Global ischaemia induces a biphasic response of the mitochondrial respiratory chain

Anoxic pre-perfusion protects against ischaemic damage

Keith VEITCH,*† Annick HOMBROECKX,* Danielle CAUCHETEUX,* Hubert POULEUR† and Louis HUE*

*Hormone and Metabolic Research Unit, International Institute of Cellular and Molecular Pathology, and †Department of Physiology, Division of Cardiology, Louvain University Medical School, avenue Hippocrate 75, 1200 Brussels, Belgium

Studies in Langendorff-perfused rat hearts have revealed a biphasic response of the mitochondrial respiratory chain to global ischaemia. The initial effect is a 30–40% increase in the rate of glutamate/malate oxidation after 10 min of ischaemia, owing to an increase in the capacity for NADH oxidation. This effect is followed by a progressive decrease in these oxidative activities as the ischaemia is prolonged, apparently owing to damage to Complex I at a site subsequent to the NADH dehydrogenase component. This damage is exacerbated by reperfusion, which causes a further decrease in Complex I activity and also decreases the activities of the other complexes, most notably of Complex III. Perfusion for up to 1 h with anoxic buffer produced only the increase in NADH oxidase activity, and neither anoxia alone, nor anoxia and reperfusion, caused loss of Complex I activity. Perfusing for 3–10 min with anoxic buffer before 1 h of global ischaemia had a significant protective effect against the ischaemia-induced damage to Complex I.

INTRODUCTION

Cardiac tissue function can be fully recovered upon reperfusion if the periods of ischaemia due to coronary occlusion are relatively short [1]. Longer periods of ischaemia cause irreversible tissue injury, hence myocardial infarction, and this damage is exacerbated by reoxygenation during reperfusion, the oxygen paradox [2]. These conditions of severe tissue damage have been associated with functional and morphological defects in the mitochondrial population [2,3]. Indeed, the transition from reversible to irreversible ischaemia has been suggested to depend on the functional state of mitochondria [1,4], and restoration of oxidative metabolism determines functional recovery [5].

Decreased mitochondrial State 3 oxygen consumption with NAD⁺-linked substrates has been demonstrated after ischaemia in several animal models [3,6–10]. This effect is apparently due to loss of activity of the Complex I component (NADH-ubiquinone reductase) of the mitochondrial electron-transport chain [6,11,12]. Different interventions such as perfusion with Ca²⁺-channel blockers and β -adrenergic-receptor antagonists to improve post-ischaemic function have been shown to protect mitochondria against ischaemic damage in perfused rabbit hearts [7]. In dog hearts, improvement of post-ischaemic function after treatment with exogenous superoxide dismutase has been associated with decreased mitochondrial free-radical production [13]. Short periods (10–15 min) of anoxic pre-perfusion have been shown to improve post-ischaemic functional recovery in rat hearts [14,15], but the effects of such treatment on mitochondrial function are not known.

We have examined the activities of the respiratory-chain Complexes I, II, III and IV in mitochondria from perfused rat hearts subjected to either global ischaemia or anoxic perfusion to determine whether different forms of damage can be associated with ischaemia, anoxia and/or reoxygenation. We have also determined whether the functional protection afforded by anoxic pre-perfusion extends to the mitochondrial damage. Our results

indicate that there are differences in sensitivity to ischaemic damage, dependent on the duration of the ischaemic episode and on the presence of oxygen. The site most sensitive to this damage is Complex I. Short periods of anoxic pre-perfusion (3–10 min) do afford some protection to Complex I during a subsequent period of global ischaemia, which may contribute to the improvement in functional recovery observed in previous reports. Furthermore, the initial mitochondrial response to hypoxia/anoxia, including the first few minutes of ischaemia, is an increase in capacity of the electron-transport chain to oxidize NADH, leading to a faster rate of mitochondrial respiration with NADH-dependent substrates.

MATERIALS AND METHODS

Chemicals

Ubiquinone-1 was generously given by Eisai Co., Tokyo, Japan. Ubiquinone-2 was generously given by Takeda Chemical Industries, Osaka, Japan. Nagarse (Protease type XXVII) and other biochemicals were purchased from either Sigma Chemical Co. (St. Louis, MO, U.S.A.) or Boehringer Mannheim G.m.b.H. (Mannheim, Germany). All chemicals were of the highest grade available from standard suppliers.

Heart perfusions

Fed male Wistar rats (150–250 g) were anaesthetized by Nembutal (70 mg/kg, intraperitoneally) at least 15 min before use. The thorax was opened and the heart rapidly removed into cold (4 °C) perfusion medium. The aorta was cannulated and perfusion started within 1 min in a Langendorff system [16] with a constant perfusion pressure of 60 cm H₂O. The perfusate was a modified Krebs–Henseleit bicarbonate buffer (pH 7.4) [17] containing 11 mM-glucose, kept at 37 °C, and continually gassed with either O₂/CO₂ (19:1) (normoxic) or N₂/CO₂ (19:1) (anoxic). All hearts were perfused in non-recirculating mode for 5 min, then for 10 min in circulating mode (total volume 100 ml) to

† To whom correspondence should be addressed.

equilibrate the tissue, before one of the following conditions was imposed. Controls were continuously perfused with normoxic buffer for 0–60 min. Ischaemia was induced by clamping off the aortic supply for the periods indicated, whereas reperfused hearts were perfused for 5 min after the period of global ischaemia shown. Anoxia was obtained by switching the aortic supply from normoxic to anoxic reservoirs. The temperature of the heart was maintained at 37 °C throughout by immersion in perfusion buffer. Coronary flow was measured in hearts perfused in a non-recirculating mode by timed collection of the coronary effluent into calibrated tubes. At the end of the respective perfusion periods, the hearts were either freeze-clamped between aluminium plates cooled in liquid N₂ for subsequent metabolite analyses, or used immediately to prepare mitochondria.

Preparation of heart mitochondrial fractions

For preparation of mitochondria, the heart was rapidly cut away and immersed in ice-cold MSME medium (220 mM-mannitol, 70 mM-sucrose, 5 mM-Mops, 1 mM-EGTA, pH 7.4). All subsequent steps were carried out at 0–4 °C. As there are two sub-populations of mitochondria in heart, only one of which is released by mechanical homogenization, we first treated the tissue with a proteinase (Nagarse), which is known to release both populations [18]. Thus the ventricles, freed of auricles and blood vessels, were minced as finely as possible with scissors, washed two or three times with fresh MSME and then incubated at 0 °C in 4–5 ml of MSME containing 0.5 mg of Nagarse/ml, to give a final ratio of 5 mg of Nagarse/g of tissue. After 5 min, 10 ml of MSME containing 15 mg of defatted BSA was added and the mixture was homogenized in a glass/Teflon homogenizer with a motor-driven pestle, then centrifuged at 600 *g* for 5 min. The supernatant was filtered through four layers of gauze and spun at 12000 *g* for 5 min to obtain a first mitochondrial pellet, which was washed in 8 ml of MSME and centrifuged at 8000 *g* for 5 min. The homogenate pellet was resuspended in 15 ml of MSME (+1 mg of BSA/ml), rehomogenized and spun again at 600 *g* for 5 min. This second supernatant was filtered through gauze and spun at 8000 *g* for 5 min to give a second mitochondrial pellet, which was combined with the first for a final wash and centrifugation at 6000 *g* for 5 min. The final pellet was resuspended in MSME at 10–25 mg of protein/ml. Protein was assayed by the Lowry method with BSA as standard.

Measurement of O₂ consumption

Mitochondrial respiration was measured at 30 °C in a Clark-type oxygen electrode (Yellow Springs Instruments, Yellow Springs, OH, U.S.A.) in 1 ml of buffer (125 mM-KCl, 2.5 mM-KH₂PO₄, 10 mM-Hepes, 5 mM-MgCl₂, 1 mM-EDTA, 2 mg of defatted BSA/ml, pH 7.2) with 5 mM-glutamate/5 mM-malate as substrates. After addition of approx. 0.2 mg of mitochondrial protein, State 3 was initiated by addition of 0.25 μmol of ADP. The oxidation rate was also measured with 0.25 mM-NADH as substrate with mitochondria lysed by three cycles of freeze-thawing (–20 °C/+20 °C) after overnight storage at –20 °C [19]. Oxygen concentration was taken as 0.45 μg-atom/ml at 30 °C.

Measurement of mitochondrial Complexes

The electron-transport-chain Complexes were assayed in mitochondria which had been stored at –80 °C. The assay conditions were designed to give *V*_{max} activities. Complexes I, II and III were assayed spectrophotometrically at 30 °C in a standard buffer (35 mM-potassium phosphate, 5 mM-MgCl₂, 2 mM-KCN, pH 7.2) with additions as noted. NADH-ubiquinone reductase (Complex I) was assayed as the rotenone-sensitive oxidation of NADH at 340 nm in 1 ml of buffer containing 2 μg of antimycin

A, 60 μM-ubiquinone-1 and 0.13 mM-NADH. This concentration of NADH, although less than that used in the oxidase assay, was sufficient to obtain *V*_{max} rates (*K*_m 5–7 μM [19]), and ensured a usable absorbance at 340 nm. The initial linear rate was monitored for 1 min after addition of 5–10 μg of mitochondrial protein, which had previously been subjected to three cycles of freeze-thawing, and a blank run after addition of 5 μM-rotenone. An absorption coefficient of 6.81 mM^{–1}·cm^{–1} was used for NADH in the presence of ubiquinone-1 [20]. Succinate-ubiquinone reductase (Complex II) was assayed by the bleaching of 2,6-dichlorophenolindophenol (DCPIP) in 1 ml of buffer, containing 88 μM-DCPIP, 2 μg of Antimycin A, 60 μM-ubiquinone-1, 25 mM-succinate and 5 μg of mitochondrial protein. This mixture was preincubated at 30 °C for 10 min before monitoring the change at 600 nm (ϵ = 21 mM^{–1}·cm^{–1}) for the next 5–8 min. Ubiquinol-cytochrome *c* reductase (Complex III) was assayed as the initial linear increase in the rate of reduction of cytochrome *c* at 550 nm (ϵ = 18.5 mM^{–1}·cm^{–1}), after addition of 5–10 μg of protein to 1 ml of buffer containing 0.5 mM-EDTA, 50 μM oxidized cytochrome *c* and 40 μmol of ubiquinol-2. Ubiquinol-2 was prepared by reduction of an 8 mM ethanolic solution of ubiquinone-2 by addition of solid borohydride, acidification with HCl and subsequent extraction in cyclohexane [21]. The final extract was redissolved at 8 mM in ethanol (pH 2 with HCl). Cytochrome *c* oxidase (Complex IV) was assayed polarographically at 30 °C in 1 ml of 250 mM-sucrose/25 mM-sodium cacodylate (pH 7.8), with 5 mM-ascorbate and 250 μM-tetramethyl-*p*-phenylenediamine dihydrochloride. Mitochondrial protein (50–100 μg) was added, and the non-enzymic rate of oxygen consumption was recorded before starting the reaction by addition of 10 μM cytochrome *c* [22].

NADH dehydrogenase and succinate dehydrogenase activities were assayed as the rates of ferricyanide reduction at 420 nm (ϵ = 1.05 mM^{–1}·cm^{–1}) in the standard buffer containing 0.5 mM-K₃Fe(CN)₆, with 0.5 mM-NADH and 25 mM-succinate as substrates respectively. The higher NADH concentration was used for this activity, as it has a greater *K*_m (70 μM) than the oxidase or Complex I activities [19]. Citrate synthase was assayed as the oxaloacetate-dependent liberation of CoA from acetyl-CoA, as described in [23]. Cytochrome contents were determined from reduced-oxidized (dithionite) difference spectra of mitochondrial suspensions (1 mg of protein/ml in MSME), calculated in accordance with [24]. Glycogen [25] and lactate [26] contents of freeze-clamped hearts were assayed in deproteinized extracts by enzymic methods. Enzyme activities are expressed as units per mg of mitochondrial protein, one unit corresponding to the reaction of 1 μmol of substrate or production of 1 μmol of product per min in the conditions described.

Results are shown as the means ± S.E.M. of at least four different preparations in each group for measurements of the coronary flow, and five mitochondrial preparations for biochemical analyses, unless specifically indicated. Statistical significances between group means were tested by an unpaired Student's *t* test.

RESULTS

Indication of functional damage after ischaemia was given by the coronary flow rates (Fig. 1). The control rate of 10.5 ± 0.3 ml/min (*n* = 27) was significantly decreased after 5 min reperfusion following periods of global ischaemia of 15 min or longer. Apart from the hearts subjected to 10 min of ischaemia, which fully recovered, there was no further improvement in these rates during reperfusion.

Mitochondrial yields, as mg of protein per g wet wt. of ventricle, were significantly decreased after all periods of ischaemia (Table 1), with a 30–40% decrease after 60 min

Fig. 1. Decrease in coronary flow rates after global ischaemia in Langendorff-perfused rat hearts

The flow rate (ml/min) was measured at 9, 5 and 1 min before the induction of global ischaemia (0 min) and then each 1 min during reperfusion after the period of ischaemia indicated: ○, control; ●, 10 min; △, 15 min; ▲, 20 min; □, 30 min; ■, 60 min. Values are means $\pm$ S.E.M. for 4–5 hearts in each group.

Table 1. Mitochondrial yields from perfused rat hearts

Mitochondria were prepared from hearts subjected to either 30 or 60 min of ischaemia, without (Ischaemia) or with (Reperused) 5 min of normoxic reperfusion. Controls were perfused normoxically for 30–60 min. The values are means $\pm$ S.E.M. for (*n*) preparations, as mg of mitochondrial protein/g of ventricle: ***P* < 0.01, ****P* < 0.001 versus control.

	Yield of mitochondrial protein (mg/g)
Control (7)	18.3 $\pm$ 0.5
30 min Ischaemia (3)	13.4 $\pm$ 1.7**
60 min Ischaemia (4)	12.8 $\pm$ 1.6**
30 min Reperused (4)	11.6 $\pm$ 1.1***
60 min Reperused (3)	11.3 $\pm$ 0.4***

Table 2. Activities of mitochondrial marker enzymes

Activities were measured in mitochondrial fractions prepared from Langendorff-perfused rat hearts subjected to 30 or 60 min of global ischaemia, and 10–60 min of global ischaemia and 5 min reperfusion with oxygenated buffer. All values are shown as the means $\pm$ S.E.M. of (*n*) preparations as units/mg of protein, and the ratio of Complex I to NADH dehydrogenase is in m-units/unit: **P* < 0.05 versus control; †*P* < 0.05 versus the equivalent period of ischaemia without reperfusion.

	Citrate synthase	Succinate dehydrogenase	NADH dehydrogenase	Complex I NADH dehydrogenase
Control (16)	1.66 $\pm$ 0.04	0.258 $\pm$ 0.012	6.22 $\pm$ 0.21	104 $\pm$ 5
30 min Ischaemia (7)	1.66 $\pm$ 0.08	0.255 $\pm$ 0.008	5.89 $\pm$ 0.17	81 $\pm$ 5*
60 min Ischaemia (8)	1.65 $\pm$ 0.10	0.224 $\pm$ 0.015	5.99 $\pm$ 0.27	61 $\pm$ 4*
10 min Reperused (6)	1.57 $\pm$ 0.09	0.238 $\pm$ 0.025	5.71 $\pm$ 0.25	110 $\pm$ 10
20 min Reperused (6)	1.64 $\pm$ 0.08	0.247 $\pm$ 0.018	6.09 $\pm$ 0.28	94 $\pm$ 8
30 min Reperused (10)	1.64 $\pm$ 0.08	0.237 $\pm$ 0.010	5.88 $\pm$ 0.21	57 $\pm$ 4*†
60 min Reperused (6)	1.39 $\pm$ 0.07*†	0.223 $\pm$ 0.013	5.63 $\pm$ 0.28	46 $\pm$ 3*†

Fig. 2. Biphasic effects of ischaemia on the State 3 oxidation rate of 5 mM-glutamate/malate in intact rat heart mitochondria

Controls were perfused with normoxic buffer for 0–60 min (□), and other hearts were made ischaemic alone (○), or were reperused for 5 min after the ischaemia (●). Activities, in ng-atoms of O · min⁻¹ · mg of protein⁻¹, are shown as means $\pm$ S.E.M. (bars) for 5–16 preparations at each point: **P* < 0.001 versus control; †*P* < 0.05 versus ischaemia alone.

of ischaemia with and without reperfusion. However, the mitochondrial fractions appeared to be equally representative of the two sub-populations found in hearts [18], as there were no major differences in the specific mitochondrial marker enzyme activities (Table 2). Citrate synthase, a matrix enzyme, was only significantly decreased by 16% (*P* < 0.01) in the most extreme condition, i.e. after 60 min of ischaemia with reperfusion. This was probably due to increased leakiness of these mitochondria, as the two inner-membrane activities assayed, succinate dehydrogenase and NADH dehydrogenase, were not significantly affected (Table 2).

Mitochondrial respiration

The duration of normoxic perfusion had no effect on the mitochondrial respiratory activity, measured as the State 3 oxidation rate with glutamate/malate. The control values given in Fig. 2 are the means of 16 hearts perfused for 0–60 min after the equilibration period. Fig. 2 illustrates the effects of ischaemia and reperfusion on this activity. After 20 min of ischaemia and reperfusion the rate was not different from controls, but longer periods of ischaemia with reperfusion caused significant decreases

Fig. 3. Biphasic effects of ischaemia on the NADH oxidase activity in lysed rat heart mitochondria

The mitochondria from Fig. 2 were lysed by three cycles of freeze-thawing, and oxygen consumption was measured with $250 \mu\text{M}$ -NADH. Other details were as in Fig. 2. Inset shows the correlation of State 3 oxidation and NADH oxidase activities.

of respiration, to 54 % and 28 % after 30 and 60 min of ischaemia respectively. These values were significantly less than those obtained after the same periods of ischaemia without reperfusion (75 % and 38 % after 30 min and 60 min respectively), suggesting that there was further damage due to reoxygenation. In contrast, after 10 min of ischaemia, when reperfusion resulted in normal rates of coronary flow (Fig. 1), a striking effect occurred, namely the mitochondria from these hearts had significantly increased rates of glutamate/malate oxidation (133 % of controls, $P < 0.001$). A similar though smaller increase was observed in mitochondria from hearts reperfused after 5 and 15 min of ischaemia (117 % and 118 % respectively, $P < 0.05$) (results not shown).

The rate of glutamate/malate oxidation in intact mitochondria may be affected by a variety of factors, including substrate transport, cofactor concentrations in the matrix, coupling to ATP formation and the activity of the respiratory chain itself. The last, as NADH oxidase, can be measured directly in mitochondria lysed by freeze-thawing, thus exposing the NADH site normally on the inner aspect of the mitochondrial inner membrane. The changes in NADH oxidase activities in the mitochondria from ischaemic and reperfused hearts are shown in Fig. 3. This clearly shows a similar pattern of responses to ischaemia and reperfusion to that observed with glutamate/malate, strongly suggesting that the effects in intact mitochondria were due to the changes in the respiratory chain. Indeed, there was an excellent correlation between the glutamate/malate rate and NADH oxidase activity (Fig. 3 inset) ($r = 0.991$, $P < 0.001$). Thus after 30 and 60 min of ischaemia NADH oxidase activity was decreased to 79 % and 49 % of control values respectively, and further decreased to 57 % and 36 % after reperfusion. The effects of shorter periods of ischaemia were the same as for glutamate/malate, i.e. 10 min of ischaemia caused a significant increase in activity (140 %, $P < 0.05$). Therefore there appears to be a biphasic response to ischaemia, the immediate effect being an increase in mitochondrial respiratory capacity for NADH, which is followed by a decrease in the NADH oxidase activity as the ischaemic period is extended. The unchanged response after 20 min would therefore be at the balance point for the stimulatory and inhibitory effects. In fact, the individual data for this time point could be divided into two groups, representing stimulated and inhibited activities respectively.

Effects of ischaemia and reperfusion on the activities of the mitochondrial complexes

In order to locate the site(s) of these effects, we measured the activities of the four complexes involved in NADH oxidase activity (Fig. 4), the results further emphasizing the differences between ischaemia and reperfusion. Complex I (NADH-ubiquinone reductase) displayed the same pattern of decreased activity after ischaemia and reperfusion as glutamate/malate and NADH oxidations, with one notable exception. Complex I activity in mitochondria from hearts reperfused after 10 min of

Fig. 4. Effects of ischaemia and reperfusion on the activities of the four respiratory-chain complexes in mitochondria from perfused rat hearts

Activities for Complexes I-III are given as units/mg of protein, and Complex IV is expressed in μg -atoms of $\text{O} \cdot \text{min}^{-1} \cdot \text{mg}$ of protein $^{-1}$: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus control. Other details were as in Fig. 2.

Table 3. Cytochrome contents of mitochondria from perfused rat hearts

Hearts were subjected to 30 or 60 min of ischaemia, with and without reperfusion. The cytochrome contents were obtained from reduced – oxidized difference spectra. Values are means $\pm$ S.E.M. for (*n*) preparations: **P* < 0.05, ***P* < 0.01, ****P* < 0.001 versus control.

	Cytochrome (pmol/mg of protein)			
	<i>c</i>	<i>c</i> ₁	<i>b</i>	<i>aa</i> ₃
Control (15)	557 $\pm$ 18	389 $\pm$ 21	333 $\pm$ 11	829 $\pm$ 23
30 min Ischaemia (4)	481 $\pm$ 22	332 $\pm$ 30	348 $\pm$ 10	830 $\pm$ 19
60 min Ischaemia (6)	419 $\pm$ 23***	285 $\pm$ 17*	274 $\pm$ 13**	742 $\pm$ 66
30 min Reperfused (6)	475 $\pm$ 21*	333 $\pm$ 27	303 $\pm$ 16	772 $\pm$ 42
60 min Reperfused (5)	375 $\pm$ 26***	273 $\pm$ 32*	247 $\pm$ 7***	628 $\pm$ 37***

ischaemia was not different from controls (99%), despite the observed 40% increase in NADH oxidase activity. Thus the decrease in activity caused by ischaemia/reperfusion could be explained by a loss of Complex I activity, but the stimulatory effect is apparently due to a separate factor.

Complex II (succinate–ubiquinone reductase) was not affected by up to 60 min of ischaemia (94%), but was significantly decreased when hearts were reperfused after this period (82%, *P* < 0.01). There was no effect of reperfusion after shorter periods of ischaemia.

Complex III (ubiquinol–cytochrome *c* reductase) was slightly more sensitive to ischaemic damage, such that the activity was not affected after 30 min of ischaemia (96% of controls), but there was a significant decrease after 60 min of ischaemia (84%, *P* < 0.05). Reperfusion, however, caused significant losses of the activity both after 30 min ischaemia (76%, *P* < 0.01) and 60 min of ischaemia (66%, *P* < 0.001). Thus Complex III appears to be particularly sensitive to reoxygenation damage.

The effects on Complex IV (cytochrome *c* oxidase) were similar to those on Complex II, i.e. no effect due to ischaemia alone (95% of controls after 60 min), but a significant decrease when hearts were reperfused after 60 min of ischaemia (81%, *P* < 0.01).

Mitochondrial cytochrome contents were also affected by ischaemia and reperfusion, and the changes were consistent with the losses of activity of the associated complexes (Table 3). There were marked losses of cytochromes *b* and *c*₁ (components of Complex III) and *c* after 60 min of ischaemia, and a further loss of cytochrome *aa*₃ (Complex IV) after reperfusion.

Thus the four Complexes display different degrees of sensitivity to both global ischaemia and reperfusion, and, although Complex III is affected by both conditions, the activity of Complex I is clearly the most sensitive to such damage, and effects on this activity can account for the decreased mitochondrial oxidation rates observed after ischaemia and reperfusion. NADH-dependent ferricyanide reduction occurs at an initial step in the complex, associated with the soluble NADH dehydrogenase component [27]. An overall loss of Complex I would therefore be expected to be reflected in similar changes in this activity. However, as shown in Table 2, the ratio of NADH–ubiquinone reductase to NADH–ferricyanide reductase activities is not constant, and reflects the decrease in Complex I activity defined in terms of mitochondrial protein. This is consistent with the loss of a specific component of Complex I subsequent to the NADH dehydrogenase.

Protection by anoxia

In order to investigate the cause of this damage, we wished to separate the various factors which may be detrimental during ischaemia, e.g. lack of oxygen, accumulation of by-products such

Table 4. Effects of anoxia on mitochondrial respiratory activities

The effects of 60 min of anoxic perfusion and 60 min of global ischaemia with 0–10 min of anoxic pre-perfusion on glutamate/malate oxidation rates in intact mitochondria, and on NADH oxidase and Complex I activities in freeze-thawed mitochondria from perfused rat hearts. Activities, as means $\pm$ S.E.M. for (*n*) preparations, are given as ng-atoms of O $\cdot$ min⁻¹ $\cdot$ mg of protein⁻¹ for glutamate/malate and NADH oxidations, and nmol of NADH $\cdot$ min⁻¹ $\cdot$ mg of protein⁻¹ for Complex I: **P* < 0.05 versus control; †*P* < 0.05 versus 60 min Ischaemia.

	Activity		
	Glutamate/ malate	NADH oxidase	Complex I
Control (16)	261 $\pm$ 5	402 $\pm$ 12	636 $\pm$ 22
60 min Anoxia (5)	373 $\pm$ 24*†	576 $\pm$ 40*†	644 $\pm$ 28†
60 min Anoxia + 5 min reperfusion (5)	368 $\pm$ 23*†	581 $\pm$ 70*†	594 $\pm$ 15†
60 min Ischaemia + anoxic pre-perfusion			
0 min (9)	99 $\pm$ 6*	196 $\pm$ 22*	351 $\pm$ 23*
1–2 min (4)	124 $\pm$ 22*	218 $\pm$ 55*	385 $\pm$ 56*
3–5 min (7)	174 $\pm$ 9*†	273 $\pm$ 28*†	458 $\pm$ 28*†
10 min (6)	195 $\pm$ 18*†	370 $\pm$ 35†	519 $\pm$ 37*†

as lactate and CO₂, and decreased pH. As the damage associated with reperfusion already suggested some role for oxygen, the effects of 60 min of perfusion with anoxic buffer were studied. Mitochondria from these hearts did not display any inhibitory effects; rather, they had significantly increased respiratory activities with glutamate/malate and NADH, similar to the results obtained from the 10 min-reperfused hearts (Table 4). Furthermore, 5 min reperfusion after 60 min of anoxia did not cause the damage observed after ischaemia. These results suggest that the damage that occurs during ischaemia is not due to the lack of oxygen. They also suggest that ischaemia sensitizes the mitochondria to the reperfusion damage.

We also studied the effects of low pH by perfusing hearts for 60 min with anoxic buffer at pH 6.6. This did not cause any apparent inhibition of Complex I (679 $\pm$ 19 nmol of NADH $\cdot$ min⁻¹ $\cdot$ mg of protein⁻¹; *n* = 3), although the rates of glutamate/malate oxidation (269 $\pm$ 37 ng-atoms of O $\cdot$ min⁻¹ $\cdot$ mg of protein⁻¹) and NADH oxidase (330 $\pm$ 49 ng-atoms of O $\cdot$ min⁻¹ $\cdot$ mg of protein⁻¹) were not increased, as would be expected after anoxia. However, perfusion at low pH resulted in very low coronary flow rates, as previously reported [28], so we were unable to distinguish between the effects of low pH and low flow in this model.

Table 5. Metabolite concentrations in perfused rat hearts

The effects of 30 min of ischaemia, with or without 10 min of anoxic pre-perfusion, on the concentrations of glycogen (μmol of glucose/g wet wt.) and of lactate (μmol /g wet wt.) in freeze-clamped perfused rat hearts. Controls were perfused for 10 min, and other hearts as shown. Values are means $\pm$ S.E.M. for three hearts in each group.

	Glycogen	Lactate
Control	10.2 $\pm$ 0.9	3.2 $\pm$ 1.8
30 min Ischaemia	1.1 $\pm$ 0.3	31.7 $\pm$ 2.0
10 min Anoxia	3.4 $\pm$ 0.5	3.2 $\pm$ 0.1
10 min Anoxia + 30 min Ischaemia	0.6 $\pm$ 0.1	20.6 $\pm$ 0.6

In order to eliminate the possibility that the factor responsible for ischaemic damage may be residual oxygen in the tissue, we attempted to purge the tissue of oxygen by perfusing with anoxic buffer for 1–10 min before 60 min of global ischaemia. Similar periods of anoxic pre-perfusion have been shown to have beneficial effects on the functional recovery after ischaemia [14]. As shown in Table 4, there was a significant protection of respiratory capacity after 3–5 min of anoxic pre-perfusion associated with decreased damage to Complex I, and greater protection after 10 min of anoxia, such that the NADH oxidase activity was no longer significantly less than controls (92%).

Neely & Grotyohann [14] showed that there was a good negative correlation between post-ischaemic recovery and tissue lactate concentrations. They suggested that the functional protection afforded by anoxic pre-perfusion is due to glycogen depletion, and hence decreased lactate accumulation due to anaerobic glycogenolysis during ischaemia. Furthermore, inclusion of lactate in the perfusion buffer, which increased tissue lactate concentrations, was antagonistic to the protective effect of anoxic pre-perfusion [14]. In our hands 10 min of anoxia caused a marked decrease ($> 65\%$) in cardiac glycogen content (Table 5), which was not associated with increased tissue levels of lactate. However, after 30 min of global ischaemia lactate was increased 10-fold, and glycogen was virtually totally depleted (10% of controls). Anoxic pre-perfusion, which caused a slight further fall in glycogen (6%), significantly decreased the lactate concentration after 30 min of ischaemia, but this lower value still represented a 6-fold increase over controls. Furthermore, perfusing for 60 min with anoxic buffer containing 30 mM-lactate did not affect the glutamate/malate oxidation rate (314 ± 47 ng-atoms of $\text{O} \cdot \text{min}^{-1} \cdot \text{mg}$ of protein $^{-1}$, $n = 3$). The protective effect of anoxic pre-perfusion on mitochondrial function does not therefore appear to be due to changes in lactate accumulation.

DISCUSSION

Previous studies have shown that global ischaemia decreased mitochondrial respiration activity in hearts from several species, particularly with NAD^+ -dependent substrates [3,5–12]. While this work was in progress, Hardy *et al.* [29] suggested that the damage is reperfusion-dependent, as they were unable to demonstrate any effect of anoxia. Our results show that such damage occurs during both the ischaemic period and the subsequent reoxygenation of the tissue. It seems evident therefore that treatments to protect mitochondria against ischaemia/reperfusion damage must be designed to protect Complex I, and for this reason it is necessary to understand the mechanism of this damage.

The opposite effects of ischaemia and anoxia indicate that lack

of oxygen is not the primary cause of the loss of Complex I activity. On the contrary, the effects observed upon reperfusion and the protection afforded by anoxic pre-perfusion strongly suggest that it is oxygen itself which is responsible. Neely & Grotyohann [14] suggested that the protection afforded by anoxic pre-perfusion was due to a depletion of tissue glycogen, thus decreasing the accumulation of lactate due to anaerobic glycogenolysis, but other reports suggest that increased myocardial glycogen protects the heart during ischaemia (see [30]). Our results show that neither lactate nor decreased pH have a detrimental effect on Complex I.

It has been shown that perfusion with exogenous superoxide dismutase improves post-ischaemic function, an effect associated with a decrease in the mitochondrial free-radical production which occurs during ischaemia [13]. A marked increase in free radicals in mitochondria has also been demonstrated to occur during the first 5 min of reperfusion [31]. Free-radical production in ischaemic myocardium has been demonstrated at very low oxygen partial pressures (1 mmHg) [32]. Anoxic pre-perfusion may decrease the tissue oxygen below this level, thus preventing free-radical production during the ischaemia. The mitochondrial matrix contains superoxide dismutase as a defence mechanism against free-radical attack, but it has been demonstrated that this activity is decreased during ischaemia [33,34]. The delay in ischaemic damage, and the apparent sensitization to reperfusion may be as a result of the depletion of this protective mechanism.

Free-radical damage of the respiratory chain has been demonstrated *in vitro* with sub-mitochondrial particles, which lose respiratory-chain activities with an order of sensitivity of Complex I $>$ Complex III $>$ Complex IV [35], i.e. the same as we have observed in perfused hearts. This is apparently due to the peroxidation of cardiolipin, which is an essential requirement for the biological activities of these complexes [36]. It is notable that the main sites of mitochondrial damage during ischaemia, i.e. Complexes I and III, are the major sources of free radicals produced by mitochondria *in vitro* [37]. NADH-dependent production of free radicals has been demonstrated cytochemically in the mitochondrial cristae of dog heart, this activity being low in normoxic tissue, markedly increased in viable ischaemic tissue and absent from severely infarcted regions [38]. This may be explained on the basis of a stimulation of free-radical production by Complex I, which is ultimately self-destructive, as these free radicals peroxidize cardiolipin. This argument may equally be applied to explain our results. The localized destruction of cardiolipin at the site of free-radical production, i.e. Complexes I and III, would explain the different sensitivities of the complexes to the same source of damage. Furthermore, as cardiolipin is not required for the activity of NADH dehydrogenase [36], this activity would not be affected.

Our observation of an increase in glutamate/malate oxidation during early ischaemia and anoxia agrees with similar increases found after short periods of hypoxia [39] and hypoperfusion [10]. Hardy *et al.* [29] did not find such an increase in hearts perfused for 40 min with anoxic buffer. We are unable to explain the differences in these results. Pelikan *et al.* [10] reported an increase of 37% in mitochondrial respiration specifically with NADH-dependent substrates and speculated that an effect on Complex I could be involved. Although the increase in respiration is specific for NADH oxidation, no change in the activity of Complex I could be detected. The increase cannot be due to the selective recovery of the more active of the two mitochondrial sub-populations found in cardiac tissue [18], as these two populations display 84% and 33% differences in the specific activities of citrate synthase and succinate dehydrogenase respectively. In our preparations these activities were unchanged in those mitochondria displaying increased mitochondrial respir-

ation or decreased activity after ischaemia/reperfusion (Table 2). However, our preliminary studies suggest an alteration of the kinetics of Complex I activity during early ischaemia.

Therefore it appears that there is biphasic response of the mitochondrial respiratory chain to cardiac ischaemia, the initial reaction being an increase in the capacity to oxidize NADH, followed by a progressive loss of the activity of Complex I owing to the destruction of a component subsequent to the soluble NADH dehydrogenase. Elimination of oxygen from the tissue by anoxic pre-perfusion decreases this damage suggesting a role of residual oxygen, the most obvious possibility being production of oxygen free radicals. Anoxic perfusion itself has no deleterious effects on the mitochondrial activities, and does not appear to sensitize the mitochondria to reoxygenation damage.

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