Kinetics of the inhibition of mitochondrial respiration by NO

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Received 29 August 1997

Abstract The kinetics of the inhibition of mitochondrial respiration by NO was examined in isolated mitochondria (here obtained from rat brown adipose tissue). The K_i of NO for the inhibition was ~27 nM; the IC_{50} of NO increased in proportion to the square of an increase in O_2 tension. The K_m of O_2 for respiration was ~16 μM; in the presence of NO, the dependence of respiration on O_2 tension had a Hill coefficient of ~2. The unusual kinetics is probably related to the ability of cytochrome *c* oxidase to use 2 NO or 1 O_2 as electron acceptor. The interaction between NO and O_2 in the control of respiration could be described by the formula $V_{O_2}(O_2, NO) = V_{O_2}max \cdot ([O_2]^2/((16\ \mu M \cdot (1+[NO]/27\ nM))^2+[O_2]^2))$. Thus, the kinetics is such that respiration in the presence of physiological levels of NO is very sensitive to decreasing O_2 tension.

Key words: Nitric oxide; Oxygen; Cytochrome *c* oxidase; Hypoxia; Non-shivering thermogenesis; Brown fat mitochondria

1. Introduction

Nitric oxide (NO) reversibly and dose-dependently attenuates or inhibits respiration in mitochondria isolated from every cell type so far examined [1–14]. This inhibition has been established to be due to NO functioning as an alternative substrate (instead of O_2) for cytochrome *c* oxidase (probably this is an evolutionary relict [15]). In this alternative reduction reaction, 2 NO yield N_2O and H_2O [7–9,16–19]. When NO functions as the substrate instead of O_2, respiration (defined as the rate of O_2 consumption) is, of course, eliminated. However, a de-energization of the mitochondria is also seen [3,20], presumably because the reduction of NO has slower kinetics than that of the normal substrate of cytochrome *c* oxidase: O_2. Electron flow through the respiratory chain – and hence proton pumping – therefore essentially ceases.

Although the degree of inhibition by NO has consistently been found to be higher at lower O_2 tensions, a more detailed study of the kinetics has not as yet been performed. We have here made a first examination of the kinetics of the inhibition. We find that the apparent affinity of NO is 3 orders of magnitude higher than that of O_2, and that the presence of NO apparently induces a pseudocooperative interaction between O_2 and the respiratory system.

2. Materials and methods

2.1. Mitochondrial preparation

Brown fat mitochondria were prepared principally as described by Cannon and Lindberg [21] from adult female Sprague-Dawley rats which had been acclimatized (one rat per cage) to 4°C (6 h light, 18 h dark) for 3 weeks. The rats had free access to food and water. Brown adipose tissue from the interscapular, periaortic, axillary and cervical deposits was dissected out. The pooled tissue was minced with scissors, and the tissue pieces were homogenized in 40 ml 250 mM sucrose solution, filtered through gauze and centrifuged at 8500×*g* for 10 min. The pellet was resuspended in sucrose and centrifuged at 800×*g* for 10 min. The resulting supernatant was then centrifuged at 8500×*g* for 10 min, and the pellet was resuspended in 100 mM KCl+20 mM Tris (pH 7.2) +0.2% fatty-acid-free bovine serum albumin. After recentrifugation at 8500×*g* for 10 min, the mitochondria were further washed and finally resuspended in KCl/Tris (without albumin). The mitochondrial protein concentration was measured with the fluorescamine method (Fluram from Fluka), and the suspension diluted to a stock concentration of 20 mg/ml.

2.2. Experimental conditions

The mitochondria, at a final concentration of 0.2 mg mitochondrial protein per ml, were added to 1.1 ml of a continuously stirred incubation medium in an air-sealed Plexiglas chamber. The medium consisted of 100 mM KCl, 20 mM Tris (pH 7.2), 2 mM $MgCl_2$, 5 mM malate, 5 mM pyruvate, 4 mM KP_i, 1 mM EDTA and 0.05% fatty-acid-free bovine serum albumin. Further additions were made with a syringe through the lid.

O_2 and NO levels in the chamber were continuously and simultaneously monitored with a Clark-type O_2 electrode and an NO electrode (ISO-NOP 200, World Precision Instruments). An incubation temperature of 32°C was chosen because the background current of the NO electrode was very temperature-sensitive and became too high at temperatures closer to 37°C. Calibration of the NO electrode was performed according to the manufacturer's instructions by using $NaNO_2$ and $KI+H_2SO_4$ to generate known amounts of NO. The calibration curves exhibited a linear correlation coefficient of 0.99 or better, up to 3 μM NO. For calibration of the O_2 electrode, air-saturated distilled water at this temperature was considered to contain 228 μM O_2. The (mutually opposing) effects of oxygen back diffusion and oxygen electrode oxygen consumption were found to influence the data points by <4% and have therefore not been compensated for. Further, we did not observe any influence of NO on the O_2 electrode, nor of O_2 on the NO electrode.

2.3. Chemicals

The NO gas solution used in the mitochondrial experiments was prepared by purging distilled water in an airtight bottle with helium gas for at least 15 min at room temperature, before gassing it with NO for a further 15 min. Aliquots of this NO-saturated NO solution were then added to the incubation chamber. The exact NO amount present was obtained from the NO electrode measurement.

EDTA, FCCP, KI, malate and pyruvate were from Sigma, $NaNO_2$ from Aldrich, and the NO and helium gases were from AGA (Lidingö).

3. Results and calculations

3.1. Inhibition of respiration by NO

For the present experiments, we used brown fat mitochondria [22,23] incubated with pyruvate/malate as substrate, in the absence of added nucleotide. Under these conditions, these mitochondria are uncoupled, due to the action of the uncoupling protein (UCP) (for review see [24]). Respiration unhampered by the activity of the ATP synthase was thus monitored. There is, however, no reason to expect that mito-

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PII S0014-5793(97)01258-1

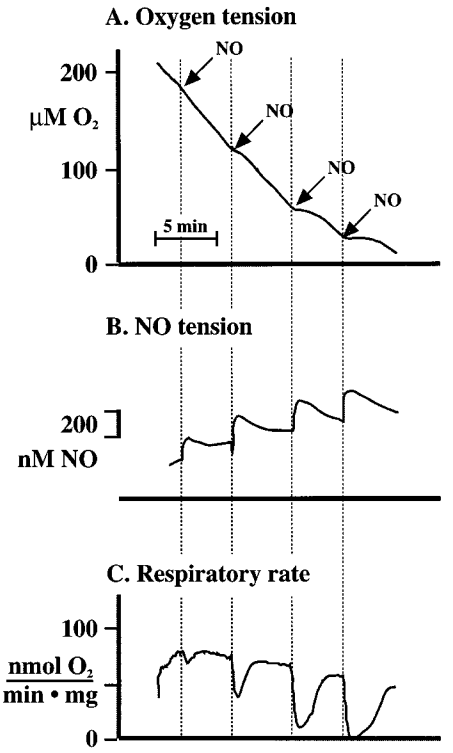

Fig. 1. Effect of NO addition at different O_2 tensions on the respiratory rate of brown fat mitochondria. Brown fat mitochondria were incubated in the presence of pyruvate+malate as substrate and in the absence of nucleotides, as detailed in Section 2. The traces represent simultaneous measurements of (A) O_2 tension, (B) NO tension and (C) mitochondrial respiratory rate in the same mitochondrial suspension. The NO electrode baseline showed some drift due to high temperature sensitivity. The mitochondrial respiratory rate (C) is the time derivative of the O_2 tension curve (A). At the indicated time points, NO-saturated water aliquots were added, each here corresponding to about 200 nM NO; for the graphs and calculations, the exact NO concentration was in each instance determined from the NO electrode calibration curve. Control additions of water had no effect (not shown).

chondria from any other source would behave significantly differently from the present mitochondria, provided they were uncoupled by an artificial uncoupler or were performing ATP synthesis at a high rate. In coupled (state 4) mitochondria, respiratory rates were too low to allow for meaningful analysis of kinetics of NO effects.

To study the effect of NO, small amounts of NO were added to the uncoupled, rapidly respiring brown fat mitochondria. A typical experiment is shown in Fig. 1. Simultaneous recordings of O_2 tension, NO tension and the rate of mitochondrial respiration are depicted.

In the presence of substrate, the mitochondria initially respired rapidly (~75 nmol O_2 per min per mg protein) (Fig. 1C), as they were in an uncoupled state. At an O_2 tension of ~190 μM (Fig. 1A), an addition of ~200 nM NO was made. As seen in Fig. 1C, this had only a weak inhibitory effect on the respiratory rate. NO disappeared during the next 2 min (Fig. 1B). A new dose of NO was then added, now at an O_2 tension of ~110 μM, and here a more marked inhibitory effect of NO addition was noted. Further additions of NO at O_2 tensions of ~50 μM or ~25 μM caused higher degrees of inhibition; at the lowest O_2 tension, respiration was fully inhibited. However, even this full inhibition was reversible when NO disappeared. In other experiments, we have observed that the recovery always coincided with the disappearance of NO and was seen to occur even after up to 11 min of complete respiratory inhibition (not shown). This inhibitory effect of NO on brown fat mitochondria, the reversibility of the effect and the increased inhibitory effect of NO with decreasing O_2 tension are all observations in line with what has been observed with mitochondria from many other sources (see Section 1).

To determine that the inhibitory effect of NO was on the respiratory system rather than on the activity of the uncou-

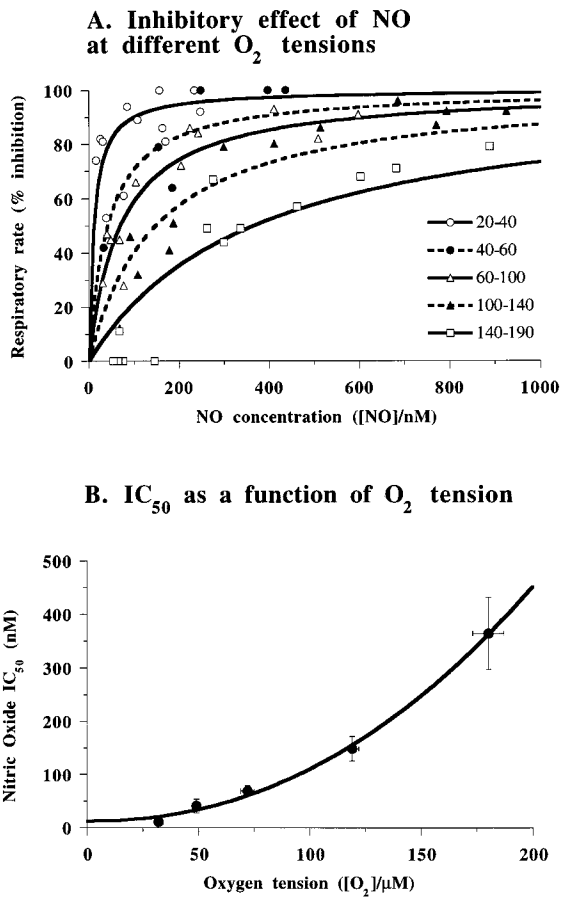

Fig. 2. The IC_{50} for NO inhibition of respiration. A: Dose-response curves for NO-induced inhibition of respiration at different O_2 concentrations. The data are from experiments as that exemplified in Fig. 1. For each NO addition, the corresponding O_2 tension was read off and binned into one of five O_2 tension groups (20–40, 41–60, 61–100, 101–140 and 141–190 μM O_2), the actual NO concentration was read off, and the relative inhibition was calculated as the decrease in respiratory rate between the rate before NO addition and the minimal rate after addition, expressed as a percentage of the rate before NO addition (control experiments had shown that the respiratory rate in the absence of NO was practically independent of O_2 tension down to very low O_2 values (<25 μM); cf. also Fig. 3A). The curves were drawn by best-fit analysis by the general curve-fit function of the KaleidaGraph application for Macintosh for a simple Michaelis-Menten equation, implying full inhibition at infinitely high NO concentrations (i.e. $y = 100 \cdot (x/(IC_{50}+x))$). The resulting data are presented in Table 1. B: The IC_{50} values as a function of O_2 tension. The values are from Table 1 and based on the data in A. The curve is drawn for best fit for a power function of the type $y = K_i \cdot (1+(x/K_m)^n)$. The resulting values were $K_i = 13 \pm 12$ nM, $K_m = 40 \pm 24$ μM and $n = 2.2 \pm 0.3$, with a correlation coefficient of 0.99. For n set at 2, the estimates were $K_i = 6 \pm 7$ and K_m 24 ± 13, with a correlation coefficient of 0.99.

pling protein (UCP), we performed control experiments in which the mitochondria were incubated in the presence of the artificial uncoupler FCCP; in these experiments any inhibitory effects on the uncoupling protein would be overrun by the artificial uncoupler. However, results very similar to those illustrated in Fig. 1 were obtained (not shown), indicating that the NO effects observed were entirely on the respiratory system.

3.2. The IC_{50} for NO inhibition of respiration

In Fig. 2A, results from a series of experiments such as those illustrated in Fig. 1 have been compiled. For each NO addition, the corresponding O_2 tension was read off and binned into one of five O_2 tension groups. Each point was then plotted in Fig. 2A. Two features are evident from Fig. 2A: there is a dose-dependent inhibitory effect of NO on brown fat mitochondrial respiration, and – in agreement with the example shown in Fig. 1 – the inhibitory effect of NO was dependent on the O_2 tension.

For each O_2 tension, the curves were initially analyzed as adhering to simple Michaelis-Menten kinetics (see, however, Section 3.4), with full inhibition occurring at infinitely high NO concentrations. The resulting inhibition curves are drawn in Fig. 2A, and the resulting data are compiled in Table 1. The calculated IC_{50} values were, as expected, lower at lower O_2 tensions. Thus, whereas at nearly air-saturated O_2 tensions (140–190 μM O_2) the IC_{50} was as high as 364 nM, at lower O_2 tensions (~50 μM) it was only 41 nM. A few estimates of IC_{50} values in other mitochondrial types have earlier been reported [1,6,8]; within experimental accuracy, the values obtained here with brown fat mitochondria are in agreement with these estimates.

In Fig. 2B, the IC_{50} values for NO from Table 1 are plotted against the actual mean O_2 tension. If NO was a simple competitor with O_2 at a single site, this representation would yield a straight line. However, as seen, the relationship is not linear. A good fit could, however, be obtained if a power function was used. The resulting power for best fit was 2.2 ± 0.3, i.e. very close to 2. As the suggested power function was so close to 2, the best fit analysis was repeated with a fixed power of 2. This also yielded a correlation coefficient of 0.99 (not shown). Thus, for any increase in O_2 tension, a quadratic increase in NO was necessary to maintain an equal degree of inhibition. Conversely, when the O_2 concentration is decreased, the NO inhibitory power is augmented in a quadratic way.

Based on the power function of this analysis, it could be suggested that the kinetics could be described as a competition between 1 O_2 and 2 NO molecules for a single binding site. In this context, it should be noted that the kinetics earlier described for NO and O_2 interaction at the binuclear center of cytochrome *c* oxidase implies that 1 O_2 competes with 2 NO for this site (see Section 1). The kinetics observed here in isolated mitochondria is therefore in very good agreement with such an interaction being the basis for the inhibition.

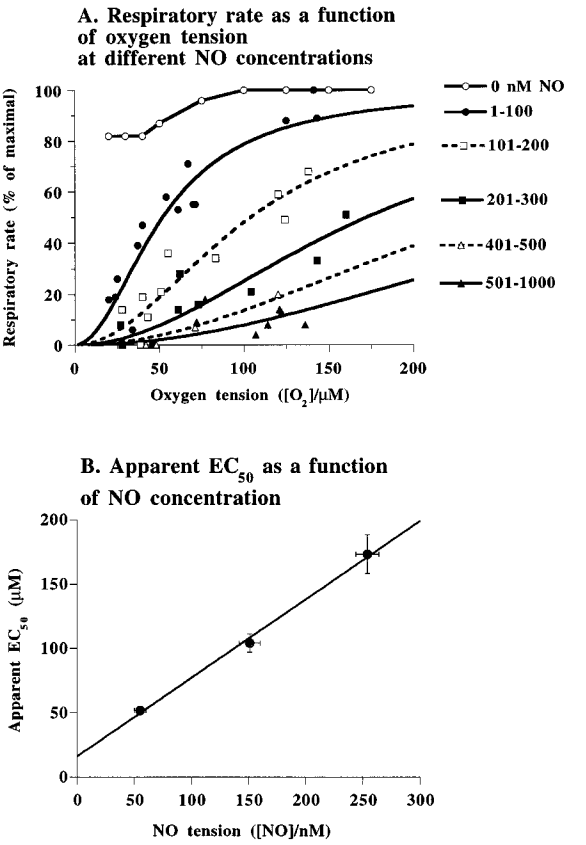

Fig. 3. A: Respiratory rate as a function of O_2 tension in the presence of different NO concentrations. The points are the same as those in Fig. 2A (except for a few outliers) but have been replotted here as a function of O_2 tension. The curves were drawn based on best-fit analysis by the general curve-fit function of the KaleidaGraph application for Macintosh for a Michaelis-Menten equation with a Hill coefficient of 2, implying full respiration at infinitely high O_2 concentrations (i.e. $y = 100 \cdot (x^2/(EC_{50}{}^2+x^2))$). The resulting values for apparent EC_{50} values were used for the plots in B. B: The apparent EC_{50} for O_2 as a function of NO concentration. The values for the apparent EC_{50} for the three lowest groups of NO concentration obtained from A were plotted against the mean NO concentration measured in each group. The line was drawn for best fit to the function $y = K_m \cdot (1+x/K_i)$. The resulting estimate of the K_m for O_2 was 16 ± 6 μM and of the K_i for NO was 27 ± 12 nM; the correlation coefficient was 0.99.

Table 1
IC_{50} for NO inhibition of mitochondrial respiration at different O_2 tensions

O_2 tension group	Actual mean O_2 tension (O_2^i) (μM)	IC_{50} (O_2^i) (nM)	*R*
20–40	32 ± 2	11 ± 3	0.33
41–60	49 ± 2	41 ± 13	0.87
61–100	72 ± 3	69 ± 10	0.92
101–140	119 ± 3	148 ± 23	0.92
140–190	180 ± 7	364 ± 67	0.90

The values are the results from the analysis of the data in Fig. 2A. The O_2 tension values are means ± S.E.M. from each group; the uncertainties given for the IC_{50} values and the correlation coefficients are those calculated by the best-fit program. The low correlation coefficient for the lowest O_2 tension group is due to the fact that even minute NO concentrations at this O_2 tension practically fully inhibited respiration, making it technically unfeasible to obtain full coverage of the inhibitory scale.

3.3. Estimation of the K_i for NO and the K_m of O_2 for the system

From the representation in Fig. 2B, values for the apparent K_m for O_2 and for the apparent K_i for NO can be obtained. Mainly because of the uncertainty introduced by the power function, the estimates for K_m or K_i based on this analysis were, however, rather imprecise ($K_i = 6 \pm 7$ nM and $K_m = 24 \pm 13$ μM; cf. legend to Fig. 2) and it was therefore considered necessary to make a second estimate of the parameters.

Therefore, the experimental data were replotted in Fig. 3A, with the respiratory rate – in the presence of different NO concentrations – as a function of O_2 tension. From this graph, it is clear that the interaction between O_2 and NO is of a competitive nature: with increasing O_2 tension, the inhibition caused by NO can be overcome; for the lowest NO concentrations (1–100 nM), the inhibition was fully eliminated at the highest O_2 tensions tested. It is also seen in the graph (curve 0 nM NO) that a direct estimate of the EC_{50} of O_2 for the reaction site cannot be made within this experimental system: it is below 30 μM.

When the points were analyzed for adherence to Michaelis-Menten kinetics, it was clear that simple kinetics (i.e. with a Hill coefficient of 1) did not give reasonable fits (not shown). When the points were instead analyzed with a free Hill coefficient, the resulting estimated Hill coefficients were close to 2 (not shown). The points were therefore finally analyzed for best fits to Michaelis-Menten kinetics with a Hill coefficient of 2; these are the curves plotted in Fig. 3A.

As the binuclear center in cytochrome *c* oxidase reacts with only one O_2 molecule (see Section 1), it may be considered surprising that the respiratory rate did not follow simple Michaelis-Menten kinetics (with a Hill coefficient of 1). However, in the present experiments, the O_2 competes with 2 NO. Thus, at a given NO concentration, the inhibitory power of the NO is diminished to a higher degree than the increase in O_2 tension, probably leading to the apparent cooperativity of O_2 in the stimulation of respiration.

The EC_{50} values from Fig. 3A for the three lowest NO concentrations (where reasonable estimates of EC_{50} values could be made) were plotted against the mean NO value measured in each group (Fig. 3B). As seen, the relationship was linear, and from this graph, K_i for NO and K_m for O_2 in the system could be obtained with reasonable precision: the K_i for NO was 27 ± 12 nM and the K_m for O_2 was 16 ± 6 μM. These values are also fairly close to those obtained from the preliminary analysis in Fig. 2.

3.4. The interaction between NO and O_2

The kinetics of a simple competition reaction can generally be described with the formula:

$$V = V_{max} \cdot (S/(K_m \cdot (1 + I/K_i) + S)) \quad (1)$$

Based on the two analyses above, the interaction between NO and O_2 in the control of respiration of these mitochondria can adequately be described with Eq. 1 in the following form:

$$V_{O_2}(O_2, NO) = V_{O_2}max \cdot ([O_2]^2/((16\ \mu M \cdot (1 + [NO]/27\ nM))^2 + [O_2]^2) \quad (2)$$

When this equation was used to predict the respiratory rate under conditions of different O_2 and NO tensions, values close to those experimentally obtained in Fig. 2A and 3A were obtained (not shown). It may be noted that the relationship in Fig. 2A between NO concentration and inhibition plotted with Eq. 2 will not follow simple Michaelis-Menten kinetics with a Hill coefficient of 1 (as that initially used for analysis); rather a positive cooperative relationship is obtained, which better fits the experimental points.

Eq. 2 thus probably is an adequate description of the interaction between NO and O_2, not only in the control of respiration in brown adipose tissue mitochondria, but – as this probably represents a general cytochrome *c* oxidase reaction – in any type of mitochondria. The formula has, however, limitations in that it predicts a Hill coefficient of 2 for the interaction of O_2 with cytochrome *c* oxidase, even in the absence of NO, and in that the predicted apparent affinity of the mitochondria for O_2 in the absence of NO is somewhat lower than that experimentally observed under such conditions (cf. e.g. Fig. 3A, curve 0 nM NO).

4. Discussion

4.1. The kinetics of O_2 and NO interaction in the control of mitochondrial respiration

In the present study, we have examined the kinetics of the inhibition by NO of respiration in isolated mitochondria. Brown fat mitochondria were used but there is no reason to expect that the behavior of other types of mitochondria would be qualitatively or quantitatively different.

The effects of NO observed here were reversible, and it is therefore likely that they represent effects of NO itself at the cytochrome *c* oxidase complex [25,26]. This localization of the effect is also supported by the competitiveness with O_2 which would not be expected at other sites in the respiratory chain. Inhibitory effects caused by NO treatment but being due to peroxynitrite formation would occur at other localizations in the respiratory chain but would not be reversible [25,26].

We found that the K_i of NO for inhibition of respiration was as low as ~27 nM but that the IC_{50} in the presence of O_2 increased with the square of the increase in O_2 tension. We estimated the K_m of O_2 for respiration in this system to be ~16 μM; this may be considered low in comparison with the K_D obtained for cytochrome *c* oxidase in direct studies of O_2 interaction (which is in the millimolar range [27]) but in the present studies, it is the kinetics of the entire respiratory system that is followed. Further, we found that in the presence of NO, the dependence of respiration on O_2 tension had a Hill coefficient of 2. The interaction between NO and O_2 in the control of respiration could thus be described by Eq. 2 above.

Qualitatively, the kinetics can be understood by the dual function of the binuclear reaction center in cytochrome *c* oxidase. This center may either reduce $O_2 \rightarrow 2H_2O$ or $2NO \rightarrow N_2O + H_2O$ (see Section 1), and the higher power kinetics can be explained by these alternative reactions. We have, however, not directly studied cytochrome *c* oxidase reactions in the present study, but rather the reaction in its biological context within the mitochondrion.

Quantitatively, we have found that the apparent affinities of the alternative substrates NO and O_2 are very different: the affinity of NO is three orders of magnitude higher than that of the normal substrate O_2.

Functionally, the kinetics is thus such that in the presence

of NO, respiration becomes much more sensitive to decreasing O_2 tension than would be initially supposed.

4.2. The physiological significance of the NO/O_2 interaction

Although it is clear from the results here, and those of others, that experimental conditions can be constructed in which NO significantly attenuates respiration, it may be discussed whether such conditions occur in vivo – i.e. whether a combination of a sufficiently high NO concentration with a sufficiently low O_2 tension occurs within tissues.

Concerning the NO concentration, tissue values quoted range from a few nM up to as much as 1000 nM, even in non-pathological cases (as detailed in e.g. [4]), i.e. exactly the range covered in the present investigation.

The O_2 tension in equilibrium with air is about 200 μM. The arterial tension values are somewhat lower, and tissue O_2 tension has been found to be between 30 and 60 μM [28], i.e. at the lower end of the range covered in the present investigation. Tension within cells may be even lower. Thus, probably only the left parts of the curves drawn in Fig. 3A cover an area of physiological interest. As seen, at these concentrations, the inhibitory potential of NO is very high. The suggestion would therefore be that respiration in many tissues is probably constantly attenuated by NO. A prediction of this tenet is that the total metabolism of an animal should be increased when NO synthase is inhibited. This has in fact been shown to be the case on addition of the NO synthase inhibitor nitro-L-arginine [29].

Whether the attenuating effect of NO on respiration is physiologically meaningful or not, is not easily answered. It may be argued that NO inhibition of respiration is meaningful in that it decreases O_2 utilization exactly when O_2 tensions are low, and O_2 should thus be 'protected'. However, in reality this is a form of self-strangulation that does diminish tissue O_2 consumption but – as electron transport is apparently slower with NO as the electron acceptor than with O_2 as acceptor – does so at the risk of mitochondrial de-energization [3,20]. Such a de-energization may have injurious effects on cells and organisms, e.g. due to Ca^{2+} release (which has been observed as an effect of NO treatment [20,30]). The realization that cytochrome *c* oxidase probably developed from NO reductase [15] may imply that the binding of NO to cytochrome *c* oxidase is simply an evolutionary relict, and that the resulting attenuation or inhibition of respiration is an effect to which organisms have adapted.

4.3. The significance of NO for non-shivering thermogenesis in brown adipose tissue

Although the kinetics described above is probably general and applicable to mitochondria from most sources, the possibility exists that the phenomenon analyzed is also of particular interest in relation to brown adipose tissue function (for review see [31]).

This is because the cells of this tissue – as earlier suggested [32] – possess NO synthase [33]. An increased expression of the NO synthase in vivo is induced in brown adipose tissue by cold exposure, and in vitro in cultured brown fat cells by noradrenaline treatment [33]. The NO synthase induced is of the i-type [33], which is generally thought to produce sustained micromolar NO concentrations [34]. It is thus very likely that brown fat cells – and hence their mitochondria – are exposed to NO at relatively high concentrations.

The interaction of NO with brown fat mitochondrial respiration described here should probably be understood in relation to other effects of NO in this tissue. Thus, e.g. in a situation in which tissue O_2 tension starts to become low, it may be advantageous for the tissue to direct the remaining O_2 towards the NO synthase instead of using it for respiration; such a redirection would be caused by the NO inhibition of respiration demonstrated here. The continued activity of the NO synthase would therefore lead to a self-promoting process, yielding increasing levels of NO in the tissue. Also the inhibitory effect of NO on the brown fat plasma membrane non-selective cation channels [32] may augment substrate supply to the NO synthase (as discussed elsewhere [35]). The blood flow to the tissue, and hence the O_2 supply, is under NO control [36,37]. Thus, as O_2 tension decreases, the resulting increased NO levels may increase blood flow to the tissue; tissue blood flow would therefore then be under metabolic control, as experimentally demonstrated by Ma and Foster [38]. This may be a self-balancing process, because oxyhemoglobin is thought to be the major mechanism for the inactivation of NO in vivo [39,40]. The increased blood flow caused by increasing levels of NO would therefore result in decreasing levels of NO and thus also alleviate the inhibition of respiration caused by NO.

The direct inhibitory effect of NO on respiration may also be discussed in two other connections. One is the absence of parasympathetic innervation in brown [41,42] and white [43] adipose tissue; only sympathetic nerves are found. Normally the sympathetic and parasympathetic systems have physiologically antagonistic effects but the NO produced upon adrenergic stimulation may perhaps be considered a substitute for the parasympathetic system in that it leads to self-regulation of the respiratory rate of the tissue. This may be also work in white adipose tissue which also possesses NO synthase [44].

The other connection is the hypersensitivity of non-shivering thermogenesis to hypoxia. It is clear from the above that a tissue becomes particularly sensitive to the inhibitory effects of NO when the O_2 tension in the tissue becomes very low. In experimental hypoxic conditions (~half normal O_2 tension), non-shivering thermogenesis is practically eliminated whereas basal metabolism is not much affected [45–47] (nor apparently is blood flow through brown adipose tissue [46]). It may therefore be suggested that the selective inhibition of non-shivering thermogenesis in the hypoxic state may be a consequence of an NO inhibition of brown fat mitochondrial respiration at low O_2.

Acknowledgements: The authors would like to thank Lars Hiertas Fund for the stipend to A.K. for the purchase of the ISO-NO electrode, Agneta Bergström for the isolation of mitochondria, and Barbara Cannon for valuable discussions. The study was supported by a grant from the Swedish Natural Science Research Council, including a postdoctoral fellowship to A.M.

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