Mitochondrial ATP production is necessary for activation of the extracellular-signal-regulated kinases during ischaemia/reperfusion in rat myocyte-derived H9c2 cells

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To search for the stimuli involved in activating the mitogen-activated protein kinases (MAPKs) during ischaemia and reperfusion, we simulated the event in a system *in vitro* conducive to continuous and non-invasive measurements of several major perturbations that occur at the time: O₂ tension, mitochondrial respiration and energy status. Using H9c2 cells (a clonal line derived from rat heart), we found that activation of the extracellular signal-regulated MAPKs (ERKs) on reoxygenation was abolished if the mitochondria were inhibited prior to and during reoxygenation. Re-introduction of O₂ *per se* is therefore not sufficient to activate the ERKs. Recovery and maintenance of cellular ATP levels by mitochondrial respiration is necessary, although ATP recovery alone is not sufficient. ERK activation by H₂O₂, but not phorbol esters, was also sensitive to mito-

chondrial inhibition. Thus, reoxygenation and H₂O₂-mediated oxidative stress share a mechanism of ERK activation that is ATP- or mitochondrion-dependent, and this common feature suggests that the reoxygenation response is mediated by reactive oxygen species. A correlation between ERK activity and ATP levels was also found during the anoxic phase of ischaemia, an effect that was not due to substrate limitation for the kinases. Our results reveal the importance of cellular metabolism in ERK activation, and introduce ATP as a novel participant in the mechanisms underlying the ERK cascade.

Key words: anoxia, MAPK, mitochondrial inhibitor, reactive oxygen species, oxidative stress.

INTRODUCTION

All three mitogen-activated protein kinase (MAPK) subfamilies have been implicated in the responses to ischaemia and reperfusion in heart, brain, kidney and liver [1–7]. The general pattern comprises activation of the p38 kinases during ischaemia, followed by the extracellular-signal-regulated kinases (ERKs) and the stress-activated protein kinases (SAPKs) upon reperfusion. However, it has been difficult to identify the stimuli for these signalling cascades as many changes occur during ischaemia and reperfusion, e.g. perturbations to O₂ tension (*p*O₂), cellular metabolism, ATP levels, pH, ion gradients and the production of reactive oxygen species (ROS). Of these events, ROS production has received particular attention as the ERKs and SAPKs are known to be activated by oxidative stresses, such as H₂O₂ [2,5,8–10]. This has led to the suggestion that the ROS produced at reperfusion [11–14] mediate the ERK/SAPK response. As H₂O₂ is easily used *in vitro*, much research on the MAPKs has been done using exposure to H₂O₂ as a model for the oxidative stress that occurs on reperfusion [2,5,9,10]. This assumes, however, that ROS are the main stimuli for these kinases. Notwithstanding the possible importance of ROS, another major event that occurs during reperfusion, and which may also have implications for MAPK activation, is metabolic recovery.

Perturbations to cellular metabolism are a distinguishing feature of ischaemia and reperfusion. Oxidative metabolism is compromised as O₂ is depleted, but recovers upon reperfusion

and reoxygenation. An indication that cellular metabolism may play a role in the reperfusion activation of the MAPKs comes from studies in which the metabolic inhibitors, cyanide (CN) and 2-deoxyglucose, were used to simulate the metabolic inhibition aspect of ischaemia. Metabolic recovery following removal from such ‘chemical anoxia’ led to activation of the ERKs and/or the SAPKs in kidney epithelial cells [6] and cardiac myocytes [15]. This implies that metabolic recovery may contribute to the reperfusion activation of the ERKs/SAPKs in an ischaemic event, and so it may be metabolism rather than (or in combination with) ROS that activates the MAPKs. However, the chemical anoxia model cannot be used to discriminate between a metabolic- or ROS-based mechanism of MAPK activation due to the confounding factors that the application of CN in the presence of O₂ enhances the mitochondrial production of ROS [13,16], and the effect of any such ROS is potentiated as the protective enzymes catalase [17] and superoxide dismutase [18] are inhibited by CN. Also, chemical anoxia does not involve the dramatic changes in *p*O₂ that are such definitive events in ischaemia and reperfusion, and which may be crucial for signal transduction in terms of ROS and/or cellular O₂ sensors.

For these reasons, the activation of the MAPKs seen on metabolic recovery from chemical anoxia may not apply to the anoxia/reoxygenation situation. Here, we set out to delineate the role of metabolic inhibition and recovery in the activation of the MAPKs during ischaemia and reperfusion, and to see whether O₂ was a direct stimulus or had an indirect role via its

Abbreviations used: CN, cyanide; ERK, extracellular-signal-regulated kinase; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; MAPK, mitogen-activated protein kinase; MBP, myelin basic protein; MEK, MAPK or ERK kinase; *p*O₂, oxygen tension; ROS, reactive oxygen species; SAPK, stress-activated protein kinase; *V*_{O₂}, rate of oxygen consumption.

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participation in the production of ROS and/or metabolic recovery. We simulated ischaemia/reperfusion *in vitro* in a physiologically meaningful sense, collected precise and accurate data on crucial parameters (pO_2 , metabolic rates, ATP) and related the changes to the activity of the ERKs. The ERKs were chosen due to their known responses to metabolic and oxidative stresses, and because they are activated early in reperfusion at a time when metabolic recovery is also occurring. We also investigated a previously reported phenomenon that has never been explained, that of a decrease in basal ERK activity during ischaemia [1,10] or chemical anoxia [6,15].

EXPERIMENTAL PROCEDURES

Materials and cell culture

Bovine brain myelin basic protein (MBP), myxothiazol, antimycin A, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) and PMA were from Sigma.

H9c2 cells, a clonal line derived from rat heart (ATCC CRL-1446; ATCC, Rockville, MD, U.S.A.), were grown in Dulbecco's modified Eagles medium with 10% fetal calf serum, 100 units/ml penicillin and 0.1 mg/ml streptomycin. At confluency, serum was reduced to 1% for 24 h, then eliminated for a further 24 h. Cells were trypsinized, washed and resuspended in sterile incubation buffer (25 mM Hepes, 125 mM NaCl, 5 mM KCl, 0.5 mM $MgCl_2$, 0.9 mM $CaCl_2$, 5 mM glucose, 2 mM sodium acetate, 2 mM glutamine and 0.5 mM lactate, pH 7.4 at 37 °C, 300 mOsm) at 1×10^6 – 2×10^6 cells/ml and transferred to glass O_2 electrode chambers (Strathkelvin Instruments, Glasgow, Scotland, U.K.) maintained at 37 °C. Viability as measured with 0.4% eosin was at least 85%. Chambers typically contained 3–4 ml of cells, and were stirred continuously.

Simulated ischaemia/reperfusion incubations with or without mitochondrial inhibitors

After 15–20 min of pre-incubation, an O_2 electrode (Strathkelvin Instruments) was inserted into the chamber to seal the cell suspension from air and to measure pO_2 continuously. The pO_2 data were collected using a MacLab system. During the initial period of progressive hypoxia (about 30 min) and the ensuing anoxic period, samples (cells+medium) were obtained via a stainless-steel tube built into the electrode [19] to avoid perturbing the pO_2 of the suspension. Two samples were collected at each time point, one directly into cold perchloric acid/EDTA (0.6 M/5 mM final concentrations) for ATP and lactate assays, and another frozen immediately in liquid N_2 for ERK assays. After 30 min of anoxia, the electrode was removed to bring about an immediate and spontaneous reoxygenation of the suspension and a return to normoxic pO_2 (140–160 μ M) by 3 min. Incubations were continued for 30 min.

To distinguish between the effect of O_2 *per se* and metabolic recovery during reoxygenation, metabolic recovery was prevented by injecting mitochondrial inhibitors (1 μ M myxothiazol or antimycin, final concentration) or solvent (0.7 mM DMSO or 0.9 mM ethanol, final concentration) into the anoxic chambers about 5 min before reoxygenation. Alternatively, the uncoupler FCCP (2 μ M final concentration) was used. The possible dependence of metabolic recovery on ERK activation was also investigated by treating cells with the MEK (MAPK or ERK kinase) inhibitor PD98059 (50 μ M final concentration; Calbiochem, San Diego, CA, U.S.A.), or solvent (7 mM DMSO, final concentration) for 10 min, then conducting the hypoxia/anoxia/reoxygenation protocol in the continued presence of the drug.

To delineate further the role of ATP recovery in ERK activation, the period immediately following reoxygenation was investigated in more detail. Cells were subjected to hypoxia/anoxia/reoxygenation, then inhibited with 1 μ M antimycin or myxothiazol at either 30, 60 or 90 s post-reoxygenation.

Normoxic incubations with mitochondrial inhibitors

All concentrations given here are final. Mitochondrial inhibitors (1 μ M myxothiazol or antimycin, or 1 mM KCN) or the uncoupler FCCP (1–4 μ M) were added to normoxic cells to assess the effects of the drugs on the rate of oxygen consumption (V_{O_2}), ATP levels, lactate production and ERK activity. Initial samples and intermittent V_{O_2} measurements were taken during 15–30 min of pre-incubation. Inhibitors or solvent (0.7 mM DMSO, 0.2–0.9 mM ethanol or saline) were then added and sampling continued for 40 min. V_{O_2} was remeasured to determine the effectiveness of the drugs. For the experiments with H_2O_2 , cells were pre-incubated for 15 min with 1 μ M antimycin or myxothiazol, or solvent, then treated with 100 μ M H_2O_2 or water for 30 min. The PMA experiments were performed on cells subjected previously to the simulated ischaemia/reperfusion protocol. Cells were taken from the control and inhibited chambers about 40 min after reoxygenation (with or without inhibitors) and treated with 100 nM PMA or solvent (1.4 mM DMSO) for 30 min at 37 °C.

In-gel MBP kinase assays

After collection into liquid N_2 , samples were stored at –80 °C. Cells were lysed on ice for 30–45 min in lysis buffer [final concentrations: 1% Triton X-100, 20 mM Tris (pH 6.8), 50 mM NaF, 20 mM β -glycerophosphate, 0.1 mM $NaVO_4$, 4 mM EDTA, 2 mM EGTA, 1 mM benzamidine, 10 μ g/ml aprotinin, 20 μ g/ml leupeptin and 1 mM PMSF], then centrifuged (10000 g, 10 min, 4 °C). Supernatants were transferred to loading buffer [final concentrations: 60 mM Tris (pH 6.8), 1% SDS, 10% glycerol and 5% β -mercaptoethanol] and fully denatured (90 °C, 3 min). Proteins were separated by SDS/PAGE with 10% gels containing 0.5 mg/ml MBP. ERK activity was determined by the in-gel MBP-kinase method [2]. Dried gels were exposed to Fuji imaging plates for 6–16 h, and the plates processed in a Fuji BAS1000 Imager. Bands corresponding to ERK1 and ERK2 were quantified by densitometry using a MacBAS program. Quantification was validated by standard curves, which were linear within the range studied.

Western-blot analysis

Cell extracts were separated by SDS/PAGE (10 or 12.5% gels) and transferred to nitrocellulose membranes (Hybond ECL, Amersham) using a semi-dry transfer apparatus (Bio-Rad). Membranes were blocked with 5% skimmed milk and probed with primary antibodies from Zymed Laboratories (ERK1/2) or New England Biolabs (all others). Horseradish peroxidase-linked secondary antibodies (Pierce) were used for detection by enhanced chemiluminescence (LumilightPlus from Roche, or Super Signal from Pierce). Films were processed conventionally.

ATP and lactate measurement

Perchloric acid extracts were neutralized and assayed for ATP and lactate by enzyme-linked spectrophotometric assays ([20], pages 121–122 and 188–189) on a SPECTRAMax 340 microplate spectrophotometer (Molecular Devices, Sunnyvale, CA, U.S.A.). ATP levels have been presented as percentages of the initial levels

(see the Figure legends). The actual ATP content of the H9c2 cells was 12.49 ± 0.45 nmol/ 10^6 cells ($\pm$ S.E.M.; $n = 16$), which is similar to a previous report (10 nmol/ 10^6 cells [21]). Using our estimated cell volume for these cells of $3 \mu\text{l}/10^6$ cells and a cell water content of 70 %, the normal intracellular concentration of ATP is about 6 mM.

Statistical analysis

All results are expressed as means $\pm$ S.E.M. ($n = 3$ or more). Comparisons were made using two-tailed paired or unpaired t tests, or a one-way repeated-measures ANOVA with either Fisher's protected least-squares difference (PLSD) or Dunnett's two-tailed test of significance. Differences were considered significant at $P < 0.05$.

RESULTS

Simulated ischaemia/reperfusion affects cell metabolism and ERK activity

The simulated ischaemia/reperfusion protocol commenced with an initial normoxic/hypoxic period as the cells gradually consumed all dissolved O_2 to reduce O_2 concentration from about $140 \mu\text{M}$ down to $0 \mu\text{M}$. During this period, ATP turnover (using a P/O_2 ratio of 4.5, and 1 mol of ATP/mol of lactate) was 1317 ± 106 nmol of ATP/ 10^6 cells per h (all metabolic rates are $n = 5$), comprising 98 % oxidative metabolism (285 ± 21 nmol of O_2 / 10^6 cells per h) and 2 % glycolytic (33 ± 10 nmol of lactate/ 10^6 cells per h). Neither ATP levels (Figure 1a) nor ERK activity (Figures 1b and 1c) changed during this initial period. At the onset of anoxia, glycolysis was stimulated 8.5-fold (282 ± 44 nmol of lactate/ 10^6 cells per h) to maintain ATP turnover at 21 ± 1 % of the oxidative rate. ATP levels started to decrease by 1 min of anoxia (results for 1–10 min not shown), and progressively declined over the next 15–20 min to reach 31 ± 1 % of initial levels at 30 min of anoxia (Figure 1a). ERK activity declined in a similar manner, decreasing to 30 ± 2 % (ERK1) at 30 min of anoxia (Figures 1b and 1c). This effect was specific for the ERKs as the activity of the 38-kDa MBP kinases (the p38 MAPKs) either increased or remained steady during hypoxia and anoxia (Figure 2), whereas other unidentified bands (Figure 1b) were not affected. As the pattern of activity of the p38 MAPKs was not as consistent as that of the ERKs, the results were not quantified.

Upon reoxygenation, V_{O_2} recommenced at 88 ± 3 % of the initial (normoxic/hypoxic) rate to enable ATP levels to recover to 79–88 % of initial values over the next 30 min (Figure 1a). Total ATP turnover (1204 ± 115 nmol of ATP/ 10^6 cells per h) and lactate production (73 ± 40 nmol/ 10^6 cells per h) returned to rates that were not significantly different from initial rates. Reoxygenation also caused rapid and short-term activation of the ERKs, with a peak between 5 and 7 min and a return to initial levels by 20 min (Figures 1b and 1c). The peak activation of ERK1 was 8.6-fold higher than the immediate anoxic value, or 2.6-fold higher than the initial control level (Figure 1c).

The activity profiles of the ERKs were confirmed by changes in phosphorylation status (Figure 3b). The specific activator of the ERKs, MEK1/2, was also dephosphorylated during anoxia, and reactivated upon reoxygenation (Figure 3d). As the reactivation of MEK preceded that of the ERKs with an earlier peak at 3–5 min of reoxygenation (Figure 3d), this confirmed that MEK was responsible for the changes in the ERKs. Total expression of ERK and MEK did not change (Figures 3a and 3c).

Incubation with $50 \mu\text{M}$ PD98059 reduced basal ERK activity to 50 % of the control before anoxia (results not shown, $n = 2$).

Figure 1 Effects of simulated ischaemia/reperfusion with or without mitochondrial inhibitors on ATP and ERK1/2 activity

Cells were subjected to hypoxia/anoxia/reoxygenation, $\pm 1 \mu\text{M}$ antimycin or myxothiazol added during anoxia. Samples were assayed for ATP and ERK1/2 activity. (a) ATP. Black bars, control cells; white bars, cells + inhibitors; $n = 5$; the values at 20 min hypoxia were set at 100%. (b) Phosphorylation of MBP by ERK1/2 in an in-gel MBP kinase assay from a representative experiment with antimycin. The other bands are unidentified MBP kinases. Molecular-mass markers (mw) are 66 and 46 kDa. (c) Densitometric quantification ($n = 5$, from three experiments with antimycin and two with myxothiazol; 20 min hypoxia set at 100%) of ERK1 (■) and ERK2 (□) activity (solid lines, control cells; dashed lines, cells + inhibitors) from the in-gel assays. For (a) and (c), comparisons within the control time course were made using ANOVAs with a Dunnett test (*, significantly different to 10 min hypoxia, $P < 0.05$; the 20 min hypoxia time-points were discarded as they were set to 100%). Paired t tests were used to compare the inhibited cells with control values at the same time point (** $P < 0.05$).

Anoxia suppressed ERK activity in both control and PD98059-treated cells. Upon reoxygenation, the ERKs in PD98059-treated cells were activated but only to 25 % of the activation seen in control cells. ATP recovery and cell metabolism (V_{O_2} , lactate production and ATP) were unaffected by PD98059 (results not shown).

Figure 2 Effects of simulated ischaemia/reperfusion on p38 MBP kinase activity

Cells were subjected to hypoxia/anoxia/reoxygenation and samples were assayed by in-gel MBP kinase assays. The activity of the p38 MBP kinase was compared with ERK1/2 activity. Results in (a) and (b) are from separate experiments taken from those described in Figure 1, and are typical of the pattern of activity of p38 MBP kinase during the transition from hypoxia (hyp) to anoxia (anox) and reoxygenation (reox).

Figure 3 Effects of simulated ischaemia/reperfusion $\pm$ antimycin on the phosphorylation states of ERK1/2 and MEK

Cells were subjected to hypoxia (hypox)/anoxia (anox)/reoxygenation (reox), $\pm 1 \mu\text{M}$ antimycin added during anoxia. Samples were extracted and analysed by Western blotting for (a) total ERK1/2, (b) phospho-ERK1/2, (c) total MEK1/2 and (d) phospho-MEK1/2. Results are representative of three experiments with antimycin and two with myxothiazol.

Mitochondrial inhibitors abolish the ERK reoxygenation response

If antimycin or myxothiazol was added prior to reoxygenation, the inhibited cells retained an anoxic pattern of metabolism despite being reoxygenated, both in terms of glycolytic rate (321 ± 57 nmol of lactate/ 10^6 cells per h) and ATP levels (Figure 1a). Activation (Figures 1b and 1c) and phosphorylation (Figures 3b and 3d) of the ERKs and MEK upon reoxygenation were also completely abolished in inhibited cells. Addition of FCCP prior to reoxygenation enhanced V_{O_2} by 2–3-fold (compared with initial V_{O_2}) following reoxygenation but it had the same effects on ATP and ERK activation as the inhibitors in Figure 1 (results not shown).

Figure 4 Recovery of ATP during early reoxygenation (0–6 min), and effects of mitochondrial inhibitors added at 30–90 s reoxygenation

Cells were subjected to hypoxia/anoxia/reoxygenation, $\pm 1 \mu\text{M}$ antimycin or myxothiazol added after 30 s ($\bullet$), 60 s ($\times$) or 90 s ($\triangle$) reoxygenation. Samples were assayed for ATP ($n = 3$ –5) with the value at mid-hypoxia set at 100%. Only reoxygenation data between 0 and 6 min are shown; hypoxic and anoxic results were similar to Figure 1(a). Control cells ($\square$) received solvent at 0 min. ANOVAs and Fisher's PLSD tests were used to compare ATP in the control time course with the control sample taken at 5 min (a, $P < 0.05$), and to compare ATP in the inhibited time courses with the sample taken at the time of initial addition of inhibitor (b, c and d, compared with appropriate control values at 30, 60 and 90 s respectively, $P < 0.05$).

ATP recovery and ERK/MEK activation during early reoxygenation

At 30–40 s following reoxygenation, levels of ATP had recovered to $77 \pm 3\%$ of initial levels, which was significantly less than the value at reoxygenation times of 60–70 s ($85 \pm 1\%$) and 5 min ($88 \pm 2\%$, Figure 4). As there was no significant difference between the latter two values, ATP recovery appeared complete by 40–60 s. Activation of the ERKs lagged behind ATP recovery, with ERK activity remaining at anoxic values up to 1.5 min after reoxygenation (Figure 5a). This was confirmed by Western-blot analysis, with phosphorylation of the ERKs seen only after 1.7 min of reoxygenation, although phosphorylation of MEK was apparent by 30 s and had increased by 1.2 min (Figure 5b). Total expression of ERK (Figure 5b) and MEK (results not shown) was unchanged.

Mitochondrial inhibition at 30, 60 or 90 s post-reoxygenation interrupted ATP recovery and immediately caused ATP to decrease towards anoxic levels (Figure 4), where it remained for the next 30 min (results not shown). Inhibition at 30 or 60 s also greatly suppressed ERK activation (Figure 5a) and resulted in less phosphorylation of ERK and MEK compared with the control (Figure 5c). When inhibitors were added at 90 s of reoxygenation, there was a more pronounced ERK activation (Figure 5a) and corresponding increases in the phosphorylation states of both ERK and MEK (Figure 5c), but levels were still lower compared with the control in both cases.

ERK activity and ATP are correlated during mitochondrial inhibition

As shown in Figure 1, both ATP levels and ERK activity decreased simultaneously during anoxia to about 30% of initial levels. This apparent relationship was investigated further using

Figure 5 Activation of ERK1/2 and MEK during early reoxygenation (0–7 min), and effects of mitochondrial inhibitors added at 30–90 s reoxygenation

Cells were subjected to hypoxia (h)/anoxia (a)/reoxygenation (reox), $\pm 1 \mu\text{M}$ antimycin or myxothiazol added after 30, 60 or 90 s of reoxygenation. **(a)** Samples were assayed for ERK1/2 activity, with activity at mid-hypoxia set at 100%. Only reoxygenation data are shown; hypoxia and anoxia were similar to Figure 1(c). Results are for ERK1 ($n = 3-5$), with inhibitors added at either 30 s ($\bullet$), 60 s ($\times$) or 90 s (Δ) reoxygenation. Control cells ($\square$) received solvent at 0 s. Results for ERK2 were similar (not shown). ANOVAs and Fisher's PLSD tests were used to compare ERK activity in the control time course with control at 0 min (a, $P < 0.05$), and to compare activity in the inhibited time courses with control at the time of initial addition of inhibitor (b, c and d, compared with control values at 30, 60 and 90 s respectively, $P < 0.05$). **(b)** Western analysis of total ERK1/2 and phosphorylated ERK1/2 and MEK1/2 during hypoxia, anoxia and 0–5 min of reoxygenation in a representative experiment ($n = 3-5$). **(c)** Western analysis of phosphorylated ERK1/2 and MEK1/2 during hypoxia, anoxia and early reoxygenation in control cells [reox(c)] or in cells inhibited with $1 \mu\text{M}$ antimycin at 60 or 90 s [reox(60), reox(90)]. Data are representative of three experiments. Results for 30 s (not shown) resembled those for 60 s.

mitochondrial inhibitors instead of anoxia to deplete ATP: $1 \mu\text{M}$ myxothiazol, $1 \mu\text{M}$ antimycin or 1 mM CN inhibited V_{O_2} immediately by $99 \pm 0.6\%$ ($n = 7$), stimulated lactate production (results not shown) and caused progressive reductions in both ATP and ERK activity (Figure 6). The relationship between ATP and the ERKs was significant ($P < 0.001$). The decreases were not due to cell death because: first, the anoxic data were collected from cells that were subsequently reoxygenated and regained 79–88% of initial ATP (Figure 1a); secondly, the rate

Figure 6 Correlation between ATP and ERK activity during anoxia or mitochondrial inhibition

Cells were subjected to mitochondrial inhibitors ($\blacksquare$; from five experiments with 1 mM CN, $1 \mu\text{M}$ myxothiazol or $1 \mu\text{M}$ antimycin) or anoxia ($\square$; from 12 experiments). Samples were collected at 1–40 min anoxia or post-inhibition, assayed for ATP and ERK1/2 activity and expressed relative to the initial values prior to inhibition or anoxia. The percentage ERK1 and ERK2 activities were combined to give an average for each point. The line was plotted using all data and has a correlation coefficient of 0.943 ($n = 98$, $P < 0.001$).

Figure 7 ERK activation by PMA is not affected by mitochondrial inhibitors

Control (Cont) and mitochondrially inhibited (Inh) cells were stimulated for 5 min with 100 nM PMA (P) or solvent (1.4 mM DMSO, D), and assayed for ERK1/2 activity. **(a)** Phosphorylation of MBP by ERK1/2 in an in-gel MBP kinase assay. **(b)** Western analysis of total ERK1/2 and phospho-ERK1/2. Results in **(a)** and **(b)** are representative of three experiments with $1 \mu\text{M}$ myxothiazol. **(c)** Densitometric quantification ($n = 3$) of PMA-induced ERK1/2 activity from the in-gel assays. Activity in PMA-stimulated control or inhibited cells was expressed as a percentage of the activity in DMSO-treated control or inhibited cells, respectively. There were no significant differences between control and inhibited cells for either myxothiazol (MX) or antimycin (AM) (paired t test, $P > 0.05$).

of lactate production was similar in anoxic and inhibited cells, and was linear throughout the incubations; and thirdly, cell viability was not different from control cells ($> 85\%$ by eosin

Figure 8 ERK activation by H₂O₂ is prevented by mitochondrial inhibitors

Control and mitochondrially inhibited cells were stimulated with 100 μ M H₂O₂ and assayed for ERK1/2 activity. (a) Phosphorylation of MBP by ERK1/2 in an in-gel MBP kinase assay. Cells were treated with 0.7 mM DMSO (Control, Control + H₂O₂) or 1 μ M myxothiazol (Inhib + H₂O₂) at -15 min, followed by 100 μ M H₂O₂ (Control + H₂O₂, Inhib + H₂O₂) or water (Control) at 0 min. Similar results were obtained in two experiments with 1 μ M antimycin. (b) Densitometric quantification of ERK1 activity from the in-gel assays ($n = 3$); results for ERK2 were similar (not shown). Control ($\square$) and mitochondrially inhibited cells ($\triangle$; 1 μ M antimycin or myxothiazol indicated by black arrow) were stimulated with 100 μ M H₂O₂ (white arrows) and compared with control ($\blacksquare$) or mitochondrially inhibited cells ($\blacktriangle$) that did not receive H₂O₂. Control cells at 0 min were set as 100%. At each time point, t tests were used to compare H₂O₂-stimulated cells (control or inhibited) with their respective non-stimulated cells (* $P < 0.05$). Note that H₂O₂ activation at 3 min could not be compared as there was no corresponding control.

staining). Controls showed no effects of solvents on V_{O_2} , ATP or ERK activity. The uncoupler FCCP also depressed both ATP and ERK activity (results not shown).

PMA, but not H₂O₂, stimulates the ERKs in mitochondrially inhibited cells

Antimycin or myxothiazol (1 μ M) did not prevent phosphorylation and activation of the ERKs by 100 nM PMA (Figure 7). The activation in inhibited cells started from a lower basal level of ERK activity due to suppression by the inhibitors (Figures 7a and 7b), but the fold activation was the same as in the control (non-inhibited) cells (Figure 7c). Total ERK levels were unchanged (Figure 7b), indicating that the differences were not due to changes in expression or degradation of the ERKs in the inhibited cells. Also, viability tests with eosin showed that the lower basal activity in inhibited cells was not due to cell death. The time course of activation by PMA was also unaffected by inhibition, with a peak at 5–10 min and a return to basal levels

by 15–20 min (results not shown). Controls showed no effect of DMSO on ERK activity. In contrast to the PMA results, activation by 100 μ M H₂O₂ was absent in inhibited cells, compared with maximal activation of $189 \pm 13\%$ (ERK1) in control cells (Figure 8). ATP, V_{O_2} and lactate production were not affected by the addition of H₂O₂ (results not shown).

DISCUSSION

Following previous reports of ERK activation during metabolic recovery from chemical anoxia [6,15], we first set out to resolve whether a similar relationship exists during metabolic recovery from physical anoxia in an ischaemia/reperfusion event, and secondly what the implications are for the current theory that the reperfusion activation of the ERKs has an ROS-based mechanism. We used H9c2 cells, a myocyte line that suited our *in vitro* system, and which showed similar ERK responses to oxidative and metabolic stresses (Figures 1 and 8) to those seen in whole-organ [1,5,6] and primary cell preparations [6,9,15].

Whereas the chemical anoxia and H₂O₂ studies [5,6,8–10,15] showed that metabolic recovery and oxidative stress could individually activate the ERKs, we show here the relationship between these two stimuli and ERK activation when they occur simultaneously during reoxygenation. By preventing metabolic recovery with mitochondrial inhibitors, we found that activation of the ERKs upon reoxygenation was also completely abolished (Figures 1 and 3). This relationship was one of ERK dependence on ATP recovery as ATP recovery occurred first (Figures 4 and 5a), and the MEK inhibitor PD98059 reduced ERK activation by 75%, yet did not affect ATP recovery. Thus recovery of ATP, or another event associated with the resumption of mitochondrial respiration, is necessary for ERK activation at reoxygenation. This shows that the physical re-introduction of O₂ is not a sufficient stimulus for the ERKs, that O₂ *per se* is not a signalling molecule and that O₂ sensors do not play a direct role in ERK activation. Instead, O₂ participates indirectly by enabling the resumption of mitochondrial respiration, and it is the latter that is required for ERK activation.

Our next step was to determine which particular aspect of mitochondrial respiration was the crucial stimulus. We have presented ATP recovery here as it is the most obvious and important aspect, and also because there appears to be a relationship between cellular ATP levels and the ERKs (Figure 6) that is not due to substrate limitation (see below). However, there are three other aspects that our data allow us to discuss: mitochondrial electron flow, pH and the production of ROS. The first of these, resumption of electron flow through the respiratory chain, may be ruled out as FCCP also suppressed ERK activation despite enhancing the respiration rate upon reoxygenation. The second event, a sudden change in pH that can occur at reperfusion following the wash-out of lactic acid accumulated during ischaemia, may also be eliminated as we did not replace the buffer upon reoxygenation.

The third aspect, the production of ROS, is important given the proposed ROS-based mechanism of ERK activation. Although the mitochondrial respiratory chain is considered the major producer of ROS during reperfusion [11,13,16], there are other possible sources, such as xanthine oxidase [12] and NADPH oxidase (present in H9c2 cells [21]). Our results (Figures 1 and 3) preclude a role for non-mitochondrial ROS in ERK activation during reperfusion as the inhibitors used would not have affected ROS from other sources. Our data are consistent with a mitochondrial source of ROS but only if it is assumed that ROS are not produced by inhibited mitochondria. However, the inhibitors used here act at different sites in the electron transport

chain and so have opposite effects, i.e. antimycin and CN enhance mitochondrial ROS production [13,16], whereas myxothiazol and FCCP reduce it [11,16]. Since all these prevented ERK activation, our data would seem to suggest that mitochondrial ROS are also not the stimulus. However, the activation of the ERKs by H_2O_2 was also blocked in mitochondrially inhibited cells (Figure 8), whereas stimulation by PMA was still possible (Figure 7). This is also seen in neonatal rat cardiomyocytes, i.e. mitochondrial inhibitors eliminated activation of the ERKs by H_2O_2 , but did not affect activation by other stimuli such as PMA, phenylephrine or hyperosmotic shock [21a]. Taken together, the data suggest that reoxygenation and H_2O_2 share a similar pathway of ERK activation which distinguishes them from all other stimuli tested. Since the effects of H_2O_2 are thought to be mediated by oxidative stress and ROS, these results suggest strongly that ERK activation in reoxygenation is also ROS-mediated, but that the signalling mechanism is dependent on normal levels of (mitochondrially produced) ATP and/or functioning coupled mitochondria.

To confirm and look further at this dependence of ERK activation on ATP and/or the mitochondria, we added the mitochondrial inhibitors in the immediate post-reoxygenation period. Whereas the results in Figures 1 and 3 suggest that ATP recovery is necessary, we found that it was not a sufficient stimulus because inhibition at 30 or 60 s post-reoxygenation, i.e. after ATP recovery had occurred, also prevented ERK activation (Figures 4 and 5a). Since ROS are also produced early in reoxygenation (peaking at 10–40 s [12,14]), the results of the 30- and 60-s inhibition experiments (Figure 5a) indicate that ROS are not a sufficient stimulus either. This confirms that continuation of the signal-transduction mechanism requires normal ATP and/or functioning mitochondria after the initial stimulus has occurred.

Interestingly, such a requirement appears only to be necessary at a crucial period, 60–90 s post-reoxygenation. This is seen in cells inhibited at 90 s of reoxygenation (Figure 5a). Comparison of the ATP and ERK time courses in these incubations reveals that following the addition of inhibitors at 90 s, ATP decreased while ERK activity continued to rise (see the periods 2–5 min in Figures 4 and 5a). This indicates that an ATP- or mitochondrion-dependent step occurs between 60 and 90 s, but once past this critical point, ERK activation can progress without further such requirements. The long delay between the first signs of phosphorylation of MEK (< 30 s) compared with ERK activation (> 1.7 min; Figures 5a and 5b) indicates that such an event may be the interaction between MEK and ERK. This step involves separation of ERK from its cytoplasmic anchor MEK [22], followed by translocation of phosphorylated ERK into the nucleus [23]. It has been reported recently that both the cytoplasmic localization of MEK and the nuclear translocation of ERK are sensitive to ATP depletion by mitochondrial inhibition [24].

Whereas the reoxygenation activation of the ERKs has been studied widely, much less attention has been paid to the decrease in ERK activity during anoxia, shown here (Figures 1 and 3) and in previous studies [1,6,10,15]. The ERK/ATP correlation (Figure 6) provides an explanation for these results as ATP would have decreased in each of the above cases. The effect is not due to the limited availability of ATP as a substrate for ERK or MEK as the decreases in ERK activity were always reflected by lower phosphorylation states of both these kinases (Figures 3 and 5). ATP would thus have to be substrate-limiting for kinase-activators above the level of MEK, such as the MEK kinases, Raf or MEKK. However, the correlation between ATP and ERK activity was evident at 80 % of normal ATP levels (Figure

6), at which time ATP would have been about 5 mM in these cells (assuming no compartmentation). As this is more than 10-fold the $K_{m,ATP}$ of c-Raf-1 (12 μ M [25]) and MEKK (11 μ M [26]), these kinases should be operating at their V_{max} and would thus be insensitive to changes in ATP concentration. Substrate limitation is also unlikely to be responsible for the absence of ERK activation during reoxygenation in inhibited cells (Figure 1c), as ATP levels were still high (about 2 mM) in those cells. Also, p38 MBP kinase activity was not inhibited by anoxia (Figure 2), which showed that upstream activators and other MAPK pathways were not restricted by the lower ATP levels. Finally, mitochondrial inhibition did not prevent ERK activation by PMA (Figure 7). We emphasize here that the inhibited cells which were stimulated 3-fold by PMA were the same cells that did not respond at all to reoxygenation. Thus the effect of ATP on ERK activity appears to be specific for the ERKs and distinct from its role as a substrate in the MAPK cascade. We cannot, however, rule out the possibility of ATP compartmentation and/or substrate limitation at other steps such as the nuclear-translocation events [24].

A final interpretation of our data is that the suppression of basal ERK activity during anoxia and mitochondrial inhibition indicates a basal activity comprising two constitutive pathways, one of which is sensitive to mitochondrial inhibition. Different stimuli would then act via one or both of these pathways. Thus in inhibited cells, PMA was able to activate the ERKs by the mitochondrion-insensitive pathway (Figure 7), whereas the absence of ERK activation by reoxygenation or H_2O_2 (Figures 1 and 8) indicates that these stimuli required the mitochondrion-sensitive pathway. These results show that sensitivity to mitochondrial inhibition may distinguish between two different mechanisms of ERK activation. The broader implications are that cellular metabolism (or perturbations to it) should be considered when studying the ERKs, and that the mitochondria may be a fruitful area for future research into the mechanisms of ERK activation.

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