Copper-Induced Proline Synthesis is Associated with Nitric Oxide Generation in *Chlamydomonas reinhardtii*

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Excess copper affects the growth and metabolism of plants and green algae. However, the physiological processes under Cu stress are largely unknown. In this study, we investigated Cu-induced nitric oxide (NO) generation and its relationship to proline synthesis in *Chlamydomonas reinhardtii*. The test alga accumulated a large amount of proline after exposure to relatively low Cu concentrations (2.5 and 5.0 $\mu$M Cu$^{2+}$). A concomitant increase in the intracellular NO level was observed with increasing concentrations of Cu applied. Data analysis revealed that the endogenous NO generated was positively associated with the proline level in Cu-stressed algae. The involvement of NO in Cu-induced proline accumulation was confirmed by using an NO-specific donor, sodium nitroprusside (SNP), and an NO scavenger cPTIO [2-(4-carboxy-2-phenyl)-4,4,5,5-tetramethylinidazoline-1-oxyl-3-oxide]. Pre-treatment with 10 $\mu$M SNP increased the proline accumulation in Cu-treated cells by about 1.5-fold, while this effect could be blocked by addition of 10 $\mu$M cPTIO. We further investigated the effect of Cu and NO on the activity and transcript amount of 1-pyrroline-5-carboxylate synthetase (P5CS, EC 2.7.2.11), the key enzyme of proline biosynthesis, and observed that application of SNP was able to stimulate the P5CS activity and up-regulate the expression of P5CS in the Cu-treated algae. These results indicate that Cu-responsive proline synthesis is closely related to NO generation in *C. reinhardtii*, suggesting the regulatory function of NO in proline metabolism under heavy metal stress.

**Keywords:** *Chlamydomonas reinhardtii* — Copper — Nitric oxide — Proline.

Abbreviations: cPTIO, 2-(4-carboxy-2-phenyl)-4,4,5,5-tetramethylinidazoline-1-oxyl-3-oxide; DAF-2DA, 4,5-diamino-fluorescein diacetate; NO, nitric oxide; NOS, nitric oxide synthase; P5CS, 1-pyrroline-5-carboxylate synthetase; ROS, reactive oxygen species; RT–PCR, reverse transcription–PCR; SNP, sodium nitroprusside.

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**Introduction**

Copper (Cu) is one of the essential micronutrients and plays essential roles in metal homeostasis and normal metabolism in plants. It is involved in a wide range of biological processes. For example, Cu is required as a cofactor of superoxide dismutase (EC 1.1.5.1.1). It also participates in electron transfer reactions of photosynthesis in the form of plastocyanin (Yruela et al. 2000). However, Cu at high levels becomes strongly phytotoxic to cells and causes inhibition of plant growth or even death. Recent studies have demonstrated that excess Cu in plant cells may activate molecular oxygen and generate reactive oxygen species (ROS) (Mocquot et al. 1996, Chen et al. 2000). Cu-induced generation of hydrogen peroxide, hydroxyl radicals and other ROS has been directly correlated with damage to membrane lipids and proteins (Murphy and Taiz 1997, Wang et al. 2004). One of the major responses of plants to Cu toxicity is the generation of proline, which is possibly associated with the protection of plant cells against oxidative damage and with signal transduction (Mehta and Gaur 1999, Bačkor et al. 2004, Choudhary et al. 2007). Although several mechanisms responsible for tolerance to Cu have been proposed, the biological process by which proline mediates Cu-induced toxicity is largely unknown.

It is known that green algae and higher plants may accumulate proline when subjected to environmental stresses such as heavy metals (Wu et al. 1998, Mehta and Gaur 1999, Tripathi and Gaur 2004), osmotic stress (Siripornadulsil et al. 2002, Slama et al. 2006), high salinity (Abrahám et al. 2003, Miller et al. 2005) and light-induced stress (Abrahám et al. 2003). In the most cases, accumulation of proline is believed to be an adaptive response of plants against stressful environments, and proline is considered as a signal/regulatory molecule able to activate multiple physiological or molecular responses (Taylor 1996, Öztürk and Demir 2002). It is suggested that proline improves the salt tolerance of *Pancratium maritimum* by protecting the protein turnover machinery from stress damage (Kheder et al. 2003). Proline protects Rubisco (Solomon et al. 1994) from NaCl-induced toxicity, and nitorgenase activity from drought stress (Pedersen et al. 1996). Accumulation of proline under stress can be achieved by either increases in synthesis or reduction of degradation.
(Hong et al. 2000). Recent studies have shown that the increased activity of Δ1-pyrroline-5-carboxylate synthetase (P5CS), an enzyme that catalyzes proline biosynthesis, was detected in higher plants exposed to Cu (Chen et al. 2001). Transgenic algae expressing the P5CS gene show more free proline and bind more Cd in the cells than the wild type, thus conferring plant tolerance to Cd toxicity (Siripornadulsil et al. 2002). Silencing expression of proline dehydrogenase (EC 1.5.99.8), the enzyme catalyzing proline oxidative degradation, resulted in accumulation of free proline in tobacco cells and consequently contributed to osmotic tolerance of the cells (Tateishi et al. 2005). These results indicate that proline may not only act as an active osmoprotectant, but may also facilitate detoxification of heavy metals.

Nitric oxide (NO) is a biologically active gaseous molecule and has received growing attention due to its association with plant responses to pathogen attack, hypersensitivity or programmed cell death (Delledonne et al. 1998, de Pinto et al. 2002, Neill et al. 2003). It is also shown to be involved in mediation of environmental stresses such as chilling (Neill et al. 2002, 2003), salt (Uchida et al. 2002, Zhao et al. 2001) and heavy metals (Hsu and Kao 2004, Wang and Yang 2005). In animals, biosynthesis of NO is regulated by nicotinic oxide synthase (NOS, EC 1.14.13.39). In plants, however, the existence of NOS has been a controversial issue (Crawford et al. 2006, Zemot et al. 2006) since the identification of the putative NOS-like enzyme, AtNOS1, in Arabidopsis (Guo et al. 2003). However, the activity of NOS for NO synthesis can be detected (Sakihama et al. 2002, Neill et al. 2003). A well established NO production system in plants is assimilatory nitrate reductase, which is to some extent structurally similar to NOS in animal tissues (Tischner et al. 2004).

NO is proposed to be one of the important second messengers in plant cells (Neill et al. 2002). Although several lines of evidence in green algae have indicated that NO may also function as a signaling molecule and indirectly mediate ROS levels in the cascade of events leading to alterations of antioxidative responses (Tischner et al. 2004), the relationship between heavy metal-induced proline accumulation and intracellular NO production has not been established. On the above grounds, we hypothesized that NO regulates Cu-induced proline accumulation in Chlamydomonas reinhardtii. The aim of this study was to characterize NO generation in C. reinhardtii under Cu stress and find out whether NO regulates proline accumulation in the alga. The outcome of the study may improve our understanding of heavy metal stress-related aspects of the NO regulatory network which controls plant responses to heavy metals.

**Results**

**Copper uptake and alga growth**

To understand the pattern of Cu accumulation in C. reinhardtii, an initial time-dependent accumulation experiment was performed. As shown in Fig. 1A, Cu accumulation in cells occurred upon the addition of Cu (5 μM) to the medium. The linear increase in Cu uptake lasted for 30 min. After that, the uptake rate saturated. The maximum content of Cu in the alga exposed to 5 μM Cu for 2 h was 0.22 fmol cell

Fig. 1 Intracellular accumulation of Cu by C. reinhardtii. (A) Time-course of Cu uptake. The culture was incubated with 5 μM Cu added to the medium. At different time intervals, a 10 ml aliquot was collected and intracellular Cu was determined in the EDTA-washed pellet. (B) Intracellular accumulation of Cu at varying external Cu concentrations. The intracellular Cu content was determined after 1 h of incubation. Vertical bars represent the SD. Asterisks indicate that mean values are significantly different between the Cu treatments and control (P<0.05).
2h after Cu treatment. After that time, however, the level gradually decreased. Accumulation of proline in Cu-treated alga exhibits a concentration-dependent change with Cu (Fig. 3B). Initially, the level of proline increased with Cu up to 5 µM. However, raising the concentration of Cu beyond this resulted in a decrease in the proline level.

**Generation of endogenous NO under Cu exposure**

Intracellular and released NO were measured in relation to the time of Cu exposure. Treatment of *C. reinhardtii* cells with 5 µM Cu induced a rapid accumulation of intracellular NO (Fig. 4A). The amount of intracellular NO increased with the time of Cu exposure. The peak level was observed at 60 min, with the NO content being approximately 11.4-fold higher than the control. Following that point, the level of intracellular NO gradually declined. Similarly, the amount of NO diffused into the external medium increased considerably during the first 60 min. The high level of release of NO remained until 120 min, and then decreased (Fig. 4B). We also investigated the effect of Cu concentration on NO generation in *C. reinhardtii*. Positive linear relationships were established with varying Cu concentrations in the medium and NO level in cells, as well as that diffused in the external medium (Fig. 4C).

To confirm whether the Cu-induced production of proline in *C. reinhardtii* was associated with endogenous NO concentrations, the histochemical detection of NO was performed using an NO fluorescent probe, 4,5-diaminofluorescein diacetate (DAF-2DA). Our analysis reveals that DAF-2DA-dependent fluorescence was
enhanced by exposure to Cu of around 5 μM (Fig. 5), showing time- or concentration-dependent changes.

Effect of exogenous NO on proline accumulation

To understand further the role of NO in mediating the Cu-induced proline production, an exogenous NO donor, sodium nitroprusside (SNP), was applied to investigate the effect of NO. Pre-incubation of *C. reinhardtii* with 10 μM SNP for 30 min increased the proline level of Cu-treated cells by about 1.5-fold (Fig. 6A, B). However, in the absence of Cu, NO hardly affected the proline accumulation, and, in addition, the SNP analog potassium ferricyanide, which is unable to release NO, had no effect on proline accumulation. In contrast, pre-treatment with an NO scavenger, cPTIO [2-(4-carboxy-2-phenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide], could completely cancel the Cu-induced proline accumulation (Fig. 6A, C). The effects of both SNP and the NO scavenger on the Cu-induced proline accumulation appear to be concentration dependent (Fig. 6B, C). For example, pre-treatment with 2.5, 5 and 10 μM CPTIO reduced the proline level of Cu-treated cells by 12, 50 and 80%, respectively, as compared with the control, indicating that NO is involved in the hyperaccumulation of proline and may have a regulatory role over proline metabolism in the alga under Cu exposure.

Effect of Cu and NO on the activity and transcript amount of P5CS

Δ¹-pyrroline-5-carboxylate synthetase (P5CS, EC 2.7.2.11) is the key enzyme that catalyzes proline biosynthesis. In the presence of Cu, the activity of P5CS increased during the first 60 min. After this time, however, it declined
rapidly (Fig. 7A). In a dose–response experiment, the P5CS activity increased progressively with increasing concentrations of Cu in the medium (Fig. 7B). The highest activity of P5CS was observed at 5 μM. Further elevation of Cu levels in the medium caused a decrease in the P5CS activity.

To examine whether P5CS expression was regulated by Cu, the algae were incubated in medium with 5 μM Cu for 240 min or with 2.5–10.0 μM for 60 min. It is shown that the P5CS transcript level increased progressively during the initial 30 min exposure to Cu (Fig. 7C). After this time, however, the P5CS expression decreased to the basal level. Accumulation of P5CS transcripts was also regulated by the concentration of Cu. Treatment with 5 μM Cu increased the amount of P5CS transcript significantly (Fig. 7D), but high levels of Cu down-regulated the expression of P5CS.

To understand the role of NO in regulating the Cu-responsive production of proline, the effect of NO on the activity and transcript amount of P5CS in C. reinhardtii was investigated. In the absence of Cu, SNP did not affect the activity of P5CS significantly, while in Cu-treated algal cells, the P5CS activity was significantly enhanced by SNP pre-treatment (Fig. 8A). However, simultaneous pre-treatment with cPTIO resulted in no effect of SNP on P5CS activity. Similar to the results obtained for P5CS activity, the expression of P5CS was up-regulated by both Cu alone and SNP + Cu (Fig. 8C, D).

Discussion

In this study, we provided evidence that application of excess Cu was able to induce proline accumulation in C. reinhardtii, a eukaryotic model of a unicellular organism for studies of heavy metal homeostasis and tolerance (Hanikenne 2003). Simultaneously, intracellular NO accumulated under the Cu stress (Figs. 4, 5), suggesting that Cu-induced proline accumulation might be mediated by NO. Such an effect can be supported by the observation that treatment with SNP, a donor of NO, was able to promote the proline accumulation in the presence of Cu (Fig. 6). The NO effect is specific because an NO scavenger cPTIO could cancel the effect of SNP on the induction of proline synthesis. These results indicate that NO is probably playing important roles in regulating the algal proline accumulation and tolerance to Cu toxicity.

In higher plants, evidence has been provided that excess Cu in the growth medium induces proline accumulation (Chen et al. 2001, Siripornadulsil et al. 2002, ...
Miller et al. 2005). In this case, proline may stabilize protein complexes, act as a scavenger of oxygen free radicals or function as a signal for regulation of downstream events (Alia and Matysik 2001, Kavi et al. 2005). However, the process of signaling proline accumulation and metal tolerance under stress is not fully understood. Also, data on regulation of proline synthesis by Cu in green algae are rare. Recently, several lines of evidence indicated that proline accumulation occurred in drought-stressed plants and can be mediated by ABA-dependent and ABA-independent signaling pathways (Hare et al. 1999, Zhu 2002). ABA is known to mediate signals in plant cells subjected to environmental stresses, and these signals can bring about expression of stress-related genes followed by synthesis of compatible osmolytes such as proline (Kavi et al. 2005, Ashraf and Foolad 2007). Furthermore, ABA accumulation in plants in response to osmotic stress has been determined to regulate expression of the P5CS gene (Xiong et al. 2001). However, ABA seems insufficient for the induction of P5CS activity. In Arabidopsis, ABA induced NO synthesis in guard cells, with a concomitant response of stomatal closure (Desikan et al. 2002), suggesting that both signal molecules are physiologically related. Our results support a connection between the intracellular NO generation and proline accumulation. Whether NO signals proline accumulation may need further verification, but we can speculate here that NO may serve as one of the signal molecules in Cu-exposed cells. All these results support our hypothesis that NO may mediate the response of the algae to Cu stress.

Recent studies have demonstrated that NO can interact with ROS in various ways, in which NO may have an antioxidant function and block ROS-induced lipid peroxidation (Hsu and Kao 2004, Laspina et al. 2005, Wang and Yang 2005). Our results with C. reinhardtii revealed that application of the NO donor SNP reduced Cu-stress-induced increases in O$_2^-$ and H$_2$O$_2$, and the process was associated with a low level of lipid peroxidation (data not shown). Several reports support these results. In higher plants, NO has been shown to regulate a variety of heavy metal-induced stresses such as that due to cadmium (Hsu et al. 2004); Cu (Yu et al. 2005) and aluminum (Wang and Yang 2005). It is found that the major role played by NO is the reduction of peroxidation triggered by heavy metals. Interestingly, NO derived from SNP cannot act alone as a regulator, but it does have a protective effect in plant cells against the metal stress. The precise mechanism for this process is unknown. NO might activate antioxidative systems to scavenge ROS or directly abrogate O$_2^-$-mediated cytotoxic effects through the conversion of O$_2^-$ into ONOO$^-$ (Neill et al. 2003) and consequently protect algae against Cu-induced oxidative stress. Our results have shown that NO in the presence of Cu induced proline production. Proline is believed to protect cell membranes from salt-induced oxidative stress by removing ROS and improving the activities of various antioxidants (Yan et al. 2000, Matysik et al. 2002). Moreover, heavy metal-induced proline hyperaccumulation is also considered a possible strategy for overcoming the oxidative threat imposed by long-term exposure to elevated levels of Cu and Zn (Tripathi et al. 2006). Under these conditions, proline appears to interact synergistically with NO to operate against heavy metal-induced oxidative stress.

Proline is primarily synthesized by a short, strictly regulated cyclic pathway (proline cycle) whereby glutamate is converted to proline by the sequential action of Delta-pyrroline-5-carboxylate (P5C) synthetase (P5CS) and P5C-reductase (P5CR); P5CS produces the intermediate P5C, and P5CR reduces P5C to proline (Roosens et al. 1999). P5CS is the rate-limiting key enzyme in the synthesis route, and its expression and enzymatic activity are controlled by proline levels as well as by environmental stimuli such as salinity, drought, chilling and darkness (Alia and Matysik 2001, Ábraháam et al. 2003). To understand the biochemical mechanism by which NO regulates Cu-responsive proline generation, we assayed the enzyme for initial proline synthesis. Our results with P5CS indicate that the enzyme activity was significantly modified by exposure to Cu. P5CS was positively regulated by Cu (Fig. 7). Treatment with the NO donor SNP induced an additional increase in the activity of P5CS (Fig. 8A). Expression of P5CS in Cu-treated algae was also up-regulated by exogenous NO (Fig. 8B). These data suggest that NO-promoted P5CS activity might be responsible for the proline accumulation under Cu exposure.

In conclusion, this study has demonstrated that Cu was able to induce the production of proline in the cells of C. reinhardtii. Although Cu also induced intracellular production of NO in this study, the process whereby this occurs is unknown. Simultaneous treatment with SNP, an NO donor, induced an additional increase in proline accumulation. The improved proline level triggered by Cu exposure was closely associated with endogenous NO accumulation. Therefore, these observations could allow us to speculate that NO may serve as one of the signal components mediating Cu-induced stress in the algae.

Materials and Methods

Algae culture and treatment

Chlamydomonas reinhardtii was obtained from the Institute of Hydrobiology, the Chinese Academic of Sciences, Wuhan, China (Culture collection No. FACHB-479). Algae were grown...
at 27 ± 2 °C in BG-11 medium, with a light intensity of 80 μmol m⁻² s⁻¹ and a 14 h photoperiod. The pH of the culture medium was adjusted to 7.0. All experiments were performed with exponentially growing cultures. When treated, the algae were incubated with culture medium containing Cu (as CuSO₄.5H₂O) at 0, 1, 2.5, 5, 7.5 and 10 μM. SNP, an NO donor, and an SNP analog, sodium ferricyanide, as well as an NO scavenger, cPTIO, were used to determine whether NO was able to mediate the biological responses of the algae to Cu-induced stress.

**Copper measurement**

For the time-course study of Cu uptake, 100 ml of culture (10⁷ cells ml⁻¹) in Erlenmeyer flasks were incubated with 5 μM Cu. At pre-determined time intervals, a 10 ml aliquot was removed and cells were separated by centrifugation (4,000 × g, 5 min). The metal content of the pellet was determined following the method described below. In another set of experiments, the alga was incubated with varying concentrations of Cu (0–10 μM) in the medium for 1 h, and the intracellular metal content was determined. The intracellular Cu content of the pellet was determined following the method described previously (Bates et al. 1973). To remove extracellular metal bound on the cell surface, cells were washed with 10 ml of EDTA (2 mM) for 10 min, and washed twice with 10 ml of Millipore water. The EDTA-washed pellet was transferred to 10 ml of digestion solution containing 70% HNO₃, 30% H₂O₂ and H₂SO₄ in a 1:1:3 ratio. Digestion was carried out on a hot plate until a clear solution of about 2 ml was left. The residue was taken up in 2% (v/v) HNO₃ and the final volume was adjusted to 5 ml. The metal content of the solution was determined using an atomic absorption spectrophotometer.

**Proline determination**

Proline accumulation in *C. reinhardtii* was determined in a time-course study as well as with varying concentrations of Cu in the medium. For the time-course experiment, 100 ml of culture (10⁷ cells ml⁻¹) was treated with the LC₅₀ concentration (5 μM) of Cu. At different time intervals, a 10 ml aliquot was removed and the proline content of the pellet was determined. An experiment was also carried out to determine the accumulation of proline in *C. reinhardtii* at varying concentrations (0–10 μM) of Cu in the external medium. After 1 h of incubation, cells were harvested by centrifugation and the proline content of the pellet was determined. The free proline content of the pellet was determined by the method described previously (Bates et al. 1973).

**Determination of NO**

NO determination was performed by either a biochemical or a fluorometric approach. The biochemical measurement of generated NO was carried out by differentiating intracellular NO from NO released into the external medium. The culture was kept in darkness for 0.5 h before Cu treatment. After Cu addition, the flasks were sealed to check NO escape. The NO in the pellet (intracellular NO) and the supernatant (NO released) was separated by centrifugation at 2,000 × g for 5 min. The hemoglobin-trapping technique, based on conversion of the ferrous form of hemoglobin (oxyhemoglobin, or HbO₂) into the ferric form, methemoglobin (metHb), was used for the detection of NO in the cell homogenate and external medium (Murphy and Noack 1994).

For cellular NO visualization, cultures were incubated in darkness for 0.5 h and then the specific NO fluorescent probe DAF-2DA (10 μM) was added to the growth medium. This process allowed the uptake of DAF-2 DA into cells (Correa-Aragunde et al. 2004). After incubation for 1 h, the cells were washed twice by centrifugation (4,000 × g for 5 min) and immediately were subjected to treatment with Cu in darkness. Thereafter, the NO generated in the Cu-treated cells was visualized using a fluorescence microscope (Axio Imager. A1, Zeiss). The intracellular fluorescence of the cells was excited at 492 nm and the emission signals at 515 nm were collected.

**Assay of enzyme activity**

The activity of P5CS in *C. reinhardtii* was assayed by a method described previously (Zhang et al. 1995). In brief, P5CS was extracted in 50 mM Tris-HCl buffer (pH 7.4) containing 7 mM MgCl₂, 0.6 M KCl, 3 mM EDTA, 1 mM dithiothreitol and 5% (w/v) insoluble polyvinylpyrrolidone. Homogenates were centrifuged at 15,000 × g for 20 min. The supernatants were passed through a Sephadex G-25 column (Pharmacia Biochem) pre-equilibrated with 50 mM Tris-HCl (pH 7.4) containing 10% glycerol. The assay mixture contained 50 mM Tris-HCl buffer (pH 7.0), 50 mM L-glutamate, 20 mM MgCl₂, 10 mM ATP and 100 mM hydroxamate-HCl. The reaction was initiated by addition of enzyme extract. The amount of γ-glutamyl hydroxamate was measured at 534 nm by comparison with a standard curve of γ-glutamyl hydroxamate. One unit of P5CS was defined as the amount necessary to produce 1 μmol of γ-glutamyl hydroxamate min⁻¹ mg⁻¹ protein under the assay conditions.

**Analysis of P5CS transcript**

Total RNA was extracted from cells of algae using Trizol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. A semi-quantitative reverse transcription–PCR (RT–PCR) assay was carried out. The specific primers were designed for P5CS. The sequence was obtained from the *Chlamydomonas* Database (http://www.biology.duke.edu/chlamy). Reverse transcription was carried out at 42 °C in a 25 μl reaction mixture containing 3 μg of RNA, 0.5 μg of oligo(dT) primer [5'-GTG CCC ATC GGC GTG CTG CT-3' (sense)]; 5' CGT GTT GGC CCT GTG ATG GC-3' (antisense)], 12.5 nmol dNTPs, 12.5 U of RNase inhibitor and 5 U of AMV reverse transcriptase (TAKARA SHUZO CO. LTD, Ohtsu, Japan). The first strand cDNA was then used as a template for polymerase chain amplification and consequently to analyze transcripts. The *Chlamydomonas* actin gene was used as a control. RT–PCR products were obtained after 36 PCR cycles. The PCR products were subjected to 1% (w/v) agarose gel electrophoresis and stained with ethidium bromide. The strength of the fluorescent signal derived from ethidium bromide in each lane was determined by the software Gel-ID from Tanon Company.

**Statistical analyses**

Each result shown in the figures was the mean of at least three independent cultures that were grown with or without Cu treatments at the same time. Significant differences between treatments were evaluated statistically by standard deviation and Student’s *t*-test methods.

**Supplementary material**

Supplementary material mentioned in the article is available to online subscribers at the journal website www.pcp.oxfordjournals.org.
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**References**


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