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Impact of nitric oxide on proline and putrescine biosynthesis in *Chlamydomonas* via transcriptional regulation

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Abstract

Nitric oxide plays an important role in regulating adaption of the model alga *Chlamydomonas reinhardtii* to various environmental stresses. One response to abiotic stresses is the accumulation of protective molecules such as proline and putrescine. The NO treatment led to a significant accumulation of proline in cells. Quantitative real-time expression analysis of proline metabolic genes in NO-treated cells showed a prolonged upregulation of the gene encoding γ -glutamyl kinase 1 (GGK1) in the glutamate biosynthetic pathway. Furthermore, truncated hemoglobin 2 (THB2)-underexpressing strains with an enhanced endogenous NO demonstrated a higher proline content and *GGK1* mRNA abundances than the wild type. In contrast, transcription of the gene encoding ornithine δ -aminotransferase in the ornithine pathway of proline biosynthesis decreased after treatment with NO. This suggests the predominance of the glutamate pathway over the ornithine pathway. We also found that the expression of the proline dehydrogenase gene encoding a key enzyme in proline catabolism was downregulated in NO-treated cells. *Chlamydomonas reinhardtii* exposed to exogenous NO also showed an increased *ornithine decarboxylase 2* mRNA and putrescine content. Our findings indicate a clear link between changes in NO application and proline and putrescine content via transcriptional regulation of respective enzymes.

Additional key words: glutamyl kinase, ornithine decarboxylase, polyamines, truncated hemoglobins.

Introduction

Nitric oxide is a gaseous secondary messenger that has evolved as an important and ubiquitous signal molecule in plants (Wendehenne and Hancock 2011, Domingos *et al.* 2015). In higher plants, NO has been implicated in many physiological processes, such as development, reproduction, resistance to various abiotic and biotic stresses (Corpas *et al.* 2011, Mur *et al.* 2013, Domingos *et al.* 2015, Corpas and Barroso 2015, Farnese *et al.* 2016). In the model green alga *Chlamydomonas reinhardtii*, NO is also involved in a number of cell functions including macro-nutrient stress responses, hypoxia, and salt stress (Hemschemeier *et al.* 2013, Wei *et al.* 2014, Chen *et al.* 2016, Minaeva *et al.* 2017, De Mia *et al.* 2019, Filina *et al.* 2019). Moreover, as in higher plants (Milani *et al.* 2003), in *C. reinhardtii*, truncated hemoglobins (*e.g.* THB1 and THB2) have also been implicated in NO scavenging to protect cells from the accumulation of the radical molecule (Sanz-Luque *et al.* 2015, Minaeva *et al.* 2017, Filina *et al.* 2019). Remarkably, despite this plethora of NO effects, its

role in the potential control of the synthesis of protective organic molecules remains largely unknown.

Among many protective molecules, proline is the most widely accumulated compound in higher plants under stress conditions and this amino acid has multifunctional roles (Verbruggen and Hermans 2008, Szabados and Savouré 2010, Sharma *et al.* 2011, Dar *et al.* 2016, Signorelli 2016, Chun *et al.* 2018). In *C. reinhardtii*, proline has been shown to prime cells against salt stress (Mastrobuoni *et al.* 2012).

As in many other plants (Szepesi and Szöllősi 2018), proline is synthesized by *C. reinhardtii* from either glutamate or ornithine (Fig. 1). In higher plants, the key enzyme in the biosynthesis of proline from glutamate is a bifunctional Δ^1 -pyrroline 5-carboxylate synthase (P5CS), which catalyzes the conversion of glutamate to γ -glutamyl phosphate (Verbruggen and Hermans 2008). However, in *C. reinhardtii*, two separate enzymes, γ -glutamyl kinase and γ -glutamyl phosphate reductase, metabolize glutamate (Miyoshi *et al.* 2011). This is similar to the first two steps of the glutamate pathway in a number of bacteria that use γ -glutamyl kinase and γ -glutamyl phosphate reductase

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Abbreviations: DEA-NONOate - 2-(N,N diethylamino)-diazene-2-oxide sodium salt; GGK1 - glutamyl kinase 1; ODC2 - ornithine decarboxylase 2; OTA1 - ornithine δ -aminotransferase 1; P5C - pyrroline-5-carboxylate; TAP - tris-acetate-phosphate; THB - truncated hemoglobin; WT - wild type.

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(Commichau *et al.* 2008, Fichman *et al.* 2015). Notably, in some bacteria, osmotic stress-adaptive synthesis of proline is controlled at the level of transcription (Hoffmann and Bremer 2016). Ornithine is the alternate precursor, which can be transaminated to pyrroline-5-carboxylate (P5C) by ornithine- γ -aminotransferase (OAT) (Fig. 1). Proline accumulation in salt-stressed *C. reinhardtii* raises the question about potential stress-inducible genes encoding enzymes of glutamate and/or ornithine pathways. This conundrum has yet to be addressed experimentally.

Apart from neutrally charged protective molecules (such as proline), polyamines are also accumulated in response to stress in a variety of plants (Sengupta *et al.* 2016). The most commonly occurring polyamine is putrescine. In higher plants, putrescine is formed primarily via the decarboxylation of arginine by arginine decarboxylase and subsequent degradation of the generated agmatine (Slocum 1991). In *C. reinhardtii*, putrescine synthesis is controlled by ornithine decarboxylase (ODC) (Fig. 1; Voigt *et al.* 2000). Importantly, putrescine is accumulated in salt-treated *C. reinhardtii* cells (Mastrobuoni *et al.* 2012). Moreover, NO plays a role in adaptive response of the alga to salt stress (Chen *et al.* 2016). We hypothesize the potential relationship(s) between changes in NO content and proline and/or putrescine content via transcriptional regulation of enzyme(s).

The aims of this study were to investigate the impact of NO on transcriptional regulation of proline and putrescine biosynthesis and to establish a relationship between the proline and putrescine accumulation, the expression of genes of their biosynthetic pathways and NO content.

Materials and methods

Strains and experimental conditions: The following *Chlamydomonas reinhardtii* strains were used: wild-type cw15-325 (*mt+*, *cw15*, *arg7*), which was kindly provided by Dr. M. Schroda (University of Kaiserslautern, Germany) and transformants with reduced THB2 obtained from cw15-325 *amiTHB2-7* (*mt+*, *cw15*), *amiTHB2-17* (*mt+*, *cw15*) and *amiTHB2-22* (*mt+*, *cw15*) (Filina *et al.* 2019). Cells were grown in tris-acetate-phosphate (TAP) medium (<http://www.chlamy.org/TAP.html>) under a continuous irradiance of 20 $\mu\text{mol}(\text{photons})\text{ m}^{-2}\text{ s}^{-1}$ and a temperature of 22 °C with constant orbital agitation at 90 rpm (Fig. 1 Suppl.). The TAP medium was supplemented with 100 mg dm⁻³ arginine when required. The 2-(N,N diethylamino)-diazene-2-oxide sodium salt (DEA-NONOate, *Sigma-Aldrich*, St. Louis, USA) was added to the medium to a final concentration of 50 μM or 100 μM and then cells were incubated for 0.5, 1, 2, or 4 h. At each harvesting times, cells were washed in TAP medium and analyzed as described below. For the salt stress analysis, the cells grown in TAP medium were collected at the mid-exponential phase of growth by centrifugation (4000 g, 5 min), and then incubated in TAP with addition of NaCl to a final concentration of 150 mM. For all measurements, samples were taken from cultures 1, 2, 4, and 6 h after NaCl supply. To induce hypoxia conditions, cultures were

grown at 22 °C to log phase ($5 - 7 \times 10^6$ cells cm⁻³) in 1-dm³ flasks. Then, for experiments, 50 cm³ aliquots of cells were transferred into sealed 50-cm³ square glass bottles as described (Ostroukhova *et al.* 2017). The flasks were subsequently incubated in the dark, allowing a gradual removal of dissolved oxygen by respiratory activity. Dissolved oxygen was assayed using a Clark-type electrode to ensure that hypoxic conditions were achieved. In each experiment, the number of cells was measured employing a counting chamber and the viable cells were estimated microscopically with use of 0.0125 % (v/v) methylene blue (*DIA-M*, Moscow, Russia). Stained (non-viable) and unstained (viable) cells were observed and counted. Four hundred cells from each sample were examined for three biological replicates.

Measurement of chlorophyll, proline, and putrescine content: Determination and calculations of total chlorophyll were performed as previously described (Minaeva *et al.* 2017). The proline content was measured at 520 nm as described (Bates *et al.* 1973). For putrescine content analysis, 10⁸ cells were collected by gentle centrifugation at 4 000 g and each pellet was immediately frozen with liquid nitrogen. Metabolites were extracted and derivatized as was previously reported (Puzanskiy *et al.* 2018). Gas chromatography - mass spectrometry analysis was performed at *Agilent 5860* chromatograph (*Agilent Technologies*, Santa Clara, CA, USA) with *Agilent 5975S* mass selective detector. Separation was performed on a *J&W HP-5ms* capillary column. Helium flow rate was 1 cm³ min⁻¹. Inlet temperature was 250 °C at splitless mode. The temperature conditions of the column thermostat were the following: initial temperature of 70 °C, increased by 6 °C per min up to 325 °C. Electron impact ionization was performed at 70 V and an ion source temperature of 230 °C. To verify putrescine peaks, the standard of it was analyzed (*Sigma-Aldrich*). Arbitrary quantification was made as normalization of metabolite peak area by internal standard (tricosane) peak area. The analysis of the GC-MS data was processed using the *PARADISE* program (Johnsen *et al.* 2017) in association with *NIST MS* search program and mass-spectrometer library *NIST2010* (National Institute of Standards and Technology, Gaithersburg, MD, USA). Statistical processing was performed in the language environment *R 3.5.2 "Eggshell Igloo"* (*R Core Team* 2020). An additional post-hoc test for mean comparison confirmed significant differences between treatments (data not shown).

Gene expression analyses: The total RNA was extracted, and cDNA strands were synthesized as described previously (Minaeva *et al.* 2017). Real-time quantitative reverse transcription PCR reactions were performed on a light cycler (CFX96, *Bio-Rad*, Hercules, USA) using *SYBR Green I* following a previously reported protocol (Zalutskaya *et al.* 2016). The primer sequences are listed in Table 1 Suppl. The relative gene expression ratios were normalized with a *receptor of activated protein kinase C* (formerly termed *CBLP*) using the ΔCt and $\Delta\Delta\text{Ct}$ methods (Livak and Schmittgen 2001).

Measurement of NO: The NO content in cells was measured using 4-amino-5-methylamino-2'7'-difluorofluorescein diacetate dye (*Sigma-Aldrich*) using a microplate reader *CLARIOstar* (*BMG*, Ortenberg, Germany) as described by Filina *et al.* (2019). The selective NO scavenger 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide (cPTIO; *Sigma-Aldrich*) was used in a final concentration of 200 μ M. Three technical replicates per condition were included on each plate, and each experiment was performed three times independently.

Statistics: The values are means $\pm$ SEs of three biological replicates and three technical replicates. The differences between means were analyzed using the Student's *t*-test. Differences were considered statistically significant at $P < 0.05$ and highly significant at $P < 0.01$.

Results

To identify the potential impact of NO on proline accumulation, we used DEA-NONOate as NO donor (Floryszak-Wieczorek *et al.* 2006). The concentrations of DEA-NONOate used (50 and 100 μ M) caused a significant increase in the fluorescence, showing the formation of NO (Fig. 2A). Notably, DEA-NONOate had no such effect after preincubation of cells with 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide (cPTIO). Proline content increased progressively after addition of 50 or 100 μ M DEA-NONOate, reaching a maximum between 2 and 4 h, where we measured 4.5- to 5-fold higher proline content than in untreated cells (Fig. 2B). When *C. reinhardtii* was exposed to 100 μ M DEA-NONOate, we observed a slightly larger accumulation of proline than in the presence of 50 μ M of NO donor. Therefore, 100 μ M of DEA-NONOate was chosen for further analysis.

Next, to demonstrate the function of NO in proline accumulation, we examined the abundance of transcripts for each gene in the glutamate and ornithine pathways (Fig. 1) in DEA-NONOate-treated *versus* untreated cells. *Chlamydomonas reinhardtii* genome contains two *glutamyl kinase* (*GGK*) genes encoding γ -glutamyl kinase, the first enzyme of the proline biosynthetic pathway. We found that only *GGK1* mRNA was induced in response to DEA-NONOate treatment (Fig. 2C). In contrast, the transcript abundances of *GGK2* and *PCR1* were down-regulated. Additionally, *glutamate-5-semialdehyde dehydrogenase* (*GSD1*) showed a 4.3-fold increase in its transcript abundance after 4 h of incubation (Fig. 2C). Proline may also be synthesized from ornithine by the ornithine- δ -aminotransferase (*OTA1*) (Fig. 1), but *OTA1* expression was downregulated by NO (Fig. 2C). The results also showed that the transcription of *proline dehydrogenase 1*, which catabolizes proline into P5C, declined significantly after 1 h (Fig. 2D), suggesting inhibition of proline catabolism.

We also tested the two genes of the arginine catabolism pathway that encode arginine deiminase (*ADI1*) and ornithine carbamoyltransferase (*OTC1*), two enzymes, producing ornithine from arginine (Fig. 1). Interestingly,

ADI1 and *OTC1* genes were induced after 30 min of exposure to NO donor (Fig. 2D). In *C. reinhardtii*, ornithine also can serve as a precursor for putrescine biosynthesis. Notably, ornithine decarboxylase 2 (*ODC2*), which converts ornithine into putrescine, was induced within 1 to 4 h of incubation with DEA-NONOate (Fig. 3A). Next, we asked the question whether the increased *ODC2* mRNA correlate with a change in putrescine content. Treatment of *C. reinhardtii* cells with NO donor resulted in a 2-fold increase in putrescine over the 4-h exposure period (Fig. 3B).

To further prove the role of NO in proline accumulation, free proline content and transcription of *GGK1* were compared in wild-type and *THB2-amiRNA* strains with reduced content of truncated hemoglobin 2 (Filina *et al.* 2019). Knockdown of *THB2* results in increased NO production (Fig. 4A). Compared to the wild type (WT), proline content was increased in *THB2*-underexpressing strains (Fig. 4B). In addition, in *amiRNA-THB2* strains, *GGK1* transcriptions were higher than in parental strain (Fig. 4B).

As *GGK1* and *ODC2* are NO-inducible genes (Fig. 2C, 3A), we speculate that they may be upregulated during stresses, where NO plays a role, such as hypoxia and salt stress (Hemschemeier *et al.* 2013, Chen *et al.* 2016). Expression analysis of *GGK1* and *ODC2* showed that these genes were inducible by salt and hypoxia (Fig. 5A,C). However, there was a significant difference between the salt- and low oxygen-treated *C. reinhardtii* in *GGK1* expressions, so that, NaCl-exposed cells showed significantly more transcript abundance (with the highest induction of 2000-fold after 4 h) than hypoxia-exposed cells (with the highest induction of 5-fold after 2 h). In contrast, *ODC2* transcriptions were very similar under both stress conditions (Fig. 5A,C). Analyses of proline and putrescine content upon two stresses showed that salt treatment resulted in higher proline and putrescine content in cells in comparison with the hypoxia-exposed cells (Fig. 5B,D).

Fig. 1. Proline and putrescine biosynthesis in *Chlamydomonas reinhardtii*. Enzymes of proline and putrescine biosynthesis pathways are shown in blue and green, respectively. Red and purple colours refer to catabolic enzymes of proline and arginine, respectively.

Discussion

Higher plants accumulate proline and putrescine as protective molecules in response to various environmental stresses (Szabados and Savouré 2010, Sengupta *et al.* 2016). In *C. reinhardtii*, proline and putrescine accumulation has been reported during salt stress (Mastrobuoni *et al.* 2012) and NO is involved the signaling pathways under these conditions (Chen *et al.* 2016). Until now, the effect of NO on proline and putrescine content in *C. reinhardtii* remains unclear. Here, we provide the first evidence that NO induced both proline and putrescine accumulation.

NO donor resulted in increased proline content, which was more abundant after exposure to 100 μM compared with 50 μM of DEA-NONOate, respectively (Fig. 2B). This might be explained by the fact that 100 μM of DEA-NONOate produced higher NO amount than 50 μM of NO donor (Fig. 2A). Since elevated NO content was detected in *THB2*-under-expressing cells (Filina *et al.* 2019), we used these strains in our experiments. Our analyses showed that under nonstress conditions *THB2*-knockdown strains maintained higher NO content than WT (Fig. 4A). In agreement with these data, *THB2*-amiRNA cells showed enhanced content of proline compared to the parental strain

(Fig. 4B). The content of proline and of intracellular NO in *THB2*-knockdown cells fell within the range normally seen in WT during treatment with 100 μM of DEA-NONOate (Figs. 2 and 4). In other words, knockdown of *THB2* induced some NO accumulation leading to an increase in proline content under nonstress conditions.

In land plants, intracellular proline accumulation is determined by its biosynthesis, catabolism, and transport (Verbruggen and Hermans 2008). *C. reinhardtii* is not able to transport external proline (Muños-Blanco *et al.* 1990). Importantly, a strong correlation between *P5CS* expression and the accumulation of proline has been shown in rice and *Arabidopsis thaliana* (Hein *et al.* 2003, Szekely *et al.* 2008). Therefore, transcription of key genes in proline metabolism of *C. reinhardtii* was tested in the presence of DEA-NONOate. Our real-time qPCR analysis of DEA-NONOate-treated *C. reinhardtii* cells indicated an increase in mRNA of *GKG1*, an enzyme of glutamate biosynthetic pathway (Fig. 2C). The second NO-inducible gene, *glutamate-5-semialdehyde dehydrogenase*, showed a delayed upregulation. These results suggest that *GKG1* might be the regulatory point controlling NO-dependent proline biosynthesis at the transcriptional level. Supporting evidence for this also comes from studies in *THB2*-

Fig. 2. Effects of 2-(N,N diethylamino)-diazeneolate-2-oxide sodium salt (DEA-NONOate) on intracellular NO generation (A), proline content (B), relative expressions of genes of proline biosynthesis (C) and proline and arginine catabolism (D). A - fluorescence intensity due to NO formation was determined using 1 μM 4-amino-5-methylamino-2',7'-difluorofluorescein diacetate dye and expressed as arbitrary units per 1 μg of chlorophyll in 10^6 cells. Cell autofluorescence was subtracted from the total fluorescence obtained. The solution of 200 μM 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide (cPTIO) was added 5 min before DEA-NONOate. B - proline content is expressed as nanogrammes per 1 μg of chlorophyll in 10^6 cells. C - relative expressions were determined in the presence of 100 μM DEA-NONOate. Values are given as expressions relative to a house-keeping gene - *receptor of activated protein kinase C*, which had a value of 1. Ctrl - control (non-treated cells). Means $\pm$ SEs of three biological replicates and three technical replicates. Statistical analysis was performed by Student's *t*-test. In (A) ** - significant differences between variants without and with cPTIO at $P < 0.01$. In (C,D) *,** - significant differences between non-treated and treated cells at $P \leq 0.05$ and 0.01, respectively.

underexpressing strains where enhanced endogenous NO resulted in higher *GGK1* mRNA abundances than in

Fig. 3. Effects of 2-(N,N diethylamino)-diazene-2-oxide sodium salt (DEA-NONOate) on relative expressions of genes for putrescine biosynthesis (A) and on putrescine content (B). Relative gene expressions were determined as described in Fig. 2C. B - putrescine amount in cells without added DEA-NONOate represents control (Ctrl, set to 100 %). Means $\pm$ SEs of three biological replicates and three technical replicates. Statistical analysis was performed by Student's *t*-test. *,** - significant differences between non-treated and treated cells at $P \leq 0.05$ and 0.01 , respectively.

Fig. 4. Nitric oxide generation (A) and proline content and glutamyl kinase 1 (*GGK1*) expression (B) in parental and *THB2-amiRNA* strains of *Chlamydomonas reinhardtii*. Fluorescence intensity and proline content were expressed as described in Fig 2A,B. The relative *GGK1* expression was normalized to the expression of a receptor of activated protein kinase C and calculated using ΔC_T . Means $\pm$ SEs of three biological replicates and three technical replicates. Statistical analysis was performed by Student's *t*-test. Differences were considered statistically significant at $P < 0.01$.

WT (Fig. 4C). Notably, *PCRI* gene is repressed by NO (Fig. 2C), suggesting that like in higher plants (Filippou *et al.* 2013), Δ^1 -pyrroline-5-carboxylate reductase appeared not to be a rate-limiting factor in proline synthesis in this alga. We also found that the expression of *proline dehydrogenase* gene encoding a key-enzyme in proline catabolism is downregulated after NO-treatment (Fig. 2D). In *C. reinhardtii*, the mode of action of NO is poorly understood. The best studied NO-dependent pathway leads to the repression of genes involved in nitrate assimilation by ammonium and involves a soluble guanylate cyclase (De Montaigu *et al.* 2010, Sanz-Luque *et al.* 2016). The components of NO-based signaling pathway(s) that are involved in induction of *GGK1*, *ODC2*, and inhibition of *proline dehydrogenase 1* needs to be further elucidated.

In legume plants, the ornithine pathway (through enhanced transcription of *OAT* encoding ornithine δ -aminotransferase) may also contribute to the stress-induced proline accumulation (Armengaud *et al.* 2004). In *C. reinhardtii*, supplementation with DEA-NONOate decreased transcription of *OTAI* (Fig. 2C), indicating that this pathway is rather repressed by NO. This suggests predominance of the glutamate pathway over the ornithine pathway.

Importantly, putrescine, which is essential metabolite of *C. reinhardtii*, is synthesized in the ornithine decarboxylase (ODC) pathway (Voigt *et al.* 2004), which after deimination of arginine to citrulline and decarboxylation to ornithine, yields putrescine by action of ODCs (Fig. 1). We hypothesized that in DEA-NONOate-treated cells, ornithine may be used as a precursor not for proline but for putrescine biosynthesis. Indeed, we demonstrated that in contrast to *ADH1* and *OTC1*, *ODC2* gene is induced by NO (Fig. 3A). Additionally, NO-dependent increase in *ODC2* mRNA abundance accompanied by a 2-fold increase in putrescine content (Fig. 3B), suggesting that another protective molecule is also regulated by nitric oxide in *C. reinhardtii*. However, we cannot exclude the possibility that putrescine is accumulated, at least in part, due to other components. For example, the contribution of arginine decarboxylase to the formation of putrescine has been reported (Tassoni *et al.* 2018). Interestingly, in *Medicago truncatula*, exogenous NO controls putrescine and proline content via enzymatic and transcriptional modulation of proline and polyamine biosynthetic pathways (Filippou *et al.* 2013).

The observed NO-controlled accumulation of proline and putrescine suggest that both may come into the same stress(es) scene. Previously, it was shown that *C. reinhardtii* produced enhanced content of proline and putrescine under salt stress (Mastrobuoni *et al.* 2012). Here, we clearly demonstrated that the increased content of proline and putrescine was accompanied with the concurrent up-regulation of the *GGK1* and *ODC2* transcriptions, respectively (Fig. 5A,B). The induced expression of *GGK1* and *ODC2* genes to generate proline and putrescine in hypoxia is also documented in the present study (Fig. 5C,D). However, whether NO is essential for accumulation of proline and putrescine via up-regulation of the *GGK1* and *ODC2* genes under salt and hypoxic

Fig. 5. Effects of salt stress and hypoxia on *glutamyl kinase 1* (GGK1) and *ornithine decarboxylase 2* (ODC2) transcript accumulations (A,C), and proline and putrescine abundances (B,D). Relative gene expressions and proline content were determined as described in Fig. 2B,C. Putrescine in non-stressed cells represent control (Ctrl, set to 1.0). Means $\pm$ SEs of at least three biological replicates and three technical replicates. Statistical analysis was performed by Student's *t*-test. *,** - significant differences between non-treated and treated cells at $P \leq 0.05$ and 0.01, respectively.

stresses requires further investigation.

In conclusion, characterization of the NO-dependent biosynthesis of proline and putrescine expands our understanding of the regulatory aspects of NO signaling network in *C. reinhardtii* beyond previous findings (Hemschemeier *et al.* 2013, Wei *et al.* 2014, Chen *et al.* 2016, Minaeva *et al.* 2017, De Mia *et al.* 2019, Filina *et al.* 2019). The implication of NO in the control of the two protective molecules suggests the existence of a molecular mechanism that may coordinate different cellular responses under stress conditions.

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