Protection of respiration of a crude mitochondrial preparation in cerebral ischaemia by control of blood glucose

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SUMMARY There is evidence that systemic hyperglycaemia influences the evolution of cerebral ischaemia adversely, through enhancement of brain lactic acidosis. A potential protective regimen based on induction of mild hypoglycaemia with insulin was therefore studied. Respiratory control in brain mitochondria prepared from the ischaemic hemisphere of rats subjected to unilateral common carotid artery occlusion for one hour was abolished, but was partially preserved in insulin-treated rats ($p = 0.01$).

A considerable volume of experimental evidence suggests that the evolution of changes in the brain parenchyma resulting from cerebral ischaemia is influenced adversely by increased availability of glucose to the brain.¹⁻⁵ In man, the outcome of stroke is worse in diabetics,⁶ and also when the systemic response to stress is enhanced:⁷ one component of this response is hyperglycaemia. Such findings are frequently attributed to a direct or indirect relationship between brain lactic acidosis and severity of neuronal damage, and the present experiments were designed to test the hypothesis that such damage might be reduced by deliberate induction of mild hypoglycaemia. Respiratory control ratio of brain mitochondria was used as an *in vitro* index of irreversible ischaemic change, and was examined in the control and ischaemic hemispheres of rats subjected to unilateral common carotid artery occlusion: one group of rats received insulin at the onset of ischaemia.

Methods

Anaesthesia was induced with 4% halothane in adult Wistar rats of either sex in the weight range 250-350g. A

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tracheostomy was carried out and the rats paralysed and ventilated to physiological PaCO_2 with 60/40 $\text{N}_2\text{O}/\text{O}_2$, containing 0.5-1% halothane. Both common carotid arteries were exposed and snares applied. A pair of silver electrodes was placed over the right hemisphere for recording and processing of EEG (CFM®): EEG amplitude was recorded as the midpoint of the voltage envelope. One femoral artery and vein were cannulated for monitoring of arterial blood gases and pressure, and for intravenous infusion of drugs as required. Hypoglycaemia was induced at onset of occlusion by intraperitoneal injection of 4-6 u/Kg soluble insulin. Following definitive (right common carotid) or sham (observation only) occlusion for one hour, the brain was quickly removed after decapitation. Arterial pressure was lowered by withdrawal of blood if no unequivocal reduction in EEG amplitude was evident on visual inspection following occlusion alone. In order to assess the mitochondrial preparation, a group of rats was stunned and decapitated without prior anaesthesia or surgery, and the cerebral hemispheres pooled before homogenisation so as to obtain a larger mitochondrial pellet.

Mitochondria were prepared using the initial phase of the brain subcellular fractionation procedure of Dodd *et al.*⁹ The fresh cerebral hemispheres were homogenised in 0.32 M sucrose, and after centrifugation at 1,000 g for 10 minutes the supernatant was layered on to 0.32 M sucrose contained 8% metrizamide and centrifuged at 160,000 g for 15 minutes. The pellet obtained was washed in 150 mM KCl, recentrifuged, and resuspended in 1-2 ml 150 mM KCl, and placed in an oxygen electrode cuvette (Rank Bros., Cambridge, UK) at room temperature (24°C). 20 μl 0.1 M sodium phosphate buffer (pH 7.2) was added, followed by 40 μl 1M sodium succinate. After achievement of a stable oxygen consumption rate 5-20 μl 40 mM ADP

was added, and State 3 respiratory rate was measured. State 4 rate was assessed subsequently and respiratory control ratio (RCR) expressed in standard manner as the ratio

$$\frac{\text{state 3 rate (mV/Min)}}{\text{state 4 rate (mV/Min)}}$$

Phosphorylation ratio (P:O) was calculated from the ratio of moles of ADP introduced (assuming complete phosphorylation) to g.atoms of oxygen consumed during state 3; oxygen content of the incubation medium was taken as 0.49 μg atom per ml 150 mM KCl at 24°C and atmospheric pressure of 760 mm Hg.¹⁰

Results are expressed as arithmetic means; ranges are quoted in the text and standard deviations in the table. Respiratory control ratio and P:O ratio data were assessed by analysis of variance; differences in these variables between pairs of treatment groups were examined using the Newman-Keuls multiple range test (unequal sample sizes). Mean arterial pressure and CFM amplitude were compared in the untreated and insulin-treated occlusion groups using the Mann-Whitney U-test.

Results (table)

Decapitation (n = 6)

Respiratory control ratio from pooled hemispheres in rats not undergoing anaesthesia or surgery was 5.89 (5.00–6.8). P:O ratio was 1.98 (1.87–2.13).

Sham occlusion (n = 6)

In rats undergoing anaesthesia and surgery comparable with those procedures for rats subjected to ischaemia, respiratory control ratio was 5.56 (3.75–6.84) (pooled hemispheres, for comparison with decapitation group), and P:O ratio was 2.01 (1.32–2.52).

Unilateral carotid occlusion (n = 6)

In none of the six experiments carried out could a stable rate of oxygen consumption be established for mitochondria from the ischaemic hemisphere; in each case oxygen uptake was rapid and tended to accelerate; P:O ratios were therefore unobtainable. Respiratory control ratio in the sham occluded hemisphere was 4.31 (1.00–6.12); this value was not significantly different from RCR in decapitated or bilateral sham occluded rats (analysis of variance with Newman-Keuls' test). P:O ratio was 1.71 (1.16–2.16; n = 5; respiration uncoupled in sixth experiment).

Unilateral carotid occlusion with hypoglycaemia (n = 6)

Stable values for state 3 and state 4 respiration were obtained in four rats. In one rat, stable state 3 respiration was achieved with no deceleration of respiration, and respiratory control ratio was therefore read as unity. Mean respiratory control ratio in the ischaemic hemisphere was 2.49 (1.00–5.24; n = 5) (p < 0.05 vs. left hemisphere in untreated right carotid occlusion group; p < 0.001 vs. decapitated, sham occlusion and left hemisphere in the treated, right carotid occlusion group (analysis of variance with Newman-Keuls' test)); P:O ratio was 1.48 (1.13–2.10; n = 4). The higher incidence of coupling in the ischaemic hemisphere of insulin-treated rats as compared with untreated rats was highly significant. The significance of the difference was tested categorically (exact test, p = 0.01). Respiratory control ratio in the sham occluded hemisphere of insulin-treated rats was 5.55 (4.50–6.39; n = 5).

Table Mitochondrial respiratory control (RCR) and P:O ratios and plasma glucose before and following occlusion in brain mitochondria prepared from rats subjected to decapitation, sham carotid occlusion, right common carotid artery occlusion, and right carotid occlusion with insulin-induced hypoglycaemia

	Respiratory control ratio		P:O ratio§		Plasma glucose (mmol) Time from occlusion (min)			
					Before	5	30	60
Decapitation (n = 6)	5.89 ± 0.69*		1.98 ± 0.09*					
Sham carotid occlusion (n = 6)	5.56 ± 1.19*		2.01 ± 0.39*					
	Right	Left	Right	Left				
Unilateral carotid occlusion (n = 6)		4.31 ± 1.76	†	1.61 ± 0.44	†8.61 ± 1.48	8.69 ± 1.36	8.91 ± 1.15	9.00 ± 0.84
Unilateral carotid occlusion + insulin (n = 5)	2.49 ± 1.89	5.55 ± 0.79	1.48 ± 0.44	1.87 ± 0.23	10.18 ± 0.89	3.46 ± 0.38	3.51 ± 0.32	3.56 ± 0.75

Values are means ± 1 SD.

*both hemispheres pooled.

†unstable, rapid respiration was observed, and RCR and P:O ratio were therefore unobtainable.

‡n = 4 (separate group).

§no significant differences in P:O ratio between groups (analysis of variance).

^{||}p < 0.05 vs. left hemisphere in untreated, occlusion group; p < 0.01 vs. decapitation, sham occlusion and left hemisphere (occlusion & insulin) (analysis of variance with Newman-Keuls' test).

(a)

(b)

Fig (a) Comparison of the time courses of mean arterial pressure in untreated and insulin-treated rats subjected to right common carotid artery occlusion. There was a small but statistically significant (Mann-Whitney U-test, $p < 0.05$, 0.01) reduction in the untreated group at 55 and 60 minutes. (b) Volumes of blood withdrawn and Cerebral Function Monitor (EEG) amplitude in untreated and insulin-treated rats subjected to right common carotid artery occlusion. Cumulative blood withdrawal and reinfusion is shown for individual rats at 5 minute intervals. Blood was fully reinfused by 20 min in all experiments. EEG amplitude was better preserved in insulin-treated rats (Mann-Whitney U-test, *, $p < 0.05$, **, $p < 0.025$).

Plasma glucose

In four rats undergoing unilateral carotid occlusion,

pre-occlusion plasma glucose was 8.86 (7.33–10.78) mmol: values at 5, 30 and 60 min after occlusion were respectively 8.69 (7.05–10.33), 8.91 (7.56–10.28), and 9.04 (8.33–10.00) mmol. In the insulin treated group, pre-occlusion glucose levels were 10.18 (9.00–11.05); values at 5, 30 and 60 min after occlusion were 3.47 (2.89–3.44), 3.51 (3.11–3.83) and 3.56 (2.83–4.50).

Mean arterial pressure (fig a)

Mean arterial pressure was tabulated at 5 minute intervals, and was closely comparable in both groups subject to occlusion until the last 5 minutes of the experiment, when a small but significant reduction ($p < 0.05$, 0.01) emerged in the untreated group. Volumes of blood withdrawn are specified for those animals in which it was required in fig (b); all blood was reinfused in each case within 20 minutes of occlusion and there was no significant difference in volume bled between the groups undergoing carotid occlusion.

CFM amplitude (fig b)

CFM amplitude fell to 58% of pre-occlusion baseline after 15 minutes of ischaemia in the occluded untreated group and to 74% in the treated group ($p < 0.025$). Mean CFM amplitudes were substantially stable for the subsequent 45 minutes and the difference between groups remained significant.

Discussion

The technique for measurement of mitochondrial respiration appears reproducible in our hands, with values for respiratory control ratio and P:O ratio and ranges comparable with those obtained elsewhere.¹¹ Halothane affects mitochondrial respiration,¹² but its effects in the concentration used in these experiments, as reflected in data from sham-occluded rats, appear unremarkable.

It is often stated that unilateral common carotid artery occlusion in the rat is not associated with morphological evidence of ischaemic damage. However, in our experiments, the deliberate restriction of arterial pressure may have induced a critically greater degree of ischaemia, sufficient either itself or in combination with halothane or with the fractionation procedure, to affect mitochondrial respiration. Comparison of light and electron microscopic appearances in the periphery of zones of focal cortical ischaemia in the cat suggests that mitochondrial abnormalities can sometimes be detected when light microscopy is normal.¹³ Also, although blood flow was not measured in these studies, it is possible that the degree of ischaemia induced was of intermediate severity, comparable with that present in penumbral

territories in focal ischaemia, and that mitochondria, affected perhaps only minimally and reversibly by ischaemia, were subsequently damaged further by the fractionation procedure: measurement of *in vitro* respiration assesses the combined effects of ischaemia and of fractionation. Bovine serum albumen is often added to the suspension medium during fractionation of mitochondria, so as to reduce free fatty acid-mediated autolysis, but was not used in these studies. Although this does not appear to have influenced our control values, it is possible that *in vitro* abnormalities in ischaemic mitochondria would have been less marked had bovine serum albumen been used. Although the crude mitochondrial fraction we used contains myelin and nerve endings, the features examined in this study (RCR and P:O ratio) are essentially specific to mitochondria.

Irrespective of the mechanisms of mitochondrial damage, a topic outside the scope of this paper, the data suggest a protective effect associated with blood glucose reduction. There are several possible mechanisms of protection. The difference in arterial pressure (fig a) is unlikely to contribute substantially to the results, firstly since the terminal blood pressure in the untreated group was still well maintained, at 94 mmHg, and secondly since the duration of any difference in arterial pressure between groups was no more than 5–10 minutes: there is good evidence from several studies that duration of insult as well as severity affects recovery from an ischaemic insult, whether assessed for example from *in vitro* synaptosomal neurotransmitter uptake,¹⁴ from preservation of recovery of extracellular potassium homeostasis following reperfusion¹⁵ or from recovery of single unit activity.¹⁶ Moreover, during the period of occlusion prior to the preterminal divergence of arterial pressures there was already significantly better preservation of CFM amplitude in the insulin-treated group (fig b). Since arterial pressures during this phase were equal and occlusion techniques were uniform the insults to both groups were similar at the point of input to the intracranial circulation, and the difference in CFM most probably reflects early emergence of a protective effect which is confirmed by the mitochondrial studies. Since cerebral blood flow was not measured, several haemodynamic and rheological factors capable of improving CBF may have contributed to protection, such as better collateral perfusion relating to improved blood viscosity or red cell deformability, enhanced red cell P_{50} and hence oxygen delivery, or reduced ischaemic cerebral capillary endothelial swelling: Paljärvi and his colleagues have recently presented evidence for an adverse effect of lactic acidosis on brain microvessels as well as on the brain parenchyma.¹⁷ The present results do not therefore

distinguish between on the one hand improved oxygen delivery and on the other reduced glycolytic flux and lactate accumulation in the brain parenchyma (independent of any improvement in CBF) as protective mechanisms.

So far as we are aware this is the first study to have tested insulin-induced hypoglycaemia as a therapeutic regimen for cerebral ischaemia and to have shown potential beneficial effects. However, caution is required in the assessment of the therapeutic potential of these results, for several reasons. First, although partial recovery of mitochondrial respiration *in vitro* has been achieved, the effects of reperfusion *in vivo* have not been tested, and it is possible that the development of oedema and progression of ischaemic damage seen during reperfusion in some experimental models might not be influenced by insulin treatment in the same way as was mitochondrial respiration in the present preparation. Secondly, it may be argued that other effects of ischaemia such as those on the microcirculation are at least important as parenchymal effects in the determination of irreversibility. However, for reasons given above the protective effect demonstrated here may indeed extend to the microcirculation. Thirdly, at intermediate levels of focal cerebral ischaemia, electrophysiological function is selectively affected, with substantial preservation of structure.^{18,19} Although it is therefore possible that the therapeutic effect demonstrated might not embrace subcellular compartments more specifically related to function such as the synapse, the difference in CFM between groups suggests a degree of *in vivo* protection of function. It is of interest that the values of plasma glucose in treated rats were lower than the glucose concentration threshold in superfusate (5 mmol) at which population spike amplitude was first affected in hippocampal slices,²⁰ but were above the values of blood sugar (2 mmol) usually associated with irreversible hypoglycaemic damage. Recent analysis by Mohr of material from the US Stroke Data Bank²¹ indicates that in man mortality from acute stroke falls from 27 to 7% when admission blood sugar lies below 100 mg%. Mortality increased in association with increasing hyperglycaemia between 100 and 200 mg%. We did not examine the effects of induction of hyperglycaemia in these experiments, which were designed to test a potential therapeutic regimen. Since mitochondrial respiration in ischaemic brain was already severely affected at plasma glucose values of 8–10 mmol/l, it is unlikely that we would have been able to demonstrate an additional adverse effect of increasing plasma glucose above 8–10 mmol/l. However, in a separate study (in preparation for presentation) in cats undergoing occlusion of the middle cerebral

artery and infused with glucose after onset of the ischaemia (plasma glucose 20 mmol/l) we have found evidence of impairment of recovery of extracellular potassium homeostasis in the penumbra during reperfusion.

We conclude that, whatever the mechanism, mild insulin-induced hypoglycaemia provides some degree of protection of *in vitro* brain mitochondrial respiration and most probably of electrophysiological function against the effects of moderate degrees of cerebral ischaemia: similar studies following a period of reperfusion are required, together with measurement of CBF during ischaemia, and assessment of neurological recovery in an appropriate model.

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