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Short Communication

Nitric Oxide Does Not Inhibit Cerebral Cytochrome Oxidase *In Vivo* or in the Reactive Hyperemic Phase After Brief Anoxia in the Adult Rat

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Summary: In this study, near-infrared spectroscopy was applied to examine whether cytochrome oxidase in the rat brain is inhibited by nitric oxide *in vivo*. During normoxia, intravenous N^G-nitro-L-arginine methyl ester (L-NAME) administration significantly decreased the cerebral saturation of hemoglobin with oxygen but did not alter the cytochrome oxidase redox state. Anoxia significantly reduced the cytochrome oxidase. The time course of the recovery of the redox state during reoxygenation was not altered by L-NAME. The results suggest that in adult rats, cytochrome oxidase is not inhibited by nitric oxide, either in physiologic conditions or during reoxygenation after a brief anoxic period. **Key Words:** Nitric oxide—Near-infrared spectroscopy—L-NAME—Cytochrome oxidase—Brain—Rat.

Nitric oxide (NO) is a competitive inhibitor of cytochrome oxidase, the terminal enzyme of the electron transport chain. In cultured synaptosomes, oxygen consumption can be inhibited by NO at physiologic concentrations, particularly at low oxygen tensions (Brown and Cooper, 1994). For this reason, it has been proposed that NO could be a physiologic modulator of the oxygen affinity of cytochrome oxidase *in vivo* (Clementi et al., 1999). This has been supported by the apparent decrease in the oxygen affinity of cytochrome oxidase *in vivo* compared with mitochondrial suspensions (Hempel et al., 1977; Kreisman et al., 1981) and the increase in whole body oxygen consumption during systemic inhibition of NO synthase (NOS) (Shen et al., 1994).

Measurements of CBF and $CMRO_2$ during NO synthase inhibition in the rat (Horvath et al., 1994) and piglet (Greenberg et al., 1994) have shown decreases in CBF but no change in oxygen consumption at normocapnia. A more sensitive technique would be to measure the redox centers of cytochrome oxidase or the redox state of cytochrome *c*, which have been shown to become more reduced during inhibition in mitochondrial suspensions (Cassina and Radi, 1996) and isolated cardiomyocytes (Stumpe et al., 2001).

Using full spectral near-infrared spectroscopy, we observed in preliminary experiments that in the brain of adult rats, the copper A center (Cu_A) of the cytochrome oxidase is reduced during the reactive hyperemia after a brief period of anoxia (unpublished results). Previously it was shown that during reperfusion after ischemic hypoxia, NO tension increases to levels shown to inhibit oxygen consumption *in vitro* and that this release can be inhibited by N^G-nitro-L-arginine methyl ester (L-NAME) (Malinski et al., 1993).

The aim of this study is to determine *in vivo* whether NO inhibits cytochrome oxidase in the brain under normal physiologic conditions and during the reactive hyperemia after a brief period of anoxia.

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MATERIALS AND METHODS

Animal housing and treatment conditions complied with European Union directive 86/609 on animal welfare.

Twelve male Sprague-Dawley rats (360 to 460 g) were anesthetized with isoflurane (1.5%), intubated, and ventilated with a 30:70 oxygen-to-nitrogen gas mixture. The left femoral artery was cannulated for arterial blood sampling and monitoring of the MABP and heart rate, and the left femoral vein for injection of L-NAME solution (Sigma, Poole, Dorset, U.K.). The rats were fixed in a stereotaxic apparatus, and the parietal and temporal bones were exposed for application of the optodes as described previously (van Rossem et al., 1999*a*). After surgery, the animals were allowed to stabilize for at least 1 hour and then the rats were subjected to a 70-second period of anoxia. After 40 minutes of recovery, a second anoxic period of 70 seconds was performed followed by a 10-minute period of recovery. Then, a terminal anoxia was applied. Six rats received a 30-mg/kg bolus (intravenous) of L-NAME 30 minutes before the second anoxia. Six control animals obtained an equal quantity of saline. Blood samples for blood gas analysis (ABL505, Radiometer, Copenhagen, Denmark) were collected 1 minute before each period of anoxia.

The near-infrared spectroscopy system and algorithms have been described previously (Springett et al., 2000). Near-infrared spectra were collected contiguously with a period of 50 milliseconds and 20 spectra were averaged to give a time resolution of 1 second. Absolute changes in the concentration of oxyhemoglobin, deoxyhemoglobin, and oxidized Cu_A ($[Cu_A]$) were calculated from the change in attenuation between 780 and 900 nm (Cope et al., 1989; Matcher et al., 1995) and normalized to the baseline optical pathlength measured from the 840-nm water feature using second differential analysis (Matcher and Cooper, 1994). Assuming an 85% water content, the absolute concentration of hemoglobin was obtained from second differential analysis and normalized to the pathlength obtained from the 740-nm water feature. The absolute deoxyhemoglobin and absolute changes in oxyhemoglobin and $[Cu_A]$ were monitored online. Absolute oxyhemoglobin was back-calculated taking the value after terminal anoxia as zero. Total hemoglobin ([HbT]) was calculated as the sum of oxyhemoglobin and deoxyhemoglobin, and mean cerebral saturation of hemoglobin with oxygen ($Smco_2$) by the following formula: (oxyhemoglobin/[HbT]) × 100.

TABLE 1. *Physiologic variables before the first (pretreatment) and second (posttreatment) anoxia*

	Control (n = 6)		L-NAME (n = 6)	
	Pretreatment	Posttreatment	Pretreatment	Posttreatment
Weight (g)	424 ± 23		399 ± 25	
MABP (mm Hg)	93 ± 9	100 ± 15	83 ± 8	115 ± 5*
Arterial pH	7.43 ± 0.03	7.43 ± 0.02	7.42 ± 0.01	7.41 ± 0.02
$Paco_2$ (mm Hg)	44.5 ± 3.9	43.2 ± 3.1	44.8 ± 1.1	44.8 ± 3.1
Pao_2 (mm Hg)	118.3 ± 10.3	119.3 ± 13.6	109.9 ± 17.3	110.5 ± 13.4

Data are represented as mean ± SD. Blood pressure and blood gases were measured 1 minute before onset of anoxia.* Significantly different ($P < 0.05$) from pretreatment in the same group.

Differences between groups were evaluated using the Wilcoxon-Mann-Whitney midrank sum test. Differences within groups were evaluated using the Wilcoxon matched-pairs signed-ranks test.

RESULTS

Table 1 shows the physiologic data obtained before the pretreatment and posttreatment anoxic periods. No significant differences were found between groups at the onset of the experiment. Pretreatment near-infrared spectroscopy parameters were also comparable between groups (Table 2) except for [HbT], which was on average 10 μmol/L lower in the L-NAME group ($P = 0.041$).

During anoxia and reoxygenation, the changes in hemoglobin parameters and $[Cu_A]$ showed a similar pattern in all animals. Figure 1 shows the mean time course of these changes for the control and L-NAME–treated group, both before and after treatment. No significant differences were noticed between the control and L-NAME group. Immediately after onset of anoxia, oxyhemoglobin dropped to zero, whereas deoxyhemoglobin increased. [HbT] gradually increased during anoxia. After reoxygenation, oxyhemoglobin rapidly increased to levels more than 40 μmol/L greater than baseline level,

TABLE 2. *Near-infrared spectroscopy parameters 1 minute before and 3 and 10 minutes after onset of a 70-second period of anoxia*

	T = −1 minute		T = 3 minutes		T = 10 minutes	
	Pretreatment	Posttreatment	Pretreatment	Posttreatment	Pretreatment	Posttreatment
[Hb] (μmo/L)						
Control	27.8 ± 2.7	25.6 ± 2.6	19.1 ± 2.6	19.0 ± 2.7	28.9 ± 2.2	28.7 ± 4.3
L-NAME	25.0 ± 2.8	27.7 ± 3.2	15.5 ± 2.1†	21.9 ± 2.6*	26.1 ± 2.0†	33.0 ± 3.9*
$[HbO_2]$ (μmol/L)						
Control	82.5 ± 7.1	79.0 ± 7.5	115.6 ± 9.3	113.6 ± 8.5	75.2 ± 8.6	72.1 ± 11.3
l-NAME	75.3 ± 3.6	64.4 ± 4.6*	109.2 ± 6.8	106.4 ± 4.3	69.2 ± 2.9	65.4 ± 6.3
[HbT] (μmol/L)						
Control	110.3 ± 9.5	104.6 ± 8.7	134.7 ± 10.9	132.6 ± 9.3	104.1 ± 9.6	100.8 ± 10.4
l-NAME	99.6 ± 3.2†	92.1 ± 6.6	124.7 ± 6.6	128.2 ± 5.6	95.2 ± 3.5	98.4 ± 9.1
$Smco_2$ (%)						
Control	74.8 ± 1.0	75.4 ± 2.1	85.8 ± 1.3	85.7 ± 1.8	72.1 ± 2.5	71.3 ± 5.1
l-NAME	75.6 ± 2.3	69.9 ± 2.3*	87.5 ± 1.8	83.0 ± 1.6*	72.6 ± 1.8	66.5 ± 2.2*

Data are represented as means ± SD. [Hb], deoxyhemoglobin concentration; $[HbO_2]$, oxyhemoglobin concentration; [HbT], total hemoglobin concentration; $Smco_2$, the derived saturation of hemoglobin with oxygen; T, time relative to the onset of anoxia.

* Significantly different ($P < 0.05$) from pretreatment in the same group.

† Significantly different from pretreatment control group.

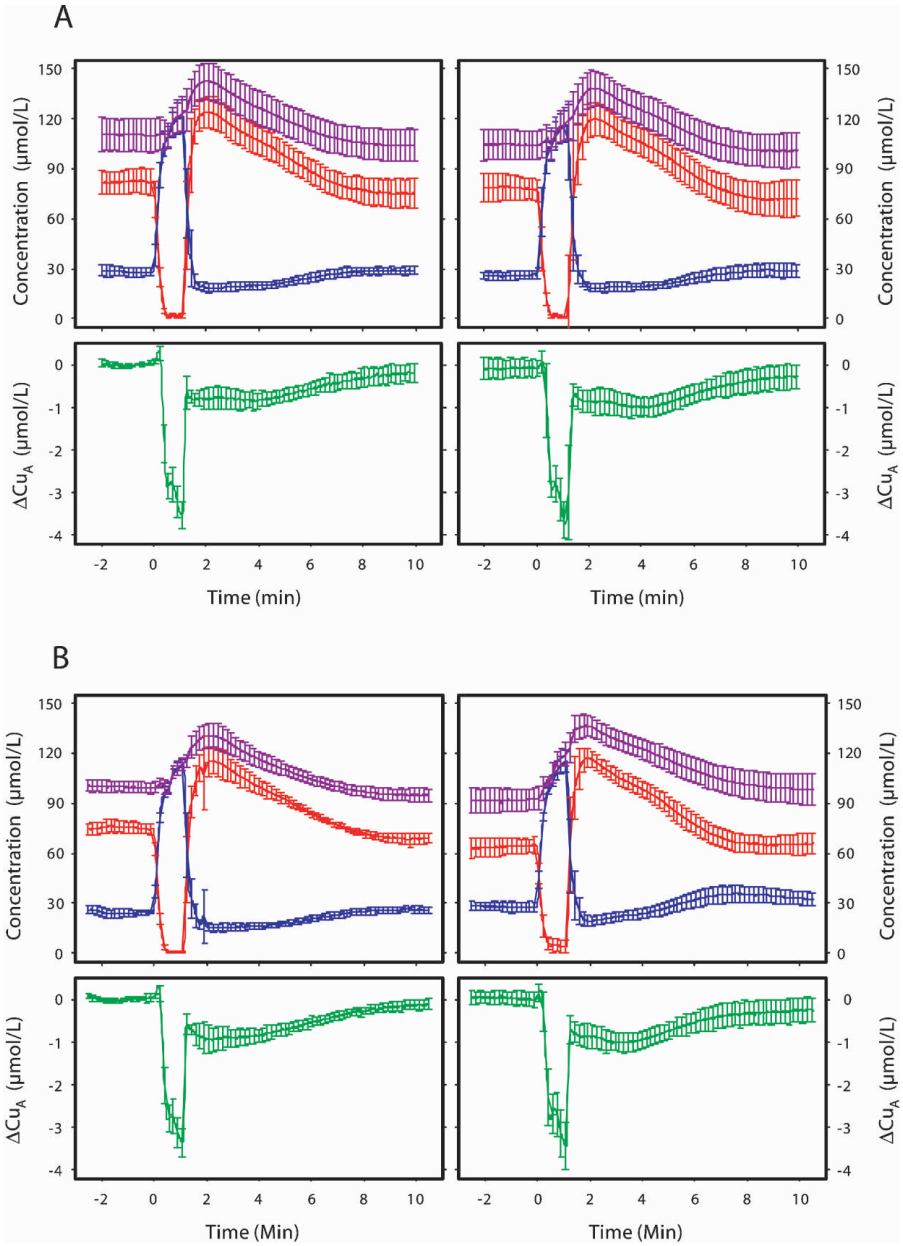

FIG. 1. Mean time course (mean ± SD) of near-infrared spectroscopy variables during and after a 70-second period of anoxia for the control **(A)** and N^G-nitro-L-arginine methyl ester (L-NAME) **(B)**–treated group. The left panels are before and the right are after administration of saline and L-NAME, respectively. In **(A)** and **(B),** upper panels show oxyhemoglobin ([HbO_2]), deoxyhemoglobin ([Hb]), and total hemoglobin ([HbT]) concentrations in red, blue, and purple respectively. Lower panels show changes in cytochrome oxidase redox state ($\Delta[Cu_A]$) in time. Time zero indicates the onset of anoxia.

whereas deoxyhemoglobin decreased to approximately 10 µmol/L less than baseline, resulting in a further increase in [HbT]. All hemoglobin parameters gradually recovered. [Cu_A] significantly changed during anoxia and reoxygenation. After the onset of the anoxia, [Cu_A] rapidly decreased to approximately 3 µmol/L below baseline level. Immediately after the onset of reoxygenation, [Cu_A] increased to approximately 1 µmol/L less than baseline level. The complete recovery of [Cu_A] took approximately as long as the recovery of the hemoglobin parameters.

In the control group, pretreatment and posttreatment values of arterial blood pressure, blood gases, [Cu_A] and brain hemoglobin parameters were comparable. Administration of L-NAME induced a significant increase in MABP ($P = 0.028$) and a significant decrease in oxyhemoglobin ($P = 0.031$) and $Smco_2$ ($P = 0.031$) (Tables 1 and 2). No changes in [Cu_A] were noticed. L-NAME did not alter the changes in near-infrared spectroscopy parameters during anoxia but induced a small but significant increase in deoxyhemoglobin during the hyperemic phase after reoxygenation (Table 2). The decrease in $Smco_2$ induced by L-NAME before anoxia was maintained during the hyperemic phase.

Table 3 shows the changes in [Cu_A] during and after anoxia. No significant differences within or between groups were observed. The decline in [Cu_A] during anoxia, plotted as a function of the $Smco_2$, was comparable before and after treatment with either saline (control) or L-NAME (Fig. 2). Because interference with other

TABLE 3. *Changes in concentration of oxidized Cu_A (μmol/L) before, during, and after a 70-second period of anoxia*

	Pretreatment	Posttreatment
T = −1 min		
Control	−0.02 ± 0.06	−0.07 ± 0.20
l-NAME	0.02 ± 0.03	0.04 ± 0.18
T = 3 min		
Control	−0.78 ± 0.22	−0.89 ± 0.30
l-NAME	−0.88 ± 0.23	−1.02 ± 0.23
T = 10 min		
Control	−0.17 ± 0.23	−0.27 ± 0.29
l-NAME	−0.15 ± 0.13	−0.27 ± 0.27
Max. change during anoxia		
Control	−3.60 ± 0.35	−3.80 ± 0.41
l-NAME	−3.40 ± 0.33	−3.45 ± 0.58

Data are represented as mean ± SD. Changes are relative to baseline level at onset of the experiment. Negative values express the reduction of Cu_A. T, time relative to the onset of anoxia. No significant differences could be observed between groups and between pre- and posttreatment.

chromophores might influence the [Cu_A] signal (Cooper et al., 1994), a decrease was regarded as significant when it exceeded 0.5 μmol/L. The critical saturation level at which the decline became significant was comparable between the first and second anoxic period in both the control group (9.69 ± 2.05% and 11.61 ± 2.11%, respectively) and the L-NAME group (10.80 ± 1.92% and 14.09 ± 7.88%, respectively). A marked increase in the critical saturation was noticed in only one animal of the L-NAME group. The statistical analysis showed no significant differences between groups.

DISCUSSION

L-NAME is a nonspecific inhibitor of NOS (Hobbs and Gibson, 1990; Rees et al., 1990). Intravenous infusion at a dose of 30 mg/kg produced a significant increase in MAPB consistent with previous reports (Buchanan and Phillis, 1993; Prado et al., 1993). In the brain, L-NAME causes vasoconstriction, increased vascular resistance, and decreased CBF (for review see Iadecola et al., 1994). In the present study, a significant decrease in oxyhemoglobin and $Smco_2$ was observed after infusion of L-NAME. This is consistent with a decrease in CBF as a result of the inhibition of cerebral NOS. Decreases in $Smco_2$ of this magnitude do not alter the Cu_A redox state (van Rossem et al., 1999*b*). Hence, any significant direct effect of L-NAME on the Cu_A redox status should be noticed as such. The absence of a change in the redox state of Cu_A after injection of L-NAME indicates that, in physiologic conditions, NO does not inhibit cytochrome oxidase.

In preliminary experiments, we observed a partial reduction of Cu_A during the hyperemic phase succeeding a brief period of anoxia (unpublished results). We postulated that during reoxygenation, NO is synthesized by constitutive NOS and contributes to the vasodilation and partial inhibition of cytochrome oxidase. Previously, it was shown that NO is synthesized during the reperfusion after middle cerebral artery occlusion (Malinski et al., 1993; Sato et al., 1994) and after 60 minutes of hypoxia (Fujisawa et al., 1999). In addition, *in vitro* experiments have shown that in isolated nerve terminals and mitochondria, NO clearly inhibits the respiration by competing with oxygen at the oxygen-binding site of cytochrome oxidase (Brown and Cooper, 1994; Brudvig et al., 1980; Cleeter et al., 1994). The present study shows that a reduction in oxidized Cu_A occurs reproducibly during and after a 70-second period of anoxia and that brain hemoglobin oxygenation and Cu_A redox state completely recover within 15 minutes after reoxygenation. Pretreatment with L-NAME did not alter the time course of changes in the Cu_A redox state during and after anoxia and did not reduce the partial inhibition of Cu_A after reoxygenation. Neither the time course nor magnitude of changes in hemoglobin concentration and

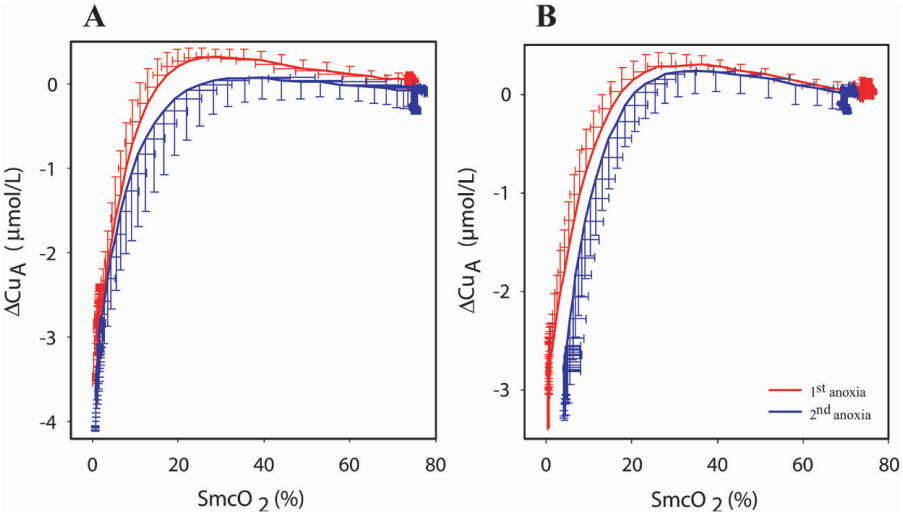

FIG. 2. Changes in cytochrome oxidase redox state (Δ[Cu_A]) observed during anoxia and expressed as a function of cerebral hemoglobin saturation ($Smco_2$) for the control **(A)** and N^G-nitro-L-arginine methyl ester (L-NAME) **(B)**–treated group. The red and blue curves were obtained, respectively, before and after administration of saline (A) and L-NAME (B). Bars indicate mean ± SD.

oxygenation were affected. This indicates that in adult rats, NO does not play a major role in the vascular responses during anoxia and reoxygenation and that NO is not significantly involved in the inhibition of cytochrome oxidase in these conditions. In contrast to middle cerebral artery occlusion (Malinski et al., 1993; Sato et al., 1994) and 60 minutes of hypoxia (Fujisawa et al., 1999), NO may not be increased after a brief period of anoxia, or the changes in concentration of NO in these conditions may not be sufficient to induce the same inhibitory effects as in *in vitro* conditions.

From our data, no conclusions can be drawn regarding the question of whether NO derived from inducible NOS inhibits respiration *in vivo*. The model we apply should be inducible NOS free because upregulation of this enzyme can only be found several hours after an insult (Chatzipanteli et al., 1999; Ikeno et al., 2000). Therefore, although NO may inhibit cytochrome oxidase *in vivo* after prolonged insults such as hypoxia–ischemia, the data presented in this article would suggest that NO derived from constitutive NOS activation does not inhibit cytochrome oxidase either under normal physiologic conditions or during the reactive hyperemia after brief anoxia. Other factors that may affect the redox state of the cytochrome oxidase during reoxygenation include mitochondrial Ca^{2+} overload, O_2 radicals, ionic shifts, acidosis, or simply an increased oxygen demand that is not matched by the oxygen delivery. Increased oxidative metabolism is likely to occur after a period of anoxia, because ionic shifts over the cell membrane have to be restored (for review, see Siesjo, 1981).

We conclude that, in adult rats, inhibition of NOS by L-NAME does not alter the vascular responses, or the partial reduction of cytochrome oxidase during and after a 70-second period of anoxia. This indicates that NO is not a major determinant of the observed alterations in these conditions.

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