Oxygen at Physiological Concentrations A Potential, Paradoxical Mediator of Reperfusion Injury to Mitochondria Induced by Phosphate

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Abstract. Cellular injury induced by reperfusion after myocardial ischemia is manifested by striking mitochondrial damage as well as other hallmarks such as contraction band necrosis. Calcium has been implicated as a mediator of irreversible cellular injury in several systems. To identify other potential mediators of the mitochondrial injury associated with reperfusion, interactions between inorganic phosphate, oxygen, and mitochondria harvested from rabbit hearts were evaluated *in vitro*. Mitochondria exhibited rapid inactivation of oxidative phosphorylation after preincubation at 25°C when phosphate and oxygen were present. Inactivation was partially but not completely precluded by EDTA, EGTA, magnesium, diltiazem, or ruthenium red, results in concert with findings of others suggesting involvement of a deleterious influx of calcium into mitochondria; exogenous calcium enhanced inactivation. However, the present data indicate that inactivation is prevented by incubation of mitochondria in the absence of oxygen, and demonstrate for the first time that injury elicited by phosphate is dependent on oxygen at physiological concentrations either because calcium and/or phosphate influx is linked to aerobic metabolism or because oxygen exerts deleterious effects on mitochondria, which may render them particularly susceptible to calcium influx. Since intracellular inorganic phosphate concentration increases markedly with ischemia, reperfusion with oxygenated medium may paradoxically augment mitochondrial injury in this setting. Thus, in the presence of increased intracellular concentrations of calcium and phosphate induced by ischemia, subsequent reestablishment of physiological levels of intracellular oxygen tension may

promote mitochondrial damage, which is known to increase with reperfusion.

Introduction

Irreversible myocardial injury induced by ischemia followed by reperfusion is associated with mitochondrial dysfunction and accumulation of dense bodies (1), events that appear to be mediated in part by calcium (2–5). In heart mitochondria, calcium uptake is mediated by a high affinity uniporter sensitive to ruthenium red (6). Efflux occurs via an electrically neutral Ca^{++} - Na^{+} exchange sensitive to high concentrations of certain calcium antagonist drugs (7). Recently, in important studies by Schwartz and his co-workers (8, 9), diminution of mitochondrial injury in response to inhibition of calcium transport has been demonstrated.

Phosphate has been implicated as an independent mediator of mitochondrial injury because of its ability to induce swelling (10, 11). Recently, dramatic increases in intracellular inorganic phosphate concentrations accompanying myocardial ischemia have been detected by nuclear magnetic resonance (12, 13). Diltiazem and verapamil, at high concentrations, inhibit phosphate-induced mitochondrial swelling and dysfunction under conditions in which substrates for oxidative phosphorylation are present (9, 14). Thus, their protective effects on jeopardized myocardium *in vivo* (15) may be mediated in part by phosphate as well as calcium.

In the course of studying the effects of recently identified fatty acid ethyl esters, myocardial metabolites of ethanol (16, 17), we found that mitochondrial function could be maintained for prolonged intervals when oxygen was excluded meticulously from incubation media containing inorganic phosphate and isolated mitochondria (18). These findings are consistent with observations made almost 30 years ago using different end-points and are generally not emphasized (19). Moreover, in numerous studies that use mitochondrial swelling as a criterion of mitochondrial injury, because it is more readily and conveniently measurable than is oxygen consumption, no direct dependency of injury on the presence of oxygen or determination of the concentration dependency of inorganic phosphate *per se* has been delineated. In view of the potential practical importance of reperfusion injury in the setting of coronary thrombolysis, this study was performed to determine whether specific con-

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centrations of oxygen may be important in mediating injury induced by inorganic phosphate under conditions simulating mitochondrial exposure to oxygen and phosphate with ischemia and reperfusion in vivo (12). Results suggest that oxygen at very low intracellular concentrations may be paradoxically instrumental to the development of mitochondrial injury in this setting.

Methods

Mitochondria were prepared daily from rabbit hearts according to standard methods (20, 21). Oxidative phosphorylation was assessed with a polarograph Yellow Springs Instrument Co. (Yellow Springs, OH) (18, 21). Rabbits were killed by cervical dislocation. Hearts were removed immediately and placed in 0.25 M sucrose, 10 mM EDTA, 0.1% BSA, pH 7.4, at 0–4°C. After the trimmed ventricles had been washed with homogenizing medium, the tissue was minced finely with a scissors and passed through a precooled, hand-operated muscle press to give a 15% (wt/vol) suspension, which was homogenized in a Potter-Elvehjem apparatus (six strokes). After filtration through cheesecloth, the suspension was centrifuged at 2,500 *g* for 10 min at 4°C. The fat on the surface of the supernate was removed with a cotton swab, the pellet discarded, and the supernatant fraction centrifuged at 9,000 *g* for 10 min. The resulting mitochondrial pellet was washed three times with 30 ml of homogenizing medium. The final pellet was collected by centrifugation at 9,000 *g* for 10 min and gently resuspended in 1 ml of homogenizing medium/g original wet weight of ventricle.

Polarographic assay of oxidative phosphorylation was performed at 25°C with a recording oxygen electrode immersed in incubation medium consisting of 250 μ l mitochondrial suspension, 250 μ l homogenizing medium, and 2.0 ml of a solution containing 112.5 mM potassium chloride, 75 mM Tris-Cl, 18.75 mM potassium monohydrogen phosphate, 7.5 mM magnesium chloride, and 10 mM EDTA, at pH 7.4. The final mitochondrial protein concentration was 1.5–2 mg/ml (18). State III respiration was initiated by addition of 700 μ l of a solution of sodium malate-pyruvate, pH 7, to give a final concentration of 21 mM, each, followed after 2 min by addition of 1.25 μ mol ADP in a volume of 50 μ l. Recordings were performed in duplicate with calculations of the rates of oxygen consumption obtained from the burst following the second ADP addition. State III rates were determined just before the addition of a second aliquot of ADP. Quality of preparations was assured by determination of respiratory control indexes and the rate of state III oxygen consumption as previously described. Preparations exhibiting coupling ratios of <4.0 or rates of maximum oxygen consumption <180 nanoatoms/min per mg were not used. In studies performed over many months, mitochondrial preparations under these conditions exhibited coupling ratios of seven to nine, maximal oxygen consumption rates of 220 nanoatoms/min per mg, and phosphate/oxygen ratios of 2.7–3.0. All experiments were performed at least in duplicate. The rate of oxygen consumption during state III respiration in control experiments was compared with those from selected conditions and expressed as a percentage; or the percentage loss of control rate was calculated. Isolation of mitochondria from other organs was performed as described in detail elsewhere (22), and were assayed identically for the purposes of phosphate inactivation studies as described above.

Incubations were performed at either 25° or 37°C with additions to the standard medium, as noted in the text. When necessary, mitochondria were sedimented after incubation at 9,000 *g* for 10 min and resuspended in fresh assay medium. Control studies were performed in

all cases to evaluate the effect of sedimentation and resuspension per se.

For anaerobic incubations, medium was first heated to boiling, cooled to room temperature, and then bubbled for 1 h with nitrogen, which was passed through a solution of 10% sodium thiosulfite. Measured oxygen tension was <1 mmHg after this treatment. This medium was used for the last resuspension of mitochondria, prepared conventionally at 4°C in the absence of phosphate, and a fourth sedimentation was performed at 9,000 *g* with subsequent resuspension in anaerobic incubation medium. To provide oxygen at intermediate concentrations, partial reoxygenation was achieved by introducing an atmosphere of selected PO₂ over the incubation medium in the recording cuvette and stirring for 5 min. The gaseous atmosphere was then excluded, the PO₂ was recorded and the incubation initiated.

Results

Protection of mitochondria by addition of substrates to deplete oxygen. At 25°C, incubation of isolated rabbit ventricular mitochondria, in standard medium (18) containing 18.75 mM phosphate, resulted in rapid impairment of oxidative phosphorylation (Fig. 1). Both the maximum rate of oxygen consumption and respiratory control were diminished in parallel, although only the data for oxygen consumption are plotted. In marked contrast, addition at time zero of 21 mM pyruvate, 21 mM malate, and 0.39 mM ADP provided complete protection against such inactivation for up to 6 h (Fig. 1, upper curve). With more prolonged incubation, deterioration of function occurred slowly, but oxidative function was still detectable after

Figure 1. Protection against mitochondrial damage by substrate addition. Fresh rabbit heart mitochondria (2 mg/ml) were incubated at 25°C in 2.0 ml buffer containing 0.25 mg bovine serum albumin, 75 mM Tris-Cl, 18.75 mM potassium monohydrogen phosphate, 7.5 mM magnesium chloride, 10 mM EDTA, and 112.5 mM potassium chloride; with (●) or without (▲) 33.5 mM sodium malate; 33.5 mM (sodium) pyruvate; and 0.6 mM ADP. After the indicated interval of preincubation, suspensions were centrifuged at 9,000 *g* for 10 min at 4°C, the supernatant

fraction discarded, the mitochondrial pellet resuspended gently, washed once with incubation medium, resuspended in a cuvette, and assayed polarographically for oxygen consumption at 25°C. State III respiration was initiated by addition of ADP, 1.25 μ mol, to the suspension. The percentage decrease of state III oxygen consumption (*V*) was calculated compared with the zero time control (*V_c*) and this parameter (*V/V_c*) plotted as a function of the duration of preincubation.

incubations for as long as 14 h (data not shown). Omission of pyruvate-malate, ADP, or both precluded any appreciable protection. It is important to note that addition of ADP alone before preincubation did not afford significant protection nor was any reversibility of inactivation obtainable. Incubation medium became anaerobic within 15 min after addition of pyruvate-malate plus ADP, because the mitochondria themselves consumed available oxygen under these conditions. Thus, the results obtained indicated that either anaerobiosis provided protection or that a metabolic product of substrate oxidation was protective.

Dependence of mitochondrial functional impairment on phosphate and oxygen. The effect of diminished oxygen concentration per se was implicated by results obtained after incubating mitochondria in the standard medium saturated with oxygen at 160 mmHg or 16 mmHg and assessing residual mitochondrial oxidative capacity after preincubation at 25°C for 2 h (Fig. 2 A). Low concentrations of added substrate provided complete protection against mitochondrial inactivation when incubations were performed at the lower oxygen tension but did not prevent inactivation at the higher oxygen tension. Since the Michaelis constant (K_m) for oxygen uptake by mitochondria is ≤ 1 mmHg (23), the results obtained that demonstrate the existence of a differential effect indicate that a deleterious effect of oxygen is the responsible factor, rather than a metabolic by-product of oxidation whose effect would have been comparable under these conditions of high and low oxygen tension with the same concentrations of substrates. No protection against in-

activation was induced at either high or low oxygen tension by indomethacin (1 mg/ml), diethylcarbamazine (2 mg/ml), catalase (1 mg/ml), or *Escherichia coli* superoxide dismutase (100 μ g/ml), agents selected because of their known effects on metabolism dependent on oxygen.

Experiments in which inorganic phosphate was omitted from the standard incubation medium demonstrated nearly complete protection of mitochondrial function under conditions that otherwise induced complete inactivation after 2 h at 25°C. In the presence of 20 mM EDTA and atmospheric oxygen tension, little loss of function occurred when phosphate concentration was <8 mM; 50% loss of function occurred when it was 13 mM; and complete loss of function occurred when it was 18 mM (Fig. 2 B). Much greater extents of inactivation would occur with lower concentrations of EDT or EGTA (see below).

Heart mitochondria were markedly more sensitive to inactivation than were mitochondria from lung, liver, or kidney (Fig. 2 C). Thus, preincubation of mitochondria from these organs at 37°C under identical circumstances in the presence of increasing concentrations of phosphate, from 0 to 18.75 mM, resulted in a series of inactivation curves. Although heart mitochondria were most sensitive, kidney mitochondria were inactivated rapidly as well with 50% reduction of the rate of state III oxygen consumption occurring at 5.6 and 10.8 mM phosphate, respectively, under these conditions. On the other hand, liver and especially lung mitochondria were remarkably insensitive, with $>80\%$ retention of state III oxygen consumption

Figure 2. (A) Effect of high and low concentrations of oxygen on impairment of mitochondrial function under conditions of high and low concentrations of substrate. Incubations were performed as described in the legend to Fig. 1 except that the oxygen tension in the incubation medium was either 160 (▲) or 16 mmHg (●) and pyruvate-malate was added to give the final concentration indicated on the abscissa. After incubation of mitochondria for 2 h at 25°C, mitochondrial function was assayed polarographically at 25°C as described in Methods. The percentage of inactivation of state III rate of oxygen consumption was determined by assuming zero inactivation was the control rate of state III and 100% inactivation occurred when no oxygen consumption occurred after ADP addition. (B) Dependence of impaired mitochondrial function on the concentration of phosphate. Incubations were performed as described in the legend to Fig. 1 except that the amount of inorganic phosphate in the preincubation suspension was varied as indicated. Percentage of inactivation of state III respiration rates was determined as described in A. (C) Susceptibility of mitochondria from different organs to phosphate-induced inactivation. Mitochondria from rabbit lung, liver, kidney, or heart were incubated at 1–2 mg/ml at 37°C for 15 min in 2.0 ml buffer containing 0.25 mg bovine serum albumin, 75 mM Tris-Cl, 1.0 mM EDTA, 112.5 mM potassium chloride, and varying amounts of phosphate as shown on the abscissa (added as potassium monohydrogen phosphate). Afterwards, the mixture was centrifugated and assayed polarographically as described in the legend to Fig. 1. Each experiment was repeated twice and the results are expressed as the percentage of control state III rate of oxygen consumption.

rates at 18.75 mM phosphate, the highest concentration tested and one that rapidly inactivates heart mitochondria even in the presence of 20 mM EDTA and 7.5 mM magnesium (See Fig. 4).

Phosphate concentration dependence of mitochondrial functional impairment under selected isoxic conditions. Experiments performed at 37°C indicated that the process of inactivation was similar in its dependencies to that at 25°C but more rapid, with complete inactivation occurring after 15 min at 37°C instead of 2 h. Subsequent experiments were performed at 37°C for convenience. In addition, the concentration of EDTA was reduced to 1 mM so that effects of calcium would not be precluded.

By varying the oxygen tension in the incubation medium from 0 to 160 mmHg and by performing incubations for 15 min at 37°C in media with selected concentrations of phosphate, we obtained a family of mitochondrial inactivation curves (Fig. 3 A). Even with high concentrations of phosphate, e.g., 20 mM, no loss of mitochondrial function occurred if the incubation medium was anaerobic (lowest curve). On the other hand, rapid

Figure 3. (A) Phosphate-induced inactivation of mitochondria at fixed oxygen concentrations of oxygen. Incubation conditions were 2.0 ml buffer containing 0.25 mg bovine serum albumin, 75 mM Tris-Cl, 112.5 mM potassium chloride, 1 mM EDTA, and fresh rabbit heart mitochondria (2 mg/ml) in the absence of added substrate. Preincubation was performed at 37°C for 15 min. The concentration of phosphate was varied as indicated on the abscissa. A family of inactivation curves was obtained for different fixed oxygen tensions, curves plotted from left to right, and those for oxygen tensions of 136, 112, 48, 16, 8, 2, and 0 mmHg, respectively. The percentage of inactivation of the rate of state III oxygen consumption is plotted as a function of the concentration of phosphate in each case. (B) Apparent K_m for oxygen for the phosphate-induced impairment of mitochondrial function. The data in A are replotted. At fixed phosphate concentrations, the P_{O_2} is plotted as a function of the percentage inhibition of state III respiration to provide an analogue of a classical velocity vs. substrate concentration plot. Then, the apparent K_m , i.e., the concentration of oxygen at which at 50% decrement of impairment of function is evident, is plotted as a function of the concentration of phosphate. As phosphate concentration increases, the apparent K_m approached 1 mmHg.

inactivation occurred with hyperbolic kinetics when oxygen tension was 136 mmHg with the concentration of phosphate as low as 1 mM. Thus, inactivation is an oxygen-dependent and phosphate-induced phenomenon. Between these two extremes of oxygen tension, a series of sigmoid-shaped curves was generated. At oxygen tensions of 2, 8, and 16 mmHg, values comparable to intracellular oxygen tension in vivo under physiological conditions, little mitochondrial dysfunction was induced until phosphate concentrations exceeded 4 mM, a supraphysiological value but one that was exceeded in ischemic and reperfused myocardium (12, 13).

The data in Fig. 3 A, analyzed according to classical kinetics, indicate an apparent K_m for oxygen of ~ 1 mmHg, a value close to that deduced for oxidative phosphorylation itself in isolated mitochondria (23). Thus, when the percentages of state III respiratory rate remaining at phosphate concentrations of 2, 4, 6, 8, 10, and 12 mM are plotted for oxygen tensions of 2, 8, 16, and 48 mmHg (from Fig. 3 A), a series of curves is obtained resembling a velocity vs. substrate concentration relationship (not shown). A plot of the oxygen tension at which 50% of that diminution in inhibition occurs provide a hyperbolic curve (Fig. 3 B). Extrapolation of the apparent K_m for oxygen at high concentrations of phosphate provides an apparent K_m of ~ 1 mmHg for oxygen in the phosphate-induced inactivation.

Effect of calcium chelators and antagonists on mitochondrial inactivation. The concentration of calcium chelators appears to affect the sensitivity of mitochondria to the oxygen-dependent, phosphate-induced effect (19) presumably by interfering with deleterious effects of calcium endogenous to the mitochondria (~ 5 nmol/mg). However, this dependency is not seen with phosphate-induced swelling (9). Accordingly, their effects were examined in the present study with respect to mitochondrial respiratory function (Fig. 4). Decreasing the concentration of EDTA from 20 to 1 mM provided progressively less protection at any given concentration of phosphate under fully aerobic conditions, with 50% inhibition evident at concentrations of

Figure 4. The dependence of mitochondrial impairment on calcium chelates. Incubations were performed in 2.0 ml buffer containing 0.25 mg bovine serum albumin, 75 mM Tris-Cl, 112.5 mM potassium chloride, and the indicated amounts of added phosphate, magnesium or chelator. Aerobic conditions were used and the concentration of chelator and phosphate varied as indicated: 1 mM EGTA (●), 1 mM EDTA (●), 5 mM EDTA (▲), 20 mM EDTA (■), and 7.5 mM magnesium chloride with 20 mM EDTA (○).

phosphate of 5, 2, 0.5, and 0.5 mM for 20, 5, and 1 mM EDTA, or 1 mM EGTA, respectively. Magnesium ion, 7.5 mM, enhanced the protective effect of 20 mM EDTA and shifted the inactivation curve even further to the right. There was also an apparent stimulation of oxygen consumption at lower phosphate concentrations that was reproducibly observed but whose basis remains unexplained.

To assess further the potential calcium dependence of the phosphate-induced, oxygen-dependent inactivation of mitochondria, we prepared mitochondria in either the presence or absence of 1 mM EDTA. Subsequently, the effects of diltiazem, 25 μ M, and ruthenium red, 5 μ M, were evaluated with mitochondria incubated for 15 min at 37°C with 7.5 mM phosphate in either 1 mM or no EDTA. Without diltiazem or ruthenium red, mitochondria exhibited a 95% loss of the rate of state III respiration. Inclusion of either diltiazem, 25 μ M, or ruthenium red, 5 μ M, in the preincubation medium provided 25–50% protection against inactivation of state III respiration. Thus, calcium appears to contribute to the oxygen-dependent, phosphate-induced inactivation of mitochondria under these conditions although it does not account for it entirely.

Because these experiments were performed in the presence of calcium endogenous to the mitochondria, but influx of calcium from blood and extravascular spaces occurs during ischemia and reperfusion, the potential deleterious effects of exogenously added calcium on the phosphate-induced, oxygen-dependent inactivation of mitochondrial respiration was characterized. In this case, mitochondria were prepared as described except that EDTA was omitted from the final two centrifugations and from the incubation at 37°C. Thus, after 15 min at 37°C in the presence of varying and increasing concentrations of phosphate under fully aerobic conditions (Fig. 5), mitochondria in the presence only of endogenous calcium concentrations lost state III oxidation capacity, with 50% reductions

Figure 5. Dependence of phosphate-induced mitochondrial inactivation on exogenous calcium. Mitochondria were isolated as described in Methods except that EDTA was omitted from the last two centrifugations at 9,000 *g*. Mitochondria were then incubated at 37°C for 15 min under aerobic con-

ditions in 2.0 ml buffer containing 0.25 mg bovine serum albumin, 75 mM Tris-Cl, 112.5 mM potassium chloride, the indicated amount of inorganic phosphate, and either no exogenous calcium or sufficient CaCl_2 to provide a final calcium concentration of 2.5 or 5.0 μ M Ca^{++} . The percentage of state III respiration rate remaining compared with control is plotted against the phosphate concentration for a given exogenous calcium concentration.

observed at ~ 6 mM phosphate. In the presence of 2.5 μ M or 5.0 μ M calcium added to the incubation, inactivation proceeded at much lower phosphate concentrations, with 50% inactivation occurring at ~ 2 and 0.1 mM phosphate, respectively. Indeed, not shown are data indicating that in the presence of 250 nM calcium in the incubation, the inactivation curve is virtually identical to that observed in the presence of 2.5 μ M calcium. These results demonstrate that exogenous calcium can participate in the inactivation process and enhance it.

Discussion

The results obtained demonstrate that the impairment of oxidative function in isolated mitochondria induced by inorganic phosphate is dependent on oxygen, compatible with results of studies using other end-points such as mitochondrial swelling (19, 24). The results define a concentration dependence of this phenomenon for both moieties. They also provide insight into conditions needed for prolonged incubations of mitochondria in studies which were otherwise impaired by "aging" and swelling of mitochondrial preparations, or concerned with configurational changes (25), ion influxes (9, 14), and other processes.

Aside from these methodological considerations, the data in Fig. 3 *A* suggest that the oxygen-dependent, phosphate-induced impairment of mitochondrial function may be important in vivo under pathological conditions. Under physiological conditions, intracellular PO_2 ranges between 2 and 10 mmHg (23) and the prevailing concentration of phosphate is 0.5 mM (12, 13). However, with ischemia, intracellular concentrations of inorganic phosphate increase markedly, depending on duration and severity of occlusion; they may exceed 5 mM. Thus, paradoxical oxygen toxicity may be striking with reperfusion when the concentration of phosphate is already high. In fact, reperfusion with oxygen-rich media has been shown to be more detrimental than reperfusion with nitrogenated media under some conditions (5).

The apparent K_m for oxygen was found to be ~ 1 mmHg for the impairment of mitochondrial function, a value close to that found by others for oxidative phosphorylation itself. Thus, mitochondrial respiration may be involved in the inactivation process induced by phosphate, just as electron transport has been implicated in phosphate-induced swelling (19, 26). The present results support the implications from experiments with cyanide or dinitrophenol-induced decreases in phosphate-induced swelling, an indirect end-point, suggesting that oxygen serves as an electron sink that is required to drive ingress of phosphate. As shown in this study, such effects may occur at physiologically relevant oxygen tensions, not only in fully aerobic buffers used in most studies in vitro. Thus, the present results demonstrate the relevance of the phosphate-induced effects to reperfusion injury heretofore unrecognized.

Since intact function is demonstrable in some swollen mitochondria (25), swelling and impaired respiratory function may therefore be dichotomous. The present analysis of functional

end-points indicates a very low apparent K_m for oxygen with respect to phosphate-induced inactivation of mitochondria and suggests that substrate oxidation may be involved in the alterations of mitochondrial function that were induced. The lack of protection against this process by indomethacin, diethylcarbamazine, catalase, and superoxide dismutase, agents that inhibit metabolic pathways known to give rise to potentially toxic products of oxygen, tend to support this conclusion or suggest that secondary metabolites of oxygen are generated in compartments not accessible to these agents.

Previous studies have shown that EDTA prevents phosphate-induced inactivation (19) but only one concentration has been used generally; nevertheless, recent results have not been confirmatory (9). Our results demonstrate that EDTA, EGTA, and magnesium retard but do not entirely prevent inactivation seen with phosphate and oxygen in a concentration-dependent manner. Thus, as suggested by others, calcium endogenous to the mitochondria may be involved in the process or responsible for independent but concomitant deleterious effects (19). Other metal ions chelated by EDTA or EGTA could be important also. Under well-defined conditions, 25 μ M diltiazem, or 5 μ M ruthenium red, partially protects mitochondria from inactivation, which further implicates calcium in the inactivation process. The responsible mechanisms remain obscure, since these agents operate principally at different sites. Phosphate may enter the mitochondria with calcium in a process dependent on electron transport associated with oxidation of substrate. Such transport at the expense of oxidative phosphorylation is well established (27, 28) and is consistent with the phosphate dependence of mitochondrial functional impairment with an apparent K_m for oxygen similar to that for respiration itself. The role of oxygen in phosphate-induced injury may be to provide an electron sink to allow transport into the mitochondrial matrix of phosphate and/or calcium, which could therefore be the primary mediators of injury. Oxygen, itself, of course could also play a direct role and its presence is required for the effects of phosphate and/or calcium to occur. The enhancement of inactivation in the presence of exogenous calcium (Fig. 5) is therefore consistent with these observations and further supports the hypothesis that during ischemia and reperfusion mitochondrial dysfunction may be enhanced.

The basis for a differential susceptibility of mitochondria from different organs is at present unclear. Studies using non-destructive methods such as ^{31}P -nuclear magnetic resonance have not been reported with intact lung, liver, or kidney to provide data for intracellular phosphate concentration in these organs. Although the primacy of calcium transport over oxidative phosphorylation appears to vary from organ to organ (29), it is actually less in heart than in liver and does not obviously explain the noted differences in phosphate-induced inactivation. Variation in yet other factors may be responsible for these differences. For example, activation of various lipases by influx of calcium, substrate specificities of such enzymes, modulation of enzymatic activities by effectors such as palmitoyl carnitine and mitochondrial reparative reserve, as well as others, may all vary

among mitochondria from different organs. Oxygen tensions within heart, liver, and kidney may vary somewhat, but the lung may be exposed to excessive concentrations of oxygen during treatment with hyperbaric oxygen and/or artificial ventilation with oxygen-enriched gas mixtures. In this regard, it is noteworthy that one of the earliest morphological signs of hyperbaric oxygen injury is mitochondrial swelling (30). Although nonspecific in nature, commonality of responses may indicate that the observations reported here may be relevant to oxygen-related injury in addition to reperfusion of ischemic myocardium.

The present findings suggest that protection of jeopardized myocardium may be conferred not only by measures favorably modifying calcium transport into or out of mitochondria but also by those directed toward modulating intracellular concentrations of phosphate and its transport. In addition, elucidating and ameliorating the apparently paradoxical, deleterious effects of oxygen on previously ischemic cells may be beneficial. Thus, at least three variables in this system—calcium, phosphate and oxygen—influence the extent of dysfunction induced. Increases or decreases in the concentration of each may counteract or augment effects associated with increases or decreases in the concentrations of others. Of course, the presence of other compounds such as lysophosphatidylcholine (31) and acyl carnitines (32) and/or lack of substrates such as adenine nucleotides (33, 34) may influence myocardial cell stability and function during and after ischemia as well.

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