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Biochimica et Biophysica Acta 1366 (1998) 291–300

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

Oxidation and reduction of cytochrome oxidase in the neonatal brain observed by in vivo near-infrared spectroscopy

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Received 25 May 1998; accepted 10 June 1998

Abstract

Near-infrared spectroscopy was used to determine the relationship between the redox state of mitochondrial cytochrome oxidase Cu_A and haemoglobin oxygenation in the isoflurane-anaesthetized neonatal pig brain. Adding 7% CO_2 to the inspired gases increased the total haemoglobin concentration by 8 μM and oxidized Cu_A by 0.2 μM. Decreasing the inspired oxygen fraction to zero for 90 s dropped the oxyhaemoglobin concentration by 27 μM and reduced Cu_A by 1.8 μM. However, no change in the Cu_A redox state was observed until oxyhaemoglobin had decreased by more than 10 μM. The response of the Cu_A redox state to these stimuli was very similar following 80% replacement of the haemoglobin by a perfluorocarbon blood substitute; this demonstrates that the results in the normal haematocrit were not a spectral artefact due to the high haemoglobin/cytochrome oxidase ratio. We conclude that the large reductions in the Cu_A redox state during anoxia are caused by a decrease in the rate of oxygen delivery to the cytochrome oxidase oxygen binding site; the small oxidations, however, are likely to reflect the effects of metabolic changes on the redox state of Cu_A, rather than increases in the rate of oxygen delivery.

Keywords: Cytochrome oxidase; Near-infrared spectroscopy; Brain; Redox state; In vivo; Anoxia

1. Introduction

In vivo cytochrome oxidase redox state has been investigated in humans and animals for several years using near-infrared (NIR) spectroscopy (NIRS) [1–4]. However, the correlation between its redox state and physiological effectors, like oxygen, substrate supply, pH, and mitochondrial inhibitors, is still controversial [5]. NIRS measures with a sub-second time resolution change in haemoglobin concentration [6,7] and oxygenation, and a unique Cu-Cu redox centre

Abbreviations: aHb, absolute Hb; CCD, charge-coupled device; CHC, total cerebral haemoglobin concentration; FiO_2, inspired oxygen fraction; Hb, deoxyhaemoglobin; HbO_2, oxyhaemoglobin; Hct, haematocrit; HR, heart rate; MABP, mean arterial blood pressure; NIR, near-infrared; NIRS, near-infrared spectroscopy; $PaCO_2$, arterial blood carbon dioxide tension; PaO_2, arterial blood oxygen tension; SaO_2, arterial blood oxygen saturation

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0005-2728/98/$ – see front matter
PII: S0005-2728(98)00129-7

of cytochrome oxidase [5,8–11] called Cu_A. Therefore, NIRS can provide information, indirectly, about the electron flux, and hence activity, of the mitochondrial respiratory chain [12]. The primary physiological parameter affecting the Cu_A centre is the intra-mitochondrial oxygen tension [13], but other factors (e.g. mitochondrial energy state and substrate delivery) can have effects on the signal in vitro [12]. Recent studies have suggested that the Cu_A signal is affected subsequent to hypoxia-ischaemic damage to the brain, as if cytochrome oxidase were being inhibited [14] by the increased nitric oxide levels present under these conditions [15].

Carbon dioxide reactivity is frequently used as a standard method to vary cerebral blood flow and oxygen delivery. In the neonatal human, it has been reported that increases in arterial CO_2 (hypercapnia) induce oxidations in cytochrome oxidase Cu_A [10]. This surprising finding was interpreted to mean that regions of the brain were oxygen limited under normocapnic conditions. However, there are concerns in interpreting this data. The decomposition of the Cu_A signal in the presence of larger changes in haemoglobin concentration and oxygenation is a non-trivial procedure [16], especially with the six wavelength photometer where no correction was made for the wavelength dependence of the in vivo optical pathlength [17].

We have therefore performed these studies of manipulating arterial oxygen and carbon dioxide tension in a well-controlled neonatal pig model, using an improved multiwavelength NIR spectrophotometer (5 nm resolution). The protocol was designed to test whether the observed Cu_A responses were an experimental artefact by partially replacing the blood (haematocrit reduced to 20% of baseline) with an artificial blood substitute (perfluorocarbon) and repeating the procedure. Although the haemoglobin concentration was five times smaller than under the control conditions, comparable oxidations of cytochrome oxidase were observed during hypercapnia. Furthermore, by comparing these findings with the changes in the cytochrome oxidase redox state observed during, and subsequent to a rapid fall in inspired oxygen, we conclude that the observed oxidations are not due to improvements in oxygen delivery to regions of the brain, but instead reflect biochemical changes acting at the level of the mitochondria.

2. Materials and methods

2.1. Anaesthesia

Seven newborn piglets of both sexes (1.6 ± 0.2 kg) were studied. Animals were sedated with Midazolam (0.2 mg/kg, i.m.). After tracheostomy, the trachea was intubated and the animals were mechanically ventilated. 3% isoflurane was used during the surgical procedure and then its level was reduced to 1.5–2%. A computer controlled gas blender [18] was used to supply the gas mixtures necessary during the experiment. The animals were checked frequently during surgery and throughout the experimental procedure to ensure an adequate level of anaesthesia. All animal procedures were performed under appropriate UK Home Office guidelines.

2.2. Surgical preparation

Details of the surgical preparation have been described previously [19]. Briefly, the umbilical artery and vein were cannulated. The venous catheter was used for fluid (gelofusine, physiological solution) administration and the arterial catheter for collecting blood samples to perform blood gas analysis. Umbilical arterial blood pressure was monitored using a strain gauge pressure transducer system (HP78201B, Hewlett-Packard, Palo Alto, CA, USA). Rectal temperature was monitored and maintained at around 38.5°C with a heating table. Bipolar electroencephalogram (Cerebral Function Monitor, Model 870, Critikon, USA) was monitored by needle electrodes placed along the lateral margins of the frontal bones. Inspired oxygen fraction (FiO_2) was 0.3–0.4 and 0.90 in normal and low haematocrit piglets, respectively. The peak inspiratory pressure and ventilation rate were adjusted in order to maintain arterial CO_2 partial pressure ($PaCO_2$) around 4.5 kPa (normocapnia).

Heart rate (HR) and arterial saturation (SaO_2) were monitored throughout the experiment using a pulse oximeter (Model 8604FO, Nonin Medical, Plymouth, MN, USA) via a probe positioned on the left foot. SaO_2 values during anoxia are not reported due to the inaccuracy of SaO_2 readings below 85%.

Mean arterial blood pressure (MABP) was maintained in the range 45–55 mmHg by infusion of plasma expander. On three out of six blood-perfluoro-

carbon exchanged piglets, MAPB was maintained in the range 40–50 mmHg by infusion of phenylephrine (Knoll, Nottingham, UK). Arterial glucose was measured (2300 STAT Plus, YSI, Yellow Springs, OH, USA) at given times and maintained in the range 4.7–6.5 mM by glucose venous infusion. Intermittent measurement of arterial O_2 partial pressure (PaO_2), $PaCO_2$, and pH were performed via arterial blood gas analysis (ABL505, Radiometer, Copenhagen, Denmark). Electroencephalogram signal amplitude was monitored in three out of seven animals, and was unchanged throughout the experiment except during a rapid fall in inspired O_2 (anoxia) when it transiently decreased to about 30% of control value. At the end of surgical preparation, antibiotics (cidomycin and benzylpenicillin) were administered.

2.3. Near-infrared spectroscopy

The piglet's head was positioned in a stereotaxic holder and two optical fibres were firmly placed over the skull, previously shaved, by two holders attached on the stereotaxic frame. The optodes (the end of the optical fibres) were placed 3.5 ± 0.2 cm apart symmetrically with respect to the midline and 1 cm posterior to the eyes. The area surrounding the optodes was painted black to ensure that any observed attenuation changes were only due to light that had passed through the animal's head from source to receiving optode.

A white light source and a cooled charge-coupled device (CCD) detector were used to measure light attenuation simultaneously between 550 and 1000 nm [20,21]. The pixel bandwidth was 0.65 nm and the slits were set to give a resolution of 5 nm. The exposure times were set to give a maximum signal of approx. 100 000 electrons per pixel (typical exposure time was 1.5 s in normal haematocrit and 0.3 s in low haematocrit piglets when the brain was more transparent). One exposure was collected every 10 s throughout the experiment. Changes in the concentration of oxyhaemoglobin (HbO_2), deoxyhaemoglobin (Hb) and the redox state of cytochrome oxidase Cu_A were calculated by fitting the attenuation changes in the region 780–900 nm using an algorithm that corrected for the wavelength dependence of optical pathlength [16]. Chromophore concentration changes were converted to units of μM by dividing by the mean baseline optical pathlength measured from the 840 nm water absorption feature [22,23]. The technique is sensitive down to less than 0.1 μM haemoglobin. Second differential spectroscopy was used to calculate the absolute Hb concentration (aHb) and the mean optical pathlength as described previously [22,23] with the following modifications: aHb and the 740 nm water pathlength were obtained simultaneously by fitting between 700 and 800 nm, and the 840 nm water pathlength by fitting between 800 and 880 nm. Water pathlengths were converted to units of cm, assuming an average water content in the neonatal brain of 80%.

2.4. Perfluorocarbon exchange transfusion

Perfluorocarbon-for-blood exchange transfusion was performed with perfluorotributylamine emulsion FC-43 (Green Cross, Osaka, Japan), while the animals breathed FiO_2 1.0. The emulsion was infused intravenously while withdrawing blood from the umbilical artery at a rate which maintained the MABP in the range 44–48 mmHg. The blood-perfluorocarbon exchange was continued until the haematocrit was reduced to about 20% of the control value. Between 240 and 300 ml of FC-43 was required to achieve this. The exchange lasted approx. 90 min and, at the end of the exchange, FiO_2 was reduced to 0.90.

2.5. Experimental protocol

A period of at least 1 h was allowed to elapse after preparation, so as to achieve stabilization.

Protocol A: pre blood-perfluorocarbon exchange, the FiO_2 was reduced to 0 for 90 s (anoxia). After a 25 min recovery period, the animals were challenged for 5 min with 7% CO_2. A 30 min recovery period followed. Piglets were challenged again for 5 min with 7% CO_2. A 30 min recovery period followed ($n = 7$).

Protocol B: the same protocol was performed 30 min after the end of the blood-perfluorocarbon exchange. In this protocol piglets were submitted to one 7% CO_2 challenge ($n = 6$).

In order to compare the chromophore changes amongst animals, the time axis of the data from each challenge was synchronized to the first rise in

total cerebral haemoglobin concentration (CHC), calculated as HbO_2+Hb, following the CO_2 increase or to the minimum value of HbO_2 following the anoxia. It should be noted that in the latter case this procedure results in a slight distortion of the later kinetic responses following anoxia. Despite the identical times of anoxia there will be slight differences in the exact time when the oxygen tension in the lungs is returned to normal. The most noticeable distortion is that each animal shows a rapid haemoglobin reoxygenation, but this appears slower in the averaged data as the reoxygenation starts at slightly different times in each animal.

Due to Cu_A signal instability during protocol B, only four piglets were considered in the results of hypercapnia and five in the anoxia challenges.

2.6. Statistics

All data are given as mean ± S.E. Analysis of variance (ANOVA), to identify significantly different treatments, as well as to compare repeated measurements, was performed by Student's *t*-test. The criterion for significance was $P < 0.01$.

3. Results

3.1. Pre exchange

All the blood gases and the physiological parameters of protocol A are reported in Table 1. PaO_2 as well as body temperature did not change significantly during hypercapnia. As expected, pH was significantly decreased and $PaCO_2$ significantly increased during hypercapnia. A transient rise in MABP occurred during both post-anoxic hyperaemia and hypercapnia. HR increased only during the hyperaemic phase following the anoxia.

Fig. 1 shows the effect of a 5 min moderate hypercapnia on the concentration changes in HbO_2, Hb, CHC and on the Cu_A redox state change. Hypercapnia induces a sub-maximal dilatation that increases blood flow [24] and this is reflected by a prompt HbO_2 and CHC rise (panel a). A delayed oxidation of Cu_A is even more evident in the single animal responses. At the end of the CO_2 challenge, Hb rapidly returned to the baseline condition whilst HbO_2 and CHC recoveries were slower. The Cu_A redox state returned to the control condition earlier than HbO_2 and CHC. Essentially, the same changes were observed on repeating the challenge (not shown).

To determine the effect of oxygen delivery on the Cu_A redox state change, a rapid anoxia was performed. The concentration changes in HbO_2, Hb, and CHC, as well as the Cu_A redox state change, are reported in Fig. 2. The illustrated cerebral HbO_2 decline and Hb rise were simultaneous with the observed fall in the SaO_2 (not shown). A small CHC rise was also observed during anoxia. The Cu_A reduction began about 20 s after the start of the HbO_2 fall. The re-establishment of the normoxic state provoked an immediate rise in HbO_2 to levels above the baseline level and a concomitant fall in Hb to below baseline levels. The overall CHC was also higher post anoxia, strongly suggestive of the rise in cerebral blood flow expected during a reactive hyperaemia. In all animals the return to the baseline values

Table 1
Blood gases and physiological parameters for the normal haematocrit piglets ($n = 7$)

	Control	Anoxia	Hyperaemia	Pre 7% CO_2	7% CO_2
pH	7.54 ± 0.02			7.53 ± 0.02	7.31 ± 0.01*,+
PaO_2 (kPa)	18.3 ± 0.8			16.6 ± 1.0	16.8 ± 0.8
$PaCO_2$ (kPa)	4.4 ± 0.2			4.3 ± 0.3	7.8 ± 0.3*,+
SaO_2 (%)	98.7 ± 0.2	0*,+	98.4 ± 0.3	98.7 ± 0.2	98.1 ± 0.1
MABP (mmHg)	47 ± 1	56 ± 2	79 ± 4*	48 ± 1	76 ± 8*,+
HR (bpm)	179 ± 4	181 ± 4	202 ± 4*	179 ± 5	170 ± 7
Temp. (°C)	38.7 ± 0.1	38.7 ± 0.1	38.7 ± 0.1	38.7 ± 0.1	38.7 ± 0.1

Values are mean ± S.E. PaO_2, arterial O_2 partial pressure; $PaCO_2$, arterial CO_2 partial pressure; SaO_2, arterial saturation; MABP, mean arterial blood pressure; HR, heart rate; bpm, beats/min.
*Different from control ($P < 0.01$).
+Different from pre 7% CO_2 ($P < 0.01$).

of Hb and HbO_2 required at least 5 min following the end of the anoxia. Cu_A oxidized promptly at the end of anoxia reaching a greater value than baseline during the hyperaemic response and quickly recovered to original values within 2 min. However, the kinetic of the Cu_A re-oxidation at the end of anoxia (Fig. 2b) was slowed by the averaging procedure described in the experimental section.

Fig. 3 shows the relationship between oxidation Cu_A and HbO_2 concentration changes obtained combining the results occurring during hypercapnia (Fig. 1) and the onset of anoxia (Fig. 2). It illustrates that the Cu_A redox state is unchanged over a wide range of brain oxygenation changes. These results are consistent with findings that oxygen delivery does not limit oxygen consumption in the brain until a critical threshold of oxygen delivery is achieved [25,26]. These data also suggest that in the piglet brain the cytochrome oxidase Cu_A is largely oxidized.

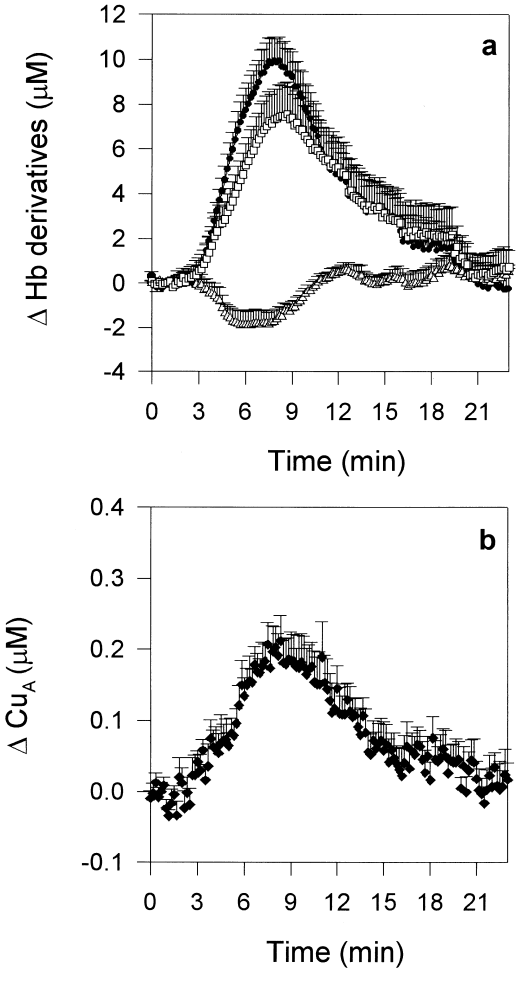

Fig. 1. Effects of moderate hypercapnia on piglet cerebral NIRS parameters. Haemoglobin derivatives (a) and Cu_A (b). Results are mean+S.E. of seven piglets. ●, HbO_2; △, Hb; □, CHC.

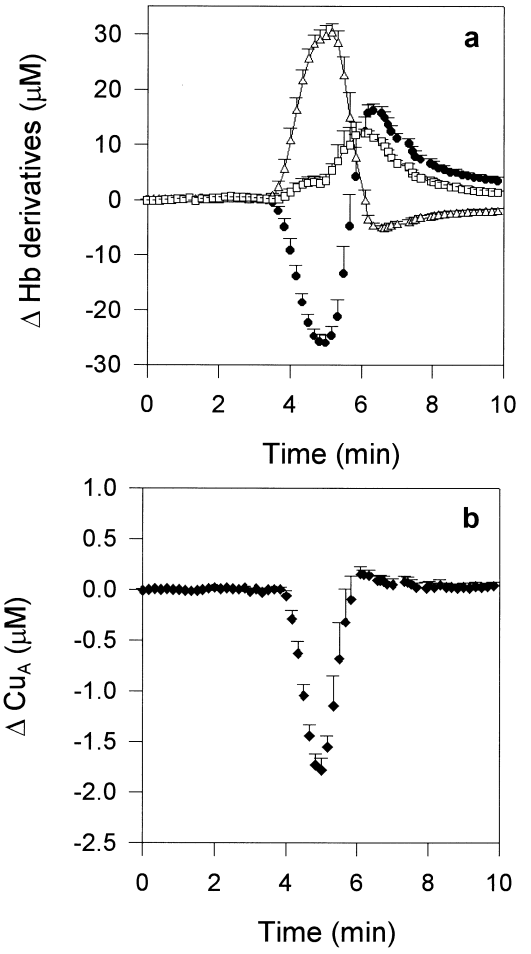

Fig. 2. Effects of anoxia and reactive hyperaemia on piglet cerebral NIRS parameters. Haemoglobin derivatives (a) and Cu_A (b). Results are mean+S.E. of seven piglets. Symbols as in Fig. 1.

3.2. Post exchange

As the Hb and HbO_2 concentrations in the brain are at least 10 times greater than those of cytochrome oxidase, the accurate detection of the small Cu_A redox changes shown in Figs. 1 and 2 is critical. In order to discount the problem of the imperfections in the deconvolution algorithm (large changes in haemoglobin concentrations may cause spurious observed changes in the Cu_A signal), six animals were partially blood-perfluorocarbon exchange transfused

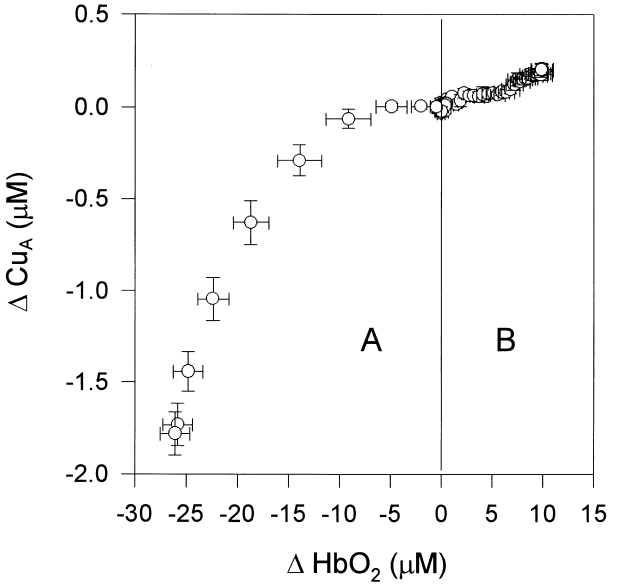

Fig. 3. The relationship between the Cu_A oxidation state and HbO_2 changes. (A) Data taken from the onset of anoxia up to the lowest reduction of Fig. 2. (B) Data taken from the first 8 min of Fig. 1. Results are mean ± S.E.

to decrease the absorption of Hb and HbO_2 in the NIR range relative to that of cytochrome oxidase. All the blood gases and the physiological parameters of protocol B are reported in Table 2. PaO_2 increased by about 45 kPa during protocol B when piglets were breathing FiO_2 0.90. The high PaO_2, the remaining haemoglobin, and the high oxygen solubility of the artificial blood substitute acted to maintain the arterial oxygen content at about 40% of that of pre-exchange conditions.

PaO_2, MABP, HR, and body temperature were unchanged during hypercapnia. As expected, pH and $PaCO_2$ were significantly decreased and increased, respectively, during hypercapnia. Brain electrical activity was unaffected by the partial blood-perfluorocarbon exchange transfusion.

Peripheral Hct was reduced on average from 26.9 ± 1.1% pre exchange to 4.6 ± 0.6% post exchange, a decrease to 17% of the control value. The optical pathlength measured at 840 nm was increased by 10% from 16.0 ± 0.6 to 18.1 ± 0.7 cm, as expected, following the exchange of the NIR absorbing haemoglobin with the NIR-transparent perfluorocarbon.

Fig. 4 shows the effect of a 5 min moderate hypercapnia on the concentration changes in HbO_2, Hb, and CHC, as well as the Cu_A redox state change in the low Hct animals. The increase in HbO_2 and CHC (panel a) was again accompanied by an oxidation of Cu_A (panel b). The amplitude of this oxidation is similar to that shown in Fig. 1b, even though the haemoglobin signals are a factor of five less.

During anoxia, as indicated by the pulse oximeter, SaO_2 dropped, and simultaneously the cerebral concentration of HbO_2 decreased and Hb rose (Fig. 5). No CHC change was observed. In the exchanged animals, Cu_A reduction began simultaneously with the HbO_2 fall suggesting, as expected, that oxygen delivery was not quite as effective as in the normal Hct animals. The re-establishment of the pre-anoxic condition provoked a rise in HbO_2 to levels above the baseline level and a concomitant fall in Hb to below baseline level. The overall CHC was also higher post anoxia. The return to the baseline values of Hb and HbO_2 occurred no sooner than 4 min after the end of anoxia. Cu_A oxidized promptly at the end

Table 2
Blood gases and physiological parameters for the low haematocrit piglets ($n = 6$)

	Control	Anoxia	Hyperaemia	Pre 7% CO_2	7% CO_2
pH	7.50 ± 0.02			7.54 ± 0.02	7.26 ± 0.02*,+
PaO_2 (kPa)	66.2 ± 2.9			63.0 ± 2.6	58.8 ± 2.3
$PaCO_2$ (kPa)	4.5 ± 0.3			4.1 ± 0.2	7.9 ± 0.5*,+
SaO_2 (%)	98.0 ± 0.4	0*,+	96.2 ± 1.0	97.8 ± 0.7	96.0 ± 1.4
MABP (mmHg)	42 ± 1	47 ± 2	57 ± 3	43 ± 1	45 ± 2
HR (bpm)	168 ± 6	172 ± 5	186 ± 7	163 ± 6	168 ± 10
Temp. (°C)	38.6 ± 0.1	38.7 ± 0.0	38.8 ± 0.1	38.7 ± 0.1	38.8 ± 0.1

Values are mean ± S.E. PaO_2, arterial O_2 partial pressure; $PaCO_2$, arterial CO_2 partial pressure; SaO_2, arterial saturation; MABP, mean arterial blood pressure; HR, heart rate; bpm, beats/min.
*Different from control ($P < 0.01$).
+Different from pre 7% CO_2 ($P < 0.01$).

of anoxia, reaching a greater value than baseline during the hyperaemic response, and quickly recovered to original values within 3 min. The similarity in the cytochrome oxidase redox state changes at vastly differing values of the CHC is good evidence that, with the algorithm we have used, the presence of haemoglobin does not interfere with the observed Cu_A redox changes.

4. Discussion

The non-invasive measurement of cytochrome oxidase redox state by NIRS has recently been reviewed [5]. The most significant physiological factor likely to affect the Cu_A redox state is the mitochondrial oxygen tension. This work demonstrates that increases and decreases in brain oxygenation over a wide range, both above and below the physiological value, have no effect on the Cu_A redox state (Fig. 3). An increased reduction of Cu_A is expected as the oxygen tension is lowered, due to a blocking of the electron exit from this centre to the binuclear haem a_3-Cu_B oxygen reduction site. However, in the normal Hct animals, only when over 50% of the normal oxyhaemoglobin in the brain became deoxygenated was significant Cu_A reduction observed (Fig. 2b). This is in agreement with previous findings in the adult rat brain [12,27–30]. It is also consistent with the finding of ourselves [5] and others [9] that in the neonatal pig

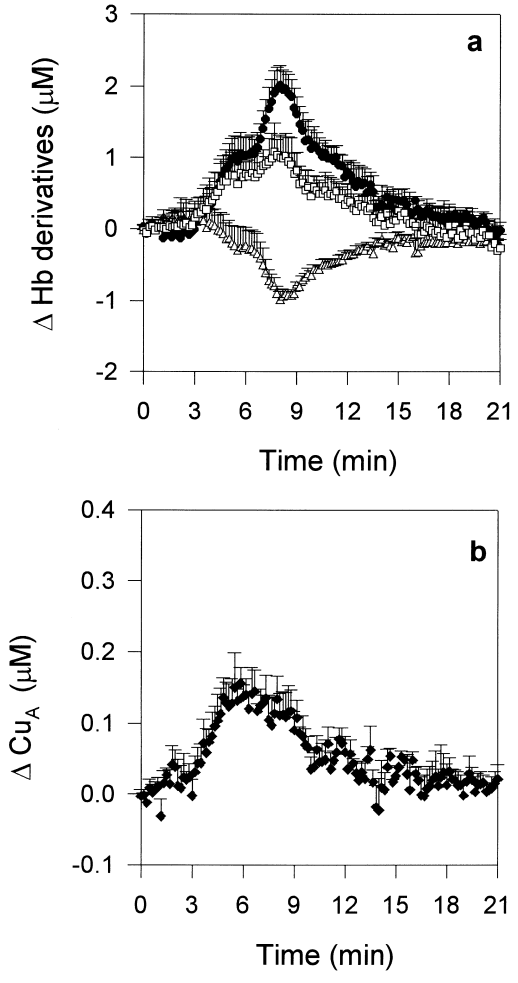

Fig. 4. Effects of moderate hypercapnia on cerebral NIRS parameters in the low haematocrit piglets. Haemoglobin derivatives (a) and Cu_A (b). Results are mean+S.E. of four piglets. Symbols as in Fig. 1.

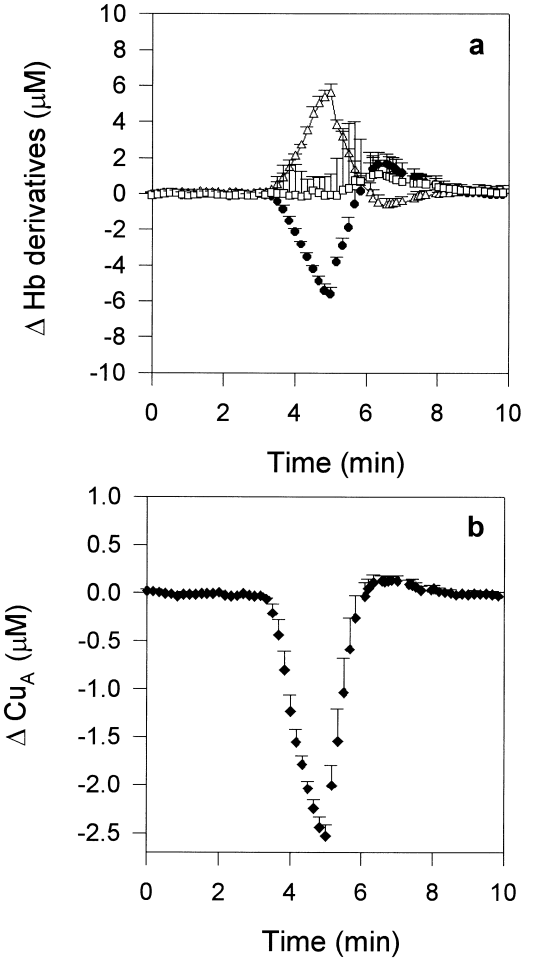

Fig. 5. Effects of anoxia and reactive hyperaemia on cerebral NIRS parameters in low haematocrit piglets. Haemoglobin derivatives (a) and Cu_A (b). Results are mean+S.E. of five piglets. Symbols as in Fig. 1.

brain a fall in the Cu_A redox state, rather than a fall in the haemoglobin oxygenation, correlates best with a decrease in the mitochondrial ATP/ADP ratio measured by ^{31}P NMR spectroscopy.

There are relatively few in vitro studies on the factors affecting the steady state reduction level of cytochrome oxidase Cu_A in turnover. However, under all the conditions studied so far (reviewed in [12]), changes in the Cu_A redox state mirror those of cytochrome *c*. This is as expected given that Cu_A is the initial electron acceptor from cytochrome *c*, and is on the same side of the mitochondrial inner membrane; hence these two metal centres sense the same membrane potential and pH gradients. Therefore, the more extensive literature [31–37] on the factors affecting the mitochondrial cytochrome *c* redox state is also likely to be relevant for the Cu_A in vivo studies described here.

The most surprising finding of this study is our confirmation of the previous results in the human neonatal brain [10], where an additional oxidation of Cu_A is seen upon increasing $PaCO_2$ (Figs. 1 and 4). The oxidation observed in this study was only a small fraction of the total cerebral cytochrome oxidase (approx. 10%, assuming that during anoxia the centre approaches full reduction). Pathlength changes, due to either increased tissue absorption induced by the increased haemoglobin concentration [28] or scattering changes, would be expected to interfere with the decomposition algorithm. We are confident that this is not happening as: (a) comparable Cu_A oxidations were observed in the low Hct animals; (b) analysis of the optical pathlength using the second differential water features at 740 and 840 nm yielded no evidence for scattering changes (not shown).

What are the possible mechanisms for an oxidation of Cu_A by an increase in $PaCO_2$? CO_2 increases cerebral blood flow (and hence oxygen delivery). One possibility is, therefore, that cytochrome oxidase is partially reduced in the normoxic condition due to lack of oxygen and, therefore, improvements in oxygen delivery will oxidize the enzyme. We think this is unlikely. Small variations of HbO_2 around the normal value do not reduce or oxidize Cu_A (Fig. 3). We only see this oxidation during the hyperaemia of hypercapnia and post anoxia when there are substantial increases in HbO_2. Therefore, the whole brain cannot be poised at 10% Cu_A reduction due to a slight oxygen limitation as, if this were the case, we would expect a linear, or near linear, relationship between haemoglobin oxygenation and the cytochrome oxidase redox state. Such a relationship is not observed in the neonatal pig (this study) or in the neonatal human [10]. Instead we would have to hypothesize a significant heterogeneity in the brain, such that 10% of the brain had complete reduction of Cu_A in the normoxic condition and that increasing CO_2 opened new pathways of oxygen delivery to these regions. We do not favour such an explanation, as it would imply a severe energy failure in a significant fraction of the brain. Furthermore, the current evidence is that, under the moderate hypercapnic conditions of this study, CO_2 does not promote active capillary recruitment into new regions of the brain [38]. Also, under such a haemodynamic mechanism, one would expect an increase in cerebral oxygen consumption following moderate hypercapnia. The general consensus is that oxygen consumption is decreased when CO_2 levels fall, but unchanged when they are increased [24].

Our data therefore suggest that the small oxidations of Cu_A require a metabolic, rather than haemodynamic explanation. Both a decrease in pH and an increase in the ADP concentration (via its effects on the proton electrochemical potential) can theoretically induce an increase in the steady state oxidation of Cu_A. In our studies, as expected, we see a significant fall in the blood pH following CO_2 challenge (Tables 1 and 2). ^{31}P-NMR spectroscopy studies on the newborn piglet indicate that, under the similar conditions of hypercapnia of this study, a significant fall in intracellular pH was observed [39]. A fall in pH has been shown to oxidize cytochrome *c* in isolated mitochondria, without affecting the oxygen consumption rate [31]. In the purified enzyme, lowering the pH oxidizes both cytochrome *c* and Cu_A by a similar amount [40]. A similar effect in vivo could also explain the Cu_A oxidation observed here (Figs. 1b and 4b). Consistent with this hypothesis is the fact that the kinetics of the Cu_A oxidation is slower in the normal Hct animal compared to the exchange animals where there is significantly more buffering capacity in the blood (haemoglobin and albumin are responsible for about 40% of total buffering by the blood). A drop in pH could also explain the oxida-

tion of Cu_A observed immediately following reoxygenation of the anoxic brain (Figs. 2b and 5b), as under these conditions significant metabolic acidosis would be expected [24]. The situation in this case is somewhat more confusing as ADP levels will also be higher immediately following anoxia and the consequent lowering of the mitochondrial membrane potential would also be expected to oxidize cytochrome *c* [31] and Cu_A [41]. Simultaneous ^{31}P-NMR and NIRS studies in the whole brain will, however, be necessary to determine whether pH or ADP concentration changes are large enough to account for the Cu_A oxidations observed in this paper.

We conclude that, as in the adult rat brain [13,27], in the normal healthy neonatal pig brain cytochrome oxidase redox states are not significantly affected by small changes in oxygen delivery. Large reductions in the Cu_A redox state reflect oxygen limitation at the binuclear haem a_3/Cu_B centre and only occur following dramatic haemoglobin desaturations. Other effectors can, however, explain small changes in the oxidation or reduction of the Cu_A centre with no simultaneous large haemoglobin oxygenation changes. These are likely to include pH, substrate supply and the presence of mitochondrial inhibitors such as nitric oxide [5].

Acknowledgements

We are grateful to Flavia Chiarotti for statistical analysis. We are grateful to the Wellcome Trust, the Royal Society, NATO (CRG.951310), Hamamatsu Photonics KK, and the European Union NIRS Concerted Action and Shared Cost Projects (BMH4-CT96.1658) for financial assistance.

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