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Nanomolar concentrations of nitric oxide reversibly inhibit synaptosomal respiration by competing with oxygen at cytochrome oxidase

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Abstract Nitric oxide (NO) reversibly inhibited oxygen consumption of brain synaptosomes. Inhibition was reversible, occurred at the level of cytochrome oxidase, and was apparently competitive with oxygen, with half-inhibition by 270 nM NO at oxygen concentrations around 145 μM and by 60 nM NO at around 30 μM O_2. Isolated cytochrome oxidase was inhibited by similar levels of NO. These levels of NO are within the measured physiological and pathological range for a number of tissues and conditions, suggesting that NO inhibition of cytochrome oxidase and the competion with oxygen may occur in vivo.

Key words: Nitric oxide; Cytochrome oxidase; Synaptosome; Oxygen

1. Introduction

Nitric oxide (NO) is a free-radical gas which acts as: (a) an intercellular messenger; and (b) a cytotoxic agent in some conditions (reviewed in [1–3]). NO produced by the endothelium is involved in the normal regulation of blood flow in many tissues. NO produced post-synaptically by some neurons may be involved in changing synaptic efficiency during memory formation in the brain. During infection/injury NO produced by a range of cells, including macrophages, may have protective and/or toxic roles. NO is toxic to many cell types at various levels. The mechanism of NO toxicity is unclear, but is thought to include damage to: mitochondrial complexes I and II, mitochondrial aconitase, glyceraldehyde-3-phosphate dehydrogenase, ribonucleotide reductase, and DNA [4]. NO levels may increase dramatically during ischaemia and/or reperfusion in brain [5], and inhibitors of nitric oxide synthase may protect against subsequent damage [2]. The neurotoxicity of glutamate has been suggested to be mediated partly by NO [1,2].

Because the cytotoxic effects of NO have been suggested to be exerted partly on mitochondrial respiration, we tested the effects of NO on the respiration of isolated nerve terminals (synaptosomes) from rat brain.

2. Materials and methods

Synaptosomes were isolated from whole brain of 3-week-old rats as in [6]. Isolated synaptosomes were incubated at 1 mg protein/ml in 125 mM NaCl, 15 mM HEPES, 10 mM glucose, 6 mM KCl, 5 mM Na-pyruvate, 1.2 mM $MgSO_4$, 1 mM $NaHPO_4$, 1 mM $CaCl_2$, 250 nM FCCP (carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone, a mitochondrial uncoupler), at pH 7.2. Incubations were in a stirred, sealed vessel thermostated at 25°C, with an oxygen electrode (Rank Brothers) and Clark-type nitric oxide-selective electrode (World Precision Instruments; based on Shibuki, 1990) inserted through a gas-tight port at the top of the vessel. The NO electrode was calibrated by two independent methods. In the first method an aliquot of NO-saturated water of known NO concentration (see below) was injected into the stirred vessel when the vessel was anaerobic, to give a known NO concentration. In the second method a known amount of potassium nitrite was injected into a stirred, anaerobic vessel containing KI, H_2SO_4, and K_2SO_4 to release stoichiometric NO (as in [8]). The two different calibrations gave the same NO-electrode response/sensitivity to within 10%. Oxygen had no detectable effect on the NO electrode, and NO had no detectable effect on the oxygen electrode. The oxygen consumption rates were corrected for a low rate of oxygen diffusion into the vessel, by adding cyanide or rotenone at the end of the experiment and following the rate of oxygen diffusion into the vessel.

In experiments with sodium nitroprusside (Fluka) NO release was caused by the addition of light. Light was supplied by a 150 W domestic light bulb at 10 cm from the incubation vessel, the light passing through two holes in a box so that the light only fell on the incubation chamber. Daylight caused significant release of NO from nitroprusside, so all extraneous light was excluded. Nitroprusside was made up fresh on the day and protected from light. In experiments with NO gas, oxygen was removed from a test tube of distilled water by bubbling with oxygen-free nitrogen gas. This was then bubbled with pure NO gas (BDH/Merck) to saturate with NO, and then sealed with a rubber seal. The concentration of NO in NO-saturated water was taken as 2.0 mM at 20°C [9].

Cytochrome oxidase was isolated by the method of Kuboyama et al. [10] with Tween-80 substituting for Emasol and incubated at 100 nM in 100 mM potassium phosphate (pH 7.0), 5 mM sodium ascorbate, 0.42 mM TMPD (*N,N,N',N'*-tetramethyl-*p*-phenylenediamin e dihydrochloride) 0.1% *n*-dodecyl β-D-maltoside, and 10 μM cytochrome *c*, at 25°C.

3. Results

We tested the effect of NO on synaptosomal respiration by measuring oxygen consumption and NO levels simultaneously, and adding either NO gas or nitroprusside. NO is released from nitroprusside when photolysed by light [11]. Fig. 1 shows that exposure to light in the presence of nitroprusside caused continuously increasing levels of NO, and increasing inhibition of the respiration rate. Turning the light off caused the NO level to decay, and the respiration rate was reactivated with the same time course as the decay of the NO level. Subsequent addition of light caused more NO release and inhibition of respiration, but (a) more NO was released for the equivalent exposure to light when the concentration of oxygen was lower, and (b) equivalent levels of NO caused higher levels of inhibition when the concentration of oxygen was lower. Half-inhibition of respiration rate occurred at about 250 nM NO at 130 μM

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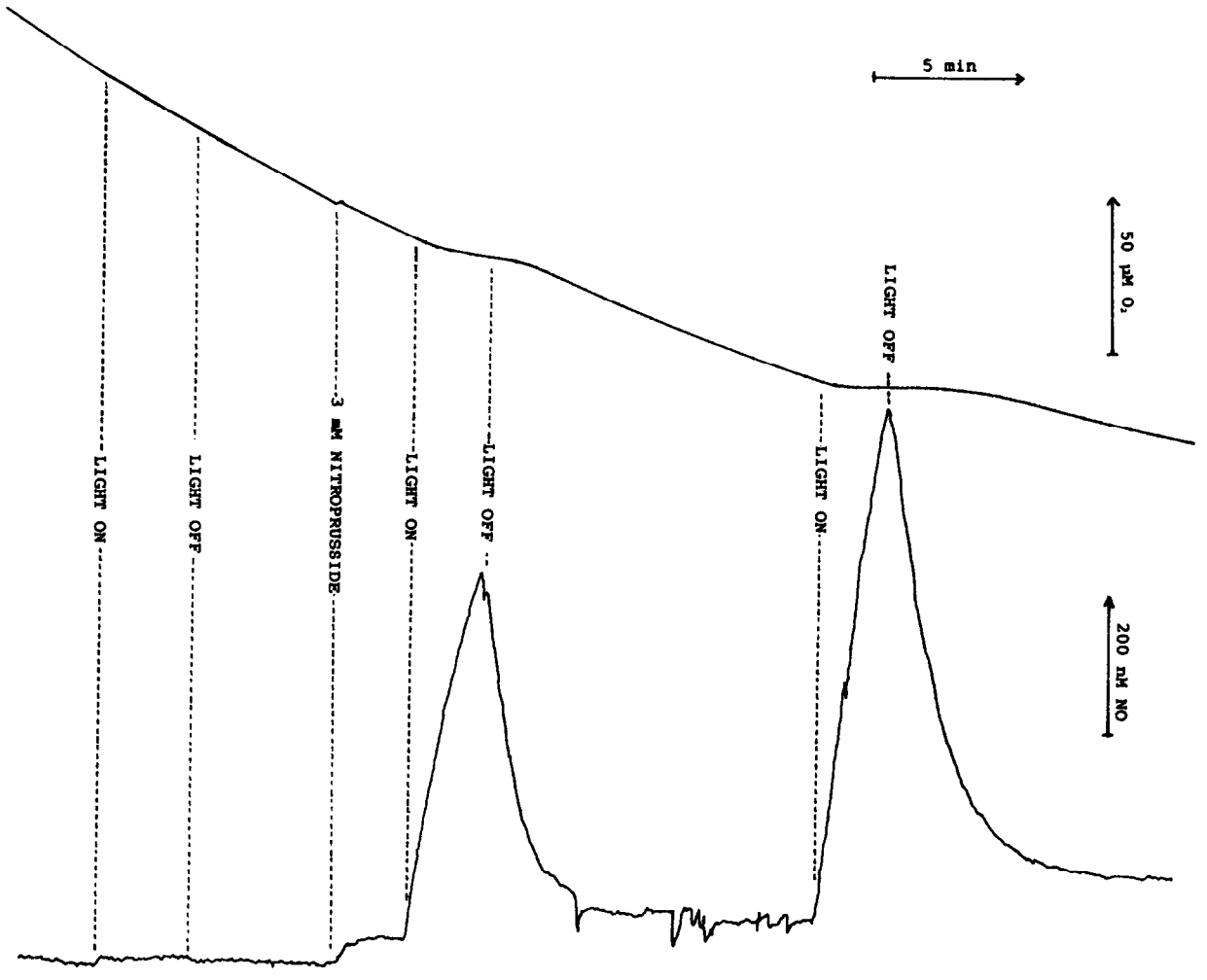

Fig. 1. Light activated NO release from nitroprusside causes reversible inhibition of synaptosomal respiration. Synaptosomes were incubated as indicated in section 2 with an oxygen electrode (upper trace) and NO electrode (lower trace).

O_2. The correlation between the levels of nitric oxide and inhibition during and after light suggests that the inhibitory agent is nitric oxide itself, and that the inhibition is rapid and reversible. The inhibition of respiration in the light was completely prevented by the presence of 15 μM haemoglobin (Sigma; undefined redox state). Since haemoglobin avidly binds NO and oxyhemoglobin inactivates NO [9], this finding again suggests that the inhibitory agent is NO. In order to determine where within the synaptosomal metabolism the NO was inhibiting respiration, we looked at the effect of NO release on the optical spectrum of the synaptosomes. The spectrum was recorded (in an Aminco DW-2000 scanning spectrophotometer) prior to and immediately after exposure to the light source in the presence of nitroprusside, and the difference spectrum is reproduced in Fig. 2. Exposure to light causes a clear reduction of cytochrome *a* (difference peak at 605 nm) and cytochrome *c* (difference peak at 550 nm). Since the synaptosomes were fully uncoupled the reduction can only be caused by an inhibition at cytochrome oxidase on the oxygen side of the a cytochromes.

We also used water saturated with NO (sometimes referred to as 'authentic' NO), prepared by bubbling pure NO gas through distilled water previously purged with oxygen-free nitrogen. Addition of this NO to the synaptosomal incubation caused immediate inhibition of respiration, which reversed as the NO was degraded (not shown). The dependence of respiration rate on NO level during the decay is plotted in Fig. 3, and was used to determine the K_i for NO. This was repeated at a lower oxygen concentration range by bubbling the incubation medium with oxygen-free nitrogen prior to addition of the synaptosomes. Fig. 3 shows that at an approximately 5-fold lower concentration of oxygen, an approximately 5-fold lower concentration of NO is required to half-inhibit respiration. The K_i was estimated to be 270 nM NO when the oxygen concentration was between 125 and 165 μM O_2, and 60 nM NO when the oxygen concentration was 18–38 μM O_2. This indicates that NO competes with oxygen, and suggests that the sight of inhibition is at the oxygen binding sight of cytochrome oxidase. Measurement of the optical spectrum of the synaptosomal incubation before and immediately after addition of NO gave a difference spectrum similar to that for nitroprusside and light, confirming that NO inhibits respiration at cytochrome oxidase on the oxygen side of cytochrome *a*.

The effect of NO on isolated cytochrome oxidase (purified from beef heart) was also measured (see Fig. 4). The addition of NO caused rapid and reversible inhibition of cytochrome oxidase at concentrations of NO similar to those causing inhi-

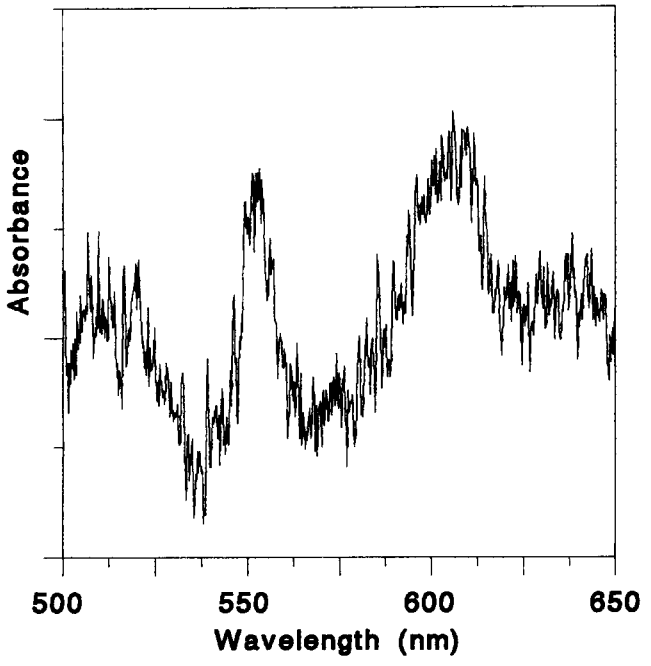

Fig. 2. Light-activated NO release from nitroprusside causes reduction of cytochromes *a* and *c* in synaptosomes. The spectra of incubated synaptosomes was recorded before and immediately after light in the presence of nitroprusside, and the difference spectrum is given above (after–before).

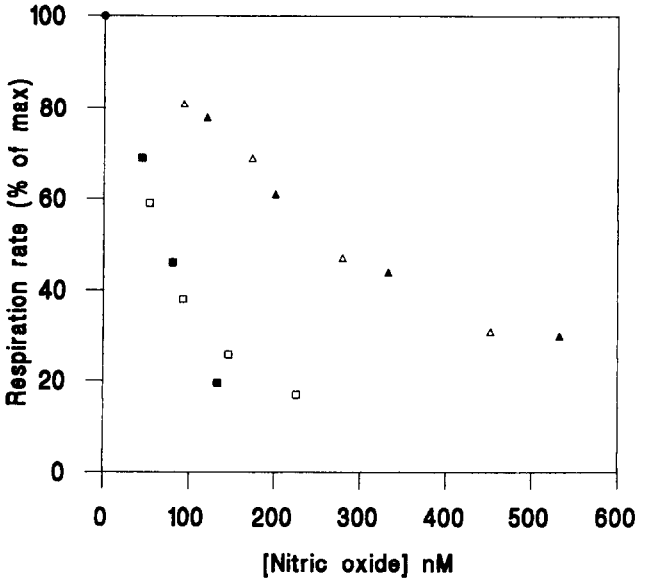

Fig. 3. Relation between NO level and inhibition of synaptosomal respiration at two different levels of oxygen. NO-saturated water was added to the synaptosomal incubation at two different levels of oxygen. The dependence of the respiration rate on the measured concentration of NO during the decay of the NO level is plotted for two different incubations at each oxygen level. The ranges of oxygen concentrations were 125–165 μM O_2 (△,▲) and 18–38 μM O_2 (□,■).

bition of synaptosomal respiration (half inhibition of oxygen consumption rate by 250 nM NO at 145 μM O_2).

4. Discussion

The results show that low levels of NO cause reversible inhibition of cytochrome oxidase probably in competition with oxygen. This is not entirely surprising as it has long been known that NO binds tightly to the reduced form of cytochrome oxidase at the oxygen binding site [12]. And it has been noted that the NO–cytochrome oxidase complex is unstable in the presence of oxygen [13], although this is probably due to the debinding of NO and reaction with oxygen. However the effects of NO on the steady-state kinetics of cytochrome oxidase in the presence of oxygen have not been investigated until recently. Cleeter et al. [14] have reported that an NO generator *S*-nitrosoglutathione reversibly inhibits respiration of isolated mitochondria from skeletal muscle at cytochrome oxidase. However the levels of NO, and the type of inhibition were not investigated. NO has been reported to inhibit mitochondrial aconitase, and complexes I and II of the respiratory chain in a manner which is only slowly reversible [4,15,16]. However, the concentrations of NO required for this type of inhibition are about 1000-fold higher than those used here. Bolaños et al. [17] have recently shown that cultured astrocytes induced to produce NO at high rates have decreased activity of cytochrome oxidase. But this was apparently irreversible damage caused by long-term exposure to NO.

We measured the K_i of NO for respiration to be 270 nM NO at 145 μM O_2 (a typical O_2 concentration for arterial blood) and 60 nM NO at 30 μM O_2 (a typical O_2 concentration for tissues). How does this compare with levels of NO produced by cells and

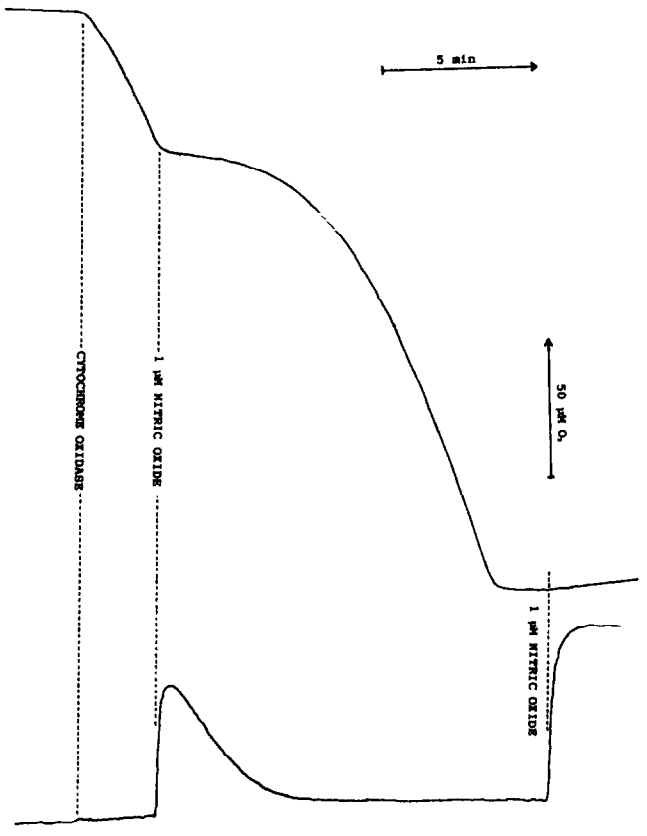

Fig. 4. Inhibition of isolated cytochrome oxidase by NO-saturated water. 100 nM cytochrome oxidase was added, followed by 1 μM NO, and when the system became anaerobic (oxygen trace flattens off) a further 1 μM NO was added to calibrate the electrode.

tissues? Reported levels measured by NO-electrode are: up to 1300 nM NO in bradykinin-stimulated aorta [18,19], 100–500 nM NO outside cultured endothelial cells [8], up to 500 nM NO from collagen-stimulated platelets in whole blood [20], and a 50 nM NO increase during electrical stimulation of brain cerebellar slices [7,21]. These levels suggest that the NO inhibition of cytochrome oxidase may be significant in some physiological conditions, and if so NO could be the first known physiological regulator to act directly on the mitochondrial respiratory chain [22]. In whole brain in vivo NO levels have been measured by electrode to be <10 nM in the basal state, 1000–4000 nM initially during ischaemia, declining in late ischaemia and then rising to about 1000 nM during reperfusion [5]. Tissue NO levels are also thought to be high after endotoxin/cytokine induction of the inducible form of NO synthase, but have not been measured as yet. 200–800 nM NO has been shown to inhibit gluconeogenesis and protein synthesis in hepatocytes [23], and this inhibition might well be mediated by NO inhibition of cytochrome oxidase.

The competition between NO and oxygen for cytochrome oxidase has one other important implication: if nanomolar NO is present in tissues the apparent K_m for oxygen of respiration in these tissues will be much higher than in isolated cytochrome oxidase or mitochondria in the absence of NO. In fact the apparent K_m for oxygen in a range of tissues has been measured to be much higher than to be expected from the in vitro K_m (see [22,24]).

A number of explanations for this phenomenon have been suggested, but it is possible that tissue NO might contribute to raising the K_m for oxygen.

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References

[1] Vincent, O.R. (1994) Prog. Neurobiol. 42, 129–160.
[2] Iadecola, C., Pelligrino, O.A., Moskowitz, O.A. and Lassen, O.A. (1994) J. Cereb. Blood Flow Metab. 14, 175–192.
[3] Bredt, O.S. and Snyder, O.H. (1994) Annu. Rev. Biochem. 63, 175–195.
[4] Nathan, C. (1992) FASEB J. 6, 3051–3064.
[5] Malinski, T., Bailey, F., Zhang, O.G. and Chopp, M. (1993) J Cereb. Blood Flow Metab. 13, 355–358.
[6] Dodd, O.R., Hardy, O.A., Oakley, O.E., Edwardson, J.A., Perry, O.K. and Delaunoy, O.P. (1981) Brain Res. 226, 107–118.
[7] Shibuki, K. (1990) Neurosci. Res. 9, 69–76.
[8] Tsukahara, H., Gordienko, O.V. and Goligorsky, O.S. (1993) Biochem. Biophys. Res. Commun. 193, 722–729.
[9] Archer, S. (1993) FASEB J. 7, 349–360.
[10] Kuboyama, M., Yong, O.C. and King, O.E. (1972) J. Biol. Chem. 247, 6375–6383.
[11] Arnold, O.P., Longnecker, O.E. and Epstein, O.M. (1984) Anesthesiology 61, 254–260.
[12] Brudvig, O.W., Stevens, O.H. and Chan, O.I. (1980) Biochemistry 19, 5275–5285.
[13] Erecinska M. and Wilson, O.F. (1980) Pharmacol. Ther. 8, 1–20.
[14] Cleeter, M.W.J., Cooper, J.M., Darley-Usmar, V.M., Moncada, S. and Scapira, A.H.V. (1994) FEBS Lett. 345, 50–54.
[15] Stadler, J., Billiar, O.R., Curran, O.D., Stuehr, D.J., Ochoa, O.B. and Simmons, O.L. (1991) Am. J. Physiol. 260, C910–C916.
[16] Stuehr, O.J. and Nathan, O.F. (1989) J. Exp. Med. 169, 1543–1555.
[17] Bolaños, O.P., Peuchen, S., Heales, O.J.R., Land, O.M. and Clark, O.B. (1994) J. Neurochem. 63, 910–916.
[18] Malinski, T. and Taha, Z. (1992) Nature 358, 676–678.
[19] Malinski, T., Taha, Z., Grunfeld, S., Patton, S., Kapturczak, M. and Tomboulian, P. (1993) Biochem. Biophys. Res. Commun. 193, 1076–1082.
[20] Malinski, T., Radomski, O.W., Taha, Z. and Moncada, S. (1993) Biochem. Biophys. Res. Commun. 194, 960–965.
[21] Shibuki, K. and Okada, D. (1991) Nature 349, 326–328.
[22] Brown, O.C. (1992) Biochem. J. 284, 1–13.
[23] Horton, O.A., Ceppi, O.D., Knowles, O.G. and Titheradge, O.A. (1994) Biochem. J. 299, 735–739.
[24] Jones, O.P. (1986) Am. J. Physiol. 250, C663–C675.