Redox behavior of cytochrome oxidase in the rat brain measured by near-infrared spectroscopy

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NEAR-INFRARED SPECTROSCOPY (NIRS), a new noninvasive technique, measures changes in the hemoglobin (Hb) oxygenation state, blood volume, and the redox state of cytochrome oxidase in tissue. This technique now finds wide clinical application. Presently, several different types of NIRS instruments are commercially available. However, the specificity and accuracy of the measurement of the redox state of cytochrome oxidase are still controversial. This is mainly attributable to the lack of valid absorption spectra for cytochrome oxidase in the near-infrared region in vivo, although many investigators have reported different spectra (3, 6, 15). Therefore, the reported absorption coefficient of copper A, which accounts for >85% of cytochrome oxidase absorption in the near-infrared spectrum (1), is ambiguous. This questions the validity of any algorithms that contain the absorption coefficient of cytochrome oxidase in simultaneous equations. Thus we have developed a simple and novel algorithm that does not contain the absorption coefficient of copper A for cytochrome oxidase measurement. The present method is an extension of our algorithm for Hb measurement (9), which has been widely employed in functional mapping studies of human brain activity (13) as well as in clinical medicine. Preliminary observations in rats (12) and adult humans (18) by this method have already been published.

In this paper, we describe the details of this method and discuss the following: 1) the redox behavior of cytochrome oxidase in the rat brain; 2) the redox state of cytochrome oxidase under normal physiological conditions; and 3) the significance of the measurement of cytochrome oxidase in living tissue.

METHODS

Theory. According to the Beer-Lambert law, the absorbance (A) of light (at a given wavelength) as it passes through a non-scattering homogeneous medium is expressed as

$$A = ECL$$  \hspace{1cm} (1)

where E is the wavelength-dependent absorption coefficient of the chromophore in the medium, C is the concentration of the chromophore, and L is the distance that light travels through the medium. The linear dependence of the changes of absorbance at a certain wavelength on concentrations of oxygenated ([HbO2]) or deoxygenated hemoglobin ([deoxy-Hb]) in tissue has been confirmed (9, 22). Thus the Beer-Lambert law can be extended to a light-scattering system like a living tissue, in which L differs markedly from the physical optical path because of the strong light scattering (4, 22).

In the near-infrared region (700–900 nm), the changes in optical density (OD) in the range shorter than 870 nm are mainly attributed to Hb. Although the small absorbance change is observed in this range in the aerobic—an aerobic spectrum of the isolated perfused rat brain, absorbance changes attributable to cytochrome oxidase are very small compared with large absorption because of Hb. In addition, wavelength-dependent absorbance changes are also very small in this range (unpublished observations). In contrast, the change in OD in the range longer than 870 nm is attributed to both Hb and oxidized cytochrome oxidase (9). Thus, when the two measuring wavelengths (λ1 and λ2) and one reference wavelength (λr) are in the range shorter than 780 nm, the OD difference between λr (the one measuring wavelength) and λn, Aλn−r – Aλr, can be written as

$$Aλn−r = Aλn = k_{n−r}[HbO2] + k_{n−r}[deoxy-Hb] + S_{n−r}$$ \hspace{1cm} (2)

where k_{n−r} and k_{r−r} are the difference absorption coefficients of HbO2 and deoxy-Hb at λn – λr, I_{n−r} is the optical path length, and S_{n−r} represents absorbance changes attributable to scattering and other chromophores at λn – λr. The optical path length varies with the scattering coefficient and the geometry of the tissue but not absorption (8, 22, 23). In our experimental conditions, however, it becomes constant and is expressed as l (see Discussion). Assuming that S_{n−r} is a constant, when the condition changes from one to the other, the change in Aλn−r is simply expressed as

$$\Delta Aλn−r = K_{n−r}[HbO2] + K_{r−r}[deoxy-Hb]$$ \hspace{1cm} (3)
where \(K_{n-r} = K_{n-r1}\) and \(K'_{n-r} = K'_{n-r1}\), which are defined as the apparent difference absorption coefficients. When \(K\) is the apparent difference absorption coefficient of either \(\text{HbO}_2\) or \(\text{deoxy-Hb}\) at an arbitrary wavelength pair \((\lambda_3, \lambda_4)\), the ratios of \(K_{n-r}\) and \(K'_{n-r}\) to \(\lambda_3\) are defined as the proportionality factors at \(\lambda_3 - \lambda_4\). The proportionality factors for \(\text{HbO}_2\) and \(\text{deoxy-Hb}\) are determined experimentally (see below). By the use of the proportionality factors, Eq. 3 can be written as

\[
\Delta A_{\lambda_{3-r}} = K(a_3 \Delta [\text{HbO}_2] + a'_3 \Delta [\text{deoxy-Hb}])
\]

where \(a_3\) and \(a'_3\) are proportionality factors \((a_3 = K_{n-r}/K, a'_3 = K'_{n-r}/K)\). The change in the absorbance at \(\lambda_3\) in the range longer than 780 nm to \(\lambda_4\) is expressed as

\[
\Delta A_{\lambda_{3-5}} = K(a_3 \Delta [\text{HbO}_2] + a'_3 \Delta [\text{deoxy-Hb}] + a''_3 \Delta [\text{cyto ox}])
\]

where \(\Delta [\text{cyto ox}]\) is the concentration change in oxidized cytochrome oxidase, and \(a''_3\) is a proportionality factor for oxidized cytochrome oxidase, which cannot be determined experimentally. The changes in \([\text{HbO}_2]\) and \([\text{deoxy-Hb}]\) are calculated from Eq. 3a as

\[
K\Delta [\text{HbO}_2] = (-a''_3 \Delta A_{\lambda_{3-5}} + a'_3 \Delta A_{\lambda_{5-730}})/(a'_3 a_2 - a'_3 a'_2)
\]

\[
K\Delta [\text{deoxy-Hb}] = (a_3 \Delta A_{\lambda_{3-5}} - a'_3 \Delta A_{\lambda_{5-730}})/(a'_3 a_2 - a'_3 a'_2)
\]

By incorporating these values into Eq. 4, we can calculate \(\Delta [\text{cyto ox}]\) by

\[
K a''_3 \Delta [\text{cyto ox}] = \Delta A_{\lambda_{3-r}} - (a_2 a'_3 - a'_2 a''_3) \Delta A_{\lambda_{5-730}}/(a'_3 a_2 - a'_3 a''_2)
\]

Because scattering effects prevent determination of the optical path length, the value of \(K\) cannot be determined. Thus the results are expressed in relative changes in absorbance units rather than in absolute concentration.

Determination of proportionality factors. Figure 1 shows optical properties of deoxygenated red blood cell suspension at three pairs of dual wavelengths in the isolated perfused rat head. A linear relationship between changes in hematocrit and absorbance difference is observed at each pair of dual wavelengths. This finding validates the assumption that the Beer-Lambert law can be extended to a living tissue. When the slope of \(\Delta A_{\lambda_{730-805}}\) in Fig. 1 is taken as 1, the relative slopes of \(\Delta A_{\lambda_{700-805}}\) and \(\Delta A_{\lambda_{780-805}}\) are 2 and 0.45, respectively. These relative slope values are proportionality factors. The linear relationship between absorbance difference and hematocrit was also obtained with any combinations of the two wavelengths in the 700- to 900-nm-wavelength range.

NIRS. We employed a four-wavelength method (3 for measuring and 1 for reference, that is, 3 pairs of dual wavelengths) to measure the three-component system ([HbO2], [deoxy-Hb], [cytochrome oxidase]). A portable apparatus was built whereby near-infrared light from a halogen lamp passed through a lens system with a rotating disc containing four interference filters (700-, 730-, 750-, and 805-nm wavelengths; 4-nm half-width) and illuminated the rat’s head 5 mm in front of an ear through use of a 4-mm-diameter light guide. Light transmitted through the cranial bone and cerebral tissue was guided through another light guide to a photomultiplier tube. The changes in light intensity at each wavelength were measured by the use of a sample-and-hold circuit and recorded after logarithmic transformation. The changes in [HbO2], [deoxy-Hb], and [cytochrome oxidase] were calculated by the following numerical formulas derived from Eqs. 5-7

\[
K\Delta [\text{HbO}_2] = -0.912 \Delta A_{\lambda_{700-750}} + 2.128 \Delta A_{\lambda_{730-750}}
\]

\[
K\Delta [\text{deoxy-Hb}] = 0.744 \Delta A_{\lambda_{700-750}} - 1.613 \Delta A_{\lambda_{730-750}}
\]

\[
K a''_3 \Delta [\text{cyto ox}] = 1.534 \Delta A_{\lambda_{700-750}} - 0.768 \Delta A_{\lambda_{730-750}} + \Delta A_{\lambda_{805-750}}
\]

In this paper, changes in \([\text{HbO}_2]\), \([\text{deoxy-Hb}]\), and [cytochrome oxidase] were expressed as a percentage of the “full-scale” value. Maximum changes caused by the aerobic (respiration with 100% O2)-to-anaerobic (respiration with 100% N2) transition were designated 100% (full scale).

Animal preparation. Male Wistar rats, weighing 180–250 g, were anesthetized by intraperitoneal injection of urethane (ethyl carbamate, 180 mg/100 g body wt). They were tracheotomized, and a femoral artery and vein were canulated to monitor arterial blood pressure (BP) and blood gases and to infuse drugs. For the electroencephalogram (EEG), skin and muscle overlying the calvarium were reflected, two bis electrocorticographs were placed symetrically on the occipital bone, and a reflectance electrode was placed on the nasal bone. Rats were paralyzed with an intravenous injection of pancronium bromide (0.02 mg/100 g body wt) and mechanically ventilated. The tidal volume and respiratory rate were adjusted to give arterial PCO2 (\(P_{\text{aCO}_2}\)) values of 37–42 Torr when animals were ventilated with air. Isolated perfused rat heads for determination of proportionality factors were prepared by the method of Inagaki and Tamura (14).

Experimental protocol. To evaluate effects of changes in oxygen delivery on cerebral oxygenation, the effects of graded hypoxia and increases in cerebral blood flow (CBF) were studied in 6 and 12 rats, respectively. Increases in CBF were induced by hypercapnia under hypoxic conditions in five rats and intravenous injection of epinephrine under hypoxic conditions in seven rats. Hypoxia was induced by
stepwise decreases of fractional inspired oxygen (FIO2) while a steady state was maintained. Hypercapnia was induced by mixing oxygen and carbon dioxide. Fractional inspired carbon dioxide (FI CO2) was increased stepwise from 0 to 16%. Epinephrine (1 µg/100 g body wt) was injected intravenously when FIO2 was 15% (mildly hypoxic conditions) and 4% (severely hypoxic conditions). Changing of inspired gas was performed after the 30-min stabilization period under respiration with 100% oxygen after surgical preparation.

RESULTS
Experiments were repeated with five to seven rats in each experiment, and results were identical within experimental error. Thus data in Figs. 1–5 are those of representative cases.

Changes in cerebral oxygenation caused by hypoxic hypoxia. Figure 2A shows changes in cerebral oxygenation, cerebral blood volume (CBV), and BP during hypoxic hypoxia. Fractional inspired oxygen (FIO2) was decreased stepwise from 100 to 0%. Changes in concentrations of oxygenated ([HbO2]), deoxygenated hemoglobin ([deoxy-Hb]), and oxidized cytochrome oxidase ([cytochrome oxidase]) are expressed as a percentage of "full-scale" value. A change in total hemoglobin concentration ([HbT]) is expressed in relative absorbance units. 1–4, EEG samples. B: changes in EEG during hypoxic hypoxia. EEG samples 1–4 were recorded at points marked on trace of relative percentage of [cytochrome oxidase] in A. LO and RO: left and right occipital regions, respectively.

Fig. 2. A: changes in cerebral oxygenation and blood pressure (BP) under hypoxic conditions in a rat. Fractional inspired oxygen (FIO2) was decreased stepwise from 100 to 0%. Changes in concentrations of oxygenated ([HbO2]), deoxygenated hemoglobin ([deoxy-Hb]), and oxidized cytochrome oxidase ([cytochrome oxidase]) are expressed as a percentage of "full-scale" value. A change in total hemoglobin concentration ([HbT]) is expressed in relative absorbance units. 1–4, EEG samples. B: changes in EEG during hypoxic hypoxia. EEG samples 1–4 were recorded at points marked on trace of relative percentage of [cytochrome oxidase] in A. LO and RO: left and right occipital regions, respectively.
stepwise decreases in FIO2 from 100 to 0%. Each EEG (samples 1–4) in Fig. 2B was measured at the point marked on the trace of the relative percentage of [cytochrome oxidase]. When FIO2 was decreased from 100 to 10%, [HbO2] decreased and [deoxy-Hb] increased reciprocally, whereas the redox state of cytochrome oxidase was not changed. Lowering FIO2 from 20 to 15% caused desynchronization on the EEG (sample 2 in Fig. 2B). When FIO2 was decreased to 10%, BP started to fall and cytochrome oxidase started to be reduced. Decreasing FIO2 further, when [cytochrome oxidase] was decreased to between 35 and 40%, resulted in the appearance of high-voltage slow waves on the EEG (sample 3 in Fig. 2B). Flattening of the EEG occurred with a delay of a few seconds after cytochrome oxidase was fully reduced under anoxic conditions (sample 4 in Fig. 2B). The behavior of absorbance change at 805 nm [isosbestic point of HbO2 and deoxy-Hb (11)] was similar to that of the change in the concentration of total hemoglobin ([HbT]), a numerical summation of [HbO2] and [deoxy-Hb]. Both changes in [HbT] and the absorbance at 805 nm are thought to reflect changes in CBV within the optical field. Under severely hypoxic conditions in which FIO2 was decreased from 6 to 0%, however, they showed different behavior: whereas [HbT] did not show any further changes, the absorbance at 805 nm decreased. This decrease in absorbance might have been due to overlap of the reduction of cytochrome oxidase. This meant that, under the condition in which cytochrome oxidase was reduced, the absorbance change at 805 nm could not be used as an indicator of changes in blood volume.

Figure 3 shows the relative oxidation state of cytochrome oxidase with respect to the relative oxygenation state of Hb obtained from six independent experiments similar to those shown in Fig. 2A. The redox state of cytochrome oxidase was unchanged and independent of changes in [HbO2], until [HbO2] decreased to ~40% in hypoxic hypoxia. In the range lower than this level of [HbO2], then, [cytochrome oxidase] was decreased almost linearly with decreases in [HbO2]. When [cytochrome oxidase] was decreased to <40%, a condition in which high-voltage slow waves appeared on the EEG, [HbO2] was decreased to between 10 and 15%.

Effects of increases in CBF on cerebral oxygenation. We examined effects of increases in CBF induced by hypercapnia under hyperoxic conditions and intravenous injection of epinephrine under hypoxic conditions. Stepwise increases in FICO2 were accompanied by decreases in FIO2 to 88% at most, at which point arterial PO2 (PaO2) and Pa CO2 were 353 and 117 Torr, respectively. Increases in FICO2 caused increases in both [HbT] and the absorbance at 805 nm (data not shown), which resulted in increases in [HbO2] to a maximum of 20% (Fig. 4). In contrast to [HbO2], no increase in [cytochrome oxidase] was observed.

When FIO2 was 15%, [HbO2] was decreased to ~65%, but [cytochrome oxidase] was not yet decreased. In these circumstances, intravenous injection of epinephrine (1 µg/100 g body wt) caused an immediate elevation of BP, and [HbO2] increased transiently, whereas further oxidation of cytochrome oxidase was not observed (Fig. 5). When FIO2 was 4%, under which condition [HbO2] decreased to ~10% and [cytochrome oxidase] decreased to ~30%, administration of the same amount of epinephrine also caused an increase in BP. The degree of an increase in BP was similar to that observed when FIO2 was 15%. Unlike the case with an FIO2 of 15%, however, an increase in [HbO2] was not observed, whereas cytochrome oxidase was markedly oxidized. [HbT] and [deoxy-Hb] increased slightly. This meant that delivery of oxygen from capillaries to mitochondria increased, which was accomplished by in-
increased flow velocity. This result also meant that the volume of the small venous vessels changed in passive response to the flow change.

**DISCUSSION**

Redox behavior of cytochrome oxidase in vivo. There are many studies on the redox behavior of cytochrome oxidase in vivo, although interpretation of the cytochrome oxidase signals shows divergence. The pioneering studies of Jöbsis et al. (16) concluded that cytochrome oxidase was partially reduced under normoxic conditions and could be oxidized by increasing oxygen availability to the brain tissue. Observations that alterations in arterial oxygen saturation (SaO2) or PaCO2 were positively related to changes both in [cytochrome oxidase] and [HbO2] in humans and animals supported this conclusion (10, 28). By contrast, in blood-free animals, in which the problem of cytochrome oxidase signals being contaminated by the more abundant Hb signals is eliminated, the reduction of cytochrome oxidase was not seen until oxygen delivery was severely impaired (7, 24). Recently, Cooper et al. (2) have also shown that, when blood is withdrawn stepwise in rats, [HbO2] falls linearly with decreases in the oxygen delivery rate to the brain, whereas the redox state of cytochrome oxidase is unchanged until the oxygen-delivery rate is reduced below about one-half of the normal physiological levels. In this study, we found that cytochrome oxidase started to be reduced when [HbO2] was decreased to ~40% in hypoxic hypoxia. The relationship between the relative oxidation state of cytochrome oxidase with respect to the relative oxygenation state of Hb (Fig. 3) was almost the same as that in the case of ischemic hypoxia observed by Cooper et al. As to other tissues, however, we observed a different relationship in cardiac tissue (17). To check the accuracy of the cytochrome oxidase measurement, we previously calibrated the oxygen dependence of the redox states of copper A and heme a3 in cytochrome oxidase of isolated mitochondria (11). The oxygen concentration at which copper A is reduced by one-half is much higher than that of Hb, by about three orders of magnitude: the oxygen concentration required for the half-maximal reduction (P50) of copper A in cytochrome oxidase is 7.5×10^{-8} M. The P50 of Hb is 4.2×10^{-5} M. Judging from this calibration, our results are acceptable.

There are several possible sources of error that interfere with the accurate measurement of the redox state of cytochrome oxidase. However, our method eliminates the most problematic source, that is, inaccuracy of the spectrum of cytochrome oxidase. In addition, classic dual-wavelength analysis provides adequate compensation for the light-scattering change of tissue itself and for instability of the photomultiplier or light source (20). One important assumption of our algorithm is that the redox change of cytochrome oxidase gives no absorbance changes in the region of 700–780 nm, as reported for the anoxic – aerobic spectrum of the blood-fluorocarbon-exchanged rat head (9). This has been questioned by the spectra obtained from Hb-free rats by Miyake et al. (21) and Wray et al. (27). Recently, we have also observed the small absorbance change in the region shorter than 780 nm in the aerobic → anaerobic spectrum of the isolated perfused rat brain. However, in the 700- to 750-nm-wavelength region, absorbance changes attributed to cytochrome oxidase were very small compared with large absorption due to Hb. In addition, wavelength-dependent absorbance changes were also very small in this region, even in the difference spectrum reported by others. Because of dual-wavelength analysis, such a small contribution may not cause significant errors. Thus our assumption is valid as long as the region of 700–750 nm is employed for measuring the Hb oxygenation state.

It is well known that scattering intensity is dependent on wavelength. Because a maximal difference between λn and λs used here is 55 nm, however, a difference in scattering intensity between two wavelengths is small. The principle of the dual-wavelength method, thus, also enables us to assume that the optical path length is constant. Under hypoxic conditions,
several changes occur in cells, such as the collapse of membrane potentials, which can produce changes in light-scattering characteristics. We have observed that marked changes in scattering intensity suddenly occurred when cytochrome oxidase was almost fully reduced under hypoxic conditions in the perfused rat head (unpublished observations). This has been also confirmed by direct measurement of the reduced-scattering coefficient of the piglet brain (29). These data indicate that scattering changes are small and can be eliminated by dual-wavelength analysis, except those under severely hypoxic conditions.

It must be noted here that the coefficients in Eq. 8, which were determined experimentally in living tissue of the rat head, contain instrumentation factors such as the half-width of the optical filters used. When a new instrument is assembled, we therefore have to reestimate these factors. This procedure is inconvenient. However, we have recently found that we can obtain identical proportionality factors in conditions in vitro by the use of red blood cell suspension (unpublished observations). Thus the in vitro data can be used in Eq. 8 when instrumentation factors change.

Redox state of cytochrome oxidase in normal physiological conditions. A general conclusion that has emerged from recent studies (2, 7, 24, 26) is that oxygen-dependent redox changes of cytochrome oxidase seem to occur only when oxygen delivery is extremely compromised. Our present data support this conclusion. However, the issue of whether or not cytochrome oxidase is partially reduced in normoxia remains to be solved. Edwards et al. (5), who reported that there was no relationship between cerebral [cytochrome oxidase] and SaO2 within the range of 85–99%, whereas increases in Paco2 from 4.3 to 9.6 kPa were accompanied by those in both [cytochrome oxidase] and [HbO2] in newborn preterm human infants, have argued that cytochrome oxidase might be partially reduced. In their study, the oxidation of cytochrome oxidase after increasing Paco2 was explained by the assumption that the copper A varied with the mitochondrial energy level in addition to the hemodynamic characteristics of the human neonatal brain. In this study, however, neither increases in Paco2 under hypoxic conditions (Fig. 4) nor intravenous injection of epinephrine under mildly hypoxic conditions (Fig. 5) caused further oxidation of cytochrome oxidase, whereas [HbO2] was increased. This discrepancy in the effect of hemodynamic changes on the redox state of cytochrome oxidase might be due to the difference in species and/or the difference between the adult and the newborn brain. However, our previous in vitro mitochondrial studies demonstrated that oxygen dependence of the redox state of copper A is independent of the mitochondrial energy state and respiratory rate (11). Thus, as for in vitro mitochondria at least, it is unlikely that the partial reduction of copper A in cytochrome oxidase occurs in normoxia by the mechanism Edwards et al. proposed (i.e., energy-dependent redox change of copper A).

Significance of measurement of redox state of cytochrome oxidase. Recently, several investigators proposed that [HbO2] may be the best indicator of impending brain hypoxia, whereas the reduction of cytochrome oxidase may be a prognosticator of irreversible brain damage because it occurs only under extreme hypoxia (2, 7). At the moment, however, NIRS does not provide quantitative information, although great efforts have been made toward quantitation. Thus the degree of cerebral hypoxia cannot be judged by near-infrared measurement of Hb oxygenation alone. When Hb oxygenation decreases in sick patients requiring intensive care, for example, we cannot decide whether we should treat them immediately without other monitoring systems. By contrast, the reduction of cytochrome oxidase preceded the appearance of high-voltage slow waves on the EEG (Fig. 2B). In hypoxic hypoxia, the appearance of these waves on the EEG indicates a decline in cerebral function. In addition, studies on the correlation of the cytochrome oxidase signal with the brain-energy states of piglets (26) and dogs (25) have shown that the reduction of cytochrome oxidase is highly correlated with a decreased brain-energy state. These data suggest that the brain works as long as cytochrome oxidase is maintained in the fully oxidized state. It therefore appears that the start of cytochrome oxidase reduction can be used as an alarm, notifying that the brain condition is metabolically and functionally critical, even though absolute values are lacking.

Before the advent of NIRS, we evaluated tissue oxygenation from several indirect variables such as PaO2. These methods, however, sometimes do not reflect tissue oxygenation correctly. As is seen in Fig. 5, intravenous injection of epinephrine was effective for increasing oxygen delivery to the brain tissue under severely hypoxic conditions. This could be confirmed by measuring the redox state of cytochrome oxidase but not by Hb. Thus direct measurement of the tissue oxygen concentration is essential for evaluating tissue oxygen sufficiency. Among the various clinical monitoring systems, only near-infrared measurement of cytochrome oxidase can meet this demand. We are now investigating clinical applications of our method to examine the value of NIRS.

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