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RESEARCH PAPER

Nitric oxide controls nitrate and ammonium assimilation in *Chlamydomonas reinhardtii*

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Abstract

Nitrate and ammonium are major inorganic nitrogen sources for plants and algae. These compounds are assimilated by means of finely regulated processes at transcriptional and post-translational levels. In *Chlamydomonas*, the expression of several genes involved in high-affinity ammonium (*AMT1.1*, *AMT1.2*) and nitrate transport (*NRT2.1*) as well as nitrate reduction (*NIA1*) are downregulated by ammonium through a nitric oxide (NO)-dependent mechanism. At the post-translational level, nitrate/nitrite uptake and nitrate reductase (NR) are also inhibited by ammonium, but the mechanisms implicated in this regulation are scarcely known. In this work, the effect of NO on nitrate assimilation and the high-affinity ammonium uptake was addressed. NO inhibited the high-affinity uptake of ammonium and nitrate/nitrite, as well as the NR activity, in a reversible form. In contrast, nitrite reductase and glutamine synthetase activities were not affected. The *in vivo* and *in vitro* studies suggested that NR enzyme is inhibited by NO in a mediated process that requires the cell integrity. These data highlight a role of NO in inorganic nitrogen assimilation and suggest that this signalling molecule is an important regulator for the first steps of the pathway.

Key words: Ammonium uptake, *Chlamydomonas*, glutamine synthetase, nitrate and nitrite uptake, nitrate reductase, nitric oxide, nitrite reductase, post-translational regulation.

Introduction

Nitric oxide (NO) has gained experimental support as an important signal molecule in many biological plant processes. NO has a relevant role in plant growth, metabolism, development, and defence processes (Corpas, 2004; He *et al*., 2004; Neill *et al*., 2008; de Montaigu *et al*., 2010; Fernández-Marcos *et al*., 2011; Yun *et al*., 2011).

Several routes have been described in NO generation in plants. Nitrate reductase (NR) is one in plants and algae, using nitrite as the substrate (Dean and Harper, 1986, 1988; Yamasaki and Sakihama, 2000; Rockel *et al*., 2002; Sakihama *et al*., 2002). In addition, other nitrite-dependent NO sources can be significant at different conditions like mitochondrial electron transport, or non-enzymatic reducing conditions (Planchet and Kaiser, 2006; Moreau *et al*., 2010). Although nitric oxide synthase has not yet been identified in plants, an arginine-dependent NO production has been reported (Corpas *et al*., 2006, 2009; Moreau *et al*., 2010). A nitric oxide synthase has been identified in the photosynthetic organism *Ostreococcus tauri* (Foresi *et al*., 2010), but as such it does not appear to be conserved in plants or in other algal species such as *Chlamydomonas*.

In plants, NO performs its function in different ways. NO, by modulating soluble guanylate cyclase (GC) activities and reactive oxygen species, affects mobilization of second messengers such as calcium and cGMP (Martínez-Ruiz *et al*., 2011). However, direct post-translational modifications

Abbreviations: CHX, cycloheximide; DEA-NONOate, 2-(*N*,*N*-diethylamino)-diazenolate 2-oxide; GC, guanylate cyclase; GS, glutamine synthetase; GSNO, *S*-nitrosoglutathione; HAAT, high-affinity ammonium transporter; HANT, high-affinity nitrate transporter; IBMX, 3-isobutyl-1-methylxanthine; NiR, nitrite reductase; NO, nitric oxide; NR, nitrate reductase; SD, standard deviation; SIN-1, 3-(4-morpholinyl) sydnonimine; SNO, sulfo-NONOate.

have also emerged as important mechanisms of regulation. *S*-Nitrosylation is a reversible process in which NO reacts with thiol groups of cysteine residues (Wang, 2006; Martínez-Ruiz *et al*., 2011). Nitrosylation and denitrosylation can be enzyme dependent (Wang, 2006; Benhar *et al*., 2009; Anand and Stamler, 2012) or independent (Fernhoff *et al*., 2009; Hill *et al*., 2010; Spadaro *et al*., 2010). Another post-translational modification mediated by NO is nitration, where NO reacts with superoxide ($O_2^{\cdot-}$) to generate peroxynitrite ($ONOO^-$), which interacts with tyrosine residues. This modification is considered irreversible in plants, but in mammals a reversible tyrosine nitration has been reported for glutamine synthetase (GS) (Görg *et al*., 2007). Endogenous glutathione can also be nitrosylated so that *S*-nitrosoglutathione (GSNO) is considered as the natural *in vivo* reservoir of NO bioactivity (Wang, 2006).

Together with nitrosylation, phosphorylation is a rapid and reversible mechanism to control protein activities. In *Arabidopsis*, nitrate and ammonium lead to different phosphorylation patterns (Engelsberger and Schulze, 2011). The high-affinity ammonium transporter (HAAT) AtAMT1.1 is inhibited by phosphorylation after ammonium or nitrate resupply (Lanquar *et al*., 2009). In *Hansenula polymorpha* the high-affinity nitrate transporter (HANT) YNT1, belonging to the NRT2 family, is phosphorylated under nitrogen deficiency (Navarro *et al*., 2008). Ubiquitination also appears to be involved in NRT2.1 degradation in *Arabidopsis* (Miller *et al*., 2007), which could be a second step after phosphorylation. Plant NR is inhibited by phosphorylation of a serine residue in a hinge domain that subsequently binds 14-3-3 proteins, thus interfering with electron transfer to the substrate (MacKintosh and Meek, 2001; Lambeck *et al*., 2012). *Chlamydomonas* NR is not modified by phosphorylation and 14-3-3 binding (Pozuelo *et al*., 2001), but this enzyme appears to be regulated by redox mechanisms (Franco *et al*., 1987). Nevertheless, plant and *Chlamydomonas* GS are phosphorylated and bind 14-3-3 proteins (Finnemann and Schjoerring, 2000; Pozuelo *et al*., 2001; Lima *et al*., 2006). Finally, nitrite reductase (NiR) does not appear to have any post-translational regulation.

At the transcriptional level, NO has been shown to participate in inhibition by ammonium of the *AMT1.1*, *AMT1.2*, *NRT2.1* and *NIA1* genes expression through a soluble GC, CYG56, in *Chlamydomonas* (de Montaigu *et al*., 2010). *AMT1.1* and *AMT1.2* encode HAATs (Gonzalez-Ballester *et al*., 2004; Ermilova *et al*., 2010) and *NRT2.1* and *NIA1* encode HANT and NR, respectively (Fernandez and Galvan, 2007, 2008). NO and cGMP amounts are increased in the cells when both nitrate and ammonium are added, giving rise to nitrate assimilation repression (de Montaigu *et al*., 2010). NO and cGMP-dependent repression of NR is not specific for *Chlamydomonas* as these second messengers also downregulate the NR genes *NIA1* and *NIA2* in *Arabidopsis* (Maathuis, 2006; de Montaigu *et al*., 2010).

Post-translational regulation by NO has been reported in other plant NRs. In wheat leaves, the enzyme is inhibited by NO (Rosales *et al*., 2010) and is activated in cabbage (Du *et al*., 2008), whereas in roots of tomato, NR activity inhibition or activation by NO depend on nitrate concentrations (Jin *et al*., 2009).

GS is regulated not only by 14-3-3 proteins but also by NO. NO triggers the tyrosine nitration of GS in *Medicago truncatula* nodules inactivating the enzyme (Melo *et al*., 2011).

In this work, the post-translational effects of NO on HAAT, high-affinity nitrate and nitrite transport (HAN/NiT), NR, NiR and GS activities in *Chlamydomonas* have been studied. The results showed that NO caused a fast, reversible, and complete inhibition of high-affinity nitrate/nitrite and ammonium transport, and a reversible and partial inhibition of NR activity dependent on cell integrity, without affecting NiR and GS activities.

Materials and methods

Strains, culture conditions, extract preparations, and chemical compounds

The *Chlamydomonas reinhardtii* wild-type strain 704 was used (cw15 arg7$^+$ *NIA1:ARS* mt$^+$) (Gonzalez-Ballester *et al*., 2005). Cells were cultured under continuous light at 23 °C in liquid medium (Harris, 2009) containing different concentrations of NH_4Cl and KNO_3, depending on the experiment, and bubbled with 3% (v/v) CO_2-enriched air. Cells were grown with 8 mM ammonium medium and induced in the indicated medium. Extracts from 10 ml of culture ($2–4\times10^6$ cells ml^{-1}) were prepared 40-fold concentrated in 50 mM Tris/HCl buffer (pH 7.5). Bacterial extracts were prepared by sonication in lysis buffer [50 mM Tris/HCl (pH 8.0), 300 mM NaCl, 10 mM imidazole].

The chemical compounds DEA-NONOate [2-(*N*,*N*-diethylamino)-diazenolate 2-oxide sodium salt], GSNO, IBMX (3-isobutyl-1-methylxanthine), ODQ [1H-(1,2,4])oxadiazolo(4,3-a) quinoxalin-1-one] and SNO (sulfo-NONOate disodium salt) all from Sigma-Aldrich, and SIN-1 [3-(4-morpholinyl) sydnonimine, hydrochloride] from Calbiochem.

Ammonium, nitrate, and nitrite uptake

The high-affinity ammonium uptake activity was determined from cells induced in nitrogen-free medium for 3 h. NH_4Cl (100 µM) was added to the medium and the ammonium remaining was determined over time, using Nessler reagents (Solorzano, 1969).

Nitrate and nitrite uptake activities were determined from cells induced in 4 mM KNO_3 for 3 h. The cells were then transferred to medium containing 50 µM KNO_3 or 100 µM KNO_2. The nitrate (Miranda *et al*., 2001) or nitrite (Snell and Snell, 1949) remaining in the medium was quantified over time according to these previously reported methods.

NADPH-NR, NiR, and GS activities

Chlamydomonas cells were induced in 4 mM KNO_3 medium for 3 h. Treatments with NO donors were then performed. Samples of 100 µl extracts, prepared from 10 ml of culture, were used to determine NADPH-NR and NiR activities according to the method of Paneque and Losada (1966) and Galvan *et al*. (1992), respectively. Enzyme activities are expressed as percentages of untreated controls. The NADPH-NR activity corresponding to 100% was 11.6 ± 3.9 mU mg^{-1} of chlorophyll. When necessary, the nitrite produced by NO oxidation was subtracted from the final amount of nitrite using appropriate controls.

NiR activity was assayed using reduced methyl viologen as an electron donor. The reaction was started by the addition of dithionite solution (2.5 mg in 100 µl of 0.29 M bicarbonate buffer, pH 7.5), and terminated with dithionite oxidation by shaking vigorously.

Nitrite was determined as indicated above. For NiR, 100% activity corresponded to 617.6 ± 67.7 mU mg^{-1} of chlorophyll.

The GS activity was assayed by measuring the ATP-dependent formation of γ-glutamylhydroxamate from L-glutamate and hydroxylamine as reported (Shapiro and Stadtman, 1970). The formed complex was determined at λ = 500 nm (ε=3.36 mM^{-1} cm^{-1}). For GS activity, 100% corresponded to 91.4 ± 4.5 mU mg^{-1} of chlorophyll.

NR purification, in vitro NADPH-NR assay, and NADPH-NR activity in bacterial cell extracts

The *Chlamydomonas NIA1* cDNA was amplified using primers NRcDNAKpnIMo (5′-GGTACCATGACCGTTGCCGAGCA-3′) and NRcDNAHindIII-3 (5′-AAGCTTCTAGAACTGGATCTGG CGGT-3′). The cDNA was cloned into pQE80L (6×His technology) and the NR protein was expressed in *Escherichia coli* strain TP1000 for 36–48 h at 23 °C in Luria–Bertani medium containing 10 μM isopropyl β-thiogalactoside and 500 μM γ-aminolevulinic acid. Cells were harvested, and His-tagged NR protein was purified from extracts using 1 ml of Ni-NTA Superflow Matrix (Qiagen) per 2 l of culture (Schwarz *et al.*, 1997).

The NADPH-NR activity of the recombinant protein was assayed as described previously (Paneque and Losada, 1966). The purified enzyme activity corresponding to 100% was 89.7 ± 6.2 mU mg^{-1} of protein.

Treatments with DEA-NONOate were also performed in bacterial cultures expressing the *Chlamydomonas* NR. Concentrated (25-fold) extracts were obtained by sonication in lysis buffer as above. NADPH-NR activity was assayed from 100 μl of extracts. The NADPH-NR activity in bacterial extract corresponding to 100% was 110.7 ± 7.5 μU mg^{-1} of protein.

Results

NO inhibits HAAT activity in a cGMP- and peroxynitrite-independent way

The NO effect on the activity of HAAT was studied. Cells were induced in a nitrogen-free medium to express these transporters (Gonzalez-Ballester *et al.*, 2004). The cells were then treated with the NO donor DEA-NONOate (Fig. 1A). The concentrations of DEA-NONOate used (2.5, 5, and 20 μM) caused a complete inhibition of uptake after just 5 min. After this time, a recovery of the uptake activity, which was concentration dependent, was observed. Sulfo-NONOate was used as a chemical control. This compound, which is unable to release NO, had no effect on the uptake activity (Supplementary Table S1 at *JXB* online). These data suggested that NO inhibits HAAT in a reversible manner. Thus, when NO disappeared or its concentration was low enough, the ammonium transport recovered the same kinetics as the control. DEA-NONOate releases NO suddenly with a short half-life of few minutes (Li and Lancaster, 2009). Therefore, HAAT uptake was efficiently blocked by DEA-NONOate and recovered after NO disappearance. The reversibility of NO inhibition was shown in experiments with cycloheximide (CHX), a protein synthesis inhibitor, under the same conditions as published previously (Díaz-Troya *et al.*, 2011). Protein synthesis inhibition did not affect uptake recovery (Supplementary Fig. S1A at *JXB* online).

Another NO donor, GSNO, was also used (Fig. 1B). GSNO at 100 μM caused complete inhibition of the ammonium uptake over long period of time (120 min) and 50 μM caused

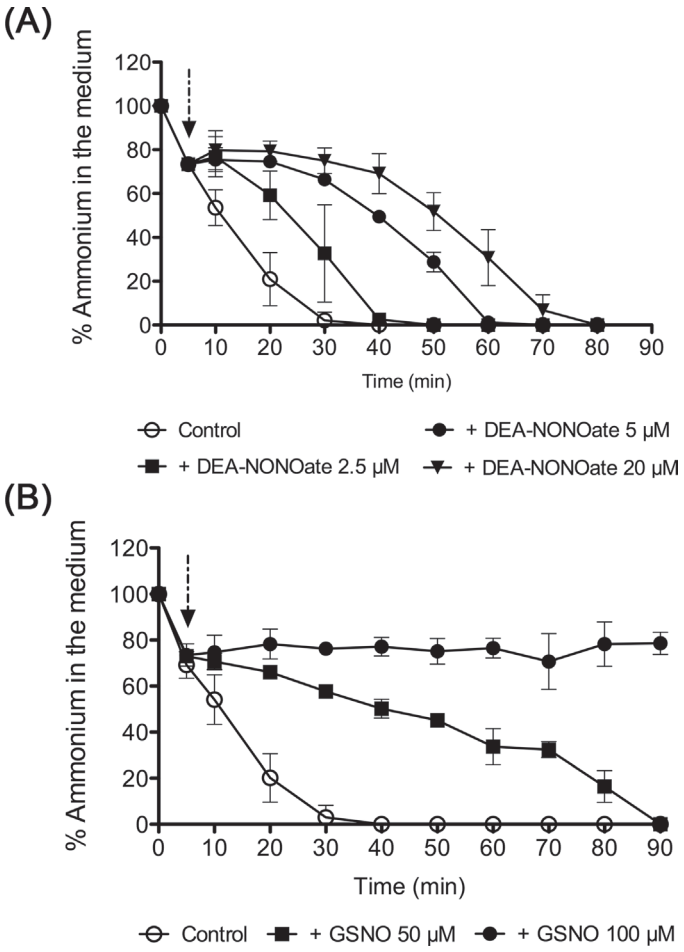

Fig. 1. NO inhibits high-affinity ammonium uptake. Ammonium uptake was determined from cells induced for 3 h in nitrogen-free medium. Uptake was initiated by adding 100 μM ammonium to the medium, and its concentration was quantified at the indicated times. The arrows show the time when treatment with NO donors DEA-NONOate (A) and GSNO (B) were applied. The error bars represent the standard deviation (SD) of three biological replicates.

partial inhibition (Fig. 1B), but below 35 μM no effect was observed (not shown). GSNO releases NO slowly (Floryszak-Wieczorek *et al.*, 2006). Thus, higher concentrations of this donor seemed to be required to provide an optimal NO concentration for HAAT inhibition over a long time period.

To elucidate whether cGMP also controls HAAT activity, the following experiments were performed. First, the effect of IBMX, a phosphodiesterase inhibitor that results in increases in cyclic nucleotides, was tested. Cells were treated for 10 min with 1 mM IBMX before ammonium uptake determination, and no inhibition of the transport was observed (Supplementary Table S1). Secondly, ODQ, a selective inhibitor of NO-activated GCs (Zhao *et al.*, 2000), was used; ODQ oxidizes the iron of the heme group in these proteins, avoiding NO binding. Thus, ODQ would prevent a cGMP increase when NO is added. The ODQ concentrations used in this work had already been shown to be effective and non-toxic in *Chlamydomonas* (de Montaigu *et al.*, 2010). When ODQ was used alone, ammonium transport was

slightly affected (Fig. 2). Treatment with both ODQ (5 µM) and DEA-NONOate (7.5 µM) caused an unexpected synergistic inhibition of ammonium uptake (Fig. 2). This higher inhibition probably occurred because ODQ is not only avoiding the NO binding to soluble GC but is also affecting other heme proteins implicated in NO detoxification. In this way, ODQ could be increasing the effective NO concentration to inhibit HAAT.

The NO effect on HAAT activity was also checked in the cyg56 mutant, which is defective in a soluble GC implicated in *AMT1.1* and *AMT1.2* repression by NO (de Montaigu *et al.*, 2010). NO inhibited HAAT activity in this mutant similarly to the wild-type strain (Supplementary Fig. S2A at *JXB* online). Therefore, experiments with IBMX and ODQ in the wild-type strain as well as the NO effect in the cyg56 mutant suggested that HAAT is inhibited by NO independently of soluble GCs.

Inside cells, NO can react with $O_2^{\cdot-}$, generating $ONOO^-$, a very reactive species that produces nitration of tyrosine residues (Moreau *et al.*, 2010; Besson-Bard *et al.*, 2012). To test the $ONOO^-$ effect on HAATs, the peroxynitrite donor SIN-1 was used. As shown in Supplementary Table S1, SIN-1 at 100 µM had no effect.

The high-affinity nitrate and nitrite uptake activities are also inhibited by NO

The NO effect on HAN/NiT was studied after nitrate induction (Galvan *et al.*, 1996). Nitrate and nitrite uptake activities were assayed in medium containing 50 µM nitrate and 100 µM nitrite, respectively (Fig. 3). A lower concentration of DEA-NONOate (5 µM) than in ammonium transport

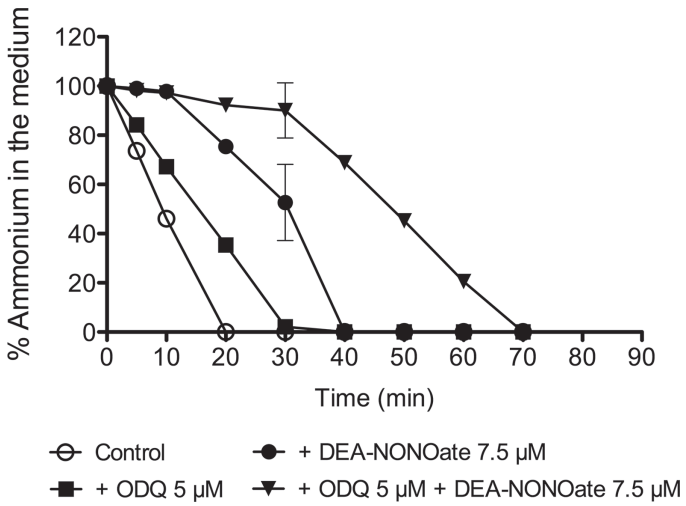

Fig. 2. NO inhibits high-affinity ammonium uptake independently of a soluble GC. Cells were induced in nitrogen-free medium for 3 h. Where indicated, cells were pre-treated with ODQ for 10 min and with DEA-NONOate for 5 min before starting the uptake measurement. Ammonium transport was started by adding 100 µM ammonium. At the indicated times, ammonium in the medium was determined. The error bars represent the SD of three technical replicates and two biological replicates.

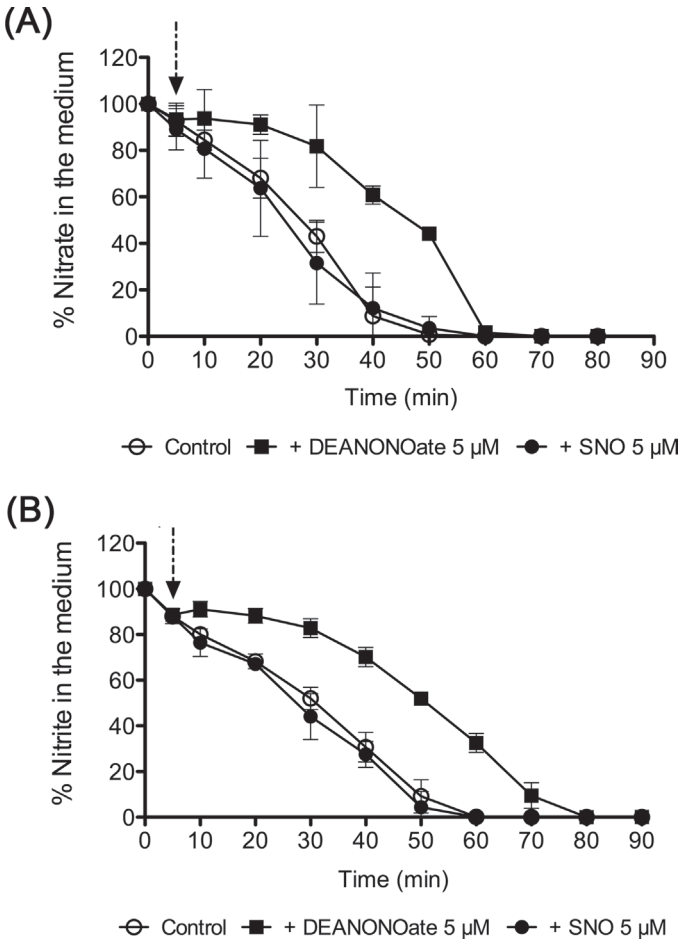

Fig. 3. NO inhibits high-affinity nitrate and nitrite uptake. Ammonium grown cells were washed and induced in nitrate medium for 3 h. After induction, the cells were washed to completely remove nitrate. Then, 50 µM nitrate (A) or 100 µM nitrite (B) was added to start the uptake. The arrows show the time when treatments with the NO donor DEA-NONOate and with sulfo-NONOate as a control were applied. At the indicated times, nitrate or nitrite remaining in the medium was determined. The error bars represent the SD of three biological replicates.

studies was used to avoid nitrite overproduction due to NO oxidation.

Similarly to the ammonium uptakes experiments, the NO donor was added 5 min after transport had started. Both nitrate and nitrite uptake activities were inhibited by NO, and after 20 min, transport activity was recovered (Fig. 3A, B). The reversibility was independent of new protein synthesis. Cultures pre-treated with CHX recovered uptake similarly to the wild-type cells (Supplementary Fig. 1B, C). Sulfo-NONOate, as a chemical control, did not show any effect.

NR activity is reversibly inhibited by NO in a cGMP- and peroxynitrite-independent way and requires cell integrity

The effect of NO on *Chlamydomonas* NR activity was studied using DEA-NONOate and GSNO. These NO donors were added to living cells, previously induced with nitrate.

The NADPH-NR activity was measured from cell extracts, after 10–60 min of treatment (Figs. 4A, B). DEA-NONOate at 20 µM produced a rapid inhibition of 60% in 10 min (Fig. 4A). None of the controls of sulfo-NONOate or nitrite affected the NR activity. The NO donor GSNO was used at 50 and 100 µM, as lower concentrations had no effect. GSNO inhibited NR activity in a time-dependent way, producing the maximal inhibition of about 60% after 40 min (Fig. 4B).

As NO inhibits NR activity, we determined whether cyclic nucleotides, derived from putative NO-dependent GCs, or peroxynitrite were involved in the NO inhibition mechanism (Fig. 4C). Treatments of *Chlamydomonas* cells with IBMX at 1 mM, for 10 or 20 min, did not inhibit the NR activity. In addition, the cyg56 mutant was used to elimiate the soluble GC CYG56 as an intermediate step generating cGMP. In the cyg56 mutant, the NR activity was affected by NO similarly to the wild-type strain (Supplementary Fig. S2B). The peroxynitrite donor SIN-1 at 100 µM also did not affect NR activity. Thus, NO seemed to inhibit the NR activity independently of cGMP or peroxynitrite.

DEA-NONOate produced a fast inhibition of NR activity at 10–20 min (Fig. 4A), and after this time, a recovery of the activity was observed (Supplementary Fig. S3 at *JXB* online). These results suggested that NO inhibits NR in a reversible way, due to the short half-life of the NO donor (DEA-NONOate). However, NR activity increased with time in untreated cultures due to *de novo* protein synthesis, and it was difficult to discriminate reversibility and new synthesis (Supplementary Fig. S3). Thus, experiments carried out as described above but including CHX were performed (Fig. 5). DEA-NONOate at concentrations of 5, 10, and 20 µM inhibited NR activity at almost the same extent after 10 min. After this time, when the NO started to disappear, NR activity was recovered. As NR protein synthesis was inhibited, the increase in activity must have been a consequence of enzyme reactivation and not due the result of *de novo* protein synthesis.

So far, NR inhibition mediated by NO has been proved in living cells. In addition, NO inhibition of NR was checked *in vitro*. DEA-NONOate (25 and 50 µM) did not affect the activity of the purified NR enzyme (Fig. 6A). This result could indicate that a cellular component mediates the NO inhibition *in vivo*. In addition, experiments with live *E. coli* cells (Fig. 6A) were performed. Cells from an *E. coli* strain overexpressing NR were treated with 100 and 200 µM DEA-NONOate for 15 min and again no inhibition was observed.

Returning to the *Chlamydomonas* system, the NO effect on NR in both living cells and extracts was compared. As shown in Fig. 6B, NO did not inhibit NR activity in extracts, in contrast to the living cells. This result indicated that a cellular component/cell structure from *Chlamydomonas* is necessary for proper NO inhibition of NR activity.

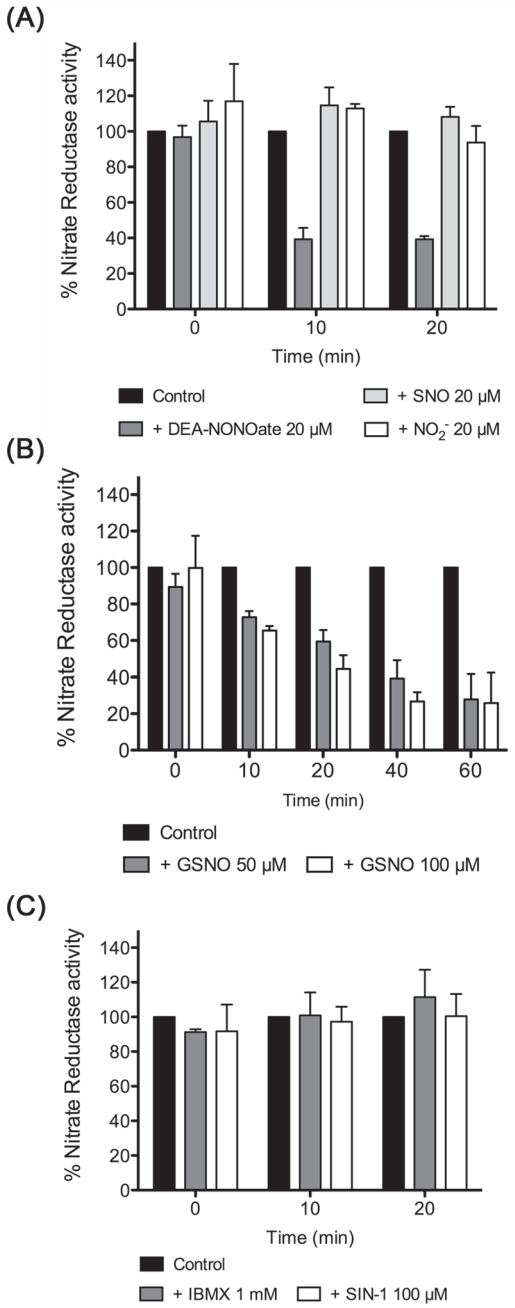

Fig. 4. *In vivo* effects of NO donors, IBMX, and peroxynitrite donor on *Chlamydomonas* NADPH-NR activity. *Chlamydomonas* cells were induced in 4 mM nitrate medium for 3 and the following chemical compounds were then added: (A) DEA-NONOate at 20 µM as a NO donor, and 20 µM SNO or 20 µM nitrite as controls; (B) 50 and 100 µM GSNO a slow NO donor; (C) 1 mM IBMX, a phosphodiesterase inhibitor, and 100 µM SIN-1, a peroxynitrite donor. At the indicated times, the cells were collected and the NADPH-NR activity was determined from the corresponding extracts. The error bars represent the SD of three biological replicates.

NO does not affect NiR and GS activities

No post-translational regulation of plastidic NiR has been reported. To check whether NO affected NiR, *Chlamydomonas* cells were treated in a similar way as in the NR experiments. After 10 and 20 min of DEA-NONOate treatment, NiR activity was not affected (Supplementary Fig. S4A at *JXB* online). High concentrations of this NO donor (up to 50 µM) were also used and no inhibition was observed.

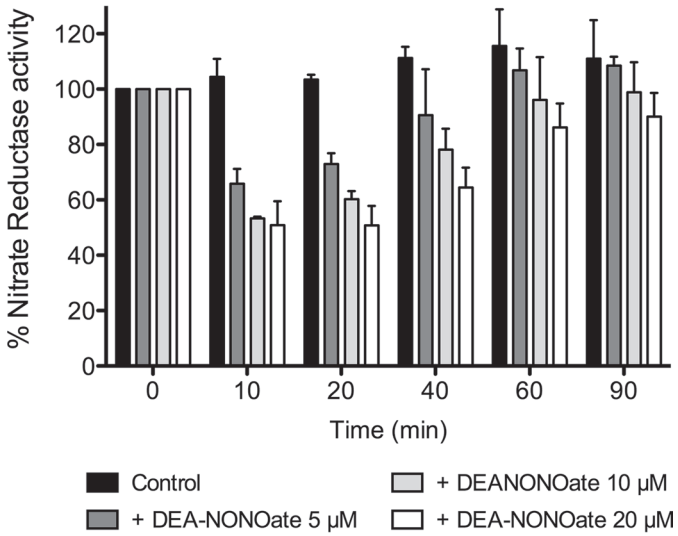

Fig. 5. NO inhibits NADPH-NR activity *in vivo* in a reversible way. *Chlamydomonas* cells were induced in 4 mM nitrate for 2 h and then CHX (10 µg ml^{-1}) was added. After 1 h in this medium of nitrate plus CHX, the cells were treated with 5, 10, or 20 µM DEA-NONOate. At the indicated times, NADPH-NR activity was determined from cell extracts. The error bars represent the SD of three biological replicates.

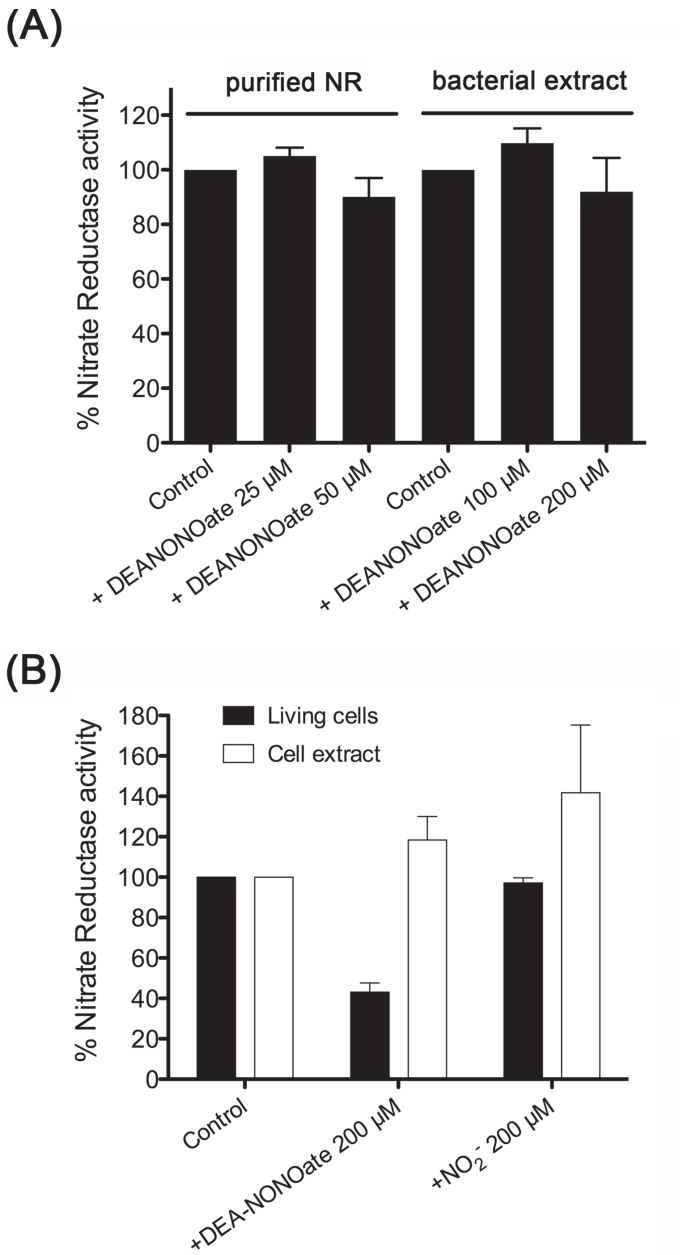

Fig. 6. Cell integrity is required for NADPH-NR inhibition by NO. (A) CrNR purified from bacteria was used for *in vitro* assays. DEA-NONOate, at the indicated concentrations, was added just before starting the assay. Cells from an *E. coli* strain overexpressing NR were treated with DEA-NONOate for 15 min, cell extracts were prepared, and NADPH-NR activity was determined. (B) *Chlamydomonas* cells were induced in nitrate for 3 h and treated for 10 min with DEA-NONOate in two ways: on living cells (filled bars) or after lysing cells (cell extracts, open bars). Controls with nitrite or without treatment were included. After treatment of living cells, these were collected and processed for extract isolations and NADPH-NR determinations. The error bars represent the SD of three biological replicates.

At least four GS isoforms are present in *Chlamydomonas*, with the plastidic isoform GS2 being the most abundant (Chen and Silflow, 1996; Fernandez and Galvan, 2008). Finally, the NO effect on GS activity was analysed. In experiments performed as for NR activity in living cells, DEA-NONOate at 20 µM did not affect total GS activity (Supplementary Fig. S4B).

These experiments with NiR and GS ruled out a total breakdown of nitrogen assimilation and demonstrated that the NO concentrations used were not affecting all nitrogen-related activities.

Discussion

This work describes the fast and reversible inhibition of HAAT and HAN/NiT by NO. The instantaneous and efficient effect on these transporters when the NO donor was added to cells indicates a post-translational regulation. The reversibility was also efficient, as the activity of these transporters was quickly recovered when the NO concentration decreased. This was highlighted by the effects of DEA-NONOate and GSNO, correlating their inhibitory effect with the persistence of the NO molecule in the cell culture. GSNO has a longer half-life as NO donor than DEA-NONOate (Floryszak-Wieczorek *et al.*, 2006; Li and Lancaster, 2009), and the inhibition produced was maintained over time. Thus, the inhibitory effect on transporters was dependent on NO donor concentration and occurred depending on its efficiency of NO release. This reversibility was independent of *de novo* protein synthesis, as demonstrated using CHX. Interestingly, GSNO allowed observation of either a complete or partial inhibition of HAAT with the different concentrations used. This could indicate a modulated regulation at specific NO concentrations. At high NO levels, the uptake could be inhibited completely, but low

amounts of NO could change the uptake kinetics, decreasing the ammonium entrance. How this modulation of activity works is unknown; however, it could confer on cells high flexibility to adapt to changing environmental conditions. Partial inhibition was not observed with DEA-NONOate due to its short half-life and its sudden release of NO.

The mechanism implicated in this NO inhibition is not clear, although our data in the wild type and mutant cyg56 eliminate, at least for HAAT, a process dependent on peroxynitrite and cGMP. Further studies are needed to understand the NO inhibition mechanism, which could be the result of a direct post-translational modification over transporters, such as *S*-nitrosylation or a modification involving some other cellular components. *In silico* studies have been carried out using the bioinformatics tool GPS-NO 1.0 (Xue *et al*., 2010). This computational prediction of *S*-nitrosylation sites identifies several conserved cysteine residues in plant and algal AMTs as targets for this modification. AMTs form trimers (Ludewig *et al*., 2003; Conroy *et al*., 2004) and use intermolecular interactions between subunits to regulate activity (Loqué *et al*., 2007). In plants, it has been shown that some cysteines located in the N-terminal region of these proteins are implicated in oligomer stabilization. In fact, substitution of these cysteines leads to a decrease in transport capacity (Graff *et al*., 2011). Curiously, some of these cysteine residues are target for *S*-nitrosylation according to GPS-NO 1.0 (Supplementary Fig. S5 at *JXB* online). Thus, NO could be modifying these cysteines, destabilizing the trimers and inhibiting the transport. In addition, according to GPS-NO 1.0, other conserved potentially nitrosylable cysteine residues have also been identified in plant and algal AMTs that could have an important role in the post-translational regulation (Supplementary Fig. S6 at *JXB* online).

On the other hand, for NRT2.1 and NRT2.2, the post-translational modification could directly affect these transporters or NAR2, which is absolutely necessary for functionality of both NRT2 proteins (Galvan *et al*., 1996; Zhou *et al*., 2000; Orsel *et al*., 2006). NRT2.2 has a predictable nitrosylable cysteine, but NRT2.1 does not (Supplementary Fig. S7 at *JXB* online). However, it is interesting that NAR2 from *Chlamydomonas* and other organisms present in their sequences one or more cysteine residues with a high score for *S*-nitrosylation according to GPS-NO 1.0 (Supplementary Fig. S8 at *JXB* online). However, our data are not enough to discard an indirect NO action triggering other kinds of mechanisms such as phosphorylation, which is already involved in regulation of HAAT and HAN/NiT. In fact, the inhibition by ammonium of the *Arabidopsis* AMT1.1 is a phosphorylation-dependent mechanism (Lanquar *et al*., 2009; Lanquar and Frommer, 2010). NO could be a signalling molecule involved in this event.

Concerning the signal that increases NO levels in the cell, it is known in *Chlamydomonas* that in nitrate-plus-ammonium-containing media, NO levels increase with ammonium concentrations (de Montaigu *et al*., 2010). This NO carries out the repression of the *AMT1.1*, *AMT1.2*, and *NRT2.1* genes through a cGMP-dependent pathway. In addition, it is known that ammonium triggers a fast inhibition of HAN/NiT in *Chlamydomonas* (Florencio and Vega, 1982; Galvan *et al*., 1991) and of AMT1.1 activity in *Arabidopsis* (Lanquar *et al*., 2009; Lanquar and Frommer, 2010). Considering the data provided in this paper, we suggest that NO could be an intermediate of the ammonium post-translational regulation so that NO would act through a slow cGMP-dependent pathway for gene repression and a fast and cGMP-independent mechanism for the regulation of the different activities.

As found for nitrate transport, NO mediated a fast and reversible inhibition of NADPH-NR, but this inhibition was not complete. From a physiological point of view, this makes sense, as NO could be detoxified by its conversion to nitrate by means of the action of different proteins with dioxygenase activity, as reported in plants, fungi, and bacteria (Ouellet *et al*., 2002; Wittenberg *et al*., 2002; Perazzolli *et al*., 2004; Schinko *et al*., 2010). Thus, it seems energetically favourable to cells saving the nitrogen used in the generation of the signal molecule. Thus, by remaining partially active, NR would carry out this assimilation function. In addition, NR inhibition was reversible and a fully active enzyme was recovered in a few minutes after NO disappearance, even in the presence of protein synthesis inhibitor. This NR inhibition occurred in a cGMP- and peroxynitrite-independent way. Thus, it is possible that the same mechanism is acting on the regulation of ammonium/nitrate transporters and NR activity.

In vascular plants, NR is regulated by phosphorylation and subsequent binding of 14-3-3 proteins (MacKintosh and Meek, 2001; Lillo *et al*., 2004). However, the serine residue implicated in this regulation is not conserved in mosses and algae as it is in *Chlamydomonas* (Pozuelo *et al*., 2001; Medina-Andrés and Lira-Ruan, 2012). NR from these organisms is regulated at a post-translational level under different conditions, such as light/dark transitions or particular nitrate, nitrite, and ammonium levels, but the mechanisms involved are unknown. Our results showed that NO inhibits NR in *Chlamydomonas*, suggesting that NO could be an intermediary player in the post-translational regulation. In the dark, the lack of photosynthetic activity decreases the reduced ferredoxin pool needed for NiR activity. Although nitrite reduction can occur in the dark (Jin *et al*., 1998), an accumulation of nitrite generated by NR would occur. Nitrite could produce NO by NR, and partial NR inhibition would prevent further nitrite accumulation, providing cells with a significant advantage as a mechanism to couple both reduction steps of NR and NiR to avoid a toxic accumulation of nitrite. Thus, this NO inhibition would be a defence mechanism in any condition leading to an uncoupling between NR and NiR, avoiding nitrate entrance and NR activity and consuming accumulated nitrite.

According to our results, NO inhibited the NR activity in intact cells but not in cell-free extracts or purified enzyme. This could indicate a signalling cascade that is not functional when cells are broken and disorganized. These results suggest an indirect effect of NO on NR. This could implicate a mediated post-translational modification as an enzyme-dependent *S*-nitrosylation of NR, as already reported for other proteins (Anand and Stamler, 2012), or a mediated phosphorylation by protein kinases activated by NO, as reported in other systems (Rabkin *et al*., 2007; Zhang *et al*., 2010; Takata *et al*., 2011). In fact, proteomic studies of nitrosylation in *Arabidopsis*

leaves have identified proteins that are nitrosylated when NO treatment is carried out on intact leaves but not when NO was added to cell-culture extracts (Lindermayr *et al*., 2005).

NR inhibition by NO is not unique to algae, as it has also been found in vascular plants such as *Triticum aestivum* (Rosales *et al*., 2010). In these organisms, NO could inhibit triggering of phosphorylation and 14-3-3 binding and/or involve an alternative regulation of the enzyme. In fact, the N-terminal region of tobacco NR has been shown to be involved in the complete inhibition during light/dark transition independently of 14-3-3 proteins and phosphorylation (Lillo *et al*., 2004).

Under the conditions studied, NO did not inhibit the activity of NiR and GS. There exists a single NiR but there are four GS in *Chlamydomonas* (Fernandez and Galvan, 2008). Thus, the lack of effect on GS activity might not be sensitive enough to reflect an effect on a minority isoform of GS. Mechanisms of post-translational regulation have not been described for NiR, which seems to be regulated only at the transcriptional level, synthesizing new protein when needed. Concerning GS, NO-dependent inhibition was not observed. However, an NO-mediated inhibition by nitration has been reported in nodules of *M. truncatula* plants grown in nitrate medium. Nevertheless, this regulation seems to be a specific regulatory mechanism to halt nitrogen assimilation in nodules when there is available nitrogen in the soil. In fact, the GS from roots was not inhibited by nitration under the same conditions (Melo *et al*., 2011).

In summary, our data show that the signalling molecule NO may have an important role in post-translational regulation of nitrate/nitrite and ammonium transporters and also of NR. Whereas the inhibitory effect on transporters can be complete, the inhibitory effect on NR activity is not. This difference is physiologically significant, allowing the assimilation of the nitrate/nitrite generated from NO and adjusting the activity of NR to the needs of the cell. This can be particularly important under nitrogen-deficiency conditions.

Supplementary data

Supplementary data are available at *JXB* online.

Supplementary Table 1. Ammonium uptake in cultures treated with several pharmacological compounds.

Supplementary Fig. S1. Reversibility of ammonium, nitrite, and nitrate uptake in cultures treated with NO and CHX.

Supplementary Fig. S2. NO inhibits ammonium uptake (a) and NR activity (b) in the cyg56 mutant.

Supplementary Fig. S3. Reversibility of NADPH-NR inhibition by NO without CHX.

Supplementary Fig. S4. NO does not inhibit NiR or GS activity.

Supplementary Fig. S5. Alignment of N-terminal sequences of AMTs and localization of nitrosylable cysteines.

Supplementary Fig. S6. Alignment of specific regions of AMTs with conserved nitrosylable cysteines.

Supplementary Fig. S7. Alignment of NRT2.1 and NRT2.2 transporter sequences from *Chlamydomonas* and *Arabidopsis*.

Supplementary Fig. S8. Alignment of NAR2 sequences from plants and algae.

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