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Stimulation of Mitochondrial Oxygen Consumption in Isolated Cardiomyocytes After Hypoxia-Reoxygenation

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An increase in mitochondrial matrix free calcium has been shown to occur during oxygen and substrate deprivation of the perfused heart which predisposes to calcium overload and inhibition of mitochondrial function on reoxygenation. In the current study we have assessed the effect of substrate free hypoxia on mitochondrial oxygen consumption and ATP synthesis in isolated rat cardiomyocytes. Myocytes were subjected to 40 min of substrate-free hypoxia and the oxygen consumption measured together with the effects on ATP and PCr synthesis. After hypoxia myocytes showed a fall in ATP to 10% of the control value. Within 5 sec of reoxygenation the ATP level recovered to a new steady state level of 30% of the original value. The rate of oxygen consumption of hypoxic/reoxygenated cells was 3–4 fold higher than that of cells maintained under normoxic controls but in the presence of oligomycin the difference was only 1.5-fold higher, indicating a greater requirement for mitochondrial synthesis of ATP following reoxygenation. Reoxygenation in the absence of extracellular Ca^{2+} resulted in a lower rate of oxygen consumption (50% of the rate measured in the presence of 1 mM-Ca^{2+}) but did not affect the steady state concentration of ATP attained 5 min after reoxygenation. These results support the idea that the increased O_2 consumption of myocytes following hypoxia/reoxygenation is due to an increased demand for ATP synthesis by mitochondria and is a response to the Na^+ and Ca^{2+} loading of the cells which occurs under these conditions. This increased demand is likely to result in a greater generation of free radicals such as superoxide by the respiratory chain which could impair cellular function over the long term.

Key words: cardiomyocytes, reperfusion damage, mitochondria, hypoxia-reoxygenation

Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenyl hydrazone

INTRODUCTION

Reoxygenation of the myocardium following extended periods of hypoxia and substrate deprivation or ischaemia results not in recovery of the tissue but in gross physical damage. This response to oxygen, the 'oxygen paradox' has been studied extensively in perfused hearts where the cell damage is characterised by mitochondrial Ca^{2+} overload and cell lysis.[1,2] Isolated myocytes do not show cell lysis under these conditions but they do undergo hypercontracture and show a marked oxygen-dependent uptake of Ca^{2+}.[3–5] They therefore provide a valuable alternative system for

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studying the mechanisms occurring during hypoxia and reoxygenation without the complication of cell lysis.

There is good evidence from both perfused hearts and isolated myocytes to show that mitochondria play a key role in the events that contribute to the oxygen paradox. Thus inhibition of mitochondrial electron transport, e.g. with either cyanide or antimycin A, results in elimination of the reoxygenation-induced cell lysis in perfused hearts and inhibition of Ca^{2+} uptake in myocytes.[1,2,5] Inhibition of mitochondrial Ca^{2+} uptake via the mitochondrial uniporter by ruthenium red reduces both damage in the perfused heart and cellular Ca^{2+} uptake in myocytes suggesting that Ca^{2+} uptake by mitochondria may be a key event in the processes leading to cell damage.[5–7]

A number of studies have provided evidence that mitochondrial electron transport is compromised by ischaemia or following hypoxia/reoxygenation[8,9,10] but to date the relevance of these observations to the functional competence of mitochondria on reoxygenation has not been established.

In the present study we have examined the behaviour of myocytes with regard to their respiration under the same conditions where Ca^{2+} uptake has been shown to occur. We have also examined the effect that Ca^{2+} uptake has on this process. Our results suggest that the behaviour of mitochondria on reoxygenation can be explained on the basis of the changed ionic status of the cell that occurs during hypoxia.

MATERIALS AND METHODS

Isolation of myocytes

Calcium tolerant myocytes were prepared from the hearts of adult rats (200–300 g) by perfusion with collagenase (Worthington type 1) essentially as described previously[5,11] with the modification that the composition of the nominally Ca^{2+}-free perfusion buffer was as follows: (mM) NaCl, 118.5; $NaHCO_3$, 14.5; KCl, 2.6; KH_2PO_4, 1.2; $MgSO_4$ 1.2; glucose, 11.0; HEPES, 10.0, pH 7.4. The preparation, after purification by sedimentation through bovine serum albumin (BSA) was allowed to sediment a further 3 times under gravity in perfusion buffer containing 1 mM-$CaCl_2$ and 0.1% (w/v) BSA (buffer A) in order to improve the percentage of rod-shaped cells. The final suspension of about 4 mls of myocytes at $0.5 - 1 \times 10^6$ rods/ml was maintained for up to an hour at 25°C under O_2/CO_2 (19:1) in buffer A until required. At the start of the experiments the myocyte preparations contained at least 70% rod shaped cells that excluded trypan blue.

Hypoxia and reoxygenation of myocytes

Myocytes were transferred to glucose-free buffer A by centrifugation (25g for 5s) followed by resuspension in the same medium: this procedure was repeated three times and was judged sufficient to reduce the glucose concentration of the bathing medium to less than 0.1 mM. Portions (1–1.8 ml depending on the nature of the experiment) of this myocyte suspension were transferred to 10 ml siliconized conical flasks and incubated at 37°C in a shaking water-bath (100 cycles/min) as previously described.[5]

Measurement of O_2 uptake

Glucose-free buffer A containing 1 μM-ruthenium red and either 0 or 1.0 mM-$CaCl_2$ was preincubated at 37°C in a shaking water-bath, allowed to equilibrate with air and then 1.5 ml was added to the chamber of the Clark-type oxygen electrode (Rank brothers, Ltd. Cambridge, U.K.). Hypoxic or normoxic myocytes (500 μl) were rapidly added from the incubation flasks and the O_2 uptake rates measured over 10–20 min. The background due to O_2 consumption by the electrode was subtracted from all subsequent measurements of oxygen uptake by the cells.

Adenine nucleotide and creatine phosphate assays

Cell samples (generally 100 µl, 1×10^6 cells/ml) were extracted with 2 vol. of ice-cold 6M-perchloric acid containing 30% (v/v) methanol. The supernatants were neutralized with 1.0M-K_2HPO_4 and the precipitated potassium perchlorate removed by centrifugation. The supernatants were stored at –20°C. HPLC analysis was carried out on a Beckman System Gold HPLC as described.[12] In experiments where both creatine phosphate and adenine nucleotides were determined on the same sample, the sample preparation was carried out as described for the creatine phosphate determinations and a portion of the sample analysed as described above. Creatine phosphate was measured by HPLC essentially as described[13] with some minor modifications. Cell samples were extracted with 0.5 vol. of ice-cold perchloric acid (1.2-M) containing 60% (v/v) methanol. Supernatants were neutralized with 1M-KOH containing 0.1M-Tris base so that the resultant pH was between 7 and 9. Samples (25 µl) of supernatant were analysed using the same HPLC system and column as that described above for the adenine nucleotides but the column was eluted isocratically with 15mM-KH_2PO_4, 2.4mM-tetrabutylammonium hydrogen sulphate (pH 3.0). This modified sample preparation method and small sample volume ensured that the creatine phosphate was eluted as a single, well-defined peak.

RESULTS

Myocyte preparations in these experiments were calcium tolerant and contained at least 70% rod-shaped cells that excluded trypan blue. The initial ATP content of the preparations was 24.2 ± 2.8 nmol/mg protein (mean ± S.S., n = 7) and the ATP:ADP ratio was 6.0 ± 2.8 (mean ± S.D., n = 7). The effect of hypoxia and subsequent reoxygenation on the ATP and creatine phosphate contents of the cells is shown in Figure 1. On hypoxia in glucose-free medium, the creatine phosphate content of the cells fell rapidly from its initial value of 44.0 + 1.8 (mean ± S.D., n = 3) nmol/mg protein to 11.9 + 0.9 (mean ± S.D., n = 3) nmol/mg protein and thereafter continued to decline steadily throughout the period of hypoxia. The ATP content of the cells remained at control levels for the first 20–25 min of the hypoxic period but then fell sharply to about 10% of this value over a period of 10–15 min. When oxygen was re-introduced after 40 min hypoxia the ATP content rose from 1.9 ± 0.18 to 9.5 ± 0.9 nmol/mg protein (mean ± S.D., n = 6). This new ATP level was established within 5 sec of admitting oxygen (the earliest

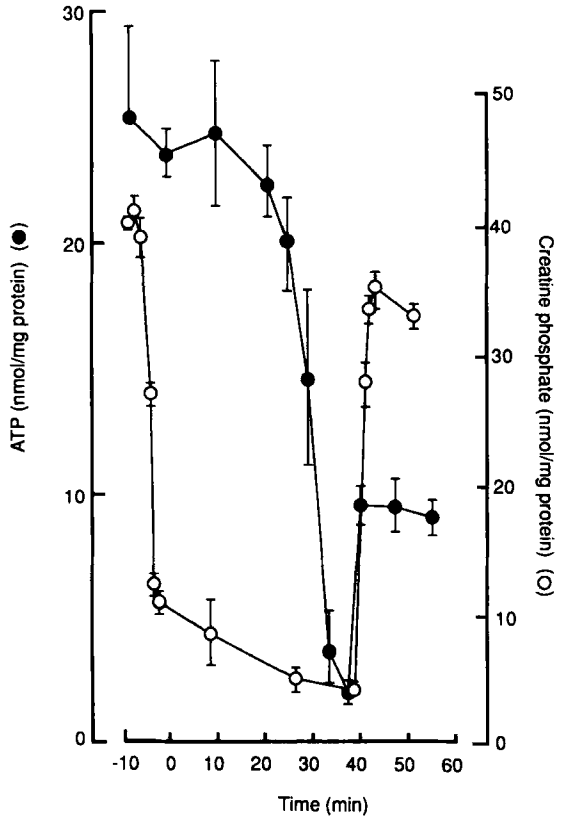

FIGURE 1 Effect of hypoxia-reoxygenation on the ATP and the creatine phosphate content of myocytes. Myocytes (2–3 mg protein/ml) were maintained under hypoxia at 37°C for 40 min and then reoxygenated. Samples were taken as indicated for ATP and creatine phosphate determination by HPLC. Values are means ± S.D. for three determinations.

practical time that samples could be taken) and was maintained for at least 10 min as was the new ATP/ADP ratio of 2.3 ± 0.5 (mean ± S.D., n = 6). In control experiments where hypoxia was sustained a gradual decline in ATP levels continued (not shown). Over the same time course, the creatine phosphate returned to 85% of the pre- hypoxic level indicating that little cell lysis occurred as a result of the hypoxia-reoxygenation treatment. This observation is consistent with our previous studies which showed that no reoxygenation-dependent release of the cytosolic enzyme creatine kinase occurs under these conditions[5] and the recovery of creatine phosphate on reoxygenation is similar to that reported by others under similar conditions.[4]

In order to characterise the behaviour of mitochondria on reoxygenation the respiration rates of normoxic and reoxygenated myocytes were compared in the oxygen electrode. When myocytes maintained in either hypoxic or normoxic conditions were incubated for 40 min in the absence of glucose and subsequently diluted four fold in the oxygen electrode (thus allowing reoxygenation of the hypoxic myocytes) it was apparent that steady state rates of oxygen consumption could be measured within about 30 sec. A typical experiment of this type, in which normoxic and hypoxic-reoxygenated cells are compared, is illustrated in Figure 2. The first few seconds of rapid O_2 consumption is due solely to the effect of adding a hypoxic solution to the chamber of the oxygen electrode. Normoxic cells showed a sustained but low rate of oxygen uptake whereas the cells previously subjected to hypoxia showed a rapid consumption of oxygen followed by a sustained high rate of uptake. The subsequent O_2 uptake of reoxygenated myocytes, which is clearly 3–4 fold higher than that of normoxic cells, was completely inhibited by rotenone showing that it is due to increased respiration in these cells (result not shown). The data from a number of experiments are summarised in Table 1. Oligomycin (35 μM), an inhibitor of the mitochondrial ATP synthase,

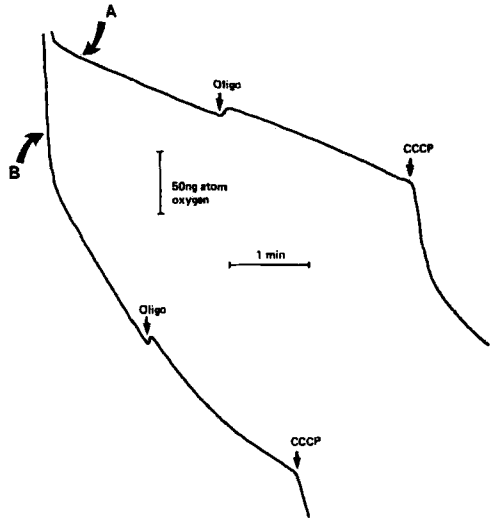

FIGURE 2 Uptake of O_2 by myocytes after 40 min normoxia (A) or 40 min hypoxia (B) in glucose-free medium. Myocytes (5 mg protein/ml) were incubated in the oxygen electrode in glucose-free buffer A containing 1.0 mM $CaCl_2$ and 1 μM ruthenium red. Further additions: Oligo, oligomycin (36 μM); CCCP, carbonyl cyanide m-chlorophenylhydrazone (1 μM).

TABLE 1 Effect of oligomycin and Ca^{2+} on O_2 consumption of reoxygenated myocytes. Rates are expressed as mean ± S.D. for the number of experiments given in parentheses. The protocols for the treatments are described in figs. 2, 3 and 4. Rates in the presence of oligomycin (36 μM) were measured 1–2 min after its addition.

	Respiration (ng atoms O/min/mg protein)	
Treatment	control	+ oligomycin
Normoxic + glucose (10 mM)	26.6 ± 2.8 (5)	22.3 ± 2.4 (6)
Normoxic – glucose	14.6 ± 1.6 (8)	14.2 ± 2.4 (6)
Reoxygenated – glucose	52.6 ± 5.6 (5)	20.2 ± 1.8 (7)
Reoxygenated + EGTA	26.0 ± 4.0 (6)	18.8 ± 2.4 (3)
Reoxygenated + EGTA + Ca^{2+}	47.6 ± 8.0 (6)	20.2 ± 2.2 (5)

reduced this O_2 utilisation of reoxygenated cells by 61.6 ± 3.5% (mean ± S.D., n = 7) indicating that approximately two thirds of the increased oxygen utilisation was coupled to ATP production. Addition of oligomycin to normoxic myocytes, however, had no measurable effect on their oxygen uptake suggesting that the rate of mitochondrial ATP production was much lower in these cells. Addition of the uncoupler CCCP resulted in comparable rates of O_2 consumption for the normoxic or reoxygenated cells suggesting that the differences observed were not due to differences in either substrate availability or competence of the electron transport chain (Figure 2).

The total ADP concentration (free and protein-bound) in the supernatant from reoxygenated myocytes was only 1–2 μM and addition of exogenous ADP (1mM) resulted in no change in the O_2 consumption of reoxygenated cells suggesting that damaged mitochondria do not contribute to the observed O_2 utilisation.

Under the conditions where myocytes show increased O_2 consumption, i.e. reoxygenation following a period of hypoxia, we have previously demonstrated that the Ca^{2+} content of the cells increases by 2–3 fold.[5] We have, therefore, examined the effect of extracellular Ca^{2+} on the O_2 utilization by reoxygenated myocytes. In these experiments myocytes were maintained in flasks under hypoxic conditions in the presence of 1mM-Ca^{2+} for 39 min and 1mM-EGTA was added 1 min before the reintroduction of O_2 to reduce the extracellular Ca^{2+} to<20 μM. The cells were then transferred to the oxygen electrode into a medium containing 1mM-EGTA (Figure 3). This procedure allowed the cells to be reoxygenated in the absence (Figure 3A) or presence (Figure 3B) of normal extracellular Ca^{2+}. The rate of oxygen utilisation under these conditions of low extracellular Ca^{2+} was greater than that observed in normoxic cells but was clearly somewhat lower than that observed in reoxygenated cells in the presence of 1 mM-Ca^{2+} (Table 1). Addition of sufficient Ca^{2+} to increase the free Ca^{2+} concentration to 1 mM (Figure 3A) resulted in a stimulation of

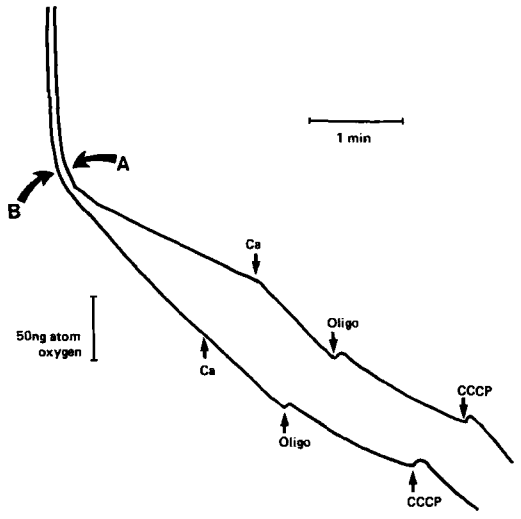

FIGURE 3 Effect of Ca^{2+} on the O_2 consumption of reoxygenated myocytes. Myocytes were maintained for 40 min under hypoxia in glucose-free medium and reoxygenated in the oxygen electrode in the presence of either 1 mM $CaCl_2$ plus 1 mM EGTA (A) or 1 mM $CaCl_2$ (B). Myocytes (2–3 mg protein/ml) were used for each of the experiments illustrated. The following additions were made as indicated; Ca, $CaCl_2$ (1 mM); Oligo, oligomycin (36 μM); CCCP, (1 μM).

the rate of oxygen consumption to that observed in control cells reoxygenated in the presence of 1 mM Ca^{2+} (Figure 3B). A similar protocol, but using cells which had been treated with oligomycin (36 μM) 5 min before reoxygenation, is illustrated in Figure 4. Addition of Ca^{2+} under these conditions did not result in a significant increase in the rate of O_2 consumption suggesting that the Ca^{2+}-stimulated increase observed was due to enhanced synthesis of ATP in the presence of Ca^{2+}.

The ATP contents of cells reoxygenated under control conditions (1 mM-Ca^{2+}) and in the presence of 1 mM-EGTA were 9.5 ± 0.9 nmol/mg protein (mean ± S.D., n = 6) and 9.5 ± 1.1 nmol/mg protein (mean ± S.D., n = 6) respectively. Thus the presumed greater rate of synthesis of ATP in the presence of Ca^{2+} was not however reflected in differences in the steady-state ATP content of the cells reoxygenated in either low or high Ca^{2+} when these were measured 5 min after reoxygenation.

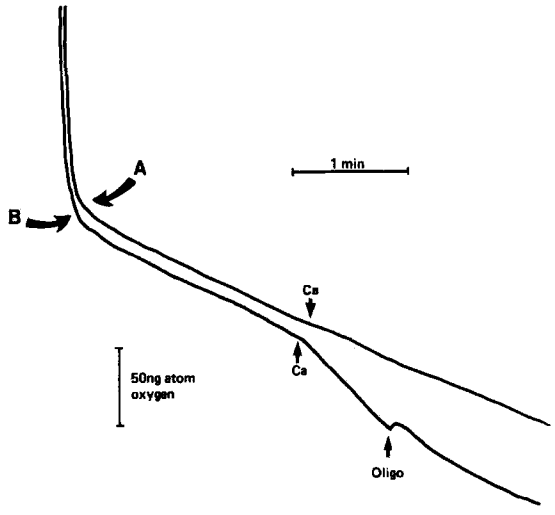

FIGURE 4 Effect of oligomycin on calcium-stimulated respiration in hypoxic myocytes. Myocytes (2–3 mg protein/ml) were used for each of the experiments illustrated. Myocytes were maintained under hypoxia in glucose free medium to which EGTA (1 mM) was added 1 min before reoxygenation. A: Oligomycin (36 μM, gassed with N_2/CO_2 to prevent concomitant addition of oxygen) was added to myocytes after 35 mins hypoxia (ie. 5 mins before reoxygenation). $CaCl_2$ (1mM) was added as indicated. B: $CaCl_2$ (1 mM) and oligomycin (36 μM) were added as indicated.

DISCUSSION

In the present study the O_2 consumption of myocytes, in the absence of exogenous substrates, was some 3–4 fold higher after a period of hypoxia followed by reoxygenation compared with that of cells maintained under normoxic conditions. This difference was observed in the absence of any exogenous substrates i.e. it occurred under conditions where perfused hearts show the oxygen paradox response and isolated myocytes show an increase in total cell Ca^{2+}.[5] Although other workers have reported a similar increase in respiration when myocytes are reoxygenated after a period of substrate-free hypoxia, these studies differ from the present study in that glucose was added to the cells before the rates of O_2 consumption were measured.[13] In these earlier studies, although mitochondrial Ca^{2+} measured at the end of the hypoxic period was not significantly different to the normoxic controls, no Ca^{2+} measurements were made following reoxygenation. It is not clear, therefore, whether or not the increase in respiration observed by these workers was associated with a change in Ca^{2+} on reoxygenation.

In the present study new steady-state levels were established within seconds of the introduction of oxygen, i.e. over a time course that could not be investigated using the techniques employed in the present study. Interestingly, however, the increased rate of O_2 consumption persisted for many minutes after the reintroduction of oxygen and corresponded to a period of time over which no changes in the steady state levels of either ATP or creatine phosphate were observed (Figures 1, 2).

Myocyte preparations, particularly after periods of hypoxia, are mixtures of cells with intact and broken sarcolemmae it was therefore important to rule out the contribution of mitochondria in 'dead' cells to these effects. In order to exclude O_2 uptake due to Ca^{2+} uptake and cycling by such mitochondria 1 μM ruthenium red was routinely included in the medium. We have previously shown[5] that this concentration of ruthenium red effectively inhibits Ca^{2+} uptake into the mitochondria of digitonin-permeabilised myocytes while having no significant effect on the reoxygenation-induced Ca^{2+} uptake by intact cells. Such mitochondria could also contribute to the oligomycin-sensitive component of the oxygen uptake but only if there were sufficient concentrations of substrates and ADP in the medium.

The creatine phosphate content of the cells recovered to some 85% of the value determined in the normoxic cells but the ATP concentration in the reoxygenated cells was only about 30% of the normoxic value. It is possible that normoxic myocytes had more glycogen stores available than those subjected to hypoxia-reoxygenation. However, it is clear that mitochondrial O_2 consumption was not limited by substrate availability under either condition (Figure 2). These observations

suggest that the failure of the ATP levels to return to the normoxic value was not due to any limitation in the ability of the mitochondria to synthesise ATP but is, as suggested by others due to the loss of adenosine from the cell during hypoxia.[4] The increased rate of O_2 utilisation with no change in ATP concentration observed in the reoxygenated cells could result either from increased turnover of ATP or from less efficient synthesis by the mitochondria (or both). Oligomycin, an inhibitor of the mitochondrial ATP synthase inhibited more than 70% of the increase in O_2 consumption indicating that most of the increase can be ascribed to an increase in ATP synthesis and suggesting that ATP turnover is increased in hypoxic/reoxygenated cells compared with normoxic cells. The observation that a large proportion of the increase is dependent on the presence of extracellular Ca^{2+} provides a clue regarding the mechanism involved. Our previous studies, in which we have measured the cell Ca^{2+} content of the cells using an identical experimental protocol for hypoxia and reoxygenation, have shown that these cells take-up Ca^{2+} on reoxygenation following hypoxia and that during the period of time over which we have measured the O_2 uptake in the present study, the intracellular Ca^{2+} in the cells is elevated compared with normal.[5] Such an increased cellular load of Ca^{2+} would be expected to result in increased ATP utilisation due to increased uptake by the sarcoplasmic reticulum and increased export from the cell via the plasma membrane Ca^{2+} ATPase. The enhanced stimulation of mitochondrial electron transport would probably also be associated with an increased generation of superoxide and data in support of this idea has been presented.[15,16]

There is good evidence to suggest that during hypoxia myocytes become loaded with sodium[17,18] and that on reoxygenation the Na^+/Ca^{2+} exchanger of the plasma membrane exchanges cytosolic Na^+ for extracellular Ca^{2+}[7,19,20] The time course of the Ca^{2+} influx and Na^+ efflux under these conditions has not been defined but it is clear that the overall Ca^{2+} content of the cells remains elevated for some time following reoxygenation. It is likely, therefore, that the cytosolic sodium concentration also exceeds the normal physiological range for a similar period and if this is the case some increased ATP utilisation will result from stimulation of the plasma membrane Na^+/K^+ ATPase. This could account for our observation that the O_2 consumption of cells reoxygenated in the absence of Ca^{2+} is higher than that of normoxic controls and that oligomycin inhibits a large part of this increase.

Under conditions where cytosolic Ca^{2+} is elevated the mitochondria become loaded with Ca^{2+}.[21,22] This clearly occurs in perfused hearts subjected to hypoxia/reperfusion where estimations of mitochondrial Ca^{2+} have been made.[23–26] Our previous results with myocytes also provide strong evidence that increases in mitochondrial Ca^{2+} occur. In a previous study we suggested that since mitochondria can take-up and release Ca^{2+} in a futile cycle at the expense of ATP synthesis such a process may reduce ATP levels under these conditions and may actually result in cell death.[7] Our present results however, which show that a large proportion of the increase in O_2 utilisation is inhibited by oligomycin, suggest that futile cycling of Ca^{2+} does not occur to any great extent under these conditions. This conclusion is consistent with the observations of other workers[25] who have shown that sodium loading of myocytes with veratridine treatment results in a very similar stimulation of respiration to the one that we have observed in this study and who concluded that Ca^{2+} cycling does not occur under those conditions. This conclusion is also supported by our finding that cyclosporin, which inhibits futile cycling of Ca^{2+} by mitochondria, has little or no effect on the oxygen dependent increase in total cell Ca^{2+} which is observed when myocytes are reoxygenated.[27]

We and others have shown that reoxygenation results in specific defects in mitochondrial functions, particularly in complex I function, which might limit the ability of the mitochondria to resynthesize ATP.[9,10] The mechanism leading to

these defects are not known but are Ca^{2+} dependent and may involve enhanced superoxide formation in the active site of this respiratory complex.[15,16]

In conclusion, therefore, we suggest that the increased respiration in cells subjected to hypoxia and reoxygenation can, to a large extent, be explained by the increased utilization of ATP associated with Na^+ loading of the cells during hypoxia and the resulting Ca^{2+} loading that occurs on reoxygenation.

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