Nitrite reductase activity is a novel function of mammalian mitochondria

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Abstract Nitrite, which is the major stable degradation product of nitric oxide, exists in all tissues capable of nitric oxide synthesis from L-arginine. The present study provides experimental evidence that nitrite in contact with respiring mitochondria accepts reducing equivalents from the ubiquinone cycle of the respiratory chain. Univalent reduction of nitrite was totally inhibited by myxothiazol. We therefore conclude on the involvement of redox cycling that ubisemiquinone is associated with the bc_1 complex. Recycling of nitric oxide degradation products via these electron carriers may become a threat to energy-linked respiration since nitric oxide in direct contact with mitochondria was shown to slow the energy-linked respiration down and to trigger a mitochondrial source for superoxide radicals. Until now, the existence of nitrite reductase activity was only demonstrated in plants and bacteria. In addition, the present observation elucidates the existence of a nitric oxide synthase-independent nitric oxide source.

Key words: Nitrite reductase; Mitochondrion; Nitric oxide; Electron paramagnetic resonance

1. Introduction

Nitric monoxide (NO, endothelium-derived relaxing factor) exerts a great variety of physiological functions. Apart from its regulation of the vascular tone, platelet aggregation and leukocyte adhesion, NO was reported to play a role in cellular signaling activities. NO is also involved in pathophysiological processes such as inflammation [1], cancer [2], ischemia [1,3] and apoptosis [4]. NO is provided by various isoforms of nitric oxide synthase (NOS) differing in their tissue localization.

The recent findings on the existence of a mitochondrial NOS were considered as a physiological control system which regulates energy-linked respiration [5]. However, NO levels required to switch the physiological function of mitochondria to pathophysiological activities are not provided by mitochondrial NOS, even under substrate saturation conditions. Pathophysiological effects of NO at the level of cell respiration therefore require NOS-independent NO sources. Zweier et al. have recently reported that nitrite (NO_2^-), which is the major breakdown product of NO in the heart, can be recycled during ischemia [6–8]. Reducing equivalents, like pyridine nucleotides, which accumulate in the reduced state, and low pH values were supposed to be involved in nitrite recycling to NO under ischemic conditions [8]. Pyridine nucleotides are co-enzymes of dehydrogenases which also form part of the mitochondrial respiratory chain. Electron carriers of the respiratory chain provide a wide span of redox potentials, many of them are thermodynamically suited for nitrite reduction. Nitrite reduction to NO requires the interaction with a one electron donor of the respiratory chain ($NO^- + 2H^+ + e^- \rightarrow NO + H_2O$). It was earlier demonstrated that ischemia affects electron transfer of the respiratory chain such that single electrons escape reducing oxygen to the superoxide radical. The most susceptible site of the mitochondrial redox chain is the bifurcated transfer of two single electrons from the ubiquinone pool to the bc_1 complex. Nitrite reduction to NO is therefore likely to occur under anaerobic conditions at the same site involved in one electron reduction of dioxygen. In the present study, we investigated whether or not electron carriers of the respiratory chain may act as nitrite reductase.

2. Materials and methods

2.1. Chemicals

Rotenone, myxothiazol, antimycin A, thenoyltrifluoroacetone (TTFA) were obtained from Sigma Chemical. Glutamic acid and malonic acid were received from Merck. Nitric monoxide gas was obtained from AGA. Other chemicals were of analytical grade purity. Hemoglobin (Hb) was prepared from bovine red blood cells as described before [9].

2.2. Preparations of mitochondria and submitochondrial particles

Rat liver mitochondria (RLM) were prepared according to Szarkowska et al. [10] and stored in buffer containing 0.25 M sucrose, 20 mM triethanolamine, 1 mM EDTA (pH = 7.25) at 0°C for 4–5 h. Submitochondrial particles (SMP) were prepared from beef hearts according to Graham et al. [11], kept at −4°C in the same buffer and thawed at the day of experiment. The concentrations of RLM and SMP in the stock solutions were 50–60 and 36 mg protein/ml, respectively. Before incubation, both SMP and RLM were diluted to a concentration of 5 mg/ml with buffer containing additionally 10 mM of potassium phosphate (pH = 7.25).

2.3. Experimental design

RLM (or SMP) alone or with different substrates was mixed with Hb and inhibitors of the respiratory chain (total sample volume was 500 μl) and kept under a flow of argon for 10 min. Substitution of oxygen by argon was facilitated using a shaking table to provide gentle mixing of the mitochondrial suspension with argon. Then, 5 μl of oxygen-free sodium nitrite ($NaNO_2$) solution was injected through a channel made in the experimental cell under anaerobic conditions. Samples were aspirated by a syringe without exposure to oxygen and kept for 2 h at room temperature (20 ± 2°C) inside a home-made anaerobic chamber. After 2 h, the samples were frozen in liquid nitrogen and subjected to electron paramagnetic resonance (EPR) measurements. The experimental medium contained 5 mg of protein/ml RLM or SMP, 0.5 mM Hb, 5 mM succinate or 5 mM of NADH or 2.5 mM glutamate plus 2.5 mM malate, 250 μM Hb, 50 μM $NaNO_2$, 0.25 M sucrose, 20 mM triethanolamine, 1 mM EDTA and 10 mM K_2HPO_4, pH = 7.25.

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Abbreviations: NO, nitric oxide; NO_2^-, nitrite; NOS, nitric oxide synthase; EPR, electron paramagnetic resonance; TTFA, thenoyltrifluoroacetone; RLM, rat liver mitochondria; SMP, submitochondrial particles; Hb, hemoglobin

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2.4. EPR measurements

EPR spectra of RLM and SMP were recorded at the liquid nitrogen temperature with a Bruker ER-200 SRP spectrometer in a quartz dewar with a finger under the following conditions: microwave frequency 9.43 GHz, modulation frequency 100 kHz, microwave power 20 mW, modulation amplitude 5 G, gain 105. The double integral of the signal was calculated and compared with that obtained from nitroso-heme complex standards. The preparation of nitroso-heme complex standards was performed as described before [12]. For this purpose, distilled water was deoxygenated by purging the water with nitrogen exhaustively. The water was then bubbled with pure NO gas for 20 min at 20 ± 2°C. The concentration of NO in a saturated solution was taken as 1.9 mM, according to the solubility of NO in water [13]. The NO-saturated water was mixed with the oxygen-free Hb solution (0.3 mM Hb in 100 mM phosphate buffer at pH = 6.0) to obtain a concentration range of 0, 20 and 40 μM nitroso-heme complex.

2.5. Statistics

Statistic parameters were calculated using Excell 5.0 (Microsoft).

3. Results

Addition of sodium nitrite to freshly isolated RLM under anaerobic conditions resulted in the formation of NO as demonstrated by the existence of the electron paramagnetic resonance (EPR)-sensitive Hb-NO adduct (Fig. 1). The involvement of electron carriers of the respiratory chain in the reduction of nitrite to NO was concluded from the observation that SMP, containing all electron carriers but, in contrast to intact mitochondria, lack endogenous substrates for respiration, were inactive in nitrite reduction unless NADH or succinate was added to start respiration. Complex I substrates (glutamate/malate, NADH) as well as succinate (complex II substrate), when used in the absence of mitochondria or submitochondrial particles, were rather ineffective in nitrite transformation to NO (Fig. 2A and B). NO formation was not observed when nitrate was used instead of nitrite (experiment not shown).

Inhibition of the electron flow from complex I into the 'Q cycle' upon the addition of rotenone (see scheme in Fig. 3) decreased NO generation from nitrite by 60% (Fig. 2A). Accordingly, when reducing equivalents were provided for the respiratory chain from the succinate-dehydrogenase complex (complex II), TTFA-induced influx inhibition slowed NO formation down by 40% (Fig. 2B). The presence of rotenone in this experiment excluded a reversed electron flow to complex I. Irrespectively of the substrate, supplying reducing equivalents for respiration inhibition of the 'Q cycle' at the oxidant site of the bc_1 complex (using antimycin A) had no effect on NO recycling from nitrite. Total suppression ($>90\%$) of nitrite transformation to NO required the functional sequestration of the bc_1 complex from the 'Q cycle' with myxothiazol.

4. Discussion

The scheme illustrates the flow of reducing equivalents from complex I and complex II substrates to the site of nitrite reduction (Fig. 3). Glutamate plus malate, the couple used to run electron flow in intact mitochondria, provides NADH for complex I via the citric acid cycle while succinate supplies reducing equivalents directly to complex II after being translocated into the matrix. In submitochondrial particles, NADH has a direct assess to complex I (Figs. 1 and 2). Both electrons from complex I and from complex II are transferred through the ubiquinone pool to complex III where one electron recycles through b-type cytochromes while the second electron is linearily conducted to the terminal electron carrier cytochrome oxidase. Rotenone, which inhibits the supply of electrons from complex I, and TTFA, which inhibits electron flow from complex II, decrease the reduction of nitrite to NO (Fig. 2). NO formation from nitrite was only observed as long as electrons had access to the bc_1 complex (complex III). Only inhibition of the electron flux to this part of the respiratory chain prevented NO formation totally. The Rieske iron-sulfur protein as well as cytochromes on the oxidant site of the Rieske center can be excluded as potential reductants of ni-

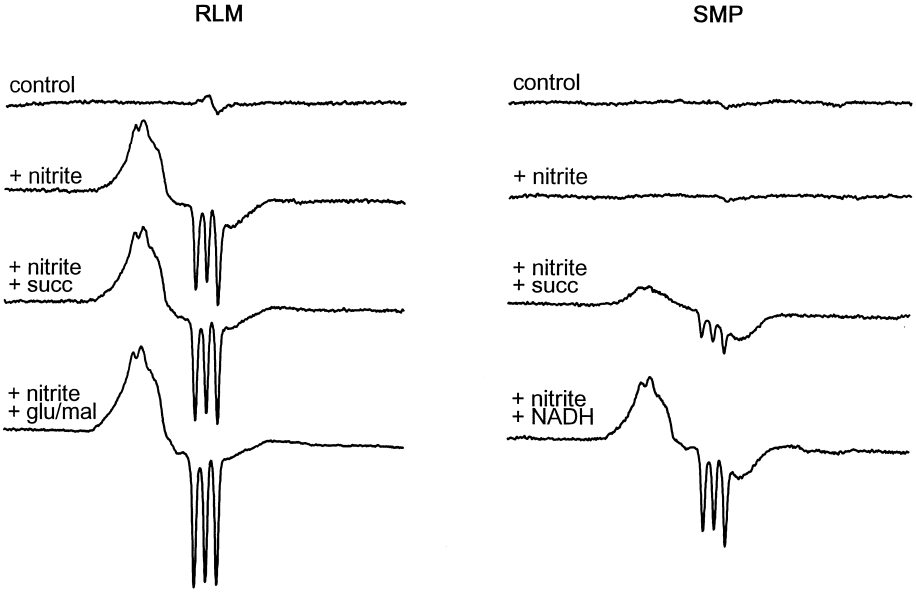

Fig. 1. EPR spectra observed after 2 h of incubation of RLM or SMP with nitrite in the presence of deoxyHb and substrates for complex I or complex II of the respiratory chain (succ = succinate, glu/mal = glutamate plus malate).

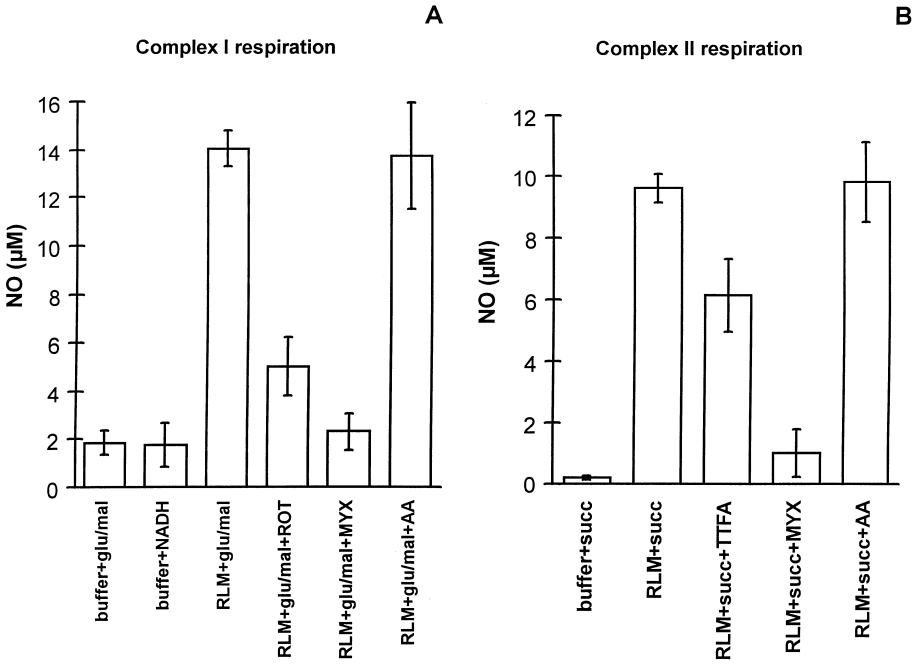

Fig. 2. Generation of NO-related EPR-sensitive Hb-NO adducts from nitrite in the presence of inhibitors preventing flow of reducing equivalents from complex I in the presence of TTFA (A) and from complex II in the presence of rotenone (B) (ROT = rotenone, MYX = myxothiazol, AA = antimycin A).

trite since the strong positive redox potentials of these electron carriers prevent an electron transfer on thermodynamic grounds.

The present study provides experimental evidence on the existence of a NOS-independent NO formation from the major metabolic NO degradation product nitrite. In contrast to all isoforms of NOS, which require L-arginine for NO synthesis, the novel NOS-independent synthase system generates NO from the stable degradation product of this bioregulator synthesized elsewhere in the tissue. A direct reduction of nitrite to NO through reductants which normally accumulate during ischemia was only of marginal significance ($< 10\%$) (see Fig. 2). Recycling of NO from nitrite requires respiring mitochondria under conditions established during ischemia, namely highly reduced electron carriers resulting from the lack of oxygen. The use of adequate inhibitors of respiration made it possible to localize the mitochondrial site of nitrite reduction (see Fig. 3). It followed from these studies that nitrite reductase activity can be attributed to the oxidant site of ubiquinol at the cytochrome bc_1 complex of the respi-

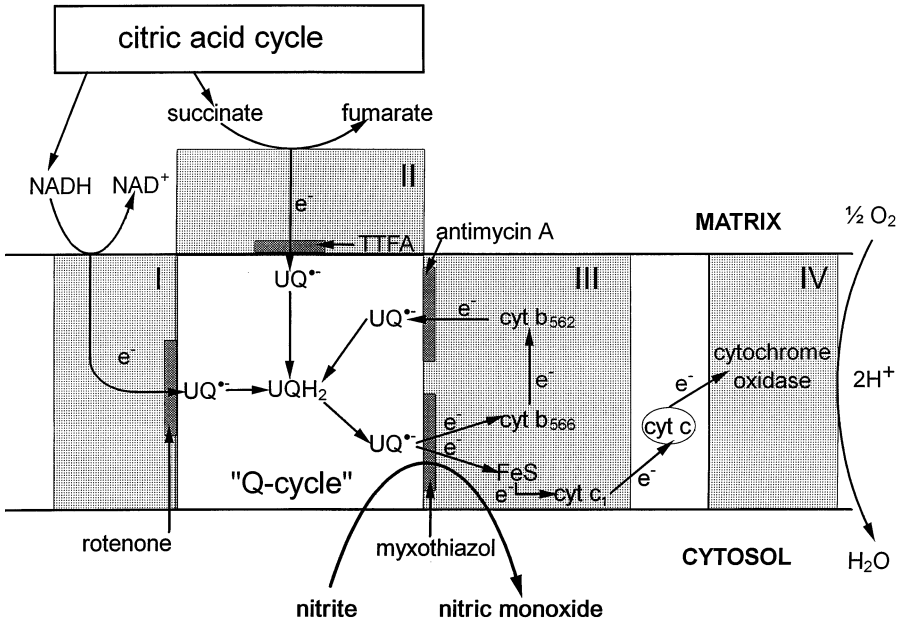

Fig. 3. Schematic presentation of the influx and flow of reducing equivalents through the various functionally complexes of the respiratory chain. The bars indicate the sites of the various inhibitors used for the localization of the nitrite reductase activity.

ratory chain (complex III) (Fig. 2). Similar to the single electron leak from the respiratory chain to dioxygen, antimycin A was rather stimulating while myxothiazol totally abolished nitrite reduction (Fig. 2). Myxothiazol inhibits the Q cycle such that reduction of the bc_1 complex from ubiquinol is prevented. The site of myxothiazol interaction is assumed to be the second electron transfer step from ubisemiquinone (Fig. 3). Myxothiazol is likely to destabilize this semiquinone species by an intervention on to its binding to the pocket of the bc_1 complex. We therefore believe that redox cycling semiquinones supply single electrons for nitrite reduction. It was surprising that complex I or complex II, both exhibiting redox potentials which are thermodynamically more appropriate for nitrite reduction, do not interact with nitrite. This is not in contradiction to the fact that inhibition of the complex I- or complex II-mediated flow of reducing equivalents into the Q cycle did not result in a total inhibition of nitrite transformation to NO. It can be assumed that despite the presence of complex I or complex II inhibitors, electrons do leak to the site where nitrite is reduced. This assumption is based on the fact that only myxothiazol, which deprives the Q cycle from the electron supply of complex I or complex II, respectively, totally suppresses nitrite reduction. Similarities with one electron reduction of nitrite suggest that the site which deviates single electrons from the respiratory chain is identical both for superoxide formation from oxygen and for NO formation from nitrite. Our study provides evidence that the redox couple between ubiquinol and complex III can be used also for cycling the NO oxidation product nitrite back to its bioactive form. This may become a significant pathophysiological event under conditions of stimulation of NO tissue levels during ischemia or external administration of NO donors. NO recycling from nitrite through mitochondria in that case may lead to deleterious NO concentrations in mitochondria becoming toxic through inhibition of energy-linked respiration at the level of cytochrome oxidase, stimulation of superoxide radical generation through inhibition of b-type cytochromes and activation of Ca^{2+}-regulated metabolic pathways by the release of Ca^{2+} from mitochondria [14,15].

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