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Nitric oxide inhibits succinate dehydrogenase-driven oxygen consumption in potato tuber mitochondria in an oxygen tension-independent manner

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NO (nitric oxide) is described as an inhibitor of plant and mammalian respiratory chains owing to its high affinity for COX (cytochrome *c* oxidase), which hinders the reduction of oxygen to water. In the present study we show that in plant mitochondria NO may interfere with other respiratory complexes as well. We analysed oxygen consumption supported by complex I and/or complex II and/or external NADH dehydrogenase in Percoll-isolated potato tuber (*Solanum tuberosum)* mitochondria. When mitochondrial respiration was stimulated by succinate, adding the NO donors SNAP (*S*-nitroso-*N*-acetyl-DL-penicillamine) or DETA-NONOate caused a 70% reduction in oxygen consumption rate in state 3 (stimulated with 1 mM of ADP). This inhibition was followed by a significant increase in the K_m value of SDH (succinate dehydrogenase) for succinate (K_m of 0.77 ± 0.19 to 34.3 ± 5.9 mM, in the presence of NO). When mitochondrial respiration was stimulated by external NADH dehydrogenase or complex I, NO had no effect on respiration. NO itself and DETA-NONOate had similar effects to SNAP. No significant inhibition of respiration was observed in the absence of ADP. More importantly, SNAP inhibited PTM (potato tuber mitochondria) respiration independently of oxygen tensions, indicating a different kinetic mechanism from that observed in mammalian mitochondria. We also observed, in an FAD reduction assay, that SNAP blocked the intrinsic SDH electron flow in much the same way as TTFA (thenoyltrifluoroacetone), a non-competitive SDH inhibitor. We suggest that NO inhibits SDH in its ubiquinone site or its Fe–S centres. These data indicate that SDH has an alternative site of NO action in plant mitochondria.

Key words: heterotrophic plant tissue, mitochondrion, nitric oxide, succinate dehydrogenase.

INTRODUCTION

NO (nitric oxide) is a free radical gas that has emerged as a critical signalling molecule in plants and that has been suggested to play an important role in different physiological events [1]. These include auxin-induced lateral root formation [2], adventitious root growth [3] and root hair development [4]. In addition, NO may be involved in abiotic and biotic stress responses [5].

NO was first discovered in animal cells where it is involved in important and diverse physiological functions, including the regulation of blood pressure and neurotransmission [6,7]. NO is greatly diffusible and reactive, but its mechanism of signalling is not well known. NO readily nitrosylates cysteine (S-nitrosylation) and tyrosine (tyrosine nitration) residues in various proteins [8,9]. Notably, S-nitrosylation has been considered to be an important prototypical redox-based, post-translational protein modification [10,11].

Metabolic alterations brought about by oxygen deprivation such as that occurring in flooded lands or deeply buried tubers and roots [12] may increase NO levels in hypoxic tissues both in animals [13] and plants [14]. There is some evidence in plants that NO production is improved in hypoxia [15], which may up-regulate the protein involved in NO metabolism [16]. The NO formation can arise from endogenous plant tissues or from exogenous sources such as soil supplementation with nitrogen fertilizers [17].

Hypoxia in the seeds of soybean (*Glycine max*) and pea (*Pisum sativum*) triggers nitrite-dependent growth in endogenous NO concentrations, whereas the increase of oxygen availability reduces endogenous NO concentrations, thereby abolishing mitochondrial and metabolic inhibition [18]. It has been proposed that nitrite, under oxygen deprivation, can act as an electron acceptor in the mitochondrial ETS (electron transfer system), providing a sink for depletion of pools of reduced NAD(P)H, thereby sustaining ATP synthesis [19]. These data suggest that metabolic NO inhibition depends on oxygen concentration and supports the view that NO competes with oxygen for the same binding site at the level of COX [cytochrome *c* oxidase (complex IV)] in mitochondria. It has already been demonstrated that NO can inhibit the cytochrome pathway in mitochondrial respiration owing to its competitiveness with oxygen at the catalytic site of cytochrome oxidase [14], indicating that the mechanism found in animals for inhibition that is dependent on the concentration of mitochondrial oxygen has also been preserved in plants. Thus it is unlikely that NO plays a role under normal or hyperoxic conditions in plant mitochondria. Nevertheless a study using purified deoxygenated cow heart ETS complexes has shown that NO can reduce succinate–cytochrome *c* reductase activity by 90%, whereas the ubiquinol–cytochrome *c* reductase is unaffected [20]. NO can also interact with different enzymes, especially those containing iron–sulfur centres, haem and cysteine [21] in their structures. Thus the enzymes of the respiratory chain

Abbreviations used: AOX, alternative oxidase; COX, cytochrome *c* oxidase; CPTIO, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide; DCPIP, dichlorophenol-indophenol; ETS, electron transfer system; faf-BSA, fatty acid-free BSA; MBM, mouse brain mitochondria; MLM, mouse liver mitochondria; NADHCO, NADH–cytochrome *c* oxidoreductase; 3-NP, 3-nitropropionic acid; PTM, potato tuber mitochondria; SCO, succinate cytochrome *c* oxidoreductase; SDH, succinate dehydrogenase; SNAP, *S*-nitroso-*N*-acetyl-DL-penicillamine; TPP, thiamine pyrophosphate; TTFA, thenoyltrifluoroacetone; WT, wild-type.

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are potential candidates for interacting with NO, owing to the presence of several iron–sulfur centres and haem in the structures of these ETS complexes [22].

In addition to complex IV, the respiratory chain complexes I, II and III are potential candidates for interaction with NO that does not depend on oxygen concentration. In the present study we investigated the effect of different concentrations of NO donors [SNAP (*S*-nitroso-*N*-acetyl-DL-penicillamine) and DETA-NONOate], as well as NO itself, on the modulation of PTM (potato tuber mitochondria) SDH (succinate dehydrogenase) and identified a potential mechanism by which NO acts on this complex. The present study shows that the electron flow in ETS is strongly blocked at the level of SDH activity, independent of oxygen levels.

MATERIALS AND METHODS

Chemicals, preparation of NO and $ONOO^-$ solutions and biological materials

ADP, ATP, faf-BSA (fatty-acid-free BSA), oligomycin, SNAP, DETA-NONOate, CPTIO [2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide], DCPIP (dichlorophenolindophenol), TTFA (thenoyltrifluoroacetone), TPP (thiamine pyrophosphate) and 3-NP (3-nitropropionic acid) were purchased from Sigma–Aldrich. Percoll was from GE Healthcare. All other reagents were analytical grade. Potato tubers (*Solanum tuberosum*) were purchased from a local supermarket.

The solution of peroxynitrite gave a final concentration of 7.3 mM based on the absorbance at 302 nm and multiplying by a molar coefficient extinction of 1300 $M^{-1} \cdot cm^{-1}$ as described previously [23]

A solution of 2 mM NO from a cylinder of medicinal NO/N_2 [NO 500 p.p.m. and N_2 qsf (quantity sufficient for)] from a mixture for hospital use from the infirmary of the National Institute of Cardiology as described previously [24]. A degraded SNAP solution was prepared by incubating this NO donor in the buffer for approximately 48 h, before adding it to the samples. This solution was used as a control for any secondary effects caused by degraded SNAP products.

Isolation of PTM by self-generated Percoll gradient

PTM were obtained as described previously [25] with some modifications, using a cold extraction buffer containing: 10 mM Hepes/Tris, pH 7.4, 0.3 M mannitol, 2 mM EGTA, 5 mM EDTA, 0.3 mM PMSF, 20 mM 2-mercaptoethanol and 0.1 % (w/v) faf-BSA. The homogenate was strained through eight layers of cheesecloth and centrifuged at 3000 ***g*** at 4 °C for 3 min. The supernatant was then centrifuged at 12000 ***g*** at 4 °C for 10 min. The mitochondrial pellet was suspended in 5 ml of ice-cold extraction buffer and layered in Hitachi P50 centrifuge tubes containing 35 ml of ice-cold extraction buffer containing 28 % (v/v) Percoll and centrifuged at 40000 ***g*** at 4 °C for 30 min. After centrifugation three major bands were obtained in sequence: the first band (fraction 1) at the top and the middle band (fraction 2) were used for further PTM isolation or for enzymatic activity determinations. Mitochondrial activity markers were found to be enriched in fraction 2. The mitochondrial fraction was removed and diluted with extraction buffer without Percoll and centrifuged twice at 12000 ***g*** at 4 °C for 10 min. The final pellet was resuspended in 0.6 ml of extraction buffer and kept in an ice-water bath. The final protein concentration varied from 10 to 20 mg/ml.

Isolation of MBM (mouse brain mitochondria) and MLM (mouse liver mitochondria)

Mitochondria from the forebrains and liver were isolated by differential centrifugation and kept at 4 °C throughout the isolation procedure. Briefly, the two tissues were rapidly removed to an ice-cold isolation buffer containing 0.32 M sucrose, 1 mM EDTA, 1 mM EGTA and 10 mM Tris/HCl (pH 7.4). After five washes to remove contaminating blood, the tissues were sliced into little pieces in isolation buffer. The tissues were manually homogenized using 11 strokes in a Teflon/glass potter. The liver homogenates were centrifuged at 600 ***g*** for 5 min using a Hitachi Himac SCR20B RPR 20-2 rotor. The supernatants were carefully removed and centrifuged again at 12000 ***g*** for 10 min. The supernatant of the second centrifugation was centrifuged at 12000 ***g*** for 10 min. The pellets obtained were re-suspended in the isolation buffer. For the isolation of brain mitochondria, the forebrain homogenate was centrifuged at 2000 ***g*** for 3 min. The supernatant was carefully removed and centrifuged again at 12000 ***g*** for 10 min. The supernatant of the second centrifugation was centrifuged at 12000 ***g*** for 10 min. The pellets obtained were re-suspended in the isolation buffer. All of the experiments with isolated mitochondria were carried out at 37 °C with continuous stirring in a respiration buffer containing 10 mM Tris/HCl, pH 7.4, 0.32 M mannitol, 8 mM inorganic phosphate, 4 mM $MgCl_2$, 0.08 mM EDTA, 1 mM EGTA and 0.2 mg/ml faf-BSA.

Oxygen uptake measurements

The oxygen consumption rates were measured polarographically using high-resolution respirometry (Oroboros Oxygraph-O2K). The electrode was calibrated between 0 and 100 % saturation with atmospheric oxygen at 28 °C for PTM or 37 °C for MBM. The PTM (0.2 mg/ml) were incubated with 2.0 ml of the standard respiration buffer containing 0.3 M mannitol, 10 mM Tris/HCl, pH 7.2, 3 mM $MgSO_4$, 10 mM NaCl, 5 mM KH_2PO_4, 0.3 mM β-NAD^+ and 0.1 % faf-BSA. The MBM (0.2 mg/ml) were incubated with 2.0 ml of the standard respiration buffer containing 10 mM Tris/HCl, pH 7.4, 0.32 M mannitol, 8 mM inorganic phosphate, 4 mM $MgCl_2$, 0.08 mM EDTA, 1 mM EGTA and 0.2 mg/ml faf-BSA. The cuvette was closed immediately before starting the experiments. Respiratory control ratio values were obtained with isolated PTM or MBM, after complex II activation by 10 mM succinate or complex I activation by 5 mM pyruvate, 1 mM malate and 10 mM TPP or external NADH dehydrogenase activation by 1 mM NADH (only in PTM). Other additions are indicated in the Figure legends.

The oxygen consumption rates are expressed as oxygen flux per mass (pmol $\cdot s^{-1} \cdot$ mg of protein^{-1}) and these values are shown in differential form (where the time difference is taken as infinitesimally small), so that the expression becomes $JO_2 = -(d[O_2]/dt)$, where JO_2 is the oxygen flux rate and t is time in seconds. This unit of oxygen consumption is found in Figures 2–5. For the sake of clarity, in some experiments the oxygen consumption is also expressed in a way that is generally found in classical work, i.e. as oxygen concentration (μM) in order to show the actual level of oxygen concentration during the course of the experiment (Figures 1 and 8).

Kinetic parameters of SDH

The activity of SDH was determined spectrophotometrically using DCPIP as an artificial electron acceptor and succinate as the substrate [26]. The assay was performed at room temperature (25° C) in 1.0 ml of reaction medium containing 20 mM phosphate buffer (pH 7.2), 0.1 % Triton X-100, 4 mM sodium azide, 5 mM

succinate, 50 μM DCPIP, from 10 to 200 μM SNAP or 100 μM DETA-NONOate, and/or 2 mM CPTIO. Blanks were obtained in the absence of succinate. The reaction was started by adding 0.1 mg of PTM and the reduction of DCPIP was monitored for 3 min at 600 nm. The SDH activity was calculated using the molar absorption coefficient of reduced DCPIP (21.0 $mM^{-1} \cdot cm^{-1}$).

The kinetic parameters were estimated by non-linear regression analysis applied to the Michaelis–Menten equation using the program package supplied by GraphPad Prism 5.0.

Kinetic parameters of SCO (succinate cytochrome *c* oxidoreductase) and NADHCO (NADH–cytochrome *c* oxidoreductase) branches of ETS

The activity of SCO was determined spectrophotometrically using reduction of cytochrome *c* with succinate as the substrate [27]. The assay was performed at room temperature (25 °C) in 1.0 ml of reaction medium containing 20 mM phosphate buffer (pH 7.2), 4 mM sodium azide, 50 mM cytochrome *c*, 10 mM succinate, from 10 to 200 μM SNAP and/or 500 μM CPTIO. Blanks were obtained in the absence of succinate. The reaction was started by adding 0.1 mg of PTM and the reduction of cytochrome *c* was monitored for 5 min at 550 nm. SCO activity was calculated using the molar absorption coefficient of reduced cytochrome *c* (19.0 $mM^{-1} \cdot cm^{-1}$). For determination of NADHCO the reaction was performed as for SCO, but the succinate was replaced by 1 mM NADH.

The kinetic parameters were estimated by non-linear regression analysis applied to the Michaelis–Menten equation using the program package supplied by GraphPad Prism 5.0.

$FADH_2/FAD^+$ status in PTM

The redox state of $FADH_2/FAD^+$ in PTM was determined by autofluorescence using excitation and emission wavelengths of 490 ± 10 nm and 530 ± 10 nm respectively [28]. The assay was performed using coupled PTM, adding 5 mM succinate to the respiration medium (see the Oxygen uptake measurements section) in the presence or absence of 100 μM SNAP, 100 μM TTFA and 10 mM 3-NP. Other additions are indicated in the Figure legends.

Potato tuber slice oxygen consumption

Small square pieces (8 mm diameter, 2 mm thickness) were cut perpendicular to the stolon-apex axis of potato tubers [29]. The tuber slices were taken from the middle of the tuber, avoiding the outer 3 mm and the tuber skin. The slices were incubated for 2 h in different conditions as shown in the Figure legend. For the oxygen consumption measurements we used 16 slices (0.3–0.45 g total slices) in 10 mM Mes/KOH, pH 6.5, buffer using high-resolution respirometry (Oroboros Oxygraph-O2K) in a chamber of 2 ml and the oxygen consumption rate was monitored.

Protein determination

The protein concentration was determined as described previously [30], using BSA as a standard.

Statistical analysis

Data were plotted with GraphPad Prism 5.0 and analysed by one-way ANOVA and a post-hoc Tukey's test. $P < 0.05$ was considered statistically different.

Table 1 Effect of NO donors and $ONOO^-$ on the respiratory rates of PTM stimulated by complex I-linked substrates

The values are means $\pm$ SEM of the respiratory rates of at least five independent PTM preparations (pmol $\cdot s^{-1} \cdot$ mg of protein^{-1}).

Respiratory state	Control	SNAP	DETA-NONOate	$ONOO^-$
Non-phosphorylation	250.4 ± 2.63	232.2 ± 5.45	248.2 ± 2.35	24.7 ± 0.85*
ADP phosphorylation	730.7 ± 25.4	747.1 ± 34.12	718.8 ± 14.25	28.7 ± 0.98*

*Significant difference between tested PTM and control PTM by ANOVA with Tukey's post-hoc test ($P < 0.05$).

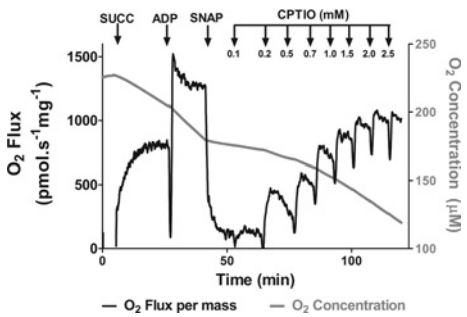

Figure 1 A typical real-time trace of the effect of the NO donor SNAP on oxygen consumption, showing flow of oxygen per mass of PTM protein and O_2 concentration levels

PTM were incubated with 2.0 ml of the standard respiration buffer (see Materials and methods section) at 28 °C. Oxygen consumption was stimulated with 10 mM succinate. The oxygen flux (pmol $\cdot s^{-1} \cdot$ mg of protein^{-1}) values are given on the left-hand axis, black trace. The oxygen concentration (μM) values are given on the right-hand axis, grey trace. SUCC, succinate.

RESULTS

NO inhibits oxygen consumption reversibly in succinate-sustained ADP-phosphorylating state respiration at high PO_2 tensions

In mammalian mitochondria it was shown previously that NO donors induce a prompt inhibition when the substrate is linked to complex I, as a result of $ONOO^-$ formation under assay conditions. When succinate is used the inhibition of respiration by the NO donor is not immediate, and instead appears after a lag phase. This result indicates that NO has to be accumulated in order to inhibit COX and causes further inhibition of mammalian mitochondria ETS that is reversed by oxymyoglobin addition [31].

In order to test the influence of NO donors and $ONOO^-$ at high oxygen concentrations (above 100 μM), the respiration of PTM supported by complex I-linked substrates (Table 1) and succinate (Figure 1) was investigated. We tested the effect of NO donors (SNAP and DETA-NONOate) in the presence of complex I-linked substrates (pyruvate/malate/TPP), external NADH dehydrogenase and succinate linked to complex II (SDH) of PTM (Figures 1 and 2, and Table 1). SNAP and DETA-NONOate did not inhibit PTM complex I, either in ADP-phosphorylating or non-phosphorylating states (Table 1). We observed that 100 μM SNAP and DETA-NONOate were both able to significantly decrease the respiratory capacity of PTM by approximately 60 % (Figures 1, 2A and 2E), when oxygen consumption was stimulated with 10 mM succinate and 1 mM ADP. This inhibition was reversed by the inclusion of 2 mM CPTIO, a NO scavenger. Under these conditions, NO donors inhibit only when complex II is contributing to ATP synthesis, an ADP-phosphorylating state. However, when the PTM was not synthesizing ATP (non-phosphorylating state), the respiration

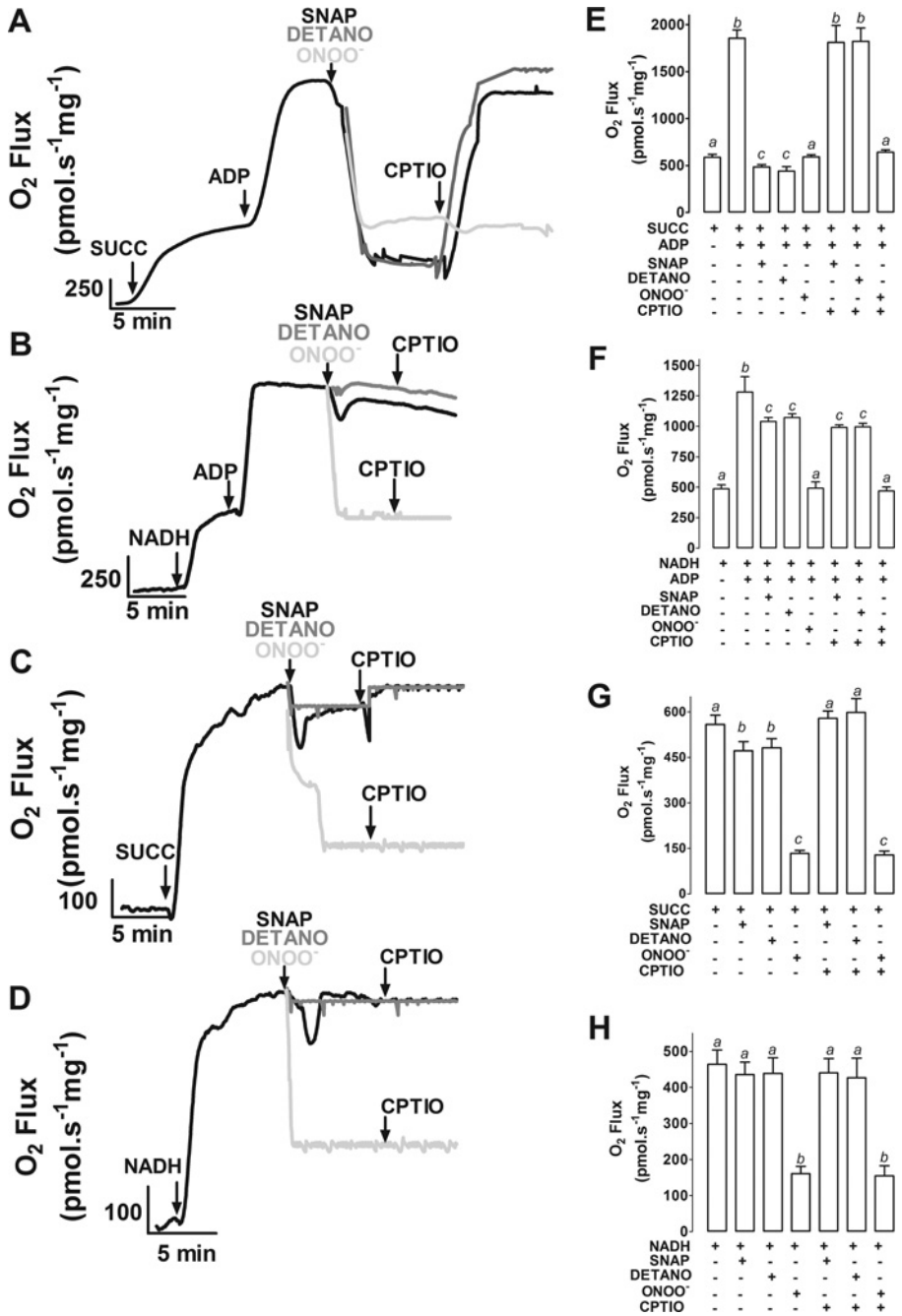

Figure 2 Effect of NO donors on oxygen consumption by PTM

PTM were incubated with 2.0 ml of the standard respiration buffer (see the Materials and methods section) at 28 °C. Oxygen consumption was stimulated with 10 mM succinate (SUCC) (**A** and **C**) or 1 mM NADH (**B** and **D**) and 1 mM ADP (**A** and **B**), or without ADP (**C** and **D**), before the addition (arrow) of 100 μM SNAP, 100 μM DETA-NONOate (DETANO) or 10 μM $ONOO^-$. (**E**), (**F**), (**G**) and (**H**) show the respiratory rates calculated from the traces in (**A**), (**B**), (**C**) and (**D**) respectively. (**E**), (**F**), (**G**) and (**H**) show means $\pm$ S.E.M. of the respiratory rates for at least five independent PTM preparations. Groups that are significantly different ($P < 0.05$ by ANOVA with Tukey's post-hoc test) are indicated by different letters above the bars.

stimulated by succinate (Figures 2C and 2G), was no longer sensitive to NO donors. When the oxygen consumption of PTM was NADH sustained, NO donors were unable to inhibit respiration in either ADP-phosphorylating (Figures 2B and 2F) or non-phosphorylating conditions (Figure 2D and 2H).

A totally different profile was observed when 10 μM $ONOO^-$ was added either to phosphorylation or non-phosphorylation oxygen consumption and, independently of substrate, used to sustain PTM respiration (Figures 2 and 3, and Table 1). In addition with $ONOO^-$, CPTIO was not able to restore the respiration level of PTM (Figures 2 and 3).

PTM is able to scavenge NO by the activity of its external NADH dehydrogenase [32]. It has been proposed that the enzyme complex present on the inner mitochondrial membrane facing the intermembrane space (referred to as external NADH dehydrogenase) of PTM, when using NADH as substrate, would decompose NO, avoiding its effects on the respiratory chain of PTM. Our intention with the experiment, the results of which are

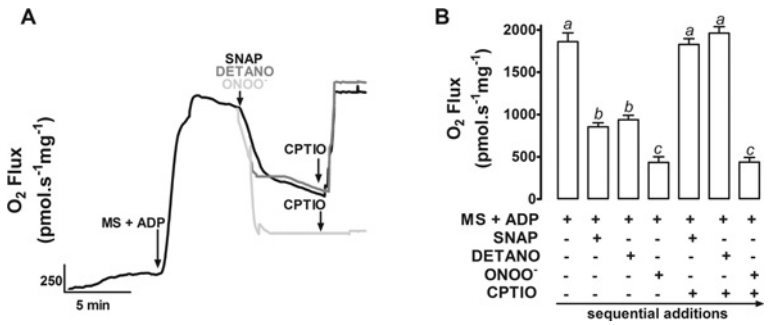

Figure 3 Effect of NO donors on oxygen consumption in PTM stimulated by multiple substrates (MS)

PTM were incubated with 2.0 ml of the standard respiration buffer (see the Materials and methods section) at 28 °C. Typical rates of oxygen consumption are shown. The PTM were stimulated by 5 mM succinate, 1 mM NADH, 5 mM pyruvate, 1 mM malate, 10 mM TPP and 1 mM ADP, before the addition (arrow) of 100 μM SNAP, 100 μM DETA-NONOate (DETANO) or 10 μM $ONOO^-$ (**A**). (**B**) Results are means ± S.E.M. of the respiratory rates for at least five independent PTM preparations. Groups that are significantly different ($P < 0.05$ by ANOVA with Tukey's post-hoc test) are indicated by different letters above the bars.

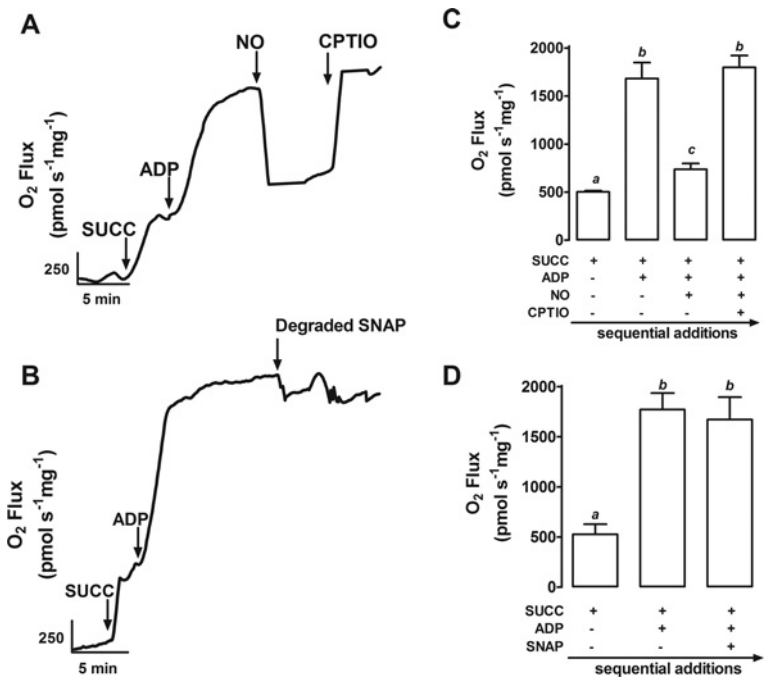

Figure 4 Effect of NO gas solution and SNAP degradation products on oxygen consumption in PTM stimulated by succinate and ADP

PTM were incubated with 2.0 ml of the standard respiration buffer (see the Materials and methods section) at 28 °C. Typical rates of oxygen consumption are shown. The PTM were stimulated by 10 mM succinate (SUCC) and 1 mM ADP and received 100 nM NO gas solution (**A**) or SNAP degradation products (**B**). At the points indicated by the arrows, 2 mM CPTIO was added. (**C**) and (**D**) Results are means ± S.E.M. of the respiratory rates for at least five independent PTM preparations from the experiments shown in (**A**) and (**B**) respectively. Groups that are significantly different ($P < 0.05$ by ANOVA with Tukey's post-hoc test) are indicated by different letters above the bars.

shown in Figure 3, was to test whether the presence of multiple substrates, including NADH, could promote full protection against NO inhibition of respiration caused by inclusion of the NO donors SNAP and DETA-NONOate, a protection that can be attributed to the presence of other substrates besides succinate. Despite the fact that different substrates were available to feed the ETS, SDH activity still contributed significantly to the PTM respiration sustained by other oxidizable substrates (pyruvate/malate/TPP and NADH). Under the conditions of this assay, NO was still able to promote a 50 % inhibition of PTM respiration. The experiment using multiple substrates was designed to look at the physiological situation when mitochondria are fed by different substrates simultaneously. In the result of the experiments we see the importance of SDH in mitochondrial respiration, where even with many substrates present; the inhibition of SDH could reduce mitochondrial respiration by 50 % (Figure 3).

To validate whether the effects of NO donors are related to NO and not to degradation products of these molecules on SDH activity, an authentic NO solution prepared with gas was tested on PTM respiration (Figures 4A and 4B). Figure 4(A) shows that addition of 0.1 μM NO inhibited the PTM respiration by 60–70 % of ADP-stimulated respiration (Figures 4A and 4B). Inclusion of CPTIO after the addition of NO gas solution reversed almost completely the inhibition promoted by NO. The NO concentration used to cause inhibition of respiration sustained by succinate in PTM was in the physiological range [31–33]. The degraded SNAP products did not cause any effect on respiration induced by

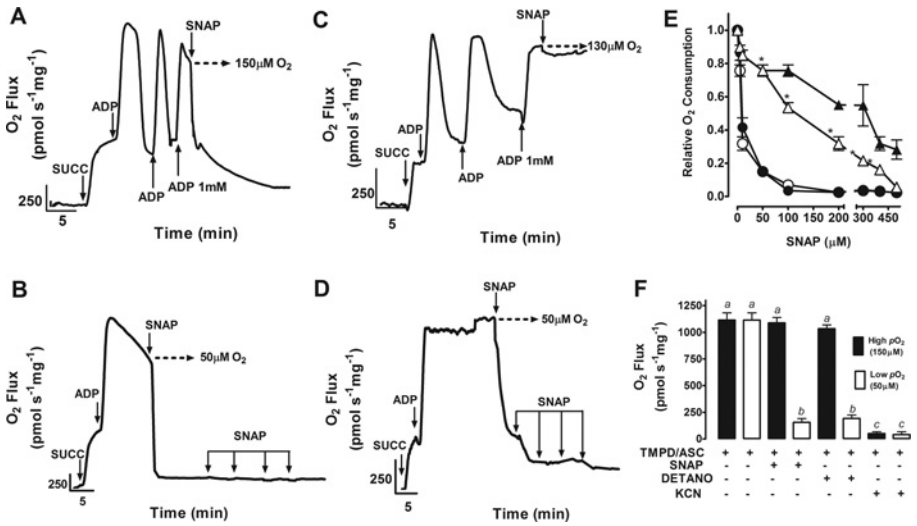

Figure 5 SNAP affects PTM and MBM respiration differentially at high (A, C and E) and low (B, D and E) PO_2 (hyperoxic condition 150 μM or normo/subnormoxic conditions 50 μM)

PTM were incubated with 2.0 ml of the standard respiration buffer (see the Materials and methods section) at 28 °C. Typical oxygen consumption traces are shown. PTM (**A** and **B**) or MBM (**C** and **D**) oxygen consumption was stimulated with 10 mM succinate and 1 mM ADP, before the addition of 100 μM SNAP (indicated by arrows). The sensitivity of PTM (circles) or MBM (triangles) in hyperoxic (**E**, closed symbols) or normo/subnormoxic (**E**, open symbols) conditions was measured in the presence of different concentrations of SNAP (from 1 to 500 μM) after mitochondrial stimulation with 10 mM succinate and 1 mM ADP. The asterisks show significant differences ($P < 0.05$ by ANOVA with Tukey's post-hoc test) between MBM high and low oxygen levels. (**F**) The effect of NO on PTM COX activity was evaluated using high-resolution respirometry in high (black bar) and low (white bars) PO_2 levels. PTM were incubated with standard respiration buffer (see the Materials and methods section) at 28 °C with 0.7 mM TMPD (*N,N,N′,N′*-tetramethyl-*p*-phenylenediamine) and 10 mM ascorbate, before the addition of 100 μM SNAP or 100 μM DETA-NONOate (DETANO) and 20 mM KCN, when indicated. Groups that are significantly different ($P < 0.05$ by ANOVA with Tukey's post-hoc test) are indicated by different letters above the bars. (**E** and **F**) Results are means ± S.E.M. of the respiratory rates for at least three independent PTM or MBM preparations. SUCC, succinate.

succinate and ADP (Figures 4B and 4D). Thus these data prove that the inhibition of succinate-sustained respirations is promoted by NO and not by products of NO donor degradation.

From all of these data, it became apparent that when NO was added to the respiration medium, in contrast with mammalian mitochondria, a direct and rapid inhibition of PTM respiration at the level of complex II was observed in the phosphorylation state (Figures 1–4). This result indicates that NO could be inhibiting the SDH directly and reversibly, because: (i) this inhibition was observed at high tensions of PO_2 (above 100 μM); and (ii) this inhibition was almost completely reversed by increasing amounts of CPTIO, a NO scavenger. This result led us to test the possibility that SDH from PTM could be a specific target for NO at high PO_2 tensions.

The inhibition of respiration by NO is independent of oxygen levels: differences between plant and animal mitochondria

We observed that SNAP caused a significant inhibition of PTM respiration regardless of whether multiple substrates or only succinate was used. This was observed at 28 °C and at reaction times when the dissolved oxygen in aqueous solution was always close to or greater than 150 μM. These conditions are far from being considered hypoxic, either for plant or animal tissues [14,33]. Thus we compared the effect of SNAP on PTM respiration by controlling the dissolved oxygen concentration (PO_2 tensions) at hyperoxic (150 μM) or normo/subnormoxic levels (50 μM). These findings were compared with those obtained using MBM, which are very sensitive to NO under hypoxic conditions (Figure 5D).

We observed that even at high PO_2 tensions in PTM, the addition of 100 μM SNAP was able to inhibit respiration by about 70 % (Figure 5A). At low PO_2 tensions this inhibition reached approximately 100 % in PTM (Figure 5B).

A completely different profile of oxygen flow was observed in MBM (Figures 5C and 5D). The respiration induced by ADP and succinate in hyperoxia (above 150 μM pO_2 level) (Figure 5C) did not decrease. However, at 50 μM PO_2 the addition of 100 μM SNAP was able to inhibit oxygen consumption by up to 70 %. Inhibition by approximately 90 % was achieved with 500 μM SNAP in respiration buffer (Figure 5D). This suggests that differences in PO_2 dependence at the level of complex IV exist (Figure 5E). In this context we also observed that, upon addition of SNAP in medium with a low PO_2, oxygen consumption mediated by the complex IV (COX) in PTM was almost equally inhibited, showing that at low tensions of oxygen, inhibition occurs at the level of COX (Figure 5F). At high PO_2 tension, the observed inhibition of PTM respiration would be mainly related to inhibition at the level of SDH activity. These data indicated that in high PO_2, NO inhibited a site in ETS that lies upstream of the COX activity complex. In the next experiments the effect of NO donors on the SCO and SDH activity were tested in order to check whether NO could inhibit the short-circuit ETS.

Succinate $FADH_2$–DCPIP oxidoreductase activity is inhibited by NO

To identify the interaction site of NO with the ETS complex enzyme we measured the succinate $FADH_2$–DCPIP oxidoreductase activity.

An analysis of the effect of 100 μM SNAP or 100 μM DETA-NONOate on succinate dependence of the SDH activity revealed that NO significantly increased the K_m value of this enzyme by more than 100-fold (Figure 6A and Table 2). Additionally we observed a significant decrease of about 30 % in the V_{max} value and in its catalytic efficiency (Figure 6A and Table 2). When these

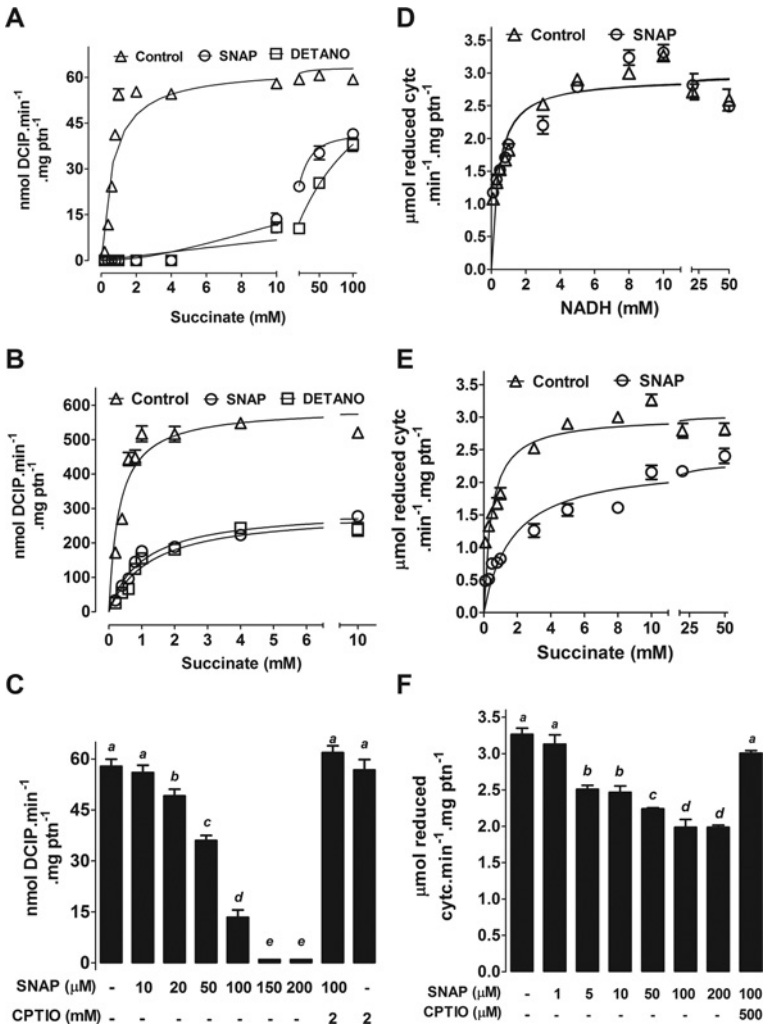

Figure 6 The effect of SNAP on the SDH (A, B and C), NADHCO (D) and SCO (E and F) activity branches of ETS

The activity of SDH was determined spectrophotometrically using DCPIP as an artificial electron acceptor and succinate as the substrate. (**A** and **B**) SDH activity was determined using from 0.1 to 100 mM (**A**) or 0.1 to 10 mM (**B**) of succinate as substrate without NO donors (△) or with 100 μM SNAP (○) or 100 μM DETA-NONOate (DETANO) (□). To activate SDH, 1 mM ATP was added to the reaction medium (**B**). (**C**) The sensitivity of a non-ATP stimulated SDH to SNAP was measured by adding different concentrations of SNAP (from 1 to 200 μM) after mitochondrial stimulation with 10 mM succinate. When necessary, 2 mM CPTIO was used as a NO scavenger. NADHCO and SCO activities were determined spectrophotometrically using the reduction of cytochrome *c* (25 °C) and 0.1 mg of PTM in 1.0 ml of reaction medium (see the Materials and methods section). NADHCO was determined using 0.1–50 μM NADH as a substrate with (○) or without (△) 100 μM SNAP (**D**). SCO was determined using 0.1–50 μM succinate as substrate with (circles) or without (triangles) 100 μM SNAP (**E**).The sensitivity of SCO to SNAP was measured by adding different concentrations of SNAP (from 1 to 200 μM) after mitochondrial stimulation with 10 mM succinate; 2 mM CPTIO was used as a NO scavenger (**F**). Groups that are significantly different ($P < 0.05$ by ANOVA with Tukey's post-hoc test) are indicated by different letters above the bars.

assays were performed in the presence of 1 mM ATP, which is an activator of SDH [34–36], the effect of NO was attenuated. Nevertheless, the enzyme was consistently inhibited by SNAP. The K_m value of SDH for succinate was significantly increased approximately 4-fold and the V_{max} and catalytic efficiency were both significantly decreased by 2-fold (Figure 6 B and Table 2).

We found that this negative modulation of NO in SDH was SNAP dose-dependent. The inhibition was reversed when CPTIO, a scavenger of free NO, was added to the reaction buffer (Figure 6C).

The SCO branch of ETS is also inhibited by NO

The effect of SNAP on SCO kinetics followed a similar pattern to that observed for the SDH activated by ATP (Figures 6E and 6F). This activity includes the path that is followed by electrons released from $FADH_2$ to be delivered to cytochrome *c*; this activity represents the results of complex II and complex III, excluding COX activity. SNAP (100 μM) significantly decreased the V_{max} value by 25 % and catalytic efficiency by 20 %, whereas it also significantly increased the K_m value for succinate by approximately 75 % (Figures 6E and 6F, and Table 3). When NADH, instead of succinate, was used as an oxidizable substrate there were no changes in these kinetic parameters upon the addition of SNAP (Figure 6 and Table 3).

NO inhibits SDH activity similarly to TTFA

To investigate the mechanism of NO inhibition we used two different classical inhibitors for this enzyme, 3-NP and TTFA. 3-NP is a competitive inhibitor of SDH that is a succinate analogue. It binds to the catalytic site of this enzyme, competing

Table 2 Comparative effect of NO donors in the kinetic parameters of PTM, MLM and MBM SDH

The values are means ± S.E.M. of the respiratory rates of at least five independent preparations.

Mitochondria	NO donor(s)	K_m (mM)	V_{max} (pmol · s^{-1} · mg^{-1})	V_{max}/K_m
PTM	SDH	0.77 ± 0.18	60.46 ± 5.05	78.51 ± 2.12
PTM	SDH and SNAP	34.61 ± 5.92*	57.46 ± 4.41	1.66 ± 0.41*
PTM	SDH and DETA-NONOate	38.22 ± 7.58*	52.45 ± 8.07	1.37 ± 0.18*
PTM	SDH and ATP	0.32 ± 0.05	592.20 ± 24.14	1850.62 ± 10.02
PTM	SDH, ATP and SNAP	1.02 ± 0.12*	297.80 ± 12.03*	291.96 ± 9.45*
PTM	SDH, ATP and DETANO	1.32 ± 0.15*	302.46 ± 10.21*	229.14 ± 14.52*
MLM	SDH	0.21 ± 0.03	21.06 ± 3.10	100.28 ± 2.12
MLM	SDH and SNAP	0.20 ± 0.03	19.50 ± 1.10	97.57 ± 1.42
MLM	SDH and DETA-NONOate	0.21 ± 0.01	22.501 ± 4.10	107.01 ± 2.46
MBM	SDH	0.29 ± 0.02	7.01 ± 0.45	24.17 ± 5.68
MBM	SDH and SNAP	0.30 ± 0.03	6.22 ± 0.49	20.73 ± 3.37
MBM	SDH and DETA-NONOate	0.28 ± 0.03	6.98 ± 0.89	24.92 ± 2.21

*Significant difference between with and without NO donor by ANOVA with Tukey's post-hoc test ($P < 0.05$).

Table 3 Effect of SNAP on kinetic parameters of PTM SCO and NADHCO

The values are means ± S.E.M. for the respiratory rates of at least five independent PTM preparations.

Reaction	K_m (mM)	V_{max} (pmol · s^{-1} · mg^{-1})	V_{max}/K_m
SCO	0.45 ± 0.05	3.01 ± 0.09	6.68 ± 0.02
SCO and SNAP	1.72 ± 0.27*	2.30 ± 0.07*	1.33 ± 0.15*
NADHCO	0.37 ± 0.06	28.51 ± 3.29	77.05 ± 4.05
NADHCO and SNAP	0.44 ± 0.08	30.59 ± 2.11	76.34 ± 2.08

*Significant difference between with and without SNAP by ANOVA with Tukey's post-hoc test ($P < 0.05$).

with succinate, and prevents the formation of $FADH_2$, thereby decreasing the $FADH_2/FAD^+$ redox ratio [37]. TTFA is a non-competitive inhibitor of SDH that prevents delivery to ubiquinone of the electron released from the $FADH_2$ [38]. TTFA inhibition is associated with an increase in the $FADH_2/FAD^+$ redox ratio.

We analysed the intrinsic fluorescence of the FAD molecule in Percoll-isolated intact PTM under different regimes of respiration (Figure 7). We observed that 100 μM SNAP was able to increase the pool of $FADH_2$ (Figure 7B), but when 3-NP was added no changes in the signal of FAD fluorescence were observed (Figure 7C). In contrast when TTFA was added to the medium we observed a typical decrease in the signal of FAD^+ and an increase in $FADH_2/FAD^+$ redox ratio similar to that found when SNAP was added (Figure 7D). The change observed with SNAP was slower than the change detected with TTFA.

NO inhibits oxygen consumption in potato tuber slices

To investigate whether the mechanism of NO modulation of plant SDH was representative of heterotrophic plant tissue *in vivo*, we measured respiration by potato tuber slices under

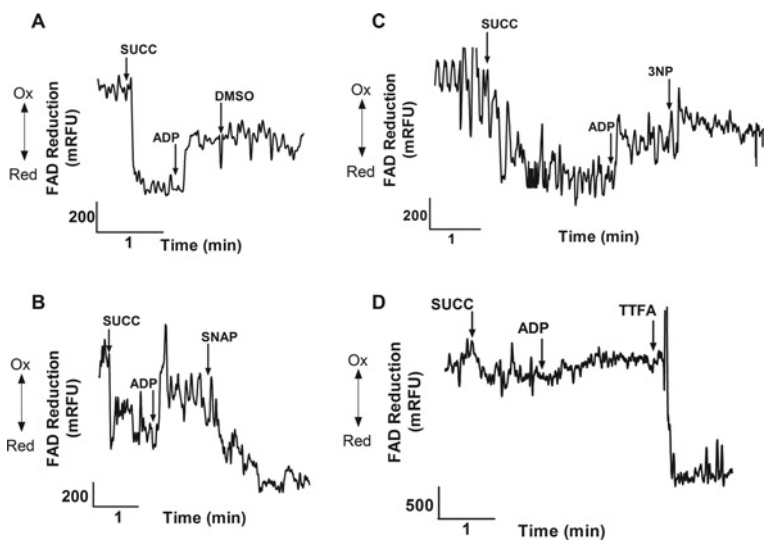

Figure 7 $FADH_2/FAD^+$ redox status in PTM

The redox status of $FADH_2/FAD^+$ in PTM was determined by auto-fluorescence using excitation and emission wavelengths of 490 ± 10 nm and 530 ± 10 nm respectively. The assay was performed using coupled PTM, and in the respiration medium (see the Materials and methods section) 5 mM succinate (SUCC) and 1 mM ADP were added in the absence, DMSO was added to reach 0.6 % (**A**) or presence of 100 μM SNAP (**B**), 10 mM 3-NP (**C**) or 100 μM TTFA (**D**).

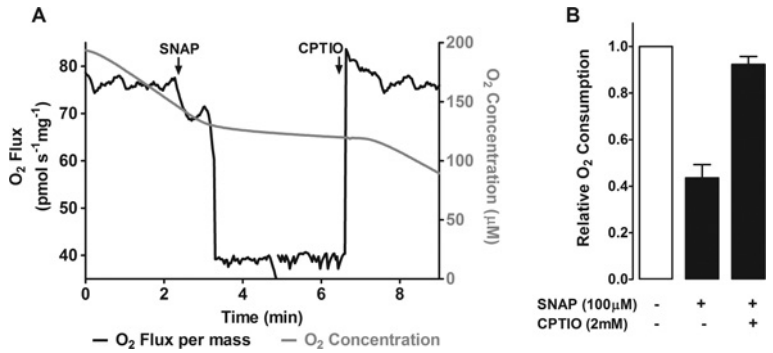

Figure 8 Effect of SNAP on oxygen consumption rate in potato tuber slices

Potato tuber slices were incubated with 2.0 ml of the standard respiration buffer (0.1 M Mes/KOH, pH 6.5) at 28 °C. The basal oxygen consumption rate was measured first with only slices in the cuvette, before the addition of 100 μM SNAP; 2 mM CPTIO was used as a NO scavenger (**A**). (**B**) Results are means ± S.E.M. of the respiratory rates for at least three independent potato tuber slice preparations.

hyperoxic conditions as an *in vitro* model of plant heterotrophic tissue organization. As a result we found that NO was able to significantly inhibit respiration by approximately 60 % in potato tuber slices (Figure 8A, black trace) at high PO_2 tensions (above 100 μM; Figure 8A, grey trace). More importantly, CPTIO was able to restore the respiratory rate to the levels seen in the control (Figure 8).

DISCUSSION

In the present study we observed the specificity of SNAP in inhibiting the SDH activity from PTM (Figure 4). This may be owing to a reaction between the binding sites for NO in one or more of the four subunits exclusively present in the plant isoform. The mammalian isoform of SDH has four subunits and contains one molecule of FAD in the SDH1 subunit facing the matrix and the outer lipid bilayer. The SDH2 subunit is buried in the inner membrane and contains a nanowire formed by iron–sulfur centres ([2Fe2S] + [4Fe4S] + [3Fe4S]) in which the electrons are channelled to ubiquinone. The SDH subunits 3/4 are in the plane of the lipid bilayer and contain a haem b group of unknown function [39]. In *Arabidopsis thaliana* SDH contains four more subunits (AtSDH5–8) associated with SDH1 and/or SDH2 [40].

The results of the present study support the view that NO reversibly inhibits electron transfer to ubiquinone, leading the FAD domain into an almost fully reduced state (Figure 7). This pattern of redox change in the FAD fluorescence signal would not expected if NO were impairing the discharge of a pair of electrons and protons from succinate to FAD directly at the SDH1 subunit. Therefore it can be concluded that NO can effectively modulate the kinetic parameters of SDH, decreasing the activity of this enzyme by lowering its affinity for succinate in a manner similar to that of the authentic SDH inhibitor TTFA. In addition, the diffusion of NO to SDH is limited by the rate of NO release from SNAP (compare Figures 7B and 7D). Thus these data suggest that, despite the fact that NO caused an apparent decrease in the SDH affinity for succinate in some assays (Figure 6A and 6B, and Table 2) No binding does not impair FAD reduction. These results agree with EPR data from purified cow heart SDH, an indication that SDH would be a probable target of NO due to the presence of iron–sulfur centres in this enzyme [20]. Despite these observations, the results from Figures 4 and 7 and Table 2 using intact PTM, MLM and MBM indicate that a different structural component containing iron–sulfur centres in PTM SDH may be involved in the greater sensitivity of complex II to NO compared with other SDH isoforms from mammals. However, further studies are necessary to identify the specific binding sites of NO in plant SDH subunits.

It had been observed [41] with rat heart and liver mitochondria that depending on their respiratory state (non-phosphorylating state of respiration supported by substrates, state 4, or phosphorylating states, state 3) the formation of superoxide anion $O_2^{\bullet -}$ is favoured (state 4). Superoxide is formed in the mitochondria from complex I and III [14]. Under this condition the presence of NO may favour the synthesis of $ONOO^-$. Peroxynitrite is very reactive and inhibits the respiration of rat heart mitochondria, probably by peroxynitrite-mediated irreversible nitration of protein complexes of ETS. In state 3 the formation of $O_2^{\bullet -}$ is not favoured and the NO inhibition is reversible and competitive with the levels of oxygen tension [41]. Curiously, in the experiments of Figure 2 with PTM a completely different dependence of respiratory states was found. In non-phosphorylating state respiration (Figures 2G and 3C) the NO donors SNAP and DETA-NONOate did not cause inhibition, although inhibition was expected from previous data [39]. A possible explanation for this observation is that the specific rate of $O_2^{\bullet -}$ formation from PTM and/or NO release from donors may not be enough to promote $ONOO^-$ synthesis. In fact, $ONOO^-$ is a potent irreversible inhibitor of the respiration of PTM, possibly by peroxynitrite-mediated oxidation of thiols, lipid peroxidation and nitration of tyrosine [42], in a non-phosphorylating state. This last observation suggests that NO itself does not inhibit SDH by some as yet unknown mechanism or conformational state of the enzyme reactive to NO.

A different and opposite situation to rat heart and liver mitochondria occurs in PTM in phosphorylating state respiration (state 3; Figures 2E and 3A), where SNAP and DETA-NONOate caused a potent inhibition, as $ONOO^-$ did for PTM state 3 respiration, indicating that the inhibitor was in fact NO. This was confirmed by adding CPTIO, which reversed the inhibition promoted by SNAP and DETA-NONOate. Similarly, inhibition by NO itself was reversed by CPTIO (Figure 4). These observations indicate that in PTM, NO modulates the SDH activity reversibly and directly. The presence of agents that bind NO, such as plant haemoglobin [14] or enzymatic systems of plant mitochondria that react with NO, such as external NAD(P)H dehydrogenase and AOX (alternative oxidase) [32], would modulate the levels of

SDH activity and PTM respiration. Finally, the SDH in state 3 is putatively stabilized in a conformational state that would favour reversible NO binding.

It has been proposed that NO has a mechanism of action in the plant mitochondria similar to that observed in cytochrome oxidase respiration in animal cells [41]. According to this model of NO action, a low oxygen tension is an obligate condition for a significant reduction in ATP synthesis and oxygen consumption, because of the competitive nature of NO inhibition and the oxygen-binding site in COX [13,43]. In the present study we observed a different modulation of respiration of PTM by NO compared with MBM, one that is present even at high oxygen concentration. The same was not observed in MBM and may represent a particular feature of plant respiration (Figure 5). There is a possibility that plants that are exposed to low oxygen tension will have both SDH and COX inhibited by NO. The inhibition of SDH at low oxygen tension could be a mechanism to prevent superoxide formation at complex III supported by succinate. At high oxygen tension only inhibition of SDH would slow the rate of succinate oxidation. Again, under this condition the superoxide anion formation would be minimized due to low reduction levels of the complex III supported by succinate and ultimately the formation of $ONOO^-$.

Previous reports have shown that SDH-dependent respiration via AOX is resistant to NO, whereas SDH-dependent respiration to COX is sensitive to NO [14]. In our preparations of PTM a low level of respiration due to AOX activity (less than 2 % of total oxygen consumption) is observed after addition of KCN ([44] and results not shown). The low sensitivity of SDH-dependent respiration via AOX to NO could be explained by the nature of the inhibitor. Peroxynitrite may be the main inhibitor of SDH under these conditions, thus the AOX activity would minimize the formation of the superoxide anion important to $ONOO^-$ synthesis [32]. However, it is important to note that even in our hands NO was not a good inhibitor of SDH-dependent respiration to COX in non-phosphorylating states (see Figures 3C and 3G). It is possible that the electron that moves from the succinate to AOX, the SDH conformation, is very similar to the state that is insensitive to NO observed in the non-phosphorylating respiration of PTM. This effect may explain, in part, the lack of NO inhibition of succinate oxidation mediated by AOX.

NO inhibition in PTM succinate-sustained respiration is more evident when compared with other substrates of ETS (Figure 2), as has been described previously [32,45]. Additionally, the specific SDH or SCO activities showed an inhibited kinetics of competitive/non-competitive pattern with succinate (Figures 6 and 7). These findings led us to the hypothesis that SDH plays a role in NO sensor bioenergetics homoeostasis for the plant cells that does not depend on oxygen availability.

The possibility of NO inhibiting the SDH system is of critical importance for soil fertilization and nitrogen status. A small, but significant, portion of nitrogen fertilizers (nitrite and nitrate) is converted into NO [46]. The current view is that the NO is lost and does not play a major biological or ecophysiological role. However, there is some evidence that the presence of nitrate in the soil increases the expression of nitrite reductase in the roots of tomato plants [47]. According to this view, the SDH activity in heterotrophic plant tissues exposed to nitrogen fertilizers would be down-regulated. In fact, we observed that about 50 % of oxygen consumption in potato tuber slices was inhibited by the presence of NO (Figure 8).

It has been shown recently that seedlings of *A. thaliana* SDH1-1/sdh1-1 mutant plants, displaying a 30 % reduction in SDH activity, grow better than the WT (wild-type) in nitrogen-limiting conditions and that the nitrate uptake in this mutant plant is more efficient than the WT, making a connection between energy metabolism and nitrogen fixation [48]. Many studies support the idea of a cross-talk between nitrogen and energy metabolism [49,50]. Together, these findings suggest that SDH activity is modulated by nitrogen fertilizers, which explains the positive growth response in plants. In addition, the suppression of SDH activity and respiration may favour the accumulation of amino acids and organic acids to support a further distribution and reallocation of carbonic skeleton to different plant parts, supporting growth and development.

Conclusion

In the present study we showed that SDH from PTM can be modulated by small amounts of NO in a reversible manner. This SDH inhibition by NO, in contrast with its inhibition of COX, occurs independently of the oxygen availability and effective PO_2. The specific binding site for NO in the SDH molecule needs to be further investigated in detail, as well as the physiological implication of these findings for plant growth and development in non-heterotrophic tissues.

AUTHOR CONTRIBUTION

Vagner Simonin performed all of the experiments and wrote the paper. Antonio Galina designed the experiments and wrote the paper.

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