Journal of Bioenergetics and Biomembranes, Vol. 30, No. 1, 1998

Reactivity of Nitric Oxide with Cytochrome *c* Oxidase: Interactions with the Binuclear Centre and Mechanism of Inhibition

Jaume Torres,[1] Chris E. Cooper,[1] Martyn Sharpe,[1] and Michael T. Wilson[1]

Received September 22, 1997; accepted September 24, 1997

Nitric oxide (NO) has recently been recognized as an important biological mediator that inhibits respiration at cytochrome *c* oxidase (C*c*O). This inhibition is reversible and shows competition with oxygen, the K_i being lower at low oxygen concentrations. Although the species that binds NO in turnover has been suggested to contain a partially reduced binuclear center, the exact mechanism of the inhibition is not clear. Recently, rapid (ms) redox reactions of NO with the binuclear center have been reported, e.g., the ejection of an electron to cytochrome *a* and the depletion of the intermediates P and F. These observations have been rationalized within a scheme in which NO reacts with oxidized Cu_B leading to the reduction of this metal center and formation of nitrite in a very fast reaction. Electron migration from Cu_B to other redox sites within the enzyme is proposed to explain the optical transitions observed. The relevance of these reactions to the inhibition of C*c*O and metabolism of NO are discussed.

KEY WORDS: Cytochrome *c* oxidase; nitric oxide; copper; inhibition; respiration; mechanism.

PROPERTIES OF NO AS A LIGAND OF HEME PROTEINS AND AS A PROBE OF THE BINUCLEAR CENTER OF CYTOCHROME *C* OXIDASE (CCO)

Wainio (1955) first reported that nitric oxide (NO) binds to reduced C*c*O. The target for NO is the oxygen binding site ($Fea_3.Cu_B$), as demonstrated by Gibson and Greenwood (1963) through the observed competition between NO and CO for the reduced enzyme. Since then, many studies have been undertaken to characterize the properties of the complex(es) formed by the enzyme and NO, recently fueled by the widespread interest in NO as a signal and regulatory molecule in biological systems.

Nitric oxide possesses several distinctive features which makes it an attractive ligand for the study of heme proteins. It binds to both ferric and ferrous states, but particularly rapidly and tightly to the latter (k_{on} typically $> 10^7\ M^{-1}s^{-1}$), where it binds with a characteristically bent Fe–NO bond (Piciulo *et al.*, 1974) similar to oxygen (Phillips, 1978). In addition, the electronic configuration of NO is almost identical to that of dioxygen except that it has one less electron, which results in an unpaired electron residing in an antibonding π^* orbital. This property can be used to transform an even-spin state into an odd-spin state observable by EPR spectroscopy. Accordingly, NO has been used extensively as a spin probe of the structure of a variety of oxygen-binding proteins (e.g., the ferrous heme in either haemoglobins or the reduced binuclear center of C*c*O) (Yonetani *et al.*, 1972; Brudvig *et al.*, 1980). In the latter, NO binds to both Cu_B^{1+} and Fe^{2+} (Brudvig *et al.*, 1980), and the corresponding infrared stretching bands of the N–O bond have been characterized by Zhao *et al.* (1994).

In addition, in enzymes where two closely associated paramagnetic metal atoms form the oxygen binding site, and antiferromagnetic exchange leaves them

[1] Department of Biological Sciences, University of Essex, Wivenhoe Park, Central Campus, CO4 3SQ, Colchester, United Kingdom.

0145-479X/98/0200-0063$15.00/0

EPR silent, binding of NO to one of them can disrupt this coupling, and leave the other metal EPR detectable. Such coupling exists in the oxidized binuclear center of C*c*O (Van Gelder and Beinert, 1969), and is broken on addition of NO to the oxidized enzyme, where it only binds Cu_B^{2+}, thus allowing the high-spin ferric ($g = 6$) signal of Fea_3 to be manifest (Stevens *et al.*, 1979).

Blokzijl-Hoffman and Van Gelder (1971) showed that NO binds in the reduced enzyme to one of the hemes (heme a_3) only. These authors extended the studies of Kon and Kataoka (1969) and interpreted the EPR signals from the adduct of NO with reduced C*c*O as a superposition of three sets of three lines arising from two equivalent nitrogens ($I=1$) interacting with the unpaired electron.

The larger of the two superhyperfine coupling constants, 21.1 G, was assigned to the N of NO and the smaller, 6.8 G, to an endogenous axial ligand. Although this ligand could have been any basic amino acid (e.g., lysine, arginine, histidine) or even tryptophan, the fact that the *g* values and hyperfine splittings were almost equal for ferrocytochrome a_3 and ferrocytochrome *c*, and that the saturation behavior for both cytochromes was identical, led them to conclude that the fifth ligand of the Fea_3 in C*c*O was also histidine.

The axial ligand to Fea_3 was positively identified as histidine by Stevens and Chan (1981), who analyzed the EPR spectrum of the ferrocytochrome a_3–NO complex following incorporation of ^{15}N-labeled histidine into yeast C*c*O. In addition, these authors found that this imidazole does not bridge the two metal centers at the Fea_3·Cu_B site but that His is the distal ligand *vis à vis* Cu_B, i.e., on the opposite side of the heme to Cu_B (Stevens *et al.*, 1979; Brudvig *et al.*, 1980). This configuration has been confirmed by the recent structure obtained by X-ray diffraction (Tsukihara *et al.*, 1996).

REDOX CHEMISTRY OF NO WITH THE BINUCLEAR CENTER OF C*c*O

The redox active metals in the binuclear center of C*c*O exhibit an extremely rich and varied chemistry in their reactions with NO and related compounds, and we believe that the redox chemistry of NO, and not only its properties as a ligand, is central to an understanding of the mode of inhibition of C*c*O by NO.

The products of the redox reactions of C*c*O with NO were first characterized by Brudvig *et al.* (1980), who showed that under anaerobic conditions C*c*O can catalyze either the reduction or oxidation of NO depending on the redox state of the enzyme. When NO was added to the reduced or oxidized enzyme, N_2O or NO_2 were detected, respectively. These authors also observed reduction of nitrite to NO using the reduced enzyme. Reduction of NO to N_2O catalyzed by C*c*O in the presence of dithionite was also detected by Zhao *et al.* (1995) through the N_2O antisymmetric stretching infrared band at 2230 cm^{-1}.

INHIBITION OF C*c*O BY NO

Since NO has been shown to be a powerful biological mediator, synthesized *in vivo* through the L-arginine:nitric oxide pathway (Moncada and Higgs, 1993), the interactions of NO with C*c*O have commanded renewed attention. The effect of NO on the activity of C*c*O in the presence of oxygen was first reported by Cleeter *et al.* (1994) who showed that an NO generator, *S*-nitrosoglutathione (SNOG), inhibited respiration reversibly in mitochondria isolated from skeletal muscle, and they identified the site of inhibition as C*c*O. The same NO generator has also been used recently to inhibit solubilized C*c*O (Torres *et al.*, 1997). A first estimation of the NO concentration necessary to inhibit C*c*O was given by Brown and Cooper (1994), who, using brain synaptosomes, reported that this reversible inhibition was oxygen concentration dependent and the concentration of NO necessary for inhibition (e.g., 270 nM at 145 μM oxygen) was within the concentration range measured for a number of tissues, especially under pathological conditions (e.g., sepsis).

Although it appears probable that NO competes with oxygen at the ferrous Fea_3 site, results using the enzyme solubilized in detergent indicated that, while the final inhibited form of the enzyme does contain the Fea_3^{2+}–NO species, the competition of O_2 and NO for ferrocytochrome a_3 may not account for the rapid onset of inhibition observed (Torres *et al.*, 1995). A mechanism was therefore postulated in which NO binds to a partially reduced binuclear center in which only Cu_B was reduced. In addition, these authors confirmed the reversibility and the oxygen dependence of the reaction. Half inhibition was obtained at a molar ratio $[O_2]/[NO] \sim 30$, a ratio that has been determined independently by Giuffre *et al.* (1996).

Giuffre *et al.* (1996) have suggested a somewhat different model for inhibition. They have also proposed

that NO binds in turnover to a partially reduced binuclear center and that the final inhibited form is the ferrocytochrome a_3–NO complex. However, these authors suggest a model in which NO binds in the half reduced binuclear center to ferrocytochrome a_3, not Cu_B^{1+}. These authors measured the "off" rate of NO from Fea_3^{2+} as 0.13 s^{-1}. Taking this value, and the "on" rate for NO binding to ferrous cytochrome a_3 as 1×10^8 M^{-1} s^{-1} (Blackmore *et al.*, 1991), the K_d for NO is 1.3 nM. For CO, which also binds to ferrous a_3 and shows competitive behavior with respect to oxygen, the measured K_i is 0.32 μM (Petersen, 1977), consistent with the measured $K_d \sim 0.31$ μM from the "on" (7×10^4 M^{-1} s^{-1}) and "off" (0.023 s^{-1}) rates for CO binding (Gibson and Greenwood, 1963). According to these values, NO should be about 200 times better as an inhibitor than CO. However, at 60 μM oxygen, 120 μM CO is required to inhibit approximately 50% of C*c*O (Davies *et al.*, 1997), whereas only 0.07 μM NO is required to have the same effect in mitochondria (Borutaite and Brown, 1996), making NO over five times more effective than is expected on the basis of the above calculations. This discrepancy however, may be resolved if, as we have recently suggested (Cooper *et al.*, 1997), the initial binding site for NO is not the ferrocytochrome a_3 but cupric Cu_B (Cu_B^{2+})(see below).

REACTIONS OF NO WITH C*c*O IN "TURNOVER" AND WITH OXYGEN INTERMEDIATES

Recent experiments (Torres *et al.*, 1996; Wilson and Torres (1997); Wilson *et al.*, 1997; Cooper *et al.*, 1997) have shown that when NO is added to the enzyme in turnover a number of distinct processes may be resolved; these include (i) rapid electron ejection from the binuclear center partially reducing cytochrome *a* and Cu_A and (ii) depletion of the oxygen intermediates P and F.

We have investigated these reactions further (Torres *et al.*, in press, 1998) by mixing NO with those enzyme species which may be prepared in a reasonably homogeneous state and which are thought to be populated in turnover, namely O, P, and F. The results may be listed as follows. (I) Upon mixing NO with the oxidized (pulsed) enzyme (O) cytochrome *a* became partially reduced (about 40–50%) as indicated (Fig. 1) by the appearance of absorption bands at 445 and 605 nm (intensity ratio 3:1) with a rate constant $k \sim 100$ s^{-1}. This reduction of cytochrome *a* was accompanied by reduction of 15–20% Cu_A.

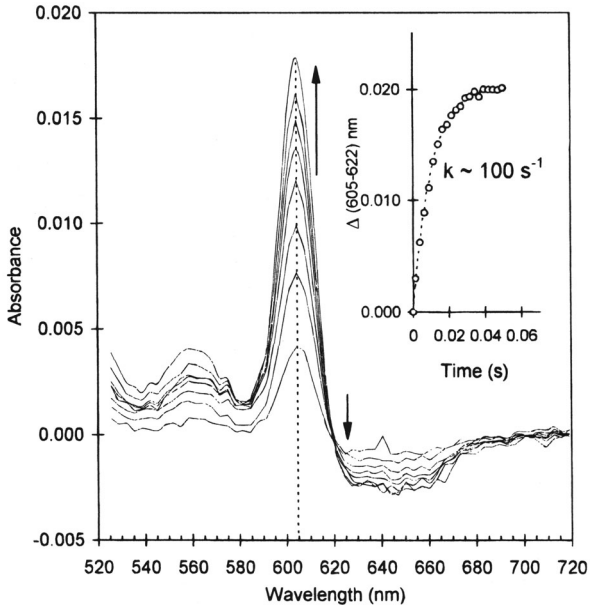


Fig. 1. Reactions of NO with species O. Difference spectra (relative to the first spectrum, collected at 1.4 ms) acquired on mixing oxidized pulsed C*c*O with NO in the stopped-flow apparatus (Applied Photophysics). Spectra are displayed at 4 ms intervals. Conditions: C*c*O, 7.5 μM in 0.1 M HEPES, 0.5% Tween 80, pH 7.4; NO, 1 mM. The inset shows the time course of cytochrome *a* reduction fitted to a single exponential.

(II) Compounds P and F were rapidly depleted on mixing with NO (Fig. 2A and B). Each of these compounds was mixed with NO in a stopped-flow apparatus in order to elucidate the kinetics of these processes:

(a) Reaction of a sample containing a substantial proportion (~80%) of compound P resulted in the *formation* of ~ 20% compound F (580 nm absorbance) within the dead time of the stopped-flow apparatus (~1.4 ms) (Torres and Wilson, 1997), whereas the band at 607 nm (P) remained unchanged over this time scale. We attribute these changes to the formation of compound F from a species distinct from P and spectrally similar to the oxidized enzyme (O). Species P (607 nm) and the newly formed species absorbing at 580 nm (F) now decayed simultaneously with a rate that was found to depend on the NO concentration. These changes, completed within ~800 ms at 1 mM NO, were closely similar in rate to the decay to O of compounds P and F seen in turnover. Some reduction of cytochrome *a* takes place simultaneously with the decay of the P and F intermediates due to a further

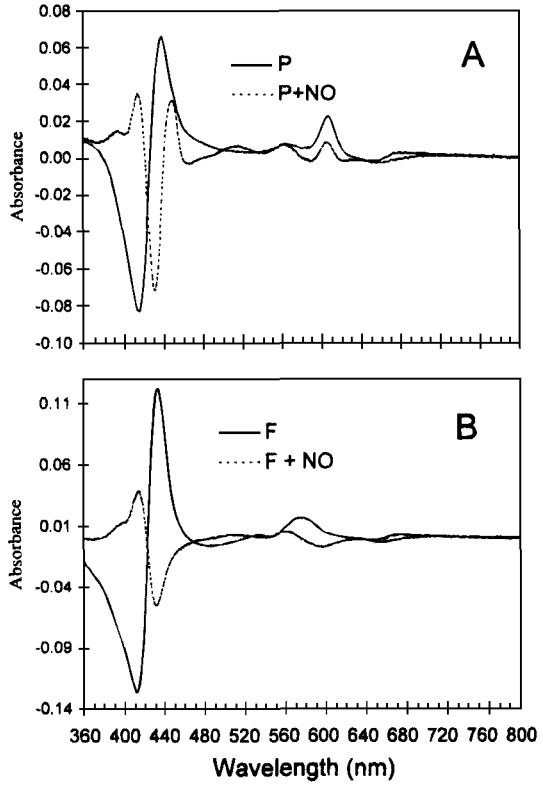


Fig. 2. Reactions of NO with P (A) and F (B). Difference spectra with respect to the oxidized enzyme O of samples containing ~ 40% P (A) and 80% F (B), before (——) and ~ 6 s after (- - - - -) the addition of 30 μM NO. C*c*O, 4.7 μM; oxygen, 240 μM.

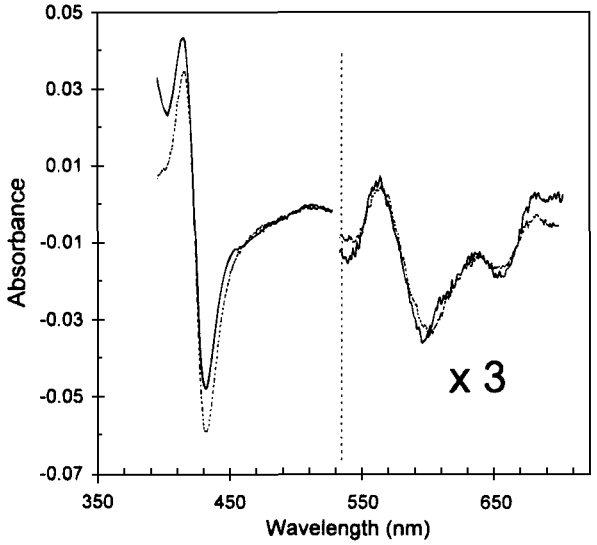


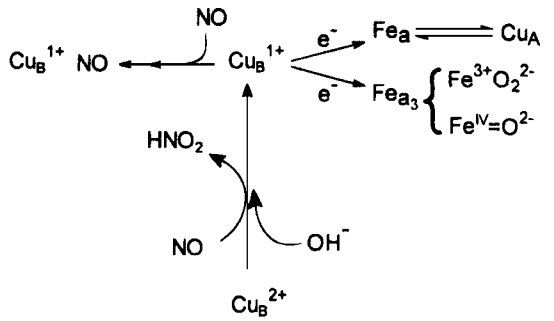
Fig. 3. Effect of nitrite binding to the oxidized binuclear center. Difference spectra versus the oxidized enzyme after the addition of 10 mM sodium nitrite to oxidized enzyme (——) compared with the difference spectrum obtained after the addition of 30 μM NO to species F, P, or O (- - - -) [in the two last cases (P, O) the contribution of cytochrome *a* reduction has been subtracted; this correction is unnecessaary when F is prepared in the presence of excess H_2O_2, see Fig 2].

reaction of NO with O, the product of the decay of P and F. (b) Compound F, prepared by the reaction between H_2O_2 and the oxidized enzyme, decayed on reaction with NO with the same rate constant (k~ 6 s^{-1} at 1 mM NO) as seen with F formed as described above (a).

(III) The final spectrum following each reaction was consistent with the presence of nitrite in the binuclear center (Fig. 3), indicating that nitrite is formed rapidly as a product of the reaction of NO with O, P, and F.

MECHANISM: NO AS A SINGLE ELECTRON REDUCTANT OF Cu_B

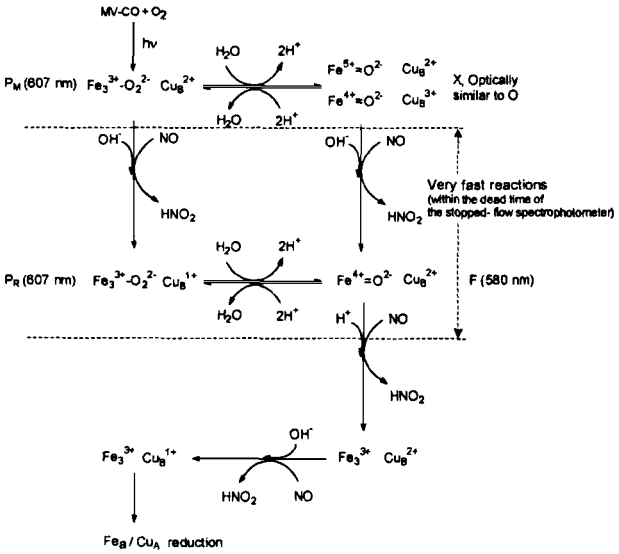
We have attempted to rationalize the disparate reactions listed above in terms of a simple underlying mechanism (see Scheme 1) the first step of which is NO binding to Cu_B^{2+}. Upon hydration of the resulting nitrosonium ($Cu_B^{1+}{\cdot}NO^+$) nitrous acid (HNO_2) is formed, leaving Cu_B reduced. The electron, residing on Cu_B, can now transfer to other redox centers within the enzyme, the destination being determined by the redox and ligand states of cytochrome a_3. For example, the electron may migrate to the Fe*a* or Cu_A sites (with O) or, alternatively, transfer to Fea_3 thereby converting F to O (Scheme 1).



Scheme 1. Postulated pathways of the reducing equivalent after reduction of Cu_B by NO.

In order to explain the formation of F within the dead time on mixing NO with samples rich in P (see IIa above), we propose that P (P_M) (Morgan *et al.* 1996) obtained from the mixed valence CO-bound

derivative (MV-CO), but also P obtained by bubbling CO in the presence of oxygen (Nicholls and Chanady, 1981), coexists with a species (X) spectrally similar to O, at the formal reduction level of peroxide (either $Fe^{4+}=O\ Cu_B^{3+}$ or $Fe^{5+}=0\ Cu_B^{2+}$, see Scheme 2). The presence of either of these species then explains the fast formation of compound F ($Fe^{4+}=O\ Cu_B^{2+}$) solely by reduction of Cu_B^{3+} to Cu_B^{2+} or of Fe^{5+} to Fe^{4+} (by electron transfer from Cu_B). A similar reaction may take place in P_M, the transition from Cu_B^{2+} to Cu_B^{1+} leading to the conversion $P_M \rightarrow P_R$, and inducing little or no spectral change (Morgan *et al.*, 1996).



Scheme 2. Possible mechanism of the interaction of NO with the different forms of the binuclear center. Only the metals in the binuclear center are depicted. The optical spectral signatures relative to the oxidized O enzyme are indicated.

This mechanism accounts, therefore, for the spectral changes that occur within the dead time after mixing P with NO, namely, the apparent nonreactivity of P_M and the formation of F from a species (X in Scheme 2) spectrally similar to O. According to this mechanism nitrite would be formed in the binuclear center within the dead time of the stopped-flow apparatus. The spectral changes we observe are also consistent with this prediction (Torres *et al.*, in press, 1998).

The postulated equilibrium between P and another species (X) at the same level of oxidation could also clarify some of the apparent inconsistencies concerning the structural assignments of the intermediates P and F. For example, the heme iron structure in compound P has been assigned as a $Fe^{3+}=O_2^{2-}$ (Wikström, 1981), $Fe^{4+}=O$ (Weng and Baker, 1991; Fabian and Palmer, 1995) and also recently $Fe^{5+}=O$ (Ogura *et al.*, 1996). This difference of opinion may be explained if a ferric peroxy and an iron oxo species coexist at the peroxide level, but whereas only one of them (ferric peroxy) can be detected optically by difference spectroscopy (at 607 nm), the stretching $\nu_{Fe=O}$ vibration of the other (iron oxo) is detected at 804 cm^{-1} by Raman spectroscopy, i.e., these two spectroscopic techniques detect different species present in the equilibrium.

To account for the simultaneous decay of P and F on reaction with NO we suggest that P_R and F are also in rapid (compared to the decay rate) equilibrium (see Scheme 2). Obviously, this equilibrium implies that the splitting of the O–O bond is reversible under these conditions, and this poses a problem from the thermodynamic point of view. This problem may be avoided if P_M and P_R contain $Fe^{5+}=O$, with Cu_B^{2+} and Cu_B^{1+}, respectively. In this case, the species that decay simultaneously are in the same oxidation state, but there is no need for reversible O–O cleavage. This model would, however, lack the ferric peroxy form, in contradiction with previous assignments (Wikström, 1981) and observations made using synthetic model compounds (Collman *et al.*, 1997), in which a peroxy species was detected during the reduction of oxygen to water.

EVIDENCE FOR THE INITIAL REACTION OF NO WITH Cu_B AND IMPLICATIONS FOR THE MECHANISM OF INHIBITION

The hypothesis that electron ejection from the binuclear center is caused by NO interacting with Cu_B, and not $Fe a_3$, is supported by several observations. Firstly, when oxidized C*c*O is incubated with NO, only Cu_B has been shown to bind this ligand (Stevens *et al.*, 1979). Secondly, we see no spectral evidence for cytochrome a_3 reduction (or ferrocytochrome a_3–NO formation) when NO reacts with O. Thirdly, we have observed a very fast reaction between NO and cupric type 2 copper in laccase, a multicopper oxidase. This reaction results in reduction of the type 2 copper, as indicated by EPR spectroscopy.

In addition, the reaction of NO with ferryl compounds in proteins where only iron is present (e.g., in myoglobin) is much slower (Gorbunov *et al.*, 1995) than the reaction of F with NO. These data support the view that Cu_B plays a key role in the mechanism by which NO reacts with F in C*c*O. Also, NO does not react with the peroxide intermediate in laccase (Torres *et al.*, unpublished results), in contrast with

the results obtained mixing P and NO, again suggesting a specific role for Cu_B.

Thus, according to this postulated redox mechanism, the apparent conflict between the observed inhibition constants and those predicted from the "on" and "off" rates of NO for ferrous a_3 (see above) can be explained taking into account that NO can also rapidly react with the fully oxidized binuclear center of C*c*O in the pulsed enzyme and that Cu_B may become reduced as a result (see above). The decrease in the K_i of NO for C*c*O may be explained by interaction of NO with the binuclear center leading to reduction and subsequent binding of a further NO molecule to Cu_B^{1+} (see Scheme 1), thus blocking the redox state of this metal, which would then constitute the real inhibitory site. Alternatively, NO may transfer rapidly from Cu_B^{1+} to cytochrome a_3 on addition of an electron to this site from cytochrome *a*/Cu_A in turn over. Thus, although formation of ferrocytochrome a_3–NO complex has been detected in the inhibitory process (Torres *et al.,* 1995) and has been suggested as the primary event in the inhibition (Giuffre *et al.,* 1996), the formation of this complex might well be a consequence rather than a cause of the inhibition.

DOES NO INHIBIT C*c*O *IN VIVO*?

Inhibition of respiration by exogenous NO has been observed in mitochondria from heart (Carr and Ferguson, 1989; Borutaité and Brown, 1996), brain (Schweizer and Richter, 1994), skeletal muscle (Cleeter *et al.,* 1994), brain nerve terminals (Brown and Cooper, 1994), and in a wide variety of cell types (Bolaños *et al.,* 1997). The first study showing that endogenous NO can inhibit C*c*O was carried out by inducing NO synthase in astrocytes and observing the inhibition of C*c*O activity (Brown *et al.,* 1995).

At organizational levels higher than cells there is as yet no fully convincing evidence that NO inhibits C*c*O. If NO inhibits C*c*O *in vivo,* one may expect NO synthase inhibitors to increase oxygen consumption. However, the data published regarding the intact heart are conflicting, with claims that NO synthase inhibitors increase (Shen *et al.,* 1994), decrease (Sherman *et al.,* 1997), or have no effect (Sadoff *et al.,* 1996) on the oxygen consumption rate.

Even in disease states where NO production is known to increase (e.g., septic shock) NO synthase inhibitors do not increase the rate of oxygen consumption (Meyer *et al.,* 1994). There are many problems with the interpretation of these *in vivo* studies. For example, a decrease in NO production by inhibitors of NO synthase may increase oxygen consumption by mitochondria but, on the other hand, tissue oxygenation may be lower, as NO is known to increase blood flow.

Also, global oxygen consumption measurements are not a good indicator of inhibition of C*c*O, as many factors other than the C*c*O activity control mitochondrial oxygen consumption *in vivo* (Brown, 1992). Measurements of the redox state of the C*c*O metal centers *in vivo* may be more revealing. Recently, near-infrared spectroscopy has been used to show that an enhanced reduction of C*c*O Cu_A follows hypoxic-ischemic damage to the neonatal pig brain, despite increases in the rate of oxygen delivery (Cooper and Springett, 1997; Springett *et al.,* 1997). This enhanced reduction is as expected if NO were inhibiting C*c*O and we are currently comparing the time course of NO production in this model with the time course of the redox state change to see if NO inhibiton is the most likely explanation.

PHYSIOLOGICAL AND PATHOPHYSIOLOGICAL ROLE OF NO INHIBITION OF C*c*O

It has been suggested that NO inhibition of C*c*O may play a normal role in controlling mitochondrial oxygen consumption (Brown, 1995); for example, it could help explain the raised K_m for oxygen of cellular oxygen consumption, compared to that of mitochondria. Increases in NO production could also bring the K_m for oxygen of cell types that sense oxygen into the physiological range, allowing C*c*O to be an oxygen sensor, e.g., in the carotid body (Wilson *et al.* 1994). These ideas are attractive, especially given the recent suggestions that mitochondria contain NO synthase activity (Bates *et al.,* 1995; Kobzik *et al.,* 1995), but as yet the *in vivo* evidence for these roles is lacking.

Finally, C*c*O inhibition by NO, although readily reversible, can have profound consequences for the cell. Inhibited mitochondria produce superoxide and this can react with NO to generate the powerful oxidant peroxynitrite ($ONOO^-$), a compound that has been clearly shown to trigger the mitochondrial permeability transition (MPT).This process leads to cytochrome *c* (Liu *et al.,* 1996; Kharbanda *et al.,* 1997) and protease release from mitochondria, and hence, triggers apoptosis (Kroemer *et al.,* 1997). Thus, inhibition of

nitric oxide by CcO may initiate a cascade of events that, through generation of $ONOO^-$, may be responsible for the role of NO in initiating apoptosis and resulting in cell death.

ACKNOWLEDGMENT

This work was supported by the Wellcome Trust, The Royal Society, The Spanish Goverment and the Biotechnology and Biological Sciences Research Council UK.

REFERENCES

Babcock, G. T., Floris, R., Nilsson, T., Pressler, M., Varotsis, C., and Vollenbroek, E. (1996). *Inorg. Chim. Acta* **243**, 345–353.
Bates, T. E., Loesch, A., Burnstock, G., and Clark, J. B. (1995). *Biochim. Biophys. Acta* **213**, 896–900.
Blackmore, R. S., Greenwood, C., and Gibson, Q. H. (1991). *J. Biol. Chem.* **266**, 19245–19249.
Blokzijl-Hoffman, and Van Gelder, B. F. (1971). *Biochim. Biophys. Acta* **234**, 493–498.
Bolaños, J. P., Almeida, A., Stewart, V., Peuchen, S., Land, J. M., Clark, J. B., and Heales, S. J.
R. (1997). *J. Neurochem.* **68**, 2227–2240.
Borutaité, V., and Brown, G. C. (1996). *Biochem. J.* **315**, 295–299.
Brown, G. C. (1992). *Biochem. J.* **284**, 1–13.
Brown, G. C. (1995). *FEBS Lett.* **369**, 136–139.
Brown, G. C., and Cooper, C. E. (1994). *FEBS Lett.* **356**, 295–298.
Brown, G. C., Bolaños, J. P., Heales, S. J. R., and Clark, J. B. (1995). *Neuroscience Lett.* **193**, 201–204.
Brudvig, G. W., Stevens, T. H., and S. I. Chan (1980). *Biochemistry* **19**, 5275–5285.
Carr, G. J., and Ferguson, S. J. (1989). *Biochim. Biophys. Acta* **1017**, 57–62.
Cleeter, M. W. J., Cooper, J. M., Darley-Usmar, V. M. Moncada, S., and Schapira, A. H. V. (1994). *FEBS Lett.* **354**, 50–54.
Collman, J. P., Fu, L., Herrmann, P. C., and Zhang, X. (1997). *Science* **275**, 949–951.
Cooper, C. E., and Springett, R. (1997). *Philos. Trans. R. Soc. London B* **352**, 669–676.
Cooper, C. E., Torres, J. Sharpe, M., and Wilson, M. T. (1977). *FEBS Lett.*, in press.
Davies, N., Trikkas, C., and Cooper, C. E. (1997). *Biochem. Soc. Trans.* **25**, 406S.
Fabian, M., and Palmer, G. (1995). *Biochemistry* **34**, 13802–13810.
Gibson, Q. H., and Greenwood, C. (1963). *Biochem. J.* **86**, 541–553
Giuffre, A., Sarti, P., D'Itri, E., Buse, G., Soulimane, T., and Brunori, M. (1996). *J. Biol. Chem.* **271**, 33404–33408.
Gorbunov, N. V., Osipov, A. N., Day, B. W., Zayas-Rivera, B., Kagan, V. E., and Elsayed, N. M. (1995). *Biochemistry* **34**, 6689–6699.
Kharbanda, S., Pandey, P., Schofield, L., Israels, S., Roncinske, R., Yoshida, K., Bharti, A., Yuan, Z. M., Saxena, S., Weichselbaum, R., Nalin, C., and Kufe, D. (1997). *Proc. Natl. Acad. Sci. USA* **94**, 6939–6942.
Kobzik, L., Stringer, B., Balligand, J.-L., Reid, M. B., and Stamler, J. S. (1995). *Biochem. Biophys. Res. Commun.* **211**, 375–381.
Kon, H., and Kataoka, N. (1969). *Biochemistry* **8**, 4757–4762.
Kroemer, G., Zanzami, N., and Susin, S. A. (1997). *Immun. Today* **18**, 44–51.
Liu, X. S., Kim, C. N., Yang, J., Jemmerson, R., and Wang, X. D. (1996). *Cell* **86**, 147–157.
Meyer, J., Lentz, C. W., Stothert, J. C., Traber, L. D., Herndon, D. N., and Traber, D. L. (1994). *Crit. Care Med.* **22**, 306–312.
Moncada, S., and Higgs, A. (1993). *N. Engl. J. Med.* **329**, 2002–2012.
Morgan, J. E., Verkhovsky, M. I., and Wikström, M. (1996). *Biochemistry* **35**, 12235–12240.
Nicholls, P., and Chanady, G. A. (1981). *Biochim. Biophys. Acta* **634**, 256–265.
Ogura, T., Hirota, S., Proshlyakov, D. A., Shinzawa-Itoh, K., Yoshikawa, S., and Kitagawa, T. (1996). *J. Am. Chem. Soc.* **118**, 5443–5449.
Petersen, L. C. (1977). *Biochim. Biophys. Acta* **460**, 299–307.
Phillips, S. E. V. (1978). *Nature* **273**, 247–248.
Piciulo, P. L., Rupprecht, G., and Scheidt, W. R. (1974). *J. Am. Chem. Soc.* **96**, 5293–5295.
Sadoff, J. D., Scholz, P. M., and Weiss, H. R. (1996). *Proc. Soc. Exp. Biol. Med.* **211**, 332–338.
Schweizer, M., and Richter, C. (1994). *Biochem. Biophys. Res. Commun.* **204**,
Shen, W., Xu, X., Ochoa, M., Zhao, G., Wolin, M. S. v., and Hintze, T. H. (1994). *Circ. Res.* **75**, 1086–1095.
Sherman, A. J., Davis, C. A., Klocke, F. J., Harris, K. R., Srinivasan, G., Yaacoub, A. S., Quinn, D. A., Ahlin, K. A., and Jang, J. J. (1997). *Circulation* **95**, 1328–1334
Springett, R. J., Penrice, J. M., Amess, P. N., Tyszczuk, L., Delpy, D. T., Wyatt, J. S., and Cooper, C. E. (1997). *Biochem. Soc. Trans.* **25**, 398.
Stevens, T. H., and Chan, S. I. (1981). *J. Biol. Chem.* **256**, 1069–1071.
Stevens, T. H., Brudwig, G. W., Bocian, D. F., and Chan, S. I. (1979). *Proc. Natl. Acad. Sci. USA* **76**, 3320–3325.
Torres, J., and Wilson, M. T. (1997). *Biochem. Soc. Trans.* **25**, 402S.
Torres, J., Darley-Usmar, V. M., and Wilson, M. T. (1995). *Biochem. J.* **312**, 169–173.
Torres, J., Cooper, C. E., and Wilson, M. T. (1996). *Biochem. Soc. Trans.* **24**, 450S.
Torres, J., Davies, N., Darley-Usmar, V. M., and Wilson, M. T. (1997). *J. Inorg. Biochem.* **66**, 207–212.
Torres J., Cooper, C. E., and Wilson, M. T. (1998). *J. Biol. Chem.* in press.
Tsukihara, T, Aoyama, H, Yamashita, E., Tomizaki, T., Yamaguchi, H., Shinzawaitoh, K., Nakashima, R, Yaono, R., and Yoshikawa, S. (1996). *Science* **272**, 1136–1144.
Van Gelder B. F., and Beinert, H. (1969). *Biochim. Biophys. Acta* **189**, 1–24.
Wainio, W. W. (1955). *J. Biol. Chem.* **212**, 723–733.
Weng, L., and Baker, G. M. (1991). *Biochemistry* **30**, 5727–5733.
Wikström, M (1981). *Proc. Natl. Acad. Sci. USA* **78**, 4051–4054.
Wikström, M., and Morgan, J. E. (1992). *J. Biol. Chem.* **267**, 10266–102731.
Wilson, M. T., and Torres, J. (1997). In *Nitric Oxide and the Cell: Proliferation, Differentiation and Death* (Moncada, S., Nistico, G., Bagetta, G., and Higgs, E. A., eds.), Portland Press, in press.
Wilson, D. F., Mokashi, A., Chugh, D., Vinogradov, S., Osanai, S., and Lahiri, S. (1994). *FEBS Lett.* **351**, 370–374.
Wilson, M. T., Torres, J., Cooper, C. E., and Sharpe, M. (1997). *Biochem. Soc. Trans.*, **25**, 905–909.
Yonetani, T., Yamamoto, H., Erman, J. E., Leigh, J. S., Jr., and Reed, G. H. (1972). *J. Biol. Chem.* **247**, 2447–2455.
Zhao, X. J., Sampath, V., and Caughey, W. S. (1994). *Biochem. Biophys. Res. Commun.* **204**, 537–543.
Zhao, X.-J., Sampath, V., and Caughey, W. S. (1995). *Biochem. Biophys. Res. Commun.* **212**, 1054–1060.