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NITRIC OXIDE POTENTLY AND REVERSIBLY DEENERGIZES MITOCHONDRIA AT LOW OXYGEN TENSION

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Nitric oxide (nitrogen monoxide, NO) at low concentrations can potently deenergize isolated liver and brain mitochondria at oxygen concentrations that prevail in cells and tissues. Deenergization is observed when mitochondria utilize respiratory substrates such as pyruvate *plus* malate, succinate, or ascorbate *plus* tetramethylphenylenediamine, but not when mitochondria are energized with ATP. The extent and duration of deenergization is determined by the concentration of NO and oxygen, and the kind of respiratory substrate. The NO-induced changes of the mitochondrial energy state are transient and are paralleled by release and reuptake of mitochondrial calcium. These findings reveal a direct action of NO on the mitochondrial respiratory chain and suggest that NO exerts some of its physiological and pathological effects by deenergizing mitochondria.

Nitric oxide (nitrogen monoxide, NO) evokes many cellular responses. It acts physiologically as neurotransmitter, regulator of blood pressure, inhibitor of platelet aggregation, and modulator of immunity and inflammation, but can also be cytotoxic, particularly at elevated concentrations [1-3]. Its targets are heme proteins, sulfhydryl-containing compounds, iron-sulfur complexes, and nucleic acids. Indirect evidence has been provided that mitochondrial functions can be affected by NO. Thus, studies with cell cultures kept at ambient oxygen tensions suggested NO-dependent inhibition of aconitase, impaired mitochondrial respiration at sites I and II, and possibly IV [4-6], and decrease of mitochondrial membrane potential ($\Delta\Psi$) as measured by rhodamine 123 fluorescence [7].

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ABBREVIATIONS:
EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; ($\Delta\Psi$), mitochondrial membrane potential; TMPD, tetramethylphenylenediamine.

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We show here that in isolated mitochondria incubated at physiological cellular oxygen tensions NO at physiological concentrations can potently and reversibly lower $\Delta\Psi$. Deenergization is observed when mitochondria are energized with respiratory substrates, but not when they are energized by ATP hydrolysis. Changes in $\Delta\Psi$ are paralleled by release and uptake of mitochondrial Ca^{2+}. These findings are relevant for a better understanding of nitric oxide's biology.

MATERIALS AND METHODS

Materials. Nitric oxide gas (98.5% pure) was obtained from Aldrich, Milwaukee, WI.. All other chemicals were purchased from standard suppliers, and were of the highest purity commercially available.

Isolation of mitochondria. Female Wistar rats of 180g body weight were used throughout. The isolation of liver mitochondria was performed by differential centrifugation [8]. Brain mitochondria were purified in a Ficoll gradient [9]. The protein content was determined by the Biuret method with bovine serum albumin as standard.

Standard incubation procedure. Mitochondria (1 mg of protein/ml) were incubated at 25°C with continuous stirring in 3 ml of buffer (210 mM mannitol, 70 mM sucrose, 5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.2).

Determination of Ca^{2+} uptake and release by mitochondria. Ca^{2+} movements across the inner mitochondrial membrane were monitored by dual-wavelength spectrophotometry at 675-685 nm in the presence of 50 µM arsenazo III [10] .

Determination of oxygen consumption. During the absorption measurements in an Aminco DW-2A spectrophotometer the oxygen concentrations were measured with the Aminco vibrating platinum electrode assembly. At the required time points, the electrodes were placed into the cuvette, thus shortly interrupting the absorption measurements, and the measured oxygen tension was recorded. The vibrating platinum electrode was calibrated with solutions adjusted to different oxygen tensions using a Clark-type oxygen electrode (Yellow Spring Instruments, Yellow Springs, OH). Respiration experiments were done with a Clark-type oxygen electrode. To this end mitochondria were incubated similar to the standard procedure in a closed chamber (1.3 ml) without oxygen supply.

Determination of the mitochondrial membrane potential. Mitochondria were incubated according to the standard procedure in the presence of 10 µM safranine T [11]. $\Delta\Psi$ was measured in an Aminco DW-2A spectrophotometer at 511-533 nm. At the end of each incubation, 1 µM of the uncoupler CCCP or oligomycin was added to verify the existence of $\Delta\Psi$. To measure $\Delta\Psi$ at low oxygen tensions, buffer was purged with a mixture of air and nitrogen under constant stirring in a closed beaker. After reaching the desired oxygen tension (measured with a Clark-type oxygen electrode), 2.8 ml were transferred into a cuvette and immediately gassed with the same air/nitrogen mixture. Thereafter, mitochondria were added, and $\Delta\Psi$ was measured as described above.

Preparation of aqueous NO solutions. NO is a colourless gas which, in the absence of oxygen, physically dissolves in aqueous solutions up to 1.9 mM at 25 °C [12]. Therefore, in our experiments buffer in a double-necked container was first deaerated by bubbling with oxygen-purged nitrogen for 60 min and then saturated with NO by bubbling for at least 30 min with NO gas, which had first been slowly directed through solid KOH in Teflon and glass tubings. To withdraw NO-saturated solution, the compartment was opened for a few seconds with constant bubbling of NO gas through the solution. This prevented entrance of oxygen, as judged from the absence of NO_X formation.

RESULTS

$\Delta\Psi$, an indicator of the energy-state of mitochondria, can be continuously monitored by following the absorption of safranine at 511-533 nm. Addition of NO-saturated buffer to rat liver mitochondria causes a transient deenergization, indicated by a decrease in safranine absorption, when mitochondria use potassium succinate as a respiratory substrate (Fig. 1A). At a given NO dose, the extent and duration of the mitochondrial deenergization increases with decreasing the oxygen tensions (Fig. 1A). At an oxygen tension similar to that found in

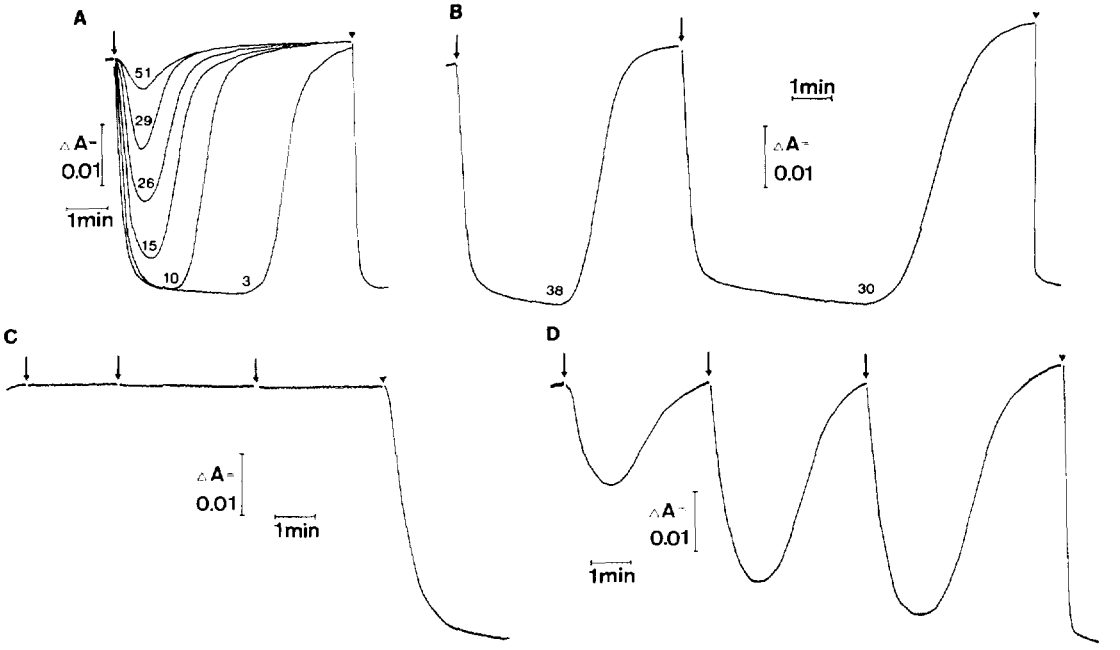

Fig. 1. Transient deenergization by NO of rat liver and brain mitochondria. Rat liver (panels A to C) or brain (panel D) mitochondria were incubated at 1mg of protein/ml in buffer in the presence of 5mM EGTA and 10μM safranine at various oxygen tensions. The membrane potential was measured by following safranine absorption at 511-533nm as described in the Methods section. (A) After energization with potassium succinate (2.5mM) in the presence of rotenone (5μM), 2μl of NO-saturated buffer was added (*arrow*) (final NO concentration 1.3μM [12]). The numbers indicate the oxygen tension (in mmHg) in the mitochondrial suspension. (B) Mitochondria were energized with 0.6M ascorbate *plus* 10mM TMPD in the presence of 5μM rotenone. Twice 2μl of NO-saturated buffer (*arrows*) were added (final NO concentration 1.3μM each), and the oxygen tension was measured as described for panel A. (C) Mitochondria were energized with 1.5mM ATP in the presence of 5μM rotenone at 30 mmHg oxygen tension. At the first two *arrows*, 10μl NO-saturated buffer (final NO concentration 6.5μM each), at the third *arrow*, 20μl of NO-saturated buffer (final NO concentration 13μM) were added. (D) Rat brain mitochondria were incubated in the presence of 5mM pyruvate *plus* 2.5mM malate at an oxygen tension of 28 mmHg. At each *arrow*, 1μl NO-saturated buffer (final NO concentration 0.65μM each) was added. At the *triangles*, 1μM CCCP (panels A, B, and D) or 1.7μg oligomycin/mg of mitochondrial protein (panel C) were added.

venous blood (about 35 mm Hg) NO at 1.3 μM (assuming a concentration of 1.9 mM for the saturated NO solution [12]) elicits a rapid and complete, but reversible deenergization of mitochondria. Increasing the amount of added NO extended the amplitude and duration of de-energization (not shown). Similar results were obtained with 5 mM pyruvate *plus* 2.5 mM malate as respiratory substrates (not shown). Liver mitochondria energized with ascorbate *plus* tetramethylphenylenediamine (TMPD) are more sensitive to NO (Fig. 1B) than mitochondria energized with succinate or pyruvate *plus* malate. Again, deenergization is completely reversible and can be repeated several times. In contrast, liver mitochondria energized with ATP are not deenergized with up to 13 μM NO even at low oxygen tension (Fig. 1C).

Very similar results were obtained with rat brain mitochondria: (i) When energized with ascorbate *plus* TMPD they were more sensitive to NO than when energized with succinate or pyruvate *plus* malate (not shown). (ii) Deenergization is completely reversible and can be evoked repeatedly (Fig. 1D). (iii) Brain mitochondria energized with ATP were not sensitive to NO (not shown).

Up to 10 mM potassium nitrite (KNO_2), which is formed in aqueous NO solutions by reaction of NO with O_2 [12], did not deenergize rat liver or brain mitochondria energized with any of the substrates used here (not shown).

Mitochondrial Ca^{2+} uptake as well as ATP synthesis is driven by $\Delta\Psi$, and deenergization of mitochondria results in the release of Ca^{2+} from the organelles. Fig. 2 shows that parallelly

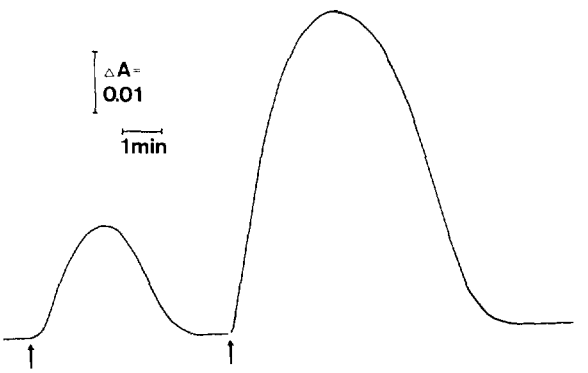

Fig. 2. Ca^{2+} uptake and release by rat liver mitochondria exposed to NO. Mitochondria at 1mg of protein/ml were incubated in buffer in the presence of 50μM arsenazo III at an oxygen tension of 30 mmHg. Ca^{2+} uptake and release were measured by following the absorption at 675-685nm as described in the Methods section. After energization with potassium succinate (2.5mM) in the presence of rotenone (5μM), twice 2μl of NO-saturated buffer (final NO concentration 1.3μM) were added (*arrows*). The mitochondrial Ca^{2+} content was 10nmol/mg of protein as determined with arsenazo III after addition of CCCP.

to the drop and rise in $\Delta\Psi$ (*c.f.*, Fig. 1A) mitochondria lose their Ca^{2+} upon addition of NO, and subsequently re-accumulate the previously released Ca^{2+}.

Also mitochondrial respiration measured with a Clark-type oxygen electrode in a closed 1.3 ml compartment without oxygenation as described in the Method section is transiently inhibited by NO (Fig. 3).

DISCUSSION

Here we show directly that NO at low micromolar and smaller concentrations and at average oxygen tensions which prevail in cells, *i.e.*, from 30 to less than 3 mm Hg [13,14], potently and reversibly deenergizes mitochondria. NO has been shown to bind to reduced cytochrome oxidase [15], and to cytochrome P450 [16, 17]. The site of NO's action in our experiments most likely is cytochrome *c* and/or cytochrome oxidase, the terminal heme protein of the respiratory chain, because (i) NO deenergizes mitochondria respiring on pyruvate *plus* malate, succinate, or ascorbate *plus* tetramethylphenylenediamine, but not when they are energized by ATP hydrolysis, and (ii) NO inhibits respiratory substrate-driven oxygen consumption by mitochondria, as was already inferred from studies with fibroblasts and cytotoxic activated macrophages [18]. This is in agreement with the results of Cleeter et al. [19] who showed that NO released from S-nitrosoglutathione (GSNO) inhibits mitochondrial respiration and cytochrome oxidase activity of isolated rat skeletal muscle mitochondria. This inhibition could be reversed by washing the mitochondria free of GSNO.

At a constant oxygen concentration, the rate of disappearance of NO in aqueous solution is proportional to the square of the NO concentration [20], *e.g.*, with an oxygen tension of 10 mm Hg and 100 μM NO, the time when the concentration of NO is reduced to 1 μM is over 5 hours. As suggested by Clarkson et al. [21] the half-life of NO of a few seconds in cells is predominantly determined by respiration-dependent NO consumption by mitochondria at NO

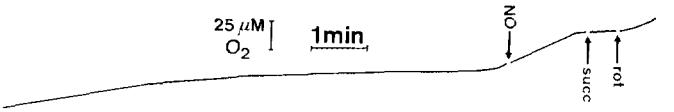

Fig. 3. Inhibition by NO of mitochondrial respiration. Rat liver mitochondria were incubated at 1mg of protein/ml in 1.3ml buffer in a closed chamber at atmospheric oxygen tension. Respiration was supported by 2.5mM potassium succinate (succ) in the presence of 5μM rotenone (rot) and measured by a Clark-type oxygen electrode as described. Where indicated, 1μl of NO-saturated buffer (final NO concentration 1.5μM) was added.

concentrations <10 μM. Together with the results presented here this shows that the reaction of NO with mitochondria may play a critical role in modulating the *in vivo* activitiy of this bioregulatory molecule at the low oxygen tensions prevailing in cells and tissues.

Deenergization of mitochondria in cells may have various consequences. Since deenergization causes a mobilization of mitochondrial and a redistribution of cellular Ca^{2+} NO may influence physiological reactions, *e.g.*, insulin secretion, by transiently triggering Ca^{2+} release from or preventing its uptake by mitochondria. Indeed, Kong *et al.* [22] have recently shown that NO increases the level of cytosolic Ca^{2+} by its mobilization from intracellular pools. On the other hand, excessive NO production and the resulting prolonged mitochondrial deenergization may cause an extended impairment of cellular Ca^{2+} homeostasis and decreased ATP levels, which can lead to necrotic and apoptotic cell death [23, 24].

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REFERENCES

[1] Moncada, S., Palmer, R.M.J. and Higgs, E.A. (1991) Pharmac. Rev. 43, 109-142.
[2] Moncada, S. and Higgs, E.A. (1993) New Engl. J. Med. 329, 2002-2012.
[3] Lowenstein, C. J., Dinerman, J.L. and Snyder, S.H. (1994) Ann. Intern. Med. 120, 227-237.
[4] Drapier, J.-C. and Hibbs, Jr., J.B. (1988) J. Immunol. 140, 2829-2838.
[5] Stadler, J., Curran, R.D., Ochoa, J.B., Harbrecht, B.G., Hoffman, R.A., Simmons, R.L. and Billiar, T.R. (1991) Arch. Surg. 126, 186-191.
[6] Tucker, S.D., Auzenne, E.J. and Sivaramakrishnan, M.R. (1993) J. Leukoc. Biol. 53, 138-143.
[7] Kurose, I., Kato, S., Ishii, H., Fukumura, D., Miura, S., Suematsu, M. and Tsuchiya, M. (1993) Hepatology 18, 380-388.
[8] Lötscher, H.R., Winterhalter, K.H., Carafoli, E. and Richter, C. (1980) J. Biol. Chem. 255, 9325-9330.
[9] Lai, J.C.K. and Clark, J.B. (1979) Meth. Enzymol. 55, 51-59.
[10] Lötscher, H.R., Winterhalter, K.H., Carafoli, E. and Richter, C. (1979) Proc. Natl. Acad. Sci. USA 76, 4340-4344.
[11] Åkerman, K.E.O. and Wikström, M.K.F. (1976) FEBS Lett. 68, 191-197.
[12] Feelisch, M. (1991) J. Cardiovasc. Pharmacol. 17, S25-S33.
[13] Gilbert, D.L. (1981) Oxygen and living processes. Springer-Verlag New York, Inc.
[14] Vanderkooi, J.M., Erecinska, M. and Silver, I.A. (1991) Am. J. Physiol. 260, C1131-C1150.
[15] Gorren, A.C.F., Van Gelder, B.F. and Wever, R. (1988) Ann. NY Acad. Sci. 550, 139-149.
[16] Khatsenko, O.G., Gross, S.S., Rifkind, A.B. and Vane, J.R. (1993) Proc. Natl. Acad. Sci. USA 90, 11147-11151.
[17] Stadler, J., Trockfeld, J., Schmalix, W.A., Brill, T., Siewert, J.R., Greim, H. and Doehmer, J. (1994) Proc. Natl. Acad. Sci. USA 91, 3559-3563.

[18] Granger, D.L., Taintor, R.R. and Hibbs, Jr., J.B. (1980) J. Clin. Invest. 65, 357-370.
[19] Cleeter, M.W.J., Cooper, J.M., Darley-Usmar, V.M., Moncada, S. and Schapira, A.H.V. (1994) FEBS Lett. 345, 50-54.
[20] Gilbert, D.L. (1994) Ann. NY Acad. Sci., in press.
[21] Clarkson, R.B., Norby, S.W., Boyer, S., Vahidi, N., Nims, R.W. and Wink, D.A. (1994) Biochim. Biophys. Acta, in press.
[22] Kong, S.K., Choy, Y.M. and Lee, C.Y. (1994) Biochem. Biophys. Res. Commun. 199, 234-240.
[23] Schanne, F.A.X., Kane, A.B., Young, E.E. and Farber, J.L. (1979) Science 206, 700-702.
[24] Orrenius, S., McConkey, D.J., Bellomo, G. and Nicotera, P. (1989) Trends Pharmacol. Sci. 10, 281-285.