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Nitric oxide degradation by potato tuber mitochondria: Evidence for the involvement of external NAD(P)H dehydrogenases

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ABSTRACT

The mechanisms of nitric oxide (NO) synthesis in plants have been extensively investigated. NO degradation can be just as important as its synthesis in controlling steady-state levels of NO. Here, we examined NO degradation in mitochondria isolated from potato tubers and the contribution of the respiratory chain to this process. NO degradation was faster in mitochondria energized with NAD(P)H than with succinate or malate. Oxygen consumption and the inner membrane potential were transiently inhibited by NO in NAD(P)H-energized mitochondria, in contrast to the persistent inhibition seen with succinate. NO degradation was abolished by anoxia and superoxide dismutase, which suggested that NO was consumed by its reaction with superoxide anion (O_2^-). Antimycin-A stimulated and myxothiazol prevented NO consumption in succinate- and malate-energized mitochondria. Although favored by antimycin-A, NAD(P)H-mediated NO consumption was not abolished by myxothiazol, indicating that an additional site of O_2^- generation, besides complex III, stimulated NO degradation. Larger amounts of O_2^- were generated in NAD(P)H- compared to succinate- or malate-energized mitochondria. NAD(P)H-mediated NO degradation and O_2^- production were stimulated by free Ca^{2+} concentration. Together, these results indicate that Ca^{2+}-dependent external NAD(P)H dehydrogenases, in addition to complex III, contribute to O_2^- production that favors NO degradation in potato tuber mitochondria.

1. Introduction

Nitric oxide (NO) has emerged as an important signaling molecule in plants, with a crucial role in several metabolic and developmental processes, such as root growth, seed germination, leaf expansion, stomata movement and senescence [1,2]. Additionally, NO is involved in plant defense against pathogens, including the hypersensitive response, the activation of some defensive genes and the production of antimicrobial compounds [3–5].

The intracellular steady-state levels of NO are determined by a balance between the rate of synthesis and degradation of this radical. Although the pathways for NO synthesis have been extensively investigated [1,6], little is known about the mechanisms by which NO is degraded in plant cells. In aqueous aerobic solutions, NO decays to nitrite through auto-oxidation [7]. However, this reaction is not fast enough to explain the extremely short half-life of NO in biological systems, indicating the existence of additional pathways for its degradation [8]. Some enzymatic mechanisms for the conversion of NO to nitrite have been identified in animals, including reactions involving lipoxygenases, prostaglandin H synthase and peroxidases (see [8] and references therein). A heme-dependent conversion of NO to nitrate, similar to the NO dioxygenase activity described in *Escherichia coli*, has also been observed in mammalian cells and plants [9].

Mitochondria are an important target for NO, which binds reversibly to the oxygen binding site of cytochrome *c* oxidase (COX or complex IV), resulting in decreased oxygen consumption [10]. This reversible inhibition of COX has been considered a physiological mechanism by which NO regulates respiration [11]. Additionally, prolonged exposure to NO can persistently inhibit respiratory activity via the nitrosation of components of the respiratory chain such as complex I [12]. Plant COX is also very sensitive to NO [13,14] which can cause cell death by adversely affecting the normal functions of plant mitochondria [15]. In view of the potentially deleterious effects of NO in mitochondria, the existence of mechanisms for controlling NO levels in this organelle may be important in regulating the signaling and toxicity of this radical.

An important mechanism for NO consumption is its non-enzymatic reaction with superoxide anion (O_2^-) to form peroxynitrite ($ONOO^-$) [16]. When mitochondrial respiration is inhibited by NO, this mechanism contributes to NO degradation in the medium, thereby reactivating the previously inhibited COX and restoring respiration [17]. Electron leakage from complex I [18], and the ubiquinone cycle and cytochrome *b* of complex III [19,20] are the main sites of O_2^- generation in the mammalian respiratory chain. The molecular mechanisms that lead to O_2^- production at these sites in the respiratory complexes are still incompletely understood [18,20].

The core of respiratory components in plant mitochondria is very similar to that in other eukaryotic mitochondria and studies suggest that complexes I and III also contribute to O_2^- production in plants [21].

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However, in addition to the standard respiratory complexes, the internal membrane of plant mitochondria contains alternative pathways for electron transport. These include the alternative oxidase (AOX) that bypasses complexes III and IV, and at least four alternative dehydrogenases that bypass complex I [22,23]. Two of these dehydrogenases face the external surface of the inner membrane and allow the direct oxidation of cytosolic NAD(P)H [23]. These alternative enzymes are non-proton pumping proteins and therefore do not contribute to energy conservation through oxidative phosphorylation. Variations in the level of expression of these dehydrogenases in different tissues, as well as during development and following exposure to different kinds of stress, suggest that these enzymes have important functions in plants [23].

Despite the singularity of mitochondria in plants and the importance of regulation of mitochondrial respiration by NO, the ability of these organelles to degrade NO has not been addressed. In this work, we show that external NAD(P)H dehydrogenases, in addition to complex III, contribute to NO degradation in potato tuber mitochondria. NAD(P)H oxidation by external dehydrogenases led to an increased generation of O_2^-, which reacted non-enzymatically with NO. This reaction competed with NO binding to COX, allowing the recovery of oxygen consumption. The role of plant mitochondrial respiratory chain in preventing the inhibitory effects of NO on respiration is discussed.

2. Materials and methods

2.1. Chemicals

Sucrose and hydrogen peroxide solution were purchased from Merck (Darmstadt, Germany). Amplex Red was from Molecular Probes (Eugene, OR). Coomassie Plus protein assay reagent was from Pierce (Rockford, IL). The other chemicals were obtained from Sigma (St. Louis, MO).

2.2. Preparation of GSNO and NO

S-Nitrosoglutathione (GSNO) was synthesized according to Mathews and Kerr [24]. Briefly, equimolar amounts of reduced glutathione and $NaNO_2$ were dissolved in an aqueous solution. The pH of the solution was adjusted to 2.0 with HCl and incubated for 15 min to allow development of the characteristic red color. Samples were then neutralized to pH 7.0 with NaOH and the concentration of GSNO was determined at 542 nm. A saturated solution of NO was prepared by bubbling NO gas through Ar-purged phosphate buffer in a rubber-sealed vial.

2.3. Preparation of mitochondria

Mitochondria were isolated from potato tubers (*Solanum tuberosum* L. cv Monalisa) obtained from a local supermarket. The tubers were peeled, cut into small cubes and then homogenized with a domestic juice extractor in buffer containing 0.4 M sucrose, 10 mM *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid) (HEPES, pH 7.6), 1 mM ethylene glycol-bis(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 0.1% bovine serum albumin (BSA) and 3 mM cysteine. The suspension was strained through four layers of gauze and the pH was adjusted to 7.1 prior to centrifugation at 1500 ×*g* for 5 min to remove debris and starch. The resulting supernatant was centrifuged at 9000 ×*g* for 15 min and the mitochondrial pellet was suspended in wash medium containing 0.25 M sucrose, 10 mM HEPES (pH 7.2), 0.1 mM EGTA and 0.1% BSA and subjected to another cycle of differential centrifugation. Mitochondrial suspensions were then combined with a solution of 21% Percoll containing 0.25 M sucrose, 10 mM HEPES (pH 7.2) and 0.3 mM EGTA and centrifuged at 39,000 ×*g* for 30 min, in an SW 41 rotor (Beckman Instruments, Palo Alto, CA). The band of mitochondria was collected with a pipette, diluted 10-fold with wash medium and centrifuged at 19,000 ×*g* for 15 min to eliminate the Percoll and collect the purified mitochondria. The use of a Percoll gradient to purify mitochondria greatly reduced the contamination by peroxisomes [25], as shown by the catalase activity in crude (1.15 μmol O_2 min^{-1} mg^{-1}) and purified (0.19 μmol O_2 min^{-1} mg^{-1}) mitochondrial suspensions. All of the steps were done at 4 °C. The mitochondrial protein concentration was determined by the Coomassie blue binding method, using BSA as the standard [26].

2.4. Nitric oxide and oxygen measurements

NO and O_2 concentrations were measured at 25 °C using the electrochemical sensors ISO-NOP and ISO-OXY-2, respectively, connected to a free radical analyzer Apollo 4000 (World Precision Instruments, Sarasota, FL). Mitochondria were incubated in a reaction medium containing 0.25 M sucrose, 10 mM phosphate buffer (pH 7.2) and 0.1% BSA. The amount of mitochondrial protein, respiratory substrate and other additions are specified in the fig. legends. The NO electrode was calibrated with *S*-nitroso-*N*-acetyl-penicillamine in 0.1 M $CuCl_2$ [27].

2.5. Measurement of the transmembrane electrical potential ($m\Delta\psi$)

The membrane potential was measured using safranine as an optical probe [28]. The changes in the absorbance at 511–533 nm were recorded using an UV-visible photodiode array spectrophotometer (MultiSpec-1500, Shimadzu Corporation, Tokyo,

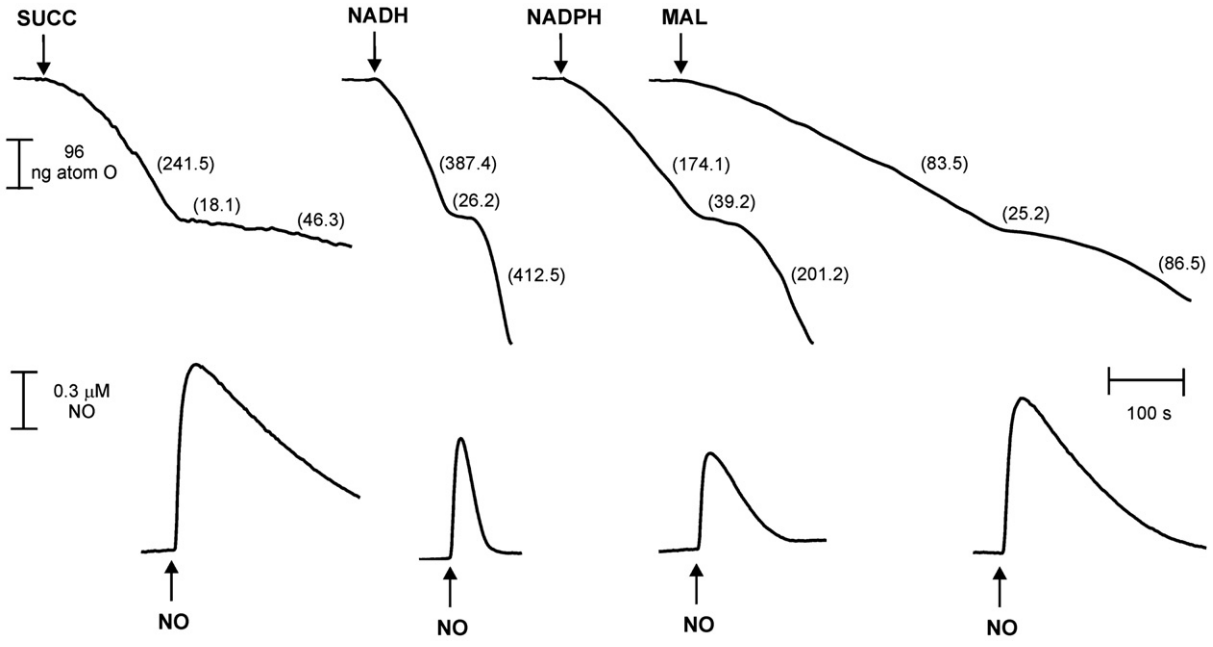

Fig. 1. NO degradation and O_2 consumption by potato tuber mitochondria energized with different respiratory substrates. Isolated mitochondria (0.25 mg/mL) were incubated in a reaction medium containing 0.25 M sucrose, 0.1% BSA and 10 mM phosphate buffer (pH 7.2) supplemented with 2 mM Mg^{2+} and 1 μmol ADP (State 3 respiration). Mitochondria were energized with 5 mM succinate (SUCC), 1 mM NADH (NADH), 1 mM NADPH and 0.5 mM Ca^{2+} (NADPH) or 10 mM malate plus 10 mM glutamate (MAL). An aliquot of NO saturated solution (~1 μM) was added after the mitochondrial suspension had consumed 50% of the O_2 in the medium. Except when malate was used, 10 μM rotenone was also added to the reaction medium. The numbers in parentheses refer to the respiratory rates expressed as ng atom O min^{-1} mg^{-1}.

Japan). Mitochondrial suspensions were incubated at 25 °C in the reaction medium supplemented with 10 μM safranine. Other additions are specified in the fig. legend.

2.6. Detection of superoxide anion production

Superoxide anion production by potato mitochondria was measured following the dismutation to H_2O_2, which was detected by the fluorogenic indicator Amplex Red in the presence of horseradish peroxidase [29]. Mitochondrial suspensions were incubated at 25 °C in reaction medium supplemented with 100 U of SOD/mL, 1 U of horseradish peroxidase/mL and 25 μM Amplex Red. Other additions are specified in the fig. legends. Fluorescence was recorded in a Hitachi F-4500 spectrofluorometer (Hitachi Ltd., Tokyo, Japan) with excitation at 563 nm and emission at 587 nm. Background fluorescence, measured in the absence of mitochondria, was discounted from the sample fluorescence. Standard curves were obtained by the sequential addition of known concentrations of H_2O_2 solution.

2.7. Statistical analysis

The results are representative of at least three mitochondrial preparations. Where appropriate, the results were expressed as the mean±standard deviation. Statistical analyses were done using Student's *t*-test with $p<0.05$ indicating significance.

3. Results

3.1. NO degradation by energized potato mitochondria: effect of different electron donors

NO degradation and its inhibitory effect on O_2 consumption by potato tuber mitochondria in state-3 conditions (ADP present) were assessed simultaneously using electrochemical sensors for NO and O_2, respectively (Fig. 1). The addition of an aliquot of an NO saturated solution (equivalent to ~1 μM) to succinate-energized mitochondria generated a peak of 0.97 μM NO that was subsequently consumed at a rate of 0.90 nmol min^{-1} mg^{-1} and caused persistent inhibition on O_2 consumption by mitochondria. In contrast, when potato mitochondria were energized with NADH or NADPH, the NO peaks were lower (0.62 μM and 0.41 μM, respectively) and the NO consumption rates were higher (3.74 nmol min^{-1} mg^{-1} and 1.63 nmol min^{-1} mg^{-1}, respectively), resulting in complete consumption of NO during the experiment. In this case, NO transiently inhibited O_2 consumption, probably because the rapid decrease in NO concentration in the reaction medium favored its release from COX [10]. Accordingly, the recovery of respiratory activity was accelerated in NADH-energized compared to NADPH-energized mitochondria. Malate-energized mitochondria had an NO consumption rate of 1.32 nmol min^{-1} mg^{-1} and a peak of 0.80 μM NO following the addition of NO to the reaction medium, indicating that NO degradation in these conditions was faster than in succinate-energized mitochondria but slower than in NAD(P)H-energized mitochondria.

When GSNO was used as an NO donor, the results were similar to those obtained with pure NO (see Fig. S1 of Supplementary material). The inhibitory effect of GSNO was reverted by the addition of the NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO), indicating that the effect of GSNO on O_2 consumption by potato tuber mitochondria was not mediated by NO-independent protein nitrosation [30]. These results indicate that NO degradation by potato tuber mitochondria can be studied using either pure NO or GSNO as an NO donor.

The effect of NO and GSNO on the membrane electrical potential ($m\Delta\psi$) of potato mitochondria was also examined (Fig. 2). Direct application of the NO saturated solution caused an intense and persistent decrease in the $m\Delta\psi$ generated by succinate oxidation, whereas the fall in the potential generated by the oxidation of NADH or NADPH was less intense and quickly reversed (Fig. 2a). Addition of the uncoupler carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP) collapsed the $m\Delta\psi$. As shown in Fig. 2b, addition of GSNO caused similar perturbations in $m\Delta\psi$ to those induced by NO solution. These results indicated that the higher rate of NO degradation in the presence of NAD(P)H (Fig. 1) was reflected in the maintenance of the $m\Delta\psi$ in energized mitochondria.

3.2. NAD(P)H and mitochondria are required for NO degradation

The NO degradation rate decreased from 1.30 nmol min^{-1} mg^{-1} to 0.60 nmol min^{-1} mg^{-1} as NADH concentration decreased from 100 μM to 10 μM. When NADH was replaced by NADPH, the NO degradation rate decreased from 1.03 nmol min^{-1} mg^{-1} to 0.38 nmol min^{-1} mg^{-1}. The kinetics of NO degradation by potato tuber mitochondria as a function of NAD(P)H concentration is shown in Fig. S2 of Supplementary material. These results show that NO degradation depends on NAD(P)H concentration in the incubation medium and that NADH is more effective than NADPH in accelerating NO degradation by potato tuber mitochondria.

The NAD(P)H-dependent NO degradation increased as a function of the mitochondrial protein concentration (not shown), and it was in the range of 1.2 nmol/min in the presence of 0.25 mg of mitochondrial protein. In the absence of mitochondria and NADH, the rate of NO decay in the incubation medium was 0.20 nmol/min and was unaltered by the addition of NADH. This much lower rate of NO decay probably reflected the spontaneous reaction of NO with oxygen to produce nitrite [7]. When GSNO was used as an NO donor, an even lower rate of NO decay (0.036 nmol/min) was detected in the absence of mitochondria and NADH. The lower spontaneous decay of NO seen with GSNO compared to NO was probably related to the continuous release of NO in the reaction medium. Together, these results indicate that NO degradation was not attributable to a non-enzymatic reaction of NAD(P)H with NO or to the auto-oxidation of NO.

3.3. NAD(P)H-dependent NO degradation is an aerobic process that is prevented by SOD

The requirement for oxygen in the NAD(P)H-dependent degradation of NO by potato tuber mitochondria was assessed as shown in Fig. 3. Potato mitochondria were energized with succinate and GSNO and NADH were added to the reaction medium before O_2 consumption by the mitochondrial suspension (Fig. 3a) or after all of the O_2 in the incubation medium had been consumed (Fig. 3b). In the presence of

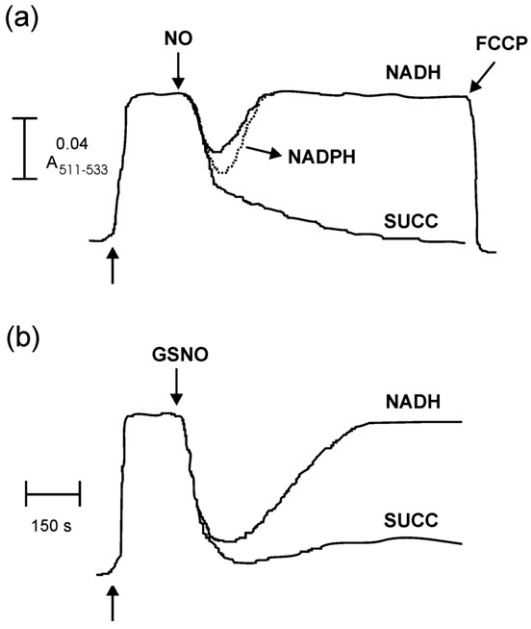

Fig. 2. Effect of NO from a pure solution (a) or from the donor GSNO (b) on the $m\Delta\psi$ of energized potato tuber mitochondria. Mitochondria (0.5 mg/mL) were incubated in reaction medium (see Fig. 1) supplemented with 10 μM rotenone and 10 μM safranine and energized with 2 mM NADH (NADH), 2 mM NADPH plus 0.5 mM Ca^{2+} (NADPH) or 10 mM succinate (SUCC), as indicated. GSNO (0.8 mM), NO (~8.5 μM) and FCCP (2 μM), were added where indicated.

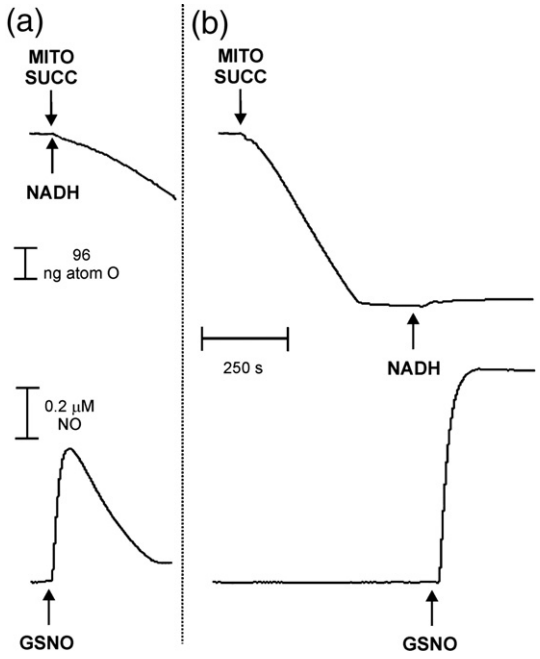

Fig. 3. The requirement for oxygen in NADH-dependent NO degradation by potato tuber mitochondria. Mitochondria (0.25 mg/mL; MITO) were incubated in reaction medium (see Fig. 1) supplemented with 2 mM Mg^{2+} and 1 μmol ADP, and energized with 5 mM succinate (SUCC). NADH (100 μM) and GSNO (100 μM) were applied (a) before O_2 consumption by the mitochondrial suspension or (b) under anaerobiosis.

O_2, GSNO generated a peak of NO (0.50 μM) that was rapidly consumed. When GSNO was added under anaerobiosis, the NO peak was much higher (0.85 μM) and the NO concentration in the reaction medium remained almost constant, indicating that NO degradation was inhibited under these circumstances. A lower rate of NO decay was expected in anaerobiosis since there is no spontaneous reaction of NO with molecular oxygen. However, the almost complete inhibition of NO degradation indicated that the detected mechanism was

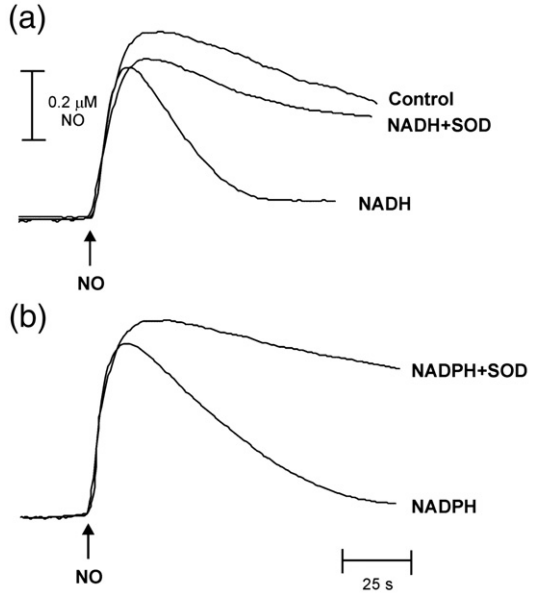

Fig. 4. Superoxide dismutase (SOD) prevents NAD(P)H-dependent NO degradation by potato mitochondria. Mitochondria (0.25 mg/mL) were incubated in the reaction medium (see Fig. 1) and an aliquot of a saturated NO solution (~1 μM) was added. NO decay was followed in the presence of 100 μM NADH (a) or NADPH (b) or mitochondria alone (control). Where indicated, 500 U of SOD/mL was used.

dependent on O_2. This result also showed that the NO degradation in these experimental conditions did not result from the action of COX [10] nor from the reduction of NO to nitroxyl anion by reduced ubiquinone [19], both of which can occur in the absence of O_2. Additionally, NO degradation by potato tuber mitochondria was not inhibited by potassium cyanide (not shown) indicating that this process was also not mediated by NO dioxygenase activity [9].

An important mechanism of NO degradation that occurs only in the presence of O_2 is its non-enzymatic reaction with O_2^- to form $ONOO^-$ [16]. As shown in Fig. 4a, when NO (~1 μM) was added to the incubation medium it was degraded by NADH-energized mitochondria at a rate of 3.77 nmol min^{-1} mg^{-1}. In the presence of superoxide dismutase (SOD), NO degradation decreased to a rate similar to that seen in the control situation (without NADH). The same inhibitory effect of SOD on NO degradation was seen when NADH was replaced by NADPH (Fig. 4b). This inhibitory effect of O_2^- dismutation by SOD on NAD(P)H-dependent NO degradation indicates that NO is mainly consumed by its reaction with O_2^-.

3.4. Effect of mitochondrial respiratory chain inhibitors on NO degradation

The foregoing results suggest that any situation that stimulates electron leakage from the respiratory chain would favor NO degradation by potato tuber mitochondria. In animals, antimycin-A (Anti-A) favors the formation of the unstable ubisemiquinone, the auto-oxidation of which generates O_2^- [31]. Anti-A was therefore used to analyze the effect of electron leakage from complex III on NO degradation by succinate-, malate- or NADH-energized potato tuber mitochondria (Fig. 5a). When

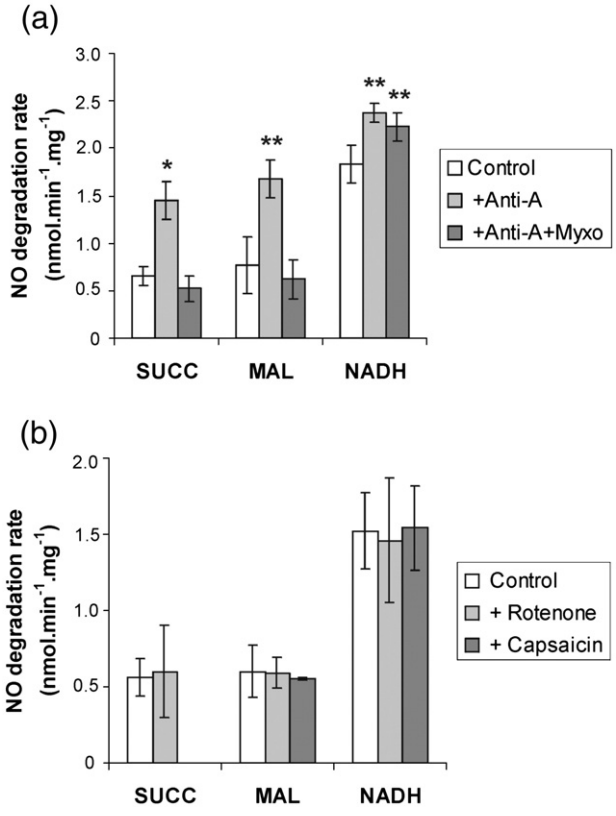

Fig. 5. Effect of inhibitors of mitochondrial respiration on NO degradation by potato tuber mitochondria. Mitochondria (0.25 mg/mL) were incubated in the reaction medium and energized with different substrates, as described in Fig. 1. The degradation of NO released from GSNO (100 μM) was followed without any further addition (control) or (a) in the presence of 10 μM Anti-A alone or with 10 μM myxothiazol or (b) in the presence of 10 μM rotenone or 60 μM capsaicin. The columns represent the mean ± standard deviation of three different mitochondrial preparations. $^{*}p<0.01$ and $^{**}p<0.05$ compared to the respective rates without inhibitor.

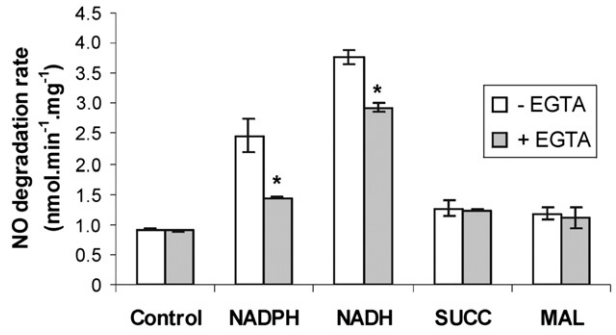

Fig. 6. Ca^{2+}-dependent NO degradation by potato tuber mitochondria. Mitochondria (0.25 mg/mL) were incubated in the reaction medium and energized with 0.1 mM NADPH (NADPH), 0.1 mM NADH (NADH), 5 mM succinate (SUCC) or 10 mM malate plus 10 mM glutamate (MAL). An aliquot of saturated NO solution was applied and NO decay was followed in the absence or presence of 0.5 mM EGTA. The control column represents NO degradation by potato mitochondria without any substrate. The columns represent the mean ± standard deviation of three experiments with a representative mitochondrial preparation. *$p<0.01$ compared to the respective rates without EGTA.

mitochondria were incubated only with succinate, a slow rate of NO consumption (0.66±0.10 nmol min^{-1} mg^{-1}) was observed. However, when incubation with Anti-A preceded the addition of GSNO, the rate of NO consumption increased to 1.45±0.21 nmol min^{-1} mg^{-1}. This enhanced rate of NO degradation was prevented by SOD (not shown). Myxothiazol inhibits the formation of the unstable ubisemiquinone, thereby preventing electron leakage from complex III [31]. The addition of myxothiazol to succinate-energized potato tuber mitochondria prevented the enhanced NO degradation seen with Anti-A and reduced the rate of NO degradation to the level seen in mitochondria incubated without substrate (0.39±0.14 nmol min^{-1} mg^{-1}). The stimulatory and inhibitory effects of Anti-A and myxothiazol, respectively, were also seen when mitochondria were energized with malate. NO degradation was also stimulated by Anti-A when NADH was used as substrate. However, in contrast to the response seen with succinate and malate, the addition of myxothiazol did not significantly inhibit the Anti-A-stimulated NO degradation, suggesting an electron leakage upstream of complex III when NADH is used as substrate. Similar results were obtained for NADPH (not shown).

Complex I is an important site of O_2^- generation in mammalian mitochondria [18] and similar formation may also occur in plants [21]. In contrast to complex III, which can release O_2^- to both sides of the inner mitochondrial membrane, O_2^- formed by complex I is released exclusively into the matrix [32]. Under our experimental conditions, NADH could favor electron leakage from complex I of a subpopulation of ruptured potato mitochondria. To test this possibility, NADH-dependent NO degradation was analyzed in the presence of rotenone and capsaicin, drugs that respectively stimulate and inhibit electron leakage from complex I [18]. As shown in Fig. 5b, neither compound had any significant effect on the rate of NO degradation by NADH-energized potato tuber mitochondria. Furthermore, the high values of respiratory controls (phosphorylating/resting respiration) of these mitochondrial suspensions (5.5–6.0 with malate and 3.6–4.0 with NADH) were indicative of a high percentage of intact mitochondria that could prevent the access of NADH to complex I. Rotenone and capsaicin also did not modify NO degradation by malate-energized mitochondria (Fig. 5b). These results indicated that the NADH-dependent NO degradation by intact potato mitochondria did not result from electron leakage from complex I. Rotenone had no effect on NO degradation by succinate-energized potato mitochondria (Fig. 5b), indicating that NO consumption, observed in these experimental conditions, was also not attributable to reverse electron transport from succinate dehydrogenase to complex I [33].

As shown in Fig. 6, Ca^{2+} chelation by EGTA significantly inhibited NO degradation by NADPH-energized mitochondria (from 2.46±0.28 nmol min^{-1} mg^{-1} to 1.44±0.02 nmol min^{-1} mg^{-1}). EGTA also inhibited NO degradation by NADH-energized potato mitochondria, but to a lesser extent (from 3.77±0.11 nmol min^{-1} mg^{-1} to 2.93±0.07 nmol min^{-1} mg^{-1}). This finding indicates that NO degradation stimulated by NADH is less dependent on Ca^{2+} than the NADPH-stimulated degradation, a characteristic that also distinguishes the external NAD(P)H dehydrogenases [21]. Accordingly, EGTA strongly inhibited the respiratory activity of NADPH-energized mitochondria (from 176.7±8.9 ng atom O min^{-1} mg^{-1} to 10.9±2.2 ng atom O min^{-1} mg^{-1}), while a lower inhibitory effect (from 372.1±1.5 ng atom O min^{-1} mg^{-1} to 258.9±11.2 ng atom O min^{-1} mg^{-1}) was observed when NADH was used as substrate. Fig. 6 also shows that EGTA did not significantly alter the rates of NO degradation by potato tuber mitochondria energized with succinate or malate; these rates were much lower than those seen with NAD(P)H-energized mitochondria and were similar to that of control mitochondria (0.91±0.02 nmol min^{-1} mg^{-1}).

Overall, these findings suggest that, in addition to the O_2^- from complex III, the extra-mitochondrial release of O_2^- formed by external dehydrogenases contributes to NO degradation when potato tuber mitochondria are energized with NAD(P)H.

3.5. Superoxide anion release by potato mitochondria

Superoxide anion generation by potato tuber mitochondria energized with different substrates was assessed based on the hydrogen peroxide (H_2O_2) concentration. As shown in Fig. 7, mitochondria energized with NADH had the highest rate of H_2O_2 production (1.07±0.02 nmol min^{-1} mg^{-1}). NADPH-energized mitochondria produced less H_2O_2 (0.43±0.05 nmol min^{-1} mg^{-1}) than with NADH. When succinate and malate were used as substrates, still lower rates were observed (0.22±0.03 nmol min^{-1} mg^{-1} and 0.24±0.02 nmol min^{-1} mg^{-1}, respectively) and these rates were similar to that of non-energized mitochondria (0.15±0.02 nmol min^{-1} mg^{-1}). For all tested substrates, the rates of H_2O_2 production were lower than the respective NO degradation rates because the monitoring of H_2O_2 concentration was carried out in the absence of NO. It is well known that the inhibitory effect of NO on respiration favors electron leakage from the respiratory chain, stimulating its own degradation. NO causes inhibition of mitochondrial electron transfer at COX and at the ubiquinone–cytochrome *b* region of the respiratory chain, the latter leading directly to an increased superoxide production [17]. These multiple inhibitory actions of NO in the respiratory chain explain the differences observed in the rates of H_2O_2 production and NO degradation.

Fig. 7 also shows that, for all tested substrates, the incubation of potato mitochondria with Anti-A practically doubled the H_2O_2 production, thereby confirming the importance of electron leakage from complex III in O_2^- generation. On the other hand, the lack of a

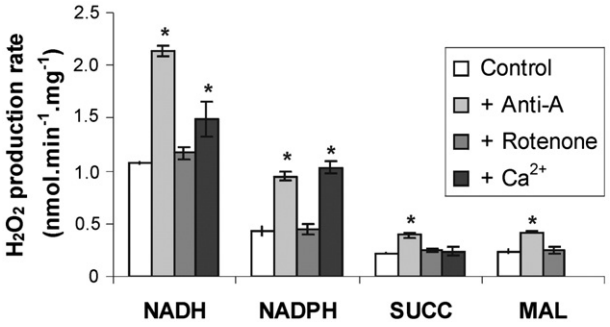

Fig. 7. Peroxide production by potato tuber mitochondria. Mitochondria (0.25 mg/mL) were incubated in the reaction medium described in Fig. 1 plus 100 U SOD/mL, 1 U horseradish peroxidase/mL and 25 μM Amplex Red, and energized with different substrates, as described in Fig. 6. The rates of H_2O_2 production in the presence of 10 μM Anti-A, 10 μM rotenone or 250 μM Ca^{2+} were compared to those without any inhibitor (Control). The columns represent the mean ± standard deviation of three experiments with a representative mitochondrial preparation. *$p<0.05$ compared to the respective rates without inhibitor.

significant effect of rotenone on H_2O_2 generation in NAD(P)H-, malate- and succinate-energized mitochondria confirmed that electron leakage from complex I, including reverse transport from complex II, was not detected in our experimental conditions, probably because the antioxidant defense mechanisms of the mitochondrial matrix can dissipate internally generated O_2^- [34]. Ca^{2+} addition to the reaction medium significantly increased the H_2O_2 production by NADPH-energized potato mitochondria, from 0.43 ± 0.05 nmol min^{-1} mg^{-1} to 1.03 ± 0.06 nmol min^{-1} mg^{-1}. In the presence of NADH, Ca^{2+} stimulated H_2O_2 production only by 40% corroborating with the lower inhibitory effect of Ca^{2+} chelation on NADH- compared to NADPH-dependent NO degradation (Fig. 6). H_2O_2 production in succinate-energized potato mitochondria was not modified by Ca^{2+}.

Overall, monitoring of H_2O_2 production gave further evidence that external NAD(P)H dehydrogenases, in addition to complex III, contribute to NO degradation by potato tuber mitochondria.

4. Discussion

The present results describe a detailed study of NO degradation by plant mitochondria. We show that mitochondria isolated from potato tubers can degrade NO under aerobiosis (Fig. 3), and this degradation was prevented by the removal of O_2^- from the reaction medium (Fig. 4). These findings indicated that the non-enzymatic reaction of NO with O_2^- accounted for the NO degradation in isolated potato mitochondria, an activity previously observed in mitochondria and submitochondrial particles of animal tissues [17,19]. The sites of electron leakage from the respiratory chain involved in NO degradation by intact potato mitochondria were identified by using various electron donors and inhibitors of mitochondrial electron transport.

Experiments with Anti-A and myxothiazol showed that extramitochondrial electron leakage from the ubiquinone cycle at complex III was an important source of O_2^- release for NO degradation (Figs. 5a and 7). Previous studies with mitochondria and submitochondrial particles of various plant species have shown the important role of complex III in O_2^- generation [21]. In plant mitochondria with stimulated AOX activity, the generation of O_2^- by complex III is reduced [35] because AOX activity attenuates the level of ubiquinone reduction [22]. Mitochondrial preparations isolated from fresh potato tubers have no AOX activity (see [36]). Hence, the effect of AOX on the mechanism of mitochondrial NO degradation could not be checked here.

In addition to electron leakage from complex III, external NAD(P)H dehydrogenases were also identified as important sites of O_2^- generation in the respiratory chain of potato tuber mitochondria. In agreement with this, higher rates of O_2^- generation were detected in NAD(P)H-energized potato mitochondria (Fig. 7), the rates of NO consumption were accelerated in NAD(P)H- compared to succinate- or malate-energized mitochondria (Figs. 1, 5 and 6), and SOD prevented the accelerated NO degradation (Fig. 4). Furthermore, NAD(P)H-dependent NO degradation, although stimulated by Anti-A, was not significantly inhibited by myxothiazol (Fig. 4a), indicating that electron leakage at sites located upstream of complex III contributed to NO degradation. The participation of Ca^{2+}-dependent external NAD(P)H dehydrogenases in this mechanism of NO degradation was further demonstrated by the observation that the rates of O_2^- generation (Fig. 7) and NO consumption (Fig. 6) increased as a function of the free Ca^{2+} in the reaction medium. Moreover, these effects of Ca^{2+} were more pronounced when NADPH was used as respiratory substrate, compared to NADH. This finding agrees with the previous observation that external NADH dehydrogenase is less dependent on Ca^{2+} than the corresponding NADPH dehydrogenase [21].

The importance of the external NADH dehydrogenase of *Saccharomyces cerevisiae* mitochondria as a source of O_2^- was suggested by Fang and Beattie [31]. An elevated O_2^- production has also been observed in potato submitochondrial particles [37] and in isolated mitochondria from green bell pepper fruit [38] in the presence of NADH as the respiratory substrate. However, in none of these studies with plants did the authors distinguish whether this enhanced production involved complex I or the activities of alternative dehydrogenases. Although complex I releases O_2^- exclusively into the matrix [32], its activity resulting from a subpopulation of ruptured potato mitochondria could contribute to NO degradation. However, the lack of effect of rotenone and capsaicin on the rate of NO degradation by NADH- or malate-energized potato mitochondria excluded this possibility (Fig. 5b). Additionally, the observation that NADH was unable to reach complex I because of the high integrity of the mitochondrial suspensions precluded any assessment of the contribution of internal NAD(P)H dehydrogenases to the NO degradation seen here. However, the participation of complex I and internal alternative dehydrogenases in NO degradation cannot be eliminated.

The accelerated degradation of NO in the presence of NAD(P)H may have an important role in regulating the signaling and toxicity of NO in plants. NO binds reversibly to COX at nanomolar concentrations and inhibits oxygen consumption and ATP synthesis in plant mitochondria [13,14]. As shown here, the degradation of NO by its reaction with O_2^- allowed the recovery of O_2 consumption (Fig. 1) and the reestablishment of the membrane potential when potato tuber mitochondria were energized with NAD(P)H (Fig. 2). This interaction with O_2^- to relieve the inhibition of COX by NO may therefore represent an alternative pathway for the use of NO by plant mitochondria. The importance of the non-enzymatic reaction of NO with O_2^- in controlling respiratory activity has previously been demonstrated in studies with mitochondria, submitochondrial particles and purified enzymes of animal origin [17,19,20]. In these studies, complex III was found to be the main source of O_2^- for NO degradation.

As demonstrated here, in addition to complex III, potato mitochondria also show electron leakage from external NAD(P)H dehydrogenases, indicating the existence of an additional mechanism for preventing the deleterious effects of NO on the respiratory activity of plant mitochondria. Many studies have focused on the potential role of these alternative dehydrogenases, acting as nonphosphorylating enzymes, in reducing the rate of free radical production by plant mitochondria. Experimental proof for this activity has been elusive and the role of these enzymes, the expression of which is altered in response to various environmental conditions and stress, has been an unsolved question [23]. The results described here have revealed a previously unrecognized role of these enzymes in plant NO metabolism.

Although the foregoing mechanism of NO degradation can recuperate mitochondrial functionality, it generates $ONOO^-$. This anion can participate in the oxidation, nitrosation and nitration of biological molecules involved in cellular signaling and, depending on the conditions, can cause also oxidative damage [16]. However, given the rates of NO and O_2^- production under physiological conditions and the extremely short half-life of $ONOO^-$, the intramitochondrial levels of $ONOO^-$ are probably very low [17,20] and unlikely to have any effect on the respiratory activity of potato mitochondria, as also reported for rat heart submitochondrial particles [17]. Recent work with animal cells suggests that rather than being a purely deleterious and cytotoxic process, the reaction of O_2^- with NO is an important regulatory mechanism that modulates signaling pathways by controlling the steady-state levels of NO and preventing the formation of peroxide and hydroxyl radical from O_2^- [39,40]. Studies with plants have also proposed that the interaction of NO with O_2^- may be important in minimizing oxidative stress [2].

The involvement of external NAD(P)H dehydrogenases in NO degradation confirms the importance of mitochondria for NO homeostasis in plants cells. Recent studies have identified mitochondria as one of the main sites of NO synthesis in plants because of their ability to reduce NO_2^- via the respiratory chain [41,42]. This NO production from NO_2^- increases in response to pathogen attack and helps to prevent the spread of pathogens [5,41]. However, the prolonged exposure of

mitochondria to this radical may lead to a persistent loss of mitochondrial functionality, as discussed above. In addition to mitochondria, NO is also produced by peroxisomes [43], chloroplasts [44], cytosol [45] and the plasma membrane [46]. The degradation of this NO by external NAD(P)H dehydrogenases may be important in preventing further inhibition of mitochondrial respiration. This mechanism of NO degradation in the vicinity of the mitochondrial membrane may contribute to NO homeostasis and maintain the overall metabolism of plant tissues that depend on mitochondria for energy, as is the case of potato tubers. However, it is important to point out that mitochondria isolated from fresh potato tubers are relatively unique among plants in lacking any AOX activity. Thus, future investigation addressing the effect of this protein on NO degradation would be relevant in order to check the extension of the present findings among other plants.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbabio.2008.02.006.

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