J Bioenerg Biomembr (2007) 39:158–166
DOI 10.1007/s10863-007-9074-1

ORIGINAL PAPER

# Nitric oxide increases oxidative phosphorylation efficiency

**Pascaline Clerc · Michel Rigoulet · Xavier Leverve · Eric Fontaine**

Received: 23 January 2007 / Accepted: 8 March 2007 / Published online: 20 April 2007


**Abstract** We have studied the effect of nitric oxide (NO) and potassium cyanide (KCN) on oxidative phosphorylation efficiency. Concentrations of NO or KCN that decrease resting oxygen consumption by 10–20% increased oxidative phosphorylation efficiency in mitochondria oxidizing succinate or palmitoyl-L-carnitine, but not in mitochondria oxidizing malate plus glutamate. When compared to malate plus glutamate, succinate or palmitoyl-L-carnitine reduced the redox state of cytochrome oxidase. The relationship between membrane potential and oxygen consumption rates was measured at different degrees of ATP synthesis. The use of malate plus glutamate instead of succinate (that changes the $H^+/2e^-$ stoichiometry of the respiratory chain) affected the relationship, whereas a change in membrane permeability did not affect it. NO or KCN also affected the relationship, suggesting that they change the $H^+/2e^-$ stoichiometry of the respiratory chain. We propose that NO may be a natural short-term regulator of mitochondrial physiology that increases oxidative phosphorylation efficiency in a redox-sensitive manner by decreasing the slipping in the proton pumps.

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**Keywords** Mitochondria · Nitric oxide · Cyanide · Slipping · Leak · Oxidative phosphorylation

**Abbreviations** NO: Nitric oxide · DPTA-NONOate: dipropylenetriamine-NONOate · EDTA: ethylene-diaminetetraacetic acid · DNP: 2,4-dinitrophenol · Pi: inorganic phosphate

## Introduction

Mitochondrial respiration is characterized by the complete reduction of molecular oxygen to water. This process involves the generation of electrons from NADH or $FADH_2$, which are produced by the oxidation of respiratory substrates. Electrons from NADH and $FADH_2$ pass through the respiratory chain and are finally accepted by oxygen, releasing energy that is stored in the form of an electrochemical proton gradient across a membrane. The transmembrane movement of protons back into mitochondria through the ATP-synthase then results in the production of ATP from ADP plus phosphate. The apparent yield of oxidative phosphorylation (i.e., the ATP synthesis rate divided by the oxygen consumption rate) is, however, not constant, but changes according to (i) the respiratory substrates, (ii) the proton gradient-consuming processes that are not linked to ATP synthesis, and (iii) the intrinsic coupling (the $H^+/2e^-$ stoichiometry) of the proton pumps (Murphy, 1989; Kadenbach, 2003).

Because electrons from NADH pass through three proton pumps (namely, complexes I, III and IV), while electrons from $FADH_2$ only pass through two proton pumps (i.e., complexes III and IV), NADH-linked substrates (e.g., malate) generate more proton gradient per oxygen consumed than $FADH_2$-linked substrates (e.g., succinate). Consequently, the yield of oxidative phosphorylation is higher with NADH- than $FADH_2$-linked substrates (Hinkle, 2005).

The electrochemical proton gradient is not totally devoted to ATP synthesis, but also serves to drive transport systems (e.g., $Ca^{2+}$ cycling) via specific transporters (Bernardi, 1999). Moreover, protons can re-enter into mitochondria specifically via uncoupling proteins (Jezek et al., 2004; Krauss et al., 2005) or unspecifically via leaks in the inner membrane (Brand, 2000). The higher these ATP synthesis-independent proton gradient-consuming processes are, the lower the yield of oxidative phosphorylation.

The mechanism by which electron flux is coupled to proton pumping inside a proton pump is not well understood. It was initially proposed that the number of protons pumped per electron transferred (the $H^+/2e^-$ stoichiometry) was constant (Hinkle et al., 1991), as is the case for any chemical reaction. However, evidence suggests that electrons can pass through a proton pump in the absence of proton movement (Murphy, 1989; Kadenbach, 2003). The change in stoichiometry for complex IV (cytochrome oxidase) has been studied in detail (Azzone et al., 1985; Murphy and Brand, 1987; Murphy and Brand, 1988; Capitanio et al., 1991; Papa et al., 1991; Capitanio et al., 1996; Ferguson-Miller and Babcock, 1996). Electron transfer through cytochrome oxidase leads to conformational changes in the proton pathway that then permits the transfer of protons against their gradient (Namslauer and Brzezinski, 2004). Thus, if electron transfer is mandatory for proton pumping, the latter is not the case for electron transfer. Indeed, several types of mutant cytochrome oxidase do not pump protons, but still transfer electrons (Namslauer and Brzezinski, 2004).

Research into the field of oxidative phosphorylation has primarily been undertaken during the second half of the last century. Many authors have reported experimental conditions, physiopathological situations or drugs that decrease oxidative phosphorylation efficiency. Much less is known about conditions in which oxidative phosphorylation efficiency is increased. In yeast mitochondria, oxidative phosphorylation efficiency increases when electron flux decreases because of a kinetic constraint on the provision of respiratory substrates (Rigoulet et al., 1993; Fitton et al., 1994). In rat liver mitochondria, either hypothyroidism (Nogueira et al., 2002) or chronic ethanol ingestion (Piquet et al., 2000) increased oxidative phosphorylation efficiency when compared with control animals. In a recent study, we have shown that oxidative phosphorylation efficiency correlates with cytochrome oxidase content (Nogueira et al., 2001). This led us to propose the existence of a compromise between flux and efficiency: high fluxes of ATP production are obtained at low yields and vice versa.

Nitric oxide (NO) is an endogenous short-lived free radical involved in numerous pathophysiological processes. NO has cardioprotective effects (Herman and Moncada, 2005; Naseem, 2005) and is also a neurotransmitter acting on normal cerebral activities (Duncan and Heales, 2005). However, excessive NO production has been implicated in central nervous system diseases (Alzheimer's disease, Parkinson's disease and multiple sclerosis) (Duncan and Heales, 2005), whereas the deregulation of endothelial NO bioavailability has been reported in a number of vascular pathologies (atherosclerosis, hypertension and septic shock) (Naseem, 2005).

NO and its derivatives are known to inhibit mitochondrial respiration (Brown, 1999). At physiological concentrations (nanomolar range), NO reversibly inhibits cytochrome oxidase in competition with oxygen. Higher (non-physiological) concentrations of NO lead to peroxinitrite production that, in turn, irreversibly inhibits complexes I (NADH oxidase) and II (Succinate deshydrogenase).

NO is formed in mammalian tissues by the enzymatic oxidation of L-arginine to citrulline (Palmer et al., 1988) via a family of NO synthase isoenzymes (Stuehr, 1999; Alderton et al., 2001). The mitochondrial NO synthase isoform is constitutively expressed and membrane-bound (Haynes et al., 2003). Recent evidence suggests that mitochondrial NO synthase physically interacts with cytochrome oxidase (Persichini et al., 2005).

Given the known effect of NO on cytochrome oxidase and the importance of cytochrome oxidase in oxidative phosphorylation efficiency, we studied the effect of NO on oxygen consumption, ATP synthesis and electrical membrane potential in isolated rat liver mitochondria. We confirm that NO induces a kinetic constraint on cytochrome oxidase, but found that this kinetic constraint decreased oxygen consumption more than ATP synthesis, resulting in an increase in oxidative phosphorylation efficiency.

## Materials and methods

Rats were killed by cervical dislocation and liver mitochondria were prepared according to standard differential centrifugation procedures in a medium containing 250 mM sucrose, 20 mM Tris-HCl (pH 7.2) and 1 mM EGTA. Experiments were carried out at 37°C in a medium containing 125 mM KCl, 5 mM Pi, 1 mM EGTA, 20 mM Tris-HCl (pH 7.2), supplemented with various respiratory substrates and inhibitors as indicated in the figure legends.

Mitochondrial oxygen consumption was measured polaro graphically using a Clark-type oxygen electrode. Electrical membrane potential was measured fluorimetrically in the presence of 0.2 $\mu$M rhodamine 123, as described in (Emaus et al., 1986), with a PTI Quantamaster C61 spectrofluorimeter (excitation-emission: 503–525 nm). The rate of ATP synthesis was followed by glucose 6-phosphate accumulation, as previously described (Fontaine et al., 1997), using an ADP regenerating system containing 20 mM glucose, 125 $\mu$M ATP, 1 mM $MgCl_2$ and increasing concentrations of hexokinase.

NO was produced directly in the incubation medium by spontaneous degradation of DPTA-NONOate. Because NO is a gas that can diffuse out of the medium, all the experiments were performed in closed chambers. In such conditions, NO accumulates with time (Hakim et al., 1996), which may affect its concentration during the experiment. To minimize such variations, a low concentration of DPTA-NONOate was used in order to decrease the rate of NO production, the required concentration of NO being obtained by changing the incubation time before starting the experiment. Surprisingly, we found that DPTA-NONOate did not affect the oxygen consumption rate at the concentration used in the presence of $MgCl_2$. Because $MgCl_2$ did not restore a normal respiratory rate previously inhibited by DPTA-NONOate, we concluded that $MgCl_2$ decreases NO production from DPTA-NONOate. Consequently, $MgCl_2$ was always added after the expected NO concentration had been reached.

Cytochrome redox status was measured at 37°C using an Analytik Jena Specord 210 double beam spectrophotometer. Cytochrome spectrums in the presence of respiratory substrate were compared to the spectrum of cytochrome fully oxidized by the addition of 0.06% of $H_2O_2$. The following wavelength pairs and absorption coefficients were used: cytochrome $c + c1$ (550–540 nm) $\varepsilon = 18\,mM^{-1}\,cm^{-1}$, cytochrome $b$ (563–575 nm) $\varepsilon = 18\,mM^{-1}\,cm^{-1}$, and cytochrome $a + a3$ (605–630 nm) $\varepsilon = 24\,mM^{-1}\,cm^{-1}$ (Ohnishi et al., 1966; Vanneste, 1966).

DPTA-NONOate was purchased from Alexis Biochemical; hexokinase, ATP, glucose-6-phosphate dehydrogenase and NAD were from Roche Diagnostic; sucrose and KCN were from Merck; rhodamine 123 was from Molecular Probes; Pi was from Carlo Erba; HCl was from Acros organics; KCl was from Prolabo, while the remaining compounds were from Sigma-Aldrich.

## Results

### Effects of NO or KCN in non-phosphorylating mitochondria

In the experiment in Fig. 1, we studied the effect of 3 respiratory chain inhibitors (namely, malonate, KCN and NO) on the relationship between the rate of oxygen consumption and electrical membrane potential in non-phosphorylating mitochondria energized with succinate. As expected (Nicholls, 1974), this relationship was not linear, indicating that the amount of oxygen consumed was not proportional to the membrane potential sustained.

Because proton efflux and proton influx are the same under steady state conditions, two mechanisms have been proposed to account for this observation (Murphy, 1989). (i) Assuming that respiratory chain stoichiometry is constant, this observation suggests that ATP synthesis-independent proton gradient-consuming processes (e.g., proton leak) are not proportional to membrane potential. With regard to electricitical activity, it has been proposed that membrane conductivity increases at high potentials. (ii) Assuming that membrane conductivity is constant (i.e., that the proton leak linearly increases with membrane potential), this observation suggests that the $H^+/2e^-$ stoichiometry of the respiratory chain changes with membrane potential. With regard to mechanical activity, it has been proposed that proton pumps had "slipped" (i.e., that electrons pass through a pump without proton transfer). It must be noted that these two mechanisms are not mutually exclusive, and that both are sustained by experimental data (Murphy, 1989; Kadenbach, 2003).

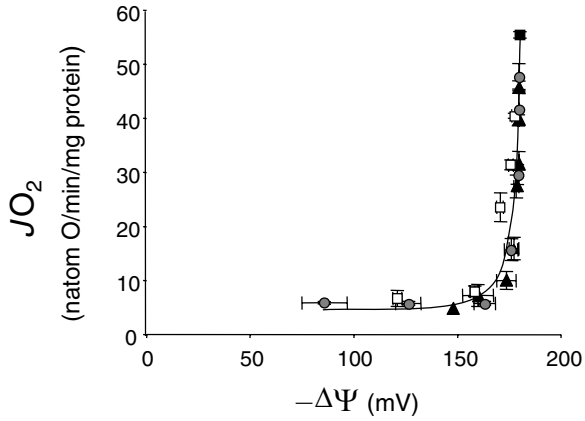


**Fig. 1** Relationship between oxygen consumption rate and electrical membrane potential in non-phosphorylating mitochondria. The incubation medium contained 125 mM KCl, 1 mM EGTA, 20 mM Tris-Hcl, 5 mM Tris-Pi, 1.25 $\mu$g/mg protein oligomycin, 0.2 $\mu$M rhodamine 123, 5 mM succinate-Tris and 1.25 $\mu$M rotenone. The final volume was 2 ml, pH 7.2, 37°C. Experiments were started by the addition of 2 mg mitochondria in the presence of increasing amounts of malonate (open symbols), KCN (closed symbols) or DPTA-NONOate (gray symbols). Oxygen consumption rate and electrical membrane potential were measured in parallel experiments. Results are mean ± SEM, $n = 3$. The closed square denotes the results obtained before the addition of inhibitors ($n = 9$)

Regardless of the mechanism(s), the fact that NO or KCN decreased oxygen consumption without any measurable effect on membrane potential strongly suggested that they decreased energy waste. Therefore, the question arose as to whether they also decreased oxygen consumption during ATP synthesis.

### Effects of NO or KCN during oxidative phosphorylation

In the experiments in Fig. 2, mitochondria were incubated in the presence of ATP, glucose, $Mg^{2+}$ and phosphate. After the addition of hexokinase, ATP and glucose were converted into glucose-6-phosphate and ADP, which, in turn, were converted to ATP by mitochondria. Depending

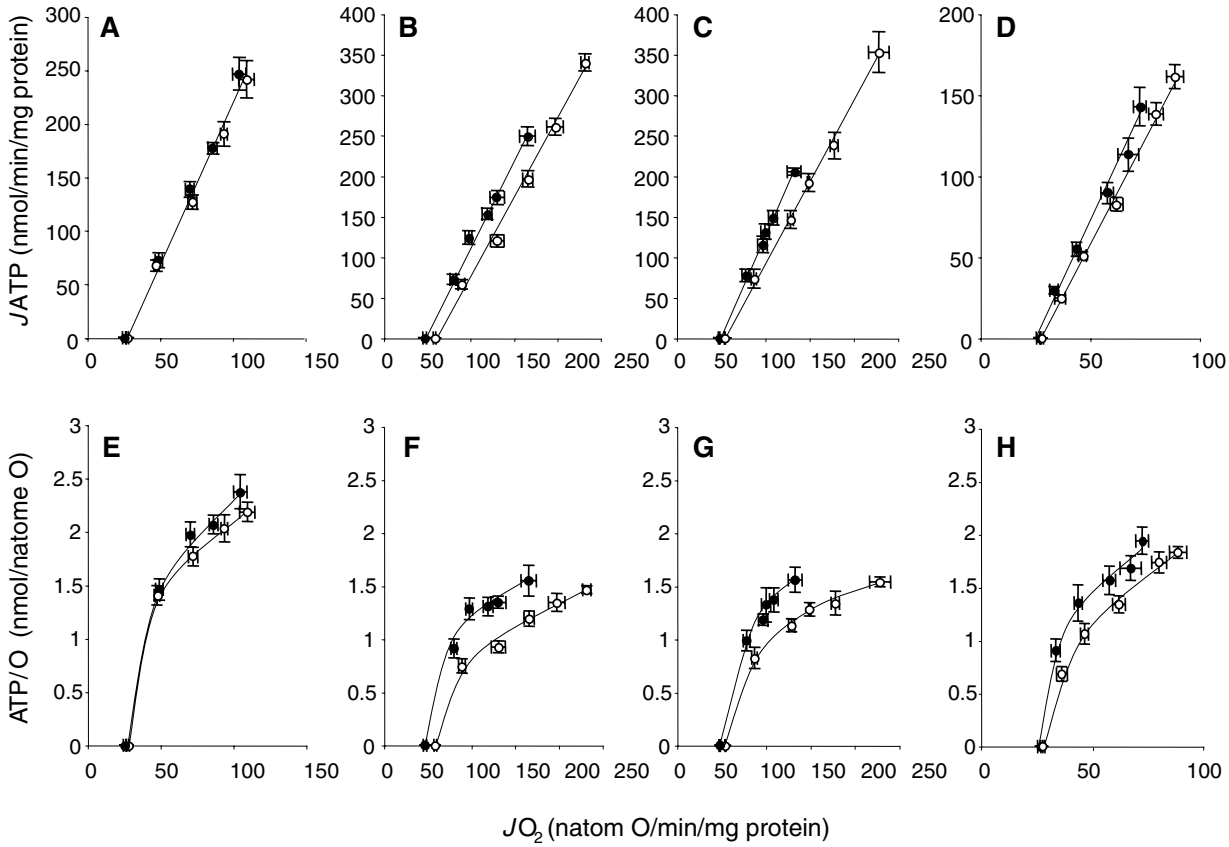


**Fig. 2** Effect of nitric oxide on oxidative phosphorylation efficiency. The incubation medium contained 125 mM KCl, 1 mM EGTA, 20 mM Tris-HCl, 5 mM Tris-Pi, 20 mM glucose, 125 $\mu$M ATP. The medium was supplemented with 5 mM glutamate-Tris plus 2.5 mM malate-Tris (panels A and E), 5 mM succinate-Tris plus 1.25 $\mu$M rotenone (panels B and F), 5 mM succinate-Tris, 5 mM glutamate-Tris and 2.5 mM malate-Tris (panels C and G) or 50 $\mu$M palmitoyl-L-carnitine (panels D and H). Experiments were started by the addition of 10 $\mu$M DPTA-NONOate (closed symbols) or vehicle (open symbols) followed 15 min later by the addition of 1 mM $MgCl_2$ and 3 mg mitochondria. Oxygen consumption rate and ATP synthesis were measured in the presence of increasing concentrations of hexokinase (0–3 U). The final volume was 2 ml, pH 7.2, 37°C. Results are mean ± SEM, $n = 10$ (A, E), $n = 12$ (B, F), $n = 4$ (C, G), $n = 7$ (D, H)

on hexokinase concentrations, different rates of oxygen consumption and glucose-6-phosphate production were reached in steady-state concentrations of ATP and ADP (Fontaine et al., 1997), implying that the rate of glucose-6-phosphate production was equivalent to the rate of ATP synthesis.

As previously reported, the relationship between oxygen consumption and the rate of ATP synthesis was linear when mitochondria were energized with NADH-linked (panel A) or $FADH_2$-linked (panel B) substrates. This relationship also remained linear when mitochondria were energized with a combination of NADH-plus $FADH_2$-linked substrates (panels C and D). Consequently, the yield of oxidative phosphorylation (i.e., the ATP/O ratio) was not constant, but increased with its rate (panels E, F, G and H). As expected, the ATP/O ratio for a given rate of oxygen consumption was always higher when mitochondria were energized with NADH-linked substrates than when mitochondria were energized with $FADH_2$-linked substrates (Hinkle, 2005).

Surprisingly, when mitochondria were incubated in the presence of malate (plus glutamate) and succinate, the yield of oxidative phosphorylation remained close to that observed with succinate alone (compare panels F and G), suggesting that malate was not oxidized. On the other hand, when mitochondria were energized with palmitoyl-L-carnitine (whose metabolism necessarily produces both NADH and $FADH_2$), the yield of oxidative phosphorylation was higher than in the presence of succinate (compare panels F and H) but lower than in the presence of malate plus glutamate (compare panels E and H).

When mitochondria energized with $FADH_2$-linked substrates (panels B, C and D) were incubated in the presence of a low concentration of NO that decreased resting respiration in the presence of succinate by 20%, the relationship between oxygen consumption and ATP synthesis remained linear, but was shifted to the left. On one hand, mild respiratory inhibition led to a decrease in the maximal rate of ATP synthesis (panels B, C and D). On the other hand, for a given

oxygen consumption rate, it increased the yield of oxidative phosphorylation (panels F, G and H).

As shown in panel A, the same concentration of NO was much less effective at inhibiting respiration when mitochondria were energized with malate plus glutamate, and did not affect the rate of ATP synthesis. Consequently, NO did not affect the yield of oxidative phosphorylation in that particular condition (panel E).

In order to understand whether the observed increase in oxidative phosphorylation efficiency was a direct effect of NO or was a consequence of cytochrome oxidase inhibition, we performed the same experiment in the presence of cyanide. As shown in Fig. 3, 15 $\mu$M KCN increased the ATP/O ratio for a given rate of oxygen consumption when mitochondria were energized with succinate or palmitoyl-L-carnitine (panels E and F), whereas it did not affect oxidative phosphorylation efficiency when mitochondria were energized with malate plus glutamate alone (panel D).

Taken together, the results from Figs. 2 and 3 indicate that a slight kinetic constraint on cytochrome oxidase led to a *decrease* in maximal ATP synthesis capacity, and to an *increase* in oxidative phosphorylation efficiency when mitochondria were incubated in the presence of $FADH_2$-linked substrates.

## Mechanism of action

As shown in Fig. 4, the addition of DNP that increases proton leak leads to a decrease in oxidative phosphorylation efficiency. Theoretically, a putative decrease in proton leak would increase oxidative phosphorylation efficiency. As expected, the oxidation of NADH-linked (instead of $FADH_2$-linked) substrates, which increases the $H^+/2e^-$ stoichiometry of the respiratory chain, led to an increase in oxidative phosphorylation efficiency. Therefore, the experiment in Fig. 4 did not discriminate an increase in respiratory chain stoichiometry from a decrease in proton leak.

In the experiment shown in Fig. 5, we studied the relationship between oxygen consumption and membrane potential at different levels of ATP synthesis. As expected, the addition of hexokinase (i.e., the stimulation of ATP production) decreased the membrane potential, which in turn increased the consumption of oxygen. Interestingly,the addi-

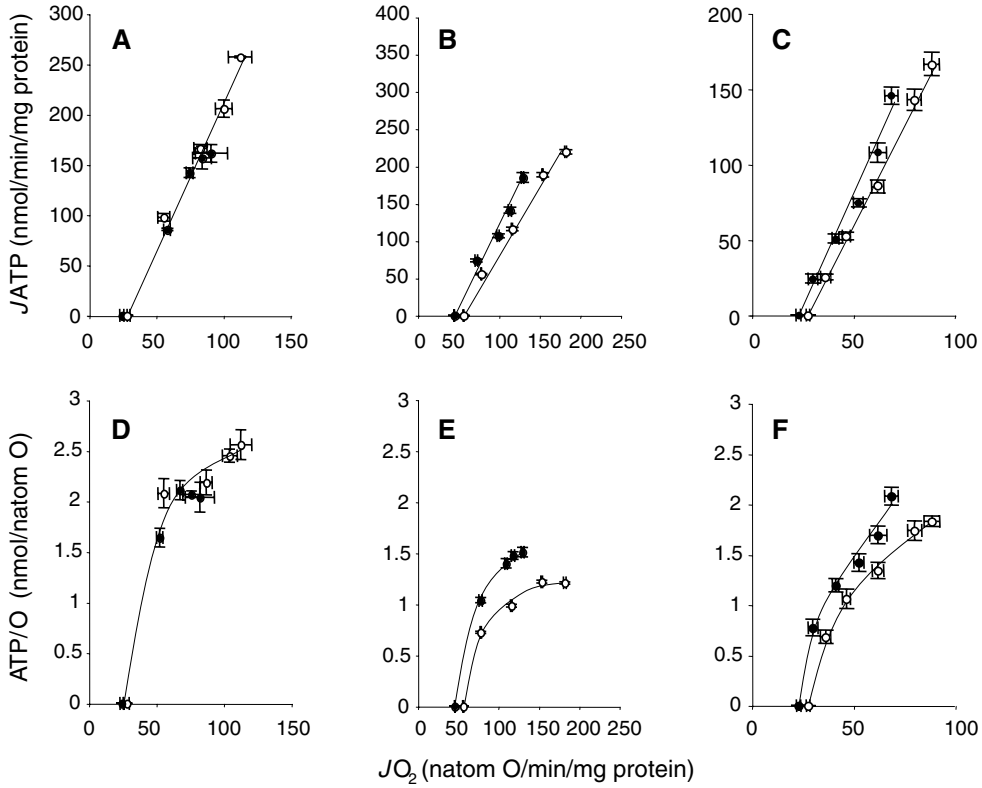


**Fig. 3** Effect of cyanide on the oxidative phosphorylation efficiency. Experimental conditions were the same as in Fig. 2. The medium was supplemented with 1 mM $MgCl_2$ and either 5 mM glutamate-Tris plus 2.5 mM malate-Tris (panels A and D), 5 mM succinate-Tris plus 1.25 $\mu$M rotenone (panels B and E) or 50 $\mu$M palmitoyl-L-carnitine (panels C and F). Experiments were started by the addition of 3 mg mitochondria in the absence (open symbols) or presence (closed symbols) of 15 $\mu$M KCN. Oxygen consumption rate and ATP synthesis were measured in the presence of increasing concentrations of hexokinase (0–3 U). The final volume was 2 ml, pH 7.2, 37°C. Results are mean ± SEM, $n = 3$ (panels A and D), $n = 10$ (panels B and E), $n = 7$ (panels C and F)

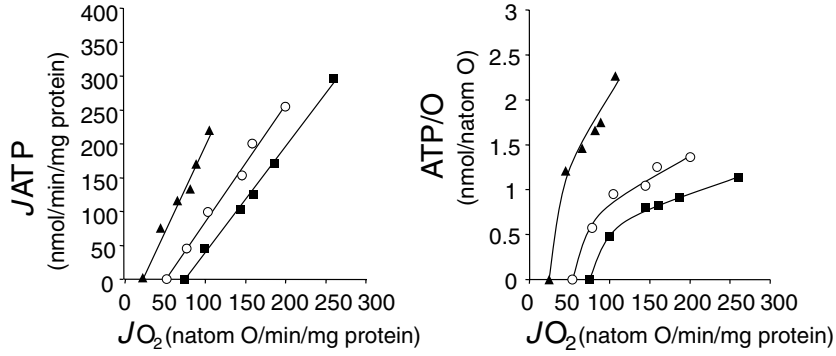


**Fig. 4** Effect of a change in respiratory chain stoichiometry and of proton leak on oxidative phosphorylation efficiency. Experimental conditions were the same as in Fig. 2. The medium was supplemented with 1 mM $MgCl_2$. Experiments were started by the addition of 2 mg mitochondria in the presence of 5 mM glutamate plus 2.5 mM malate (closed triangles), 5 mM succinate plus 1.25 $\mu$M rotenone (open circles) or 5 mM succinate, 1.25 $\mu$M rotenone and 2.5 $\mu$M DNP (closed squares). The final volume was 2 ml, pH 7.2, 37°C. Oxygen consumption rate and ATP synthesis were measured in the presence of increasing concentrations of hexokinase (0–3 U)

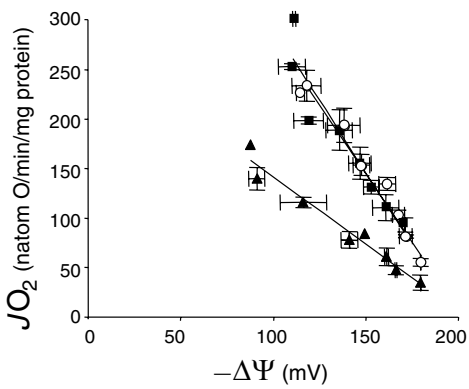


**Fig. 5** Effect of a change in respiratory chain stoichiometry and of proton leak on the relationship between oxygen consumption rate and electrical membrane potential in phosphorylating mitochondria. Experimental conditions were the same as in Fig. 4, except that the medium was supplemented with 0.2 $\mu$M rhodamine 123. Experiments were started by the addition of 3 mg mitochondria in the presence of 5 mM glutamate-Tris plus 2.5 mM malate-Tris (closed triangles), 5 mM succinate-Tris plus 1.25 $\mu$M rotenone (open circles) or 5 mM succinate-Tris, 1.25 $\mu$M rotenone and 2.5 $\mu$M DNP (closed squares). The final volume was 2 ml, pH 7.2, 37°C. Oxygen consumption rate and electrical membrane potential were measured in parallel experiments in the presence of increasing concentrations of hexokinase (0–3 U). Results are mean ± SEM, $n = 3$

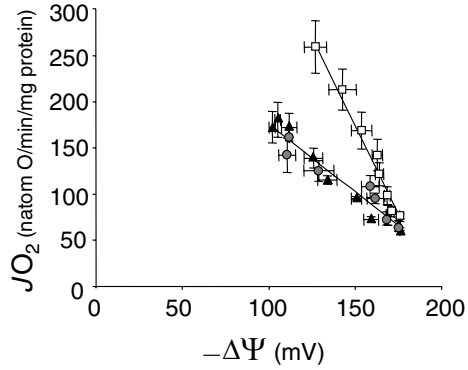


**Fig. 6** Effect of nitric oxide or cyanide on the relationships between oxygen consumption rate and electrical membrane potential in phosphorylating mitochondria. Experimental conditions were the same as in Fig. 5. The medium was supplemented with 5 mM succinate-Tris plus 1.25 $\mu$M rotenone. Experiments were started by the addition of 10 $\mu$M DPTA-NONOate (gray symbols), 15 $\mu$M KCN (closed symbols) or vehicle (open symbols) followed 15 min later by the addition of 1 mM $MgCl_2$ and 3 mg mitochondria. The final volume was 2 ml, pH 7.2, 37°C. Oxygen consumption rate and electrical membrane potential were measured in parallel experiments in the presence of increasing concentrations of hexokinase (0–3 U). Results are mean ± SEM, $n = 3$

tion of DNP that increased proton leak affected both oxygen consumption and membrane potential in the resting state, but did not affect the relationship between these two parameters. This kind of relationship was dramatically changed by the nature of the respiratory substrate: the oxygen required to maintain a given membrane potential being lower when NADH-linked substrates were oxidized (i.e., when respiratory chain stoichiometry was increased). As shown in Fig. 6, both NO and KCN dramatically changed such a relationship, suggesting that a kinetic constraint on cytochrome oxidase increases respiratory chain stoichiometry when succinate is oxidized.

In order to establish a basis for the observed lack of effect of NO or KCN on oxidative phosphorylation efficiency when $FADH_2$-linked substrates are not oxidized, we compared the redox status of the respiratory chain according to the type of respiratory substrates. As shown in Fig. 7, the absence of $FADH_2$-linked substrates decreased the amount of reduced cytochrome a + a3, whereas cytochromes b and c + c1 were not affected by the respiratory substrates. Note that at the concentration used, neither NO or KCN affected the redox status of the respiratory chain (data not shown).

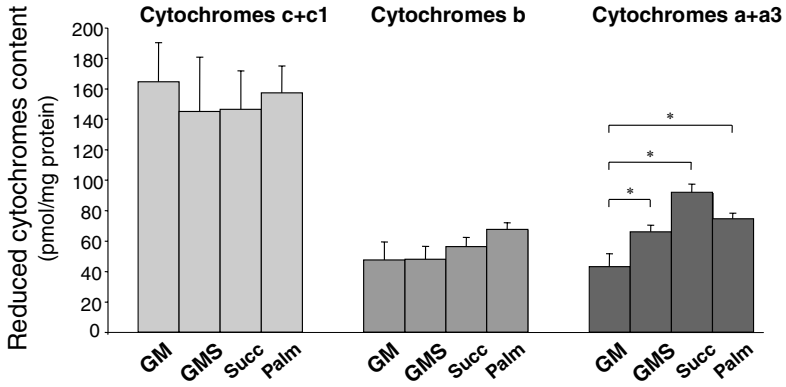


**Fig. 7** Effect of respiratory substrates on the redox state of the respiratory chain. The incubation medium contained 125 mM KCl, 1 mM EGTA, 20 mM Tris-HCl, 5 mM Tris-Pi. The medium was supplemented with 5 mM glutamate-Tris plus 2.5 mM malate-Tris (GM; $n = 4$), 5 mM succinate-Tris plus 1.25 $\mu$M rotenone (Succ; $n = 7$), 5 mM succinate-Tris and 5 mM glutamate-Tris plus 2.5 mM malate-Tris (GMS; $n = 4$) or 50 $\mu$M palmitoyl-L-carnitine (Palm; $n = 3$). Experiments were started by the addition of 5 mg mitochondria. The final volume was 1 ml, pH 7.2, 37°C. The amounts of reduced cytochromes were measured as described in 'Material and Methods' by comparing cytochromes spectrum in the presence of respiratory substrates before and after $H_2O_2$ addition. Results are mean ± SEM, $^*p < 0.05$, student's $t$ test

## Discussion

In this paper we show that a slight inhibition of cytochrome oxidase restricts maximal ATP synthesis capacity, while it decreases energy waste and increases oxidative phosphorylation efficiency when $FADH_2$-linked substrates are oxidized.

Schematically, two main mechanisms can account for a decrease in energy waste: a decrease in proton gradient consuming processes, or an increase in respiratory chain efficacy. In order to differentiate between these two mechanisms, one must take into account that the decrease in energy waste observed in this work has three characteristics: (i) it greatly depends on the nature of the respiratory substrates (Figs. 2 and 3); (ii) it affects the relationship between oxygen consumption and membrane potential in phosphorylating conditions (Fig. 6); and (iii) it does not depend on the membrane potential (see below).

In the absence of ATP synthesis, all the oxygen is consumed in the generation of the proton gradient, and the ATP/O is equal to zero. During ATP synthesis, part of the oxygen consumed remains dedicated to processes unrelated to ATP synthesis, but its proportion in the total oxygen consumption decreases when ATP synthesis increases. From the result in Fig. 1, one would expect that the oxygen consuming processes unrelated to ATP synthesis decrease with membrane potential. Note, however (see Figs. 2 and 3), that there was no convergence in the relationship between ATP synthesis and oxygen consumption in the presence or absence of NO or KCN when ATP synthesis and respiration increased (i.e., when membrane potential decreases). This implies that the oxygen consuming processes unrelated to ATP synthesis did not decrease with membrane potential in phosphorylating conditions. In other words, the oxygen consuming processes unrelated to ATP synthesis observed in Fig. 1 (under non-phosphorylating conditions) cannot simply be extrapolated under phosphorylating conditions (see also Fontaine et al., 1997).

Remarkably, the relationship between oxygen consumption and membrane potential in phosphorylating conditions was not affected by an increase in proton leak (Fig. 5). Under such conditions, for a given oxygen consumption rate, the membrane potential remained the same (Fig. 5), whereas ATP synthesis and ATP/O decreased (Fig. 4). In the absence of methods known to decrease proton leak, one can only speculate that a putative decrease in proton leak would not affect the relationship between oxygen consumption and membrane potential (i.e., that for a given oxygen consumption rate, membrane potential would remain the same but ATP synthesis and ATP/O would increase).

By contrast, an increase in respiratory chain efficiency (due to the oxidation of NADH-linked instead of $FADH_2$-linked substrates) affected the relationship between oxygen consumption and membrane potential in phosphorylating conditions (Fig. 5). Under such conditions, for a given oxygen consumption rate, the membrane potential decreased when the respiratory chain efficiency increased (Fig. 5), but ATP synthesis and ATP/O increased (Fig. 4).

As shown in Fig. 6, NO or KCN also affected the relationship between oxygen consumption and membrane potential in phosphorylating conditions when succinate was oxidized. Under such conditions, for a given oxygen consumption rate, NO or KCN decreased membrane potential (Fig. 6), but increased ATP synthesis and ATP/O (Figs. 2 and 3). Taken together, these observations strongly suggest that NO or KCN increased respiratory chain efficiency.

A large body of evidence indicates that cytochrome oxidase is able to slip (Azzone et al., 1985; Murphy and Brand,

1987; Murphy and Brand, 1988; Capitanio et al., 1991; Papa et al., 1991; Capitanio et al., 1996; Ferguson-Miller and Babcock, 1996). Therefore, we propose that NO or KCN, which inhibit cytochrome oxidase, lead to a decrease in cytochrome oxidase slippage.

Results in Fig. 7 indicate that the nature of the respiratory substrates dramatically affects the redox state of cytochrome oxidase (cytochrome a + a3). Note that the resting oxygen consumption rate in the presence of malate plus glutamate was equal to that in the presence of palmitoyl-L-carnitine (see Figs. 2 and 3), whereas the redox state of cytochrome oxidase was dramatically different (Fig. 7). This observation suggests that the redox state of the cytochrome oxidase does not simply correlate with its activity, but depends on the respiratory substrates *per se*. Although the molecular basis for this observation remains to be solved, it is striking to note that NO or KCN increased oxidative phosphorylation efficiency only when cytochrome oxidase was in a reduced state (compare Figs. 2, 3 and 7).

Because proton leak is not expected to depend on the redox state of the respiratory chain, whereas it is conceivable that the slippage of cytochrome oxidase depends on its redox state, we hypothesized that cytochrome oxidase did not slip in the presence of malate plus glutamate alone. This proposal easily accounts for the lack of effect of NO and KCN on oxidative phosphorylation efficiency in that particular condition.

An increase in oxidative phosphorylation efficiency has been previously reported during chronic ethanol ingestion or hypothyroidism (Piquet et al., 2000; Nogueira et al., 2002). In both cases, a change in cytochrome oxidase has been implicated. Indeed, chronic ethanol ingestion leads to a decrease in cytochrome oxidase activity (Piquet et al., 2000), while hypothyroidism is characterized by a dramatic decrease in cytochrome oxidase content (Nogueira et al., 2002). Conversely, hyperthyroidism or polyunsaturated fatty acid deficiency, which are characterized by an increase in cytochrome oxidase content, lead to a decrease in oxidative phosphorylation efficiency (Nogueira et al., 2001).

Beside such long-term regulations, this work reports that acute redox-sensitive cytochrome oxidase inhibition leads to a short-term increase in oxidative phosphorylation efficiency. Obviously, cyanide cannot be regarded as a physiological compound. On the other hand, NO is an endogenous compound that may physiologically increase oxidative phosphorylation efficiency in a reversible manner. In supporting a physiological role for NO in the improvement of energy metabolism, it has been reported that NO synthase inhibition in guinea pig hearts led to an increase in myocardial oxygen consumption with no difference in ATP synthesis or cardiac performance (Shen et al., 2001).

The location of mitochondrial NO synthase in close proximity to one of the target sites of NO (cytochrome oxidase) further suggests that NO is a physiological regulator of cytochrome oxidase. Deletion of the domain that anchors NO synthase in the mitochondrial outer membrane has been shown to increase oxygen consumption in HUVEC cells (Gao et al., 2004), confirming the importance of NO production in proximity to cytochrome oxidase. However, the question arises as to whether the inhibition of oxidative phosphorylation may be beneficial to a cell. Until now, all the situations that led to an increase in oxidative phosphorylation efficiency have been characterized by a restriction in maximal ATP synthesis capacity. In metabolic situations that require high levels of ATP synthesis, this may be deleterious. However, this may be helpful when the provision of oxygen limits ATP synthesis.

**Acknowledgements** This work was supported by Grants from INSERM and the Ministère de l'Enseignement de la Recherche et de la Technologie (MERT). We thank Gareth Butt for his help in correcting the manuscript.

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