolic effects of hypoxia upon heart cells. Most of these investigations have dealt with the influence of relatively long-term (up to several hours) hypoxia on tissue high-energy phosphate levels and membrane permeability, as assessed by the release of cytoplasmic enzymes such as lactic dehydrogenase (LDH) and creatine phosphokinase (CPK).

In the present study we wished to determine if short-term (5 min) deprivation of oxygen followed by reoxygenation would have an effect on myocardial substrate oxidation. If an effect was demonstrable, we wished to localize the site of alteration to the cytosol or to the mitochondria. In addition, we investigated the effects of longer exposure to oxygen deprivation for the sake of comparison. The results indicate that short-term deprivation of oxygen alters myocardial metabolism not only during the exposure, but also following reoxygenation. Anaerobic glycolysis is elevated during hypoxia, whereas both glycolysis and oxidation are impaired following reoxygenation.

Materials and Methods. Myocytes were prepared according to the method of Montini *et al.* (7). In brief, hearts were excised from pentobarbital-anesthetized, male, Sprague–Dawley rats (250–350 g body weight). Cells were obtained by perfusing the hearts with Jokliks phosphate buffer (minimum essential medium) supplemented with 8 m*M* glutamate, 1.25 m*M* $MgSO_4$, 0.1% bovine serum albumin (BSA), and 0.1% collagenase (Type II, Worthington Chemicals) followed by mincing of the ventricles and mechanical dispersion. Cells were purified in Jokliks-Mg-glutamate buffer containing 1% BSA (essentially fatty acid (FA)-free) and finally taken up in Jokliks-Mg-buffer supplemented with 5 m*M* glucose, 1% BSA

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(FA free), and 1.5 mM $CaCl_2$ (this solution is referred to as the experimental buffer). The resulting cell suspension was separated into two aliquots, which were centrifuged at low speed (50g) for 1 min. The supernatants were removed and the resulting pellets were diluted in the experimental buffer; one aliquot of cells was taken up in a buffer gassed with 100% O_2 and the other aliquot in a N_2-gassed buffer. The two cell populations were then incubated at 37°C in a Dubnoff type metabolic shaker bath at 60 cpm for 5, 30, or 60 min while gassed with either 100% O_2 or 100% N_2. In some experiments, cells were sampled at the stated time points for adenosine triphosphate (ATP), creatine phosphate (CP), lactate, and protein. In other experiments, the paired aliquots of cells were taken after the 5, 30, or 60 min incubation and were washed twice with oxygenated Jokliks buffer containing 1% BSA, 1.5 mM $CaCl_2$, and either 5 mM glucose, 1 mM pyruvate, or 1 mM octanoate. Thus, 5 mM glucose was present with the cells during the hypoxic episode and a selected substrate was present with the cells upon reoxygenation. After reoxygenation the cell suspensions were sampled for protein, ATP, and lactate levels, for oxidation of exogenously supplied ^{14}C-labeled substrate, and for viability. $^{14}CO_2$ produced from oxidation of [U-^{14}C]glucose (5 mM), [U-^{14}C]pyruvate (1 mM), or [1-^{14}C]octanoate (1 mM) was collected in hyamine hydroxide and counted by liquid scintillation spectrometry. Substrate oxidation was calculated by dividing the counts per minute of $^{14}CO_2$ by the specific activity of the exogenous substrate (counts per minute per nanomole). Viability, i.e., elongated cells as a percentage of the total cell population, was determined on cells incubated with unlabeled substrate for 30 min at 37°C. Another sample of this cell suspension was taken for the determination of lactate in order to assess the accumulation of lactate during the reoxygenation period. Oxygen consumption was measured in one series of experiments on cells exposed to O_2 or N_2 for 5 min and then reoxygenated with either a glucose-, octanoate-, or pyruvate-containing Jokliks buffer. Oxygen consumption was determined with an oxygraph and a Clark electrode and expressed as nanomoles of O_2 utilized per milligram myo-

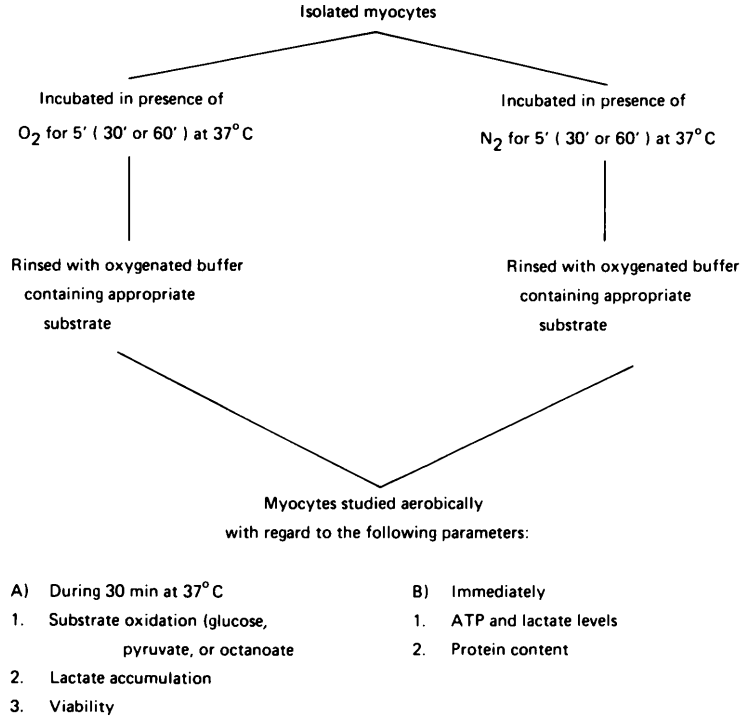

FIG. 1. Flow diagram of experiment protocol.

cyte protein. A schematic flow-chart of the oxidation studies is shown in Fig. 1. ATP, CP, and lactate levels were determined on neutralized perchloric acid extracts by fluorometry (8). Protein was determined by the Lowry method (9). Statistical significance was evaluated by the Wilcoxon signed-rank test (10) as all data were paired.

Results. In some experiments cells were sampled during the O_2 or N_2 exposure in order to determine lactate accumulation and the concentration of adenosine triphosphate (ATP) and creatine phosphate (CP). During the exposure to N_2, the cells produced lactate at a greater rate than did the oxygenated cells (Fig. 2). In addition, adenosine triphosphate (ATP) and creatine phosphate (CP) levels fell during the course of incubation with N_2 but were well maintained in the oxygenated group (Fig. 3). Evidently, in these noncontracting cells, the high-energy-phosphate requirements were met to some degree by glycolysis in the low-oxygen environment. Thus, the decrease in ATP and CP levels was not very large especially after only 5 min of oxygen deprivation.

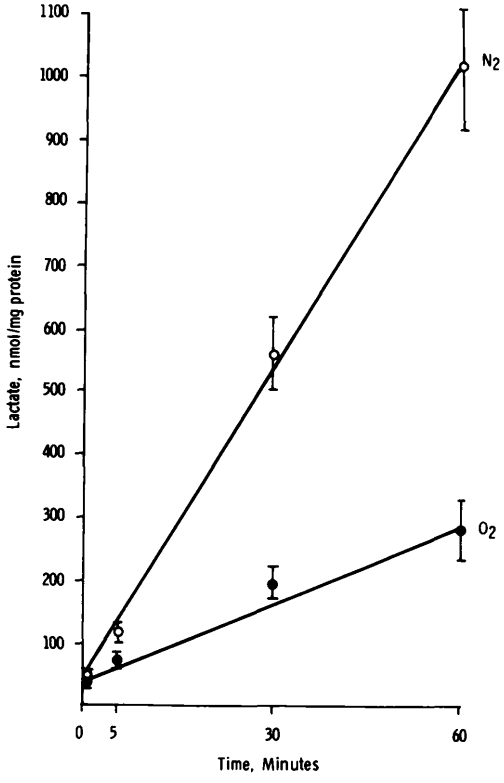

FIG. 2. The effect of N_2 on lactate accumulation. Cells were incubated in Jokliks buffer supplemented with 1% BSA, 5 m*M* glucose, 1.5 m*M* $CaCl_2$, and 1.25 m*M* $MgSO_4$ in a shaking 37°C water bath and gassed with either 100% O_2 or 100% N_2. Aliquots were taken at 5, 30, and 60 min, extracted with perchloric acid, and the neutralized supernatant assayed for lactate.

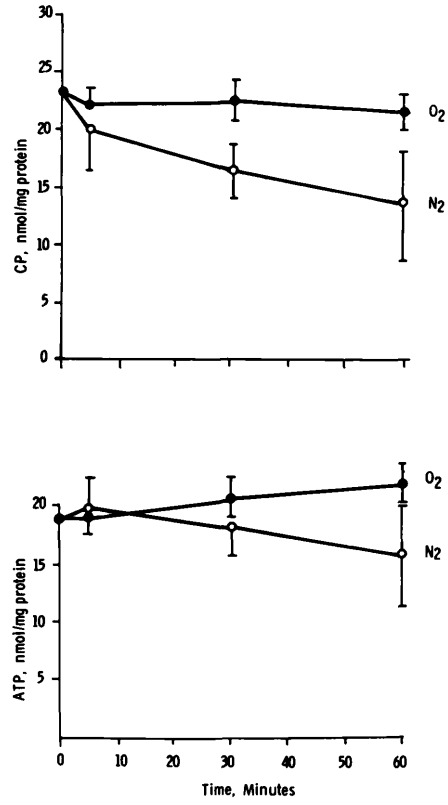

FIG. 3. The effect of N_2 on ATP and CP concentration in isolate myocytes. Cells were incubated as described in Fig. 2 and gassed with 100% O_2 or 100% N_2. Aliquots of the cells were analyzed for adenosine triphosphate (ATP) and creatine phosphate (CP).

The ability of cells to oxidize exogenously supplied substrates after exposure to low oxygen (as depicted in the flow diagram of Fig. 1) is shown in Table I. After only 5 min of N_2 exposure the rate of [^{14}C]glucose oxidation upon reoxygenation was depressed (66% of control). This depression was even greater after 30 or 60 min of hypoxia (38 and 34% of control, respectively). To determine if the defect in glucose oxidation was accompanied by a depression in the utilization of other metabolites as well, two other substrates were stud-

TABLE I. RATES OF SUBSTRATE OXIDATION (nmol/mg · min) IN CELLS EXPOSED TO 100% O_2 OR 100% N_2 FOR 5, 30, OR 60 min[a]

Substrate	5 Min	30 Min	60 Min
Glucose			
O_2	1.35 ± 0.12 (7)	1.15 ± 0.14 (6)	1.39 ± 0.15 (6)
N_2	0.89 ± 0.09	0.44 ± 0.07	0.47 ± 0.06
	P = 0.008	P = 0.016	P = 0.016
Pyruvate			
O_2	8.18 ± 1.08 (5)	6.55 ± 0.47 (5)	
N_2	7.28 ± 0.44	4.60 ± 0.44	
	P = 0.094	P = 0.031	
Octanoate			
O_2	1.03 ± 0.08 (6)	1.11 ± 0.12 (6)	1.11 ± 0.07 (5)
N_2	1.02 ± 0.11	1.16 ± 0.15	0.92 ± 0.02
	P = 0.422	P = 0.422	P = 0.031

[a] Cells were rinsed twice with oxygenated buffer containing the substrate that was to be used for oxidation studies, i.e., 5 mM glucose, 1 mM pyruvate, or 1 mM octanoate. Mean ± SEM, number of experiments in parentheses.

ied—pyruvate and octanoate. Oxidation of pyruvate, which is metabolized solely in the mitochondria, was not significantly altered after 5 min, but was diminished (72% of control) after 30 min of hypoxia. On the other hand, oxidation of the fatty acid, octanoate, which bypasses the carnitine transferase system, was not altered after 5 or 30 min and only slightly decreased (83% of control) after 60 min of hypoxia.

In addition to a depressed rate of glucose oxidation, cells exposed to hypoxic conditions for 5, 30, or 60 min demonstrated lower rates of lactate accumulation during reoxygenation with glucose as substrate (Table II). This inhibition of lactate accumulation developed only during the reoxygenation period in contrast to the elevated rate of lactate production during the 60 min of hypoxic exposure (see Fig. 2).

The glucose oxidation and lactate accumulation data indicate that glycolysis was inhibited during reoxygenation following exposure to hypoxia. Lactate is a possible candidate for causing this inhibition. However, lactate accumulation (Table II) was lower when glucose was the exogenous substrate during reoxygenation. Also, the levels of lactate present at the beginning of the oxidation study were not elevated in the N_2-treated cells (Table III) because the cells were rinsed after N_2 exposure, thus bringing about a washout of accumulated metabolites. Inhibition of glycolysis was, therefore, probably not due to lactate and low pH in the N_2-treated cells.

Mitochondrial function did not appear to be altered by short exposure of myocytes to hypoxic conditions. Oxidation of octanoate was unchanged (Table I) as was pyruvate oxidation by 5 min exposure to N_2. Thus, oxidation and the citric acid cycle, as assessed

TABLE II. RATES OF LACTATE ACCUMULATION DURING REOXYGENATION BY CELLS EXPOSED TO 100% O_2 OR 100% N_2 FOR 5, 30, OR 60 min[a]

	Rates of lactate accumulation (nmole/mg · min)		
	5 Min	30 Min	60 Min
	In the presence of glucose		
O_2	9.0 ± 0.7 (5)	7.6 ± 1.1 (4)	8.9 ± 0.9 (5)
N_2	6.6 ± 0.3	3.6 ± 0.7	4.6 ± 1.1
	P = 0.031	*P = 0.062	P = 0.031
	In the presence of pyruvate		
O_2	4.8 (2)	4.9 (2)	—
N_2	5.5	4.3	—

[a] Lactate accumulation was determined during incubation in an oxygenated buffer for 30 min at 37°C. Mean ± SEM, number of experiments in parentheses.

* Lowest P value possible for N = 4, Wilcoxon signed rank test.

TABLE III. LACTATE LEVELS IN CELLS REOXYGENATED AFTER EXPOSURE TO 100% O_2 OR 100% N_2 FOR 5, 30, OR 60 min[a]

	Lactate levels (nmole/mg)		
	5 Min	30 Min	60 Min
O_2	44.1 ± 3.8 (5)	50.4 ± 10.5 (4)	60.6 ± 21.3 (5)
N_2	33.3 ± 3.9	45.1 ± 5.4	55.3 ± 15.5

[a] Lactate levels were measured after cells (N_2- and O_2-treated) had been rinsed two times with fresh, oxygenated buffer containing glucose. Mean ± SEM, number of experiments in parentheses.

by octanoate oxidation, were unaltered by 5 or 30 min N_2 exposure and only slightly inhibited after 60 min. The depression of pyruvate oxidation after 30 min exposure was probably due to some step prior to the citric acid cycle, perhaps pyruvate dehydrogenase (PDH) or pyruvate transport. Altschuld *et al.* (3) also showed a depression in pyruvate oxidation after exposure of cells to a low-oxygen environment for as little as 10 min. Since cells were lysed with digitonin and the pyruvate concentration was 10-fold higher (10 m*M*) in that study, the inhibition was probably related to enzyme handling of pyruvate. In our study, transport as well as PDH activity may have been altered.

Cell viability was determined after 30 min of reoxygenation in order to assess the state of cells during the period over which oxidation of substrates was measured. Viability was not significantly altered until 60 min of N_2 exposure (Table IV). Therefore, the metabolic alterations noted at 30 min and particularly at 5 min cannot be attributed to decreased cell viability during the exposure to N_2 and reoxygenation.

To characterize further the status of the cells in which glucose handling is alone affected (i.e., after 5 min hypoxia), oxygen consumption and high-energy-phosphate levels were measured in cells with glucose, pyruvate, or octanoate present in the reoxygenation buffer (Table V). These results indicate that high-energy-phosphate levels are normal in cells reoxygenated after short-term hypoxia. In addition the oxygen consumption is unchanged by 5 min hypoxia. Although the rate of oxygen consumed is greatly affected by the substrate present, it is apparently not altered by short-term O_2 deprivation. Due to the depression in glucose oxidation, the percentage of oxygen used in oxidation of substrate is depressed in the N_2-treated cells. This might indicate that cytosolically "bound" NADH is limiting the rate of glucose hydrolysis.

After 30 min of N_2 exposure followed by reoxygenation, ATP levels, when glucose was the exogenous substrate, were 26.1 ± 3.2 nmole/mg protein in O_2 and 22.8 ± 1.1 in N_2-treated cells ($N = 5$). After 60 min, the N_2-exposed cells had lower ATP levels (18.8 ± 3.2 vs 29.7 ± 2.1, $N = 5$) than O_2 cells.

Discussion. The present studies demonstrated that even short-lasting oxygen deprivation followed by reoxygenation altered substrate oxidation in calcium-tolerant, noncontracting rat myocytes. Glucose oxidation and lactate production were decreased in myocytes reoxygenated following 5 min of oxygen deprivation, whereas pyruvate and octanoate oxidation were unaltered, implying that the short-term oxygen deprivation affected cytosolic, but not mitochondrial substrate utili-

TABLE IV. VIABILITY OF CELLS EXPOSED TO 100% N_2 FOR 5, 30, OR 60 min FOLLOWED BY REOXYGENATION FOR 30 min[a]

	5 Min		30 Min		60 Min	
Substrate	O_2	N_2	O_2	N_2	O_2	N_2
Glucose	70 ± 4 (7)	70 ± 4	70 ± 4 (5)	74 ± 4	—	—
Pyruvate	72 ± 2 (5)	70 ± 3	71 ± 3 (5)	66 ± 4	—	—
Octanoate	74 ± 4 (5)	74 ± 4	76 ± 3 (6)	76 ± 2	76 ± 3 (5)	68 ± 4

[a] Viability was determined after two rinses with oxygenated buffer and incubation at 37°C for 30 min in an oxygenated environment. Viability is expressed as percentage of elongated cells present in the total cell population, (N).

TABLE V. ATP, CP, O_2 CONSUMPTION AND THE PERCENTAGE OF O_2 UTILIZED FOR OXIDATION OF THE EXOGENOUS SUBSTRATE LISTED AFTER SHORT-TERM (5 min) HYPOXIA AND REOXYGENATION[a]

	Substrate	O_2	N_2
ATP (nmole/mg)	Glucose	24.7 ± 1.2[b] (6)[c]	25.3 ± 1.6 (6)
	Pyruvate	23.2 ± 1.3 (6)	25.3 ± 1.1 (5)
	Octanoate	23.9 ± 1.8 (5)	22.5 ± 0.7 (6)
CP (nmole/mg)	Glucose	29.4 ± 2.8 (6)	32.9 ± 3.0 (6)
	Pyruvate	31.7 ± 4.8 (6)	38.2 ± 3.7 (5)
	Octanoate	30.2 ± 3.6 (4)	30.9 ± 3.1 (5)
O_2 consumption (nmole/mg · min)	Glucose	15.8 ± 0.9 (5)	17.2 ± 1.3 (5)
	Pyruvate	26.7 ± 1.3 (5)	26.2 ± 2.0 (5)
	Octanoate	31.4 ± 1.8 (5)	32.5 ± 3.4 (5)
Percentage of O_2 used in oxidation of substrate	Glucose	51.4	31.1
	Pyruvate	76.6	69.6
	Octanoate	36.1	34.5

[a] Cells were treated as in Table I except that only 5 min of N_2 exposure was used. For percentage O_2 consumed in oxidation of substrate, we multiplied oxidation rates of glucose, pyruvate, and octanoate listed in Table I by 6, 2.5, and 11, respectively.

[b] Mean ± SEM.

[c] (*N*).

zation. Longer periods of oxygen deprivation decreased pyruvate oxidation as well.

ATP and CP content of the isolated myocytes was unaltered in cells exposed to 100% nitrogen for 5 min and subsequently reoxygenated. Oxygen consumption of these cells also was similar to that of control cells, regardless of the exogenous substrate present (glucose, pyruvate, or octanoate). Oxygen consumption in the presence of glucose was markedly lower than in the presence of pyruvate or octanoate as the sole exogenous substrate. A similar observation on myocytes was made by Chen *et al.* (11) when oxygen consumption in the presence of glucose alone and palmitate alone were compared. The increased O_2 consumption with palmitate as exogenous substrate, resembles the phenomenon of "oxygen wasting" (12) observed by several investigators under *in vivo* conditions in dog hearts (13, 14). Although the cause of the "oxygen wasting" in the presence of fatty acids is not known, uncoupling of oxidative phosphorylation by fatty acids or their metabolites has often been suggested. It is of interest to note that in the present studies a similar increase of oxygen consumption was also observed when 1 m*M* pyruvate was used as the sole substrate, thus making uncoupling by fatty acids an unlikely explanation for the phenomenon.

One can observe from Table V that oxidation of exogenous glucose would account for approximately half of the oxygen consumption in the oxygen exposed cells. The corresponding percentage of oxygen consumed for pyruvate oxidation was 77% and for octanoate oxidation was 36%. This fraction remained unaltered following 5 min of oxygen deprivation and reoxygenation when either pyruvate or octanoate served as the sole exogenous substrate. However, when glucose was the sole substrate, the fraction of oxygen utilized for exogenous substrate oxidation was decreased to 31%. It appears that after oxygen deprivation and reoxygenation a larger fraction of the oxidized substrate is derived from endogenous sources, when glucose was the only exogenous substrate. Furthermore, the calculated ATP production from exogenous glucose (glucose oxidized × 38) is 51.3 nmole/mg × min in the oxygenated cells, and 33.8 in cells exposed to nitrogen and subsequently reoxygenated. In comparison, 122.7 and 109.2 nmole/mg × min ATP was produced in the presence of pyruvate as the exogenous substrate (oxidation rate × 15) in O_2- and N_2-treated cells, respectively. Corresponding fig-

ures for octanoate were 62.8 and 62.2 (oxidation rate × 61). These data indicate a lesser contribution of exogenous substrate for ATP production in the nitrogen-exposed, reoxygenated cells only in the presence of glucose.

During oxygen deprivation, glucose seemed to supply enough ATP via lactate production to maintain high-energy-phosphate levels and membrane integrity for at least 5 min and probably up to 30 min in this noncontracting system. Haworth *et al.* (5) also demonstrated a similar protective effect of glucose in cells in an aerobic medium but with rotenone present. The absence of glucose resulted rather quickly in cell death. Finally, Spanier and Weglicki (6) showed a beneficial effect of glucose on cells in a N_2 environment at pH 6.5 or 6.8. This protective effect of glucose may be related to the putative role of glycolytically produced ATP in the maintenance of membrane integrity, as proposed by Opie and Bricknell (15) and others. In contrast to the very crucial role glucose plays during the oxygen deprivation, upon reoxygenation, however, glucose hydrolysis and oxidation were impaired while substrates which required only mitochondrial handling were readily oxidizable.

The mechanism of depressed glucose hydrolysis after this short oxygen deprivation is not clear. It is possible that some glycolytic enzymes leaked from the cytosol during oxygen deprivation. However, this does not seem very likely, since, cells exposed to 5 min of nitrogen or oxygen contained the same concentration of CPK at the end of the 30-min reoxygenation period (oxygenated cells = 7.17 ± 1.36 IU/mg; cells exposed to nitrogen = 7.06 ± 1.09; ($N = 4$). We postulate that an elevated cytosolic NADH/NAD ratio may be responsible for the change of glucose oxidation rate. The elevated NADH/NAD ratio that occurs during oxygen deprivation is likely to return to normal more quickly in the mitochondria than in the cytosol following reoxygenation. It is also possible that some of the NADH that accumulated during hypoxia, remained bound to glyceraldehyde-3-phosphate dehydrogenase, thus inhibiting glycolysis early during the reoxygenation period. Such a possibility is raised by the results of Mochizuki and Neely (16) who measured an early decrease in glycolytic flux upon reperfusion after 30 min of ischemia in the isolated, perfused working heart.

In the present study, mitochondrial function, as assessed by the ability of the isolated cells to oxidize exogenous ^{14}C-labeled pyruvate or octanoate, was not affected by short-term oxygen deprivation and reoxygenation, whereas cytosolic metabolism of substrate was reduced. Our results agree with the findings of Godin *et al.* (17) who measured mitochondrial and sarcolemmal ATPase activities after coronary artery ligation and reperfusion in rabbits. In contrast to the mitochondrial ATPase, the Na^+, K^+-ATPase was depressed after only 10 min of ischemia and remained depressed even after 60 min of reperfusion. Our data also agree with the conclusion of other investigators (18–20) suggesting the possibility of earlier alterations in cytosolic or sarcolemmal activity following reoxygenation in contrast to the delayed effects on mitochondrial function. The findings also call attention to the relatively long-lasting effects of a comparatively mild insult.

One can speculate that in the human myocardial cell during vasospasm, glycolytic production of ATP may be very important in maintaining cell viability. However, following vascular spasm and during reperfusion, decreased glucose hydrolysis may be detrimental if glycolytically derived ATP is important in maintaining cell membrane integrity as suggested by others (15). Furthermore, the enhancement of the glycolytic rate during a second episode of vascular spasm (occurring shortly after the first one and at the same location) could be greatly attenuated if glycogen levels were not replenished due to catecholamine release during the prior hypoxic episode. Catecholamines, via receptor mediation, inactivate glycogen synthase and thus could interfere with replenishment of glycogen stores in the myocardium (21) following an episode of vascular spasm.

These studies not only confirm the usefulness of isolated myocardial cells in studying the consequences of oxygen deprivation on myocardial metabolism independent of contractile activity, but also appear to provide a potentially useful model for studying the metabolic consequences of short-term, transient oxygen deprivation (e.g., vasospasm of small myocardial vessels).

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