The relationship of oxygen delivery to absolute haemoglobin oxygenation and mitochondrial cytochrome oxidase redox state in the adult brain: a near-infrared spectroscopy study

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Near-infrared spectroscopy was used to determine the effect of changes in the rate of oxygen delivery to the adult rat brain on the absolute concentrations of oxyhaemoglobin, deoxyhaemoglobin and the redox state of the Cu_A centre in mitochondrial cytochrome oxidase. The cytochrome oxidase detection algorithm was determined to be robust to large changes in haemoglobin oxygenation and concentration. By assuming complete haemoglobin deoxygenation and Cu_A reduction following mechanical ventilation on 100% N₂O, the absolute concentration of oxyhaemoglobin (35 μ M), deoxyhaemoglobin (27 μ M) and the redox state of Cu_A (82% oxidized) were calculated in the normal adult brain. The mean arterial blood

pressure was decreased by exsanguination. When the pressure reached 100 mmHg, haemoglobin oxygenation started to fall, but the total haemoglobin concentration and oxidized Cu_A levels only fell when cerebral blood volume autoregulation mechanisms failed at 50 mmHg. Haemoglobin oxygenation fell linearly with decreases in the rate of oxygen delivery to the brain, but the oxidized Cu_A concentration did not start to fall until this rate was 50% of normal. The results suggest that the brain maintains more than adequate oxygen delivery to mitochondria and that near-infrared spectroscopy may be a good measure of oxygen insufficiency *in vivo*.

INTRODUCTION

Mitochondrial cytochrome oxidase is responsible for > 95% of the oxygen consumption in the body and is essential for the efficient generation of cellular ATP [1]. The enzyme contains four redox-active metal centres; one of these, the Cu_A centre, has a strong absorbance in the near-infrared [2], which enables it to be detectable *in vivo* [3] by near-infrared spectroscopy (NIRS). Unlike haemoglobin, concentration changes of the total cytochrome oxidase protein occur very slowly (over days) and are therefore not easily detectable by NIRS. However, the copper centre changes its redox state quickly; this redox change is NIRS detectable. The fact that the concentration of this centre is less than 10% of that of haemoglobin [4] means, however, that its detection is not a trivial matter [5,6].

In previous articles we have discussed the factors affecting the oxidation state of cytochrome oxidase Cu_A [7], the difficulties in measuring the cytochrome oxidase NIRS signal *in vivo* [8] and its relationship to changes in cerebral ADP and ATP concentrations [9]. In this paper we demonstrate a method for testing the efficacy of cytochrome oxidase NIRS measurements *in vivo* and quantifying the absolute Cu_A redox state and absolute oxy- and deoxy-haemoglobin concentrations ([HbO₂] and [Hb] respectively) in the adult rat brain. Many factors can affect the Cu_A redox state *in vivo* [7], but the most significant is likely to be the molecular oxygen concentration. Previous cytochrome oxidase NIRS studies have only measured relative changes in chromophore concentrations and have not measured cerebral blood flow and the rate of oxygen delivery (DO₂) directly. In this paper we directly quantify the relationship between oxygen delivery to the brain, haemoglobin oxygenation and concentration, and the

cytochrome oxidase redox state. The results demonstrate that the Cu_A redox state is not affected by changes in the DO₂ to the brain under normal conditions.

EXPERIMENTAL

Animal preparation

All studies were carried out under the relevant U.K. Home Office Guidelines. Rats (Wistar) weighing 250–300 g body weight were anaesthetized, paralysed with pancuronium bromide and mechanically ventilated on 1.0% halothane/70% N₂O/30% O₂ during surgical preparations. Femoral artery and vein catheters were inserted. A catheter in a branch of the internal maxillary vein cannulated the transverse sinus for cerebral venous blood sampling. The musculature overlying the temporal plates was blunt-dissected away from the skull bilaterally to allow placement of the NIR optodes directly on the skull, and hence remove influence from muscle chromophores (e.g. myoglobin). The optodes were fixed in position with a C-clamp on the head, with the optodes attached to the clamp and placed on the lateral aspect of the skull for transillumination of the brain. Optical coupling gel was placed between the optodes and the skull. The separation between the two optodes was 1.4 cm. The rat's head was then fixed on to a micromanipulator table with tape to secure it under a microelectrode positioner. A platinum microelectrode (tip diameter 25 μ m), inserted into the confluence of the sinuses, monitored cerebral venous H₂ clearance for measurement of cerebral blood flow using a Transdyne Chemical Microsensor. The burr hole and electrode were covered with agar gel in saline. Baseline arterial blood gases were checked and found to be in the normal range for total haemoglobin (11.6 $\pm$ 1.0 g/100 ml), pH

Abbreviations used: NIRS, near-infrared spectroscopy; HbO₂, oxyhaemoglobin; Hb, deoxyhaemoglobin; CHC, cerebral haemoglobin concentration; DO₂, rate of oxygen delivery.

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(7.20 ± 0.02), pO_2 (14.7 ± 1.1 kPa), pCO_2 (14.7 ± 1.1 kPa), bicarbonate (22.9 ± 2.5 mM), base excess (-5.0 ± 2.2 mM) and mean arterial blood pressure (145 ± 18 mmHg). Mean arterial blood pressure was then lowered step-wise by removal of blood from the femoral artery. At each new blood pressure level, NIRS, cerebral blood flow and arterial blood oxygen content measurements were made (using a co-oximeter). Cerebral blood flow was measured using the hydrogen-clearance method, as previously described [10]. The DO_2 to the brain was calculated by multiplying the cerebral blood flow by the arterial oxygen content. At the end of the study the animal was killed with 100% N_2O to obtain the final deoxygenated NIR spectrum. In one animal, 0.5 mg of cyanide was injected intravenously followed by 100% N_2O to test the response of the cytochrome oxidase NIR signal to cytochrome oxidase inhibition.

NIRS measurements

The NIRS system used was as described previously [11]. Briefly, the system uses a quartz-halogen light source, a commercial grating spectrograph and a liquid-nitrogen-cooled charge-coupled-device detector to obtain continuous NIR spectra over the wavelength range 650–1000 nm, at 1.1 nm resolution. A reference spectrum was taken and changes in absorbance over the wavelength range 780–900 nm were calculated. The algorithm used to convert absorbance changes into chromophore concentration was one designed to take into account the non-linear relationship between absorbance changes and optical pathlength in a multiply scattering system [12]. However, essentially the same results were obtained with more conventional algorithms that only correct for the wavelength-dependence of the optical pathlength at the initial control situation (e.g. that designated UCLn and described in detail in [5]). Second-differential spectroscopy of the 740 nm deoxyhaemoglobin band and the 730 nm and 830 nm water bands was used to measure the absolute [Hb] concentration, assuming a cerebral water content of 80%, as described previously [11,13].

RESULTS

There has long been a debate over whether NIRS can measure cytochrome oxidase redox-state changes in the brain in the presence of the larger changes in haemoglobin oxygenation [5,6,14–17]. We recently compared algorithms for determining cytochrome oxidase redox-state changes in the presence of haemoglobin concentration changes [5] and found that they mostly showed similar qualitative trends in the cytochrome signal, although there were significant quantitative differences. However, this in itself is not evidence that the algorithms are valid, as many were based on similar assumptions. In an attempt to 'challenge' the efficacy of the algorithms further we wished to use a protocol where blood volume and oxygenation were varied dramatically, but in which cytochrome oxidase redox state was fixed. A suitable protocol involves the addition of an intravenous dose of a high, but sub-lethal, cyanide concentration. The brain is especially sensitive to this treatment as it contains only small amounts of rhodanese, the enzyme that metabolizes cyanide. Cyanide binds to haem a_3 of cytochrome oxidase, preventing oxygen reduction by electrons leaving Cu_A and haem a . Therefore both these centres should become completely reduced. This has been shown for haem a in the bloodless, perfluorocarbon-perused rat brain [18] and for Cu_A in the normal brain [19]. However, after cyanide addition, $[HbO_2]$ should rise and $[Hb]$ fall as oxygen consumption is inhibited; vasodilation will also occur, increasing the blood volume and thus the total cerebral haemoglobin concentration (CHC). If the inspired oxygen fraction

Figure 1 Effect of cyanide and anoxia on NIR chromophore changes

At the indicated time 0.5 mg of cyanide was injected intravenously and the inspired oxygen fraction was reduced to zero. Changes in $[HbO_2]$, $[Hb]$, $[HbO_2] + [Hb]$ (CHC) and [oxidized cytochrome oxidase Cu_A] (Cyt) are indicated. Note that the Cu_A concentration change (Cyt) is shown on a $10 \times$ expanded scale.

is then reduced to zero cytochrome oxidase, Cu_A should remain reduced, whereas $[HbO_2]$ should now fall and $[Hb]$ rise. Imperfections in the deconvolution algorithm would be expected to occur when large haemoglobin concentration and oxygenation changes yield spurious cytochrome oxidase concentration changes (leading to so called 'cross-talk' between the chromophores). Any cross-talk in the algorithms will be revealed if the cytochrome signal changes following anoxia. Figure 1 shows that no such change occurs and that therefore the algorithm used is robust to these extreme changes in $[Hb]$, $[HbO_2]$ and CHC.

We can therefore use this algorithm to measure the changes in haemoglobin oxygenation, haemoglobin concentration and cytochrome oxidase Cu_A redox state when the DO_2 to the brain is reduced (and hence large changes in haemoglobin oxygenation and concentration are to be expected). This is achieved by a step-wise reduction in blood pressure. Figure 2(a) shows that there is no change in $[Hb]$ or $[HbO_2]$ until the mean arterial blood pressure falls below 100 mmHg, when $[Hb]$ starts to rise and $[HbO_2]$ starts to fall. However, despite this drop in blood pressure, there is no change in the blood volume in the brain, as measured by the CHC (Figure 2b); this is due to autoregulation mechanisms in the cerebral vasculature [20]. However, these mechanisms start to fail at about 50 mmHg, leading to an observed consequent fall in CHC. In contrast to the haemoglobin oxygenation changes, the Cu_A redox state seems insensitive to the drop in blood pressure until the failure of autoregulation. Thus no change is seen in the cytochrome oxidase NIR signal (Figure 2c) until the mean arterial blood pressure drops below 50 mmHg. Throughout the blood pressure titration there is therefore a correlation between the loss of oxidized cytochrome oxidase Cu_A and the drop in CHC (Figure 2d), but no such correlation exists between the loss of oxidized Cu_A and haemoglobin oxygenation changes.

In the brain there is a well-characterized [21] biphasic response of oxygen consumption to changes in the DO_2 . As DO_2 declines there is no change in cerebral oxygen consumption until a critical rate of O_2 delivery is reached (approx. 10 ml of O_2 /min per 100 g of brain tissue). It might be expected that the Cu_A redox state would display a similar biphasic response. This has been clearly shown in a variety of studies in the blood-free, perfluorocarbon-

Figure 2 Effect of reducing blood pressure on NIR chromophores

Mean arterial blood pressure was reduced step-wise as described in the Experimental section. Data are pooled from studies on four animals. Chromophore concentration changes are plotted versus mean arterial pressure for (a) [HbO₂] and [Hb] separately; (b) [HbO₂] + [Hb] (CHC); (c) oxidized cytochrome oxidase Cu_A (Cyt). The relationship between ΔCHC and ΔCyt is plotted in (d) along with a linear least-squares regression line, $r = 0.82$.

perfused brain in both rats [6,22] and cats [6,23,24]; in the latter case, Cu_A redox state, cerebral oxygen consumption rate and somatosensory evoked potential were all unchanged until DO₂ dropped below 5 ml of O₂/min per 100 g of tissue [24]. *A priori*, one would expect the physiological oxygen carrier (haemoglobin) to be at least as efficient as perfluorocarbon emulsions, and therefore that under normal levels of oxygen delivery in the blood-perfused brain the Cu_A redox state would be relatively insensitive to small changes in DO₂.

Consistent with these ideas, as DO₂ falls, we see a complementary and linear decrease in cerebral [HbO₂] (Figure 3). However, no changes are observed in the Cu_A signal (Figure 4) until cerebral [HbO₂] has dropped to below 10 μM (when DO₂ is below 10 ml of O₂/min per 100 g of brain tissue). Thus, unlike haemoglobin oxygenation changes, Cu_A changes are a good indicator of oxygen deficiency to the brain.

The studies described here all ended with the ventilation of the animal on 100% N₂O. Under these conditions the [HbO₂] falls rapidly to a stable trough (see for example Figure 1). Given both the low K_m for oxygen consumption of mitochondrial cytochrome oxidase and the active ventilation with zero inspired oxygen fraction, under these conditions the cerebral [HbO₂] will be essentially zero. Second-differential spectroscopy at this point [11,13] reveals the absolute [Hb]. Therefore we have a point in time where we know the absolute [Hb] and the absolute [HbO₂]. As our NIRS algorithm allows us to calculate micromolar changes in both these concentrations, a back-calculation from

this point allows us to determine the absolute concentrations of Hb, HbO₂, mean cerebral oxygen saturation and the total CHC for any time point of the study. Values ($\pm$ S.D., $n = 4$) for the normal rat brain prior to blood withdrawal were calculated to be: [Hb] = $27.3 \pm 4.3 \mu\text{M}$; [HbO₂] = $34.9 \pm 6.7 \mu\text{M}$; CHC = $62.2 \pm 8.2 \mu\text{M}$; mean cerebral oxygen saturation = $55.9 \pm 6.4\%$.

As expected, these values demonstrate the larger CHC in the adult brain compared with that observed in the neonate [25], consistent with the increased cerebral blood flow [26,27]. The mean cerebral oxygen saturation (56%) is consistent with NIRS sampling of primarily the capillary/venous circulation, rather than the arterial [28]. Despite the increased flow, this saturation is lower than that observed by NIRS in the neonatal pig brain, suggesting that oxygen consumption is higher in the adult brain. Again this is as expected, given the increased rate of cerebral oxygen consumption [29] and mitochondrial content [4,30] in the adult brain compared with the neonate.

Finally, as demonstrated in Figure 1, anoxia also yields full reduction of cytochrome oxidase Cu_A. This allows us to back-calculate to the [oxidized Cu_A] in the normal state. The cytochrome oxidase content of the adult rat brain has been measured at 5.5 μM [4]. We can use this value to measure the oxidation state of Cu_A in the adult brain. The calculated value ($\pm$ S.D., $n = 4$) was $82.0 \pm 16.6\%$ oxidized. This value is only an approximation, as it assumes that the light is sensing a representative fraction of the brain; however, the finding that the brain is mostly (82%) oxidized is consistent with the difficulty in

Figure 3 Effect of reducing DO_2 on cerebral haemoglobin oxygenation

Mean arterial pressure was reduced as in Figure 2. DO_2 was measured as described in the Experimental section and the absolute $[\text{HbO}_2]$ at each blood pressure was calculated using the method described in Table 1. Data are displayed for three rats, with data for each animal represented by a different symbol.

Figure 4 Effect of reducing DO_2 on cytochrome oxidase Cu_A redox state

Data from the experiments in Figure 3 were used to compare the fall in $[\text{HbO}_2]$ with that of oxidized Cu_A .

measuring oxidations of the Cu_A signal in the brain [31,32] and the small size of these signals when they are observed. It is also consistent with measurements in intact mitochondria [33].

DISCUSSION

The findings reported here demonstrate that the cytochrome oxidase Cu_A redox state in the adult rat brain is insensitive to changes in the DO_2 to the brain, until a critical rate is reached,

whereas haemoglobin oxygenation falls linearly with drops in DO_2 . We have previously suggested this to be the case, based on the finding that, following rapid drops in the inspired oxygen fraction, the haemoglobin oxygenation always falls prior to a drop in the cytochrome oxidase redox state [8,12], but this is the first demonstration that this is clearly due to a different response of the chromophores to DO_2 . Interestingly, the $[\text{HbO}_2]$ threshold below which cytochrome oxidase changes occur in the adult brain, about $10 \mu\text{M}$, is similar for rapid and slow changes in haemoglobin oxygenation [8].

The fact that over 50% of the normal oxyhaemoglobin in the brain becomes deoxygenated prior to significant Cu_A reduction is in agreement with previous findings in the adult rat brain [7,8,12,16]. It is also consistent with our finding [9], and that of others [34], that in the neonatal brain a fall in the Cu_A redox state, not a fall in the haemoglobin oxygenation, correlates best with a decrease in cerebral ATP. It is inconsistent with the somewhat surprising finding of some authors [35,36] that the cytochrome oxidase redox state varies with small changes in oxygen delivery to the brain, and we attribute these differences to differences in the deconvolution algorithms used [5,37], rather than differences in the experimental procedures.

The critical DO_2 , below which oxygen consumption is compromised, for Cu_A reduction is similar to that we have previously observed for oxygen consumption in the rat brain [10]. A similar close link between oxygen consumption and the Cu_A redox state has been observed in some [24], but not all [38], studies in the blood-free perfluorocarbon-perfused cat brain. Comparisons between different systems are difficult to make, given the large errors in cerebral oxygen consumption measurements. We would merely like to add that in studies with the neonatal pig (C. E. Cooper, D. T. Delpy, V. Quaresima, R. Springett, M. Ferrari and E. M. Nemoto, unpublished work), we have recently shown that the cytochrome oxidase redox state is more sensitive to changes in oxygen delivery in the partially perfluorocarbon-perfused brain; oxygen delivery is lower in these systems and therefore the Cu_A is nearer its critical DO_2 than in the normal brain.

In interpreting NIRS data of cytochrome oxidase it is important to understand the nature of the signal being observed. As we have described previously [7,8], there is now very good evidence that the spectral changes seen in the 780–900 nm NIR region of mitochondria are over 80% due to cytochrome oxidase Cu_A , with the remainder being due to cytochrome *c*, cytochrome *b*, cytochrome *a* and the various oxygenated intermediates of cytochrome oxidase [39]. Large changes in this signal, as reported in this paper, therefore have to be interpreted on the basis of changes in the Cu_A redox state. Although there are relatively few *in vitro* studies on the factors affecting the steady-state reduction level of cytochrome oxidase Cu_A in turnover [7,39], the Cu_A centre in cytochrome oxidase is close to the cytochrome *c* binding site [40,41], is the initial electron acceptor from that site [42] and senses the same electrochemical potential as cytochrome *c* [43,44]. The redox potentials for the two centres are also similar [43–45]. One would therefore expect changes in the Cu_A redox state to mirror those of cytochrome *c* under different conditions. Kinetic [33], thermodynamic [43,44] and structural [40,41] data all suggest that the Cu_A centre will respond to effectors in a similar manner to cytochrome *c*, and not haem *a* or haem *a*₃, and this needs to be taken into account when comparing the ‘*in vivo*’ data reported here with published ‘*in vitro*’ data on the effects of oxygen tension and energization on mitochondrial redox centres [46–50].

There is controversy over the relationship between mitochondrial oxygen consumption and the redox state of the mitochondrial cytochromes in mitochondria, cells and *in vivo*.

Partial reduction of cytochrome *c* in cardiac myocytes [51], neuroblastoma cells [48] and mitochondria [49,50] has been seen prior to a fall in oxygen consumption by some groups, although this has been disputed by others [46]. In agreement with the former findings, it has been suggested that there is an increased reduction of Cu_A *in vivo* in completely bloodless animals, prior to any observed change in oxygen consumption [38]. Our findings suggest that in the normal rat brain the Cu_A redox state, and hence by implication that of cytochrome *c*, is insensitive to changes in DO₂ around the normal range, i.e. mitochondrial cytochrome oxidase redox states are not affected by oxygen at normal tissue oxygen tensions. However, the partial changes in cytochrome *c* reduction described above are relatively small until low oxygen tensions are reached, and it is not clear that they would be detected in the noise of our NIRS measurement.

There have been many studies using direct reflectance spectroscopy of the brain surface to measure changes in the cytochrome oxidase haem *a* redox state using visible dual-beam spectroscopy [52–54]. These data showed in general that this signal is far more sensitive to changes in oxygen delivery to the brain than the Cu_A data reported here. Much of the discrepancy is removed when improved three-wavelength algorithms were used to deconvolute the signal changes in the three chromophores (HbO₂, Hb and cytochrome oxidase) that absorb at 605 nm [55]. However, a discrepancy still exists, in that raising the inspired oxygen fraction from 0.3 to 1.0 causes a small oxidation in haem *a*, a finding we have never observed in the NIRS Cu_A signal. There are several possible explanations for these conflicting findings. First, it is important to realise that the two methods probe different regions of the brain, namely surface (visible) and deep tissue (NIR), and differences may exist in the oxygen delivery to these regions. Secondly, and perhaps more importantly, the two chromophores, haem *a* (visible) and Cu_A (NIR) have different redox potentials and respond differently to energization changes across the mitochondrial membrane. The different redox potentials mean that haem *a* is generally more reduced in the steady state than Cu_A [32,33,39], and therefore oxidations of haem *a* will be easier to see than those of Cu_A. More importantly, given the position of Cu_A on the membrane surface and haem *a* in the middle of the membrane, the two chromophores respond differently to changes in the mitochondrial membrane potential. A drop in membrane potential oxidizes Cu_A [43] and cytochrome *c* [56] but reduces haem *a* [43,56,57]. As the oxygen concentration falls, one would expect a reduction in haem *a* and an increase in the ADP/ATP ratio. This would in turn cause a drop in the mitochondrial membrane potential, further reducing haem *a*, but oxidizing Cu_A. There are therefore good biochemical reasons why the Cu_A redox state might be less sensitive to changes in the inspired oxygen fraction than that of haem *a*.

In summary, our data demonstrate that the cytochrome oxidase NIRS signal is a more sensitive indicator of brain oxygen insufficiency than changes in haemoglobin oxygenation. This signal can therefore provide a useful marker of brain dysoxia *in vivo*, and, unlike other optical sensors of brain oxygenation/oxygen state, can be readily applied in human studies.

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