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Cadmium toxicity is reduced by nitric oxide in rice leaves

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Abstract

We evaluate the protective effect of nitric oxide (NO) against Cadmium (Cd) toxicity in rice leaves. Cd toxicity of rice leaves was determined by the decrease of chlorophyll and protein contents. $CdCl_2$ treatment resulted in (1) increase in Cd content, (2) induction of Cd toxicity, (3) increase in H_2O_2 and malondialdehyde (MDA) contents, (4) decrease in reduced form glutathione (GSH) and ascorbic acid (ASC) contents, and (5) increase in the specific activities of antioxidant enzymes (superoxide dismutase, glutathione reductase, ascorbate peroxidase, catalase, and peroxidase). NO donors [N-*tert*-butyl-α-phenylnitrone, 3-morpholinosydonimine, sodium nitroprusside (SNP), and ASC + $NaNO_2$] were effective in reducing $CdCl_2$-induced toxicity and $CdCl_2$-increased MDA content. SNP prevented $CdCl_2$-induced increase in the contents of H_2O_2 and MDA, decrease in the contents of GSH and ASC, and increase in the specific activities of antioxidant enzymes. SNP also prevented $CdCl_2$-induced accumulation of NH_4^+, decrease in the activity of glutamine synthetase (GS), and increase in the specific activity of phenylalanine ammonia-lyase (PAL). The protective effect of SNP on $CdCl_2$-induced toxicity, $CdCl_2$-increased H_2O_2, NH_4^+, and MDA contents, $CdCl_2$-decreased GSH and ASC, $CdCl_2$-increased specific activities of antioxidant enzymes and PAL, and $CdCl_2$-decreased activity of GS were reversed by 2-(4-carboxy-2-phenyl)-4,4,5,5-tetramethyl-imidazoline-1-oxyl-3-oxide, a NO scavenger, suggesting that protective effect by SNP is attributable to NO released. Reduction of $CdCl_2$-induced toxicity by NO in rice leaves is most likely mediated through its ability to scavenge active oxygen species including H_2O_2.

Abbreviations: AOS – active oxygen species; APX – ascorbate peroxidase; ASC – ascorbic acid; CAT – catalase; c-PTIO – 2-(4-carboxy-2-phenyl)-4,4,5,5-tetra-methylimidazoline-1-oxyl-3-oxide; d.wt – dry weight; f.wt – fresh weight; GR – glutathione reductase; GS – glutamine synthetase; GSH – reduced glutathione; MDA – malondialdehyde; NO – nitric oxide; PAL – phenylalanine ammonia-lyase; PBN – N-*tert*-butyl-α-phenylnitrone; POX – peroxidase; SIN-1 – 3-morpholinosydonimine; SNP – sodium nitroprusside; SOD – superoxide dismutase

Introduction

Cadmium (Cd) is a heavy metal that is toxic for humans, animals and plants, and is one of the widespread pollutants with a long biological half-life (Wagner 1993). This metal enters the environment mainly from industrial process and phosphate fertilizers and is transferred to animals and humans through the food chain (Wagner 1993). Taken up in excess by plants, Cd directly or indirectly inhibits physiological processes such as respiration, photosynthesis, cell elongation, plant–water relationships, nitrogen metabolism, and mineral nutrition, resulting in poor growth and low biomass (Sanitá di Toppi and Gabbrielli 1999).

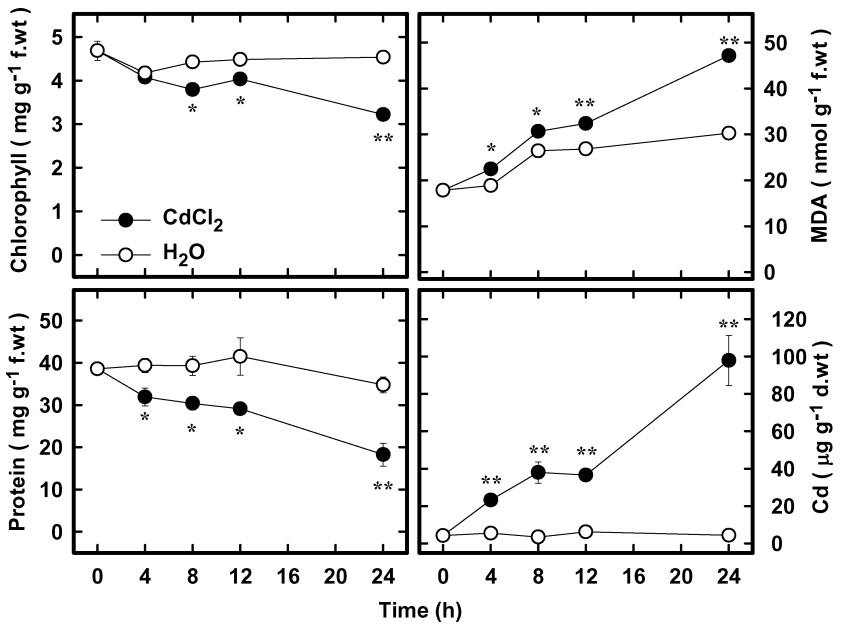

Figure 1. Changes in the contents of chlorophyll, protein, MDA, and Cd in rice leaves treated with $CdCl_2$. Detached rice leaves were treated with either water or 5 mM $CdCl_2$ in the light. * and ** represent values that are significant at $P < 0.05$ and $P < 0.01$, respectively.

Cd is a non-redox metal unable to produce active oxygen species (AOS) via Fenton and/or Haber–Weiss reactions (Sanitá di Toppi and Gabbrielli 1999). However, several reports demonstrate that Cd can indirectly promote the generation of AOS (Sandalio et al. 2001; Schützendübel et al. 2001; Olmos et al. 2003). Cd-increased lipid peroxidation has been demonstrated in *Phaseolus vulgaris* roots and leaves (Chaoui et al. 1997), *Helianthus annuus* leaves (Gallego et al. 1996), *Pisum sativum* shoot and root tissues (Lozano-Rodriguez et al. 1997), and *Oryza sativa* leaves (Chien et al. 2002). Thus, Cd leads to an oxidative stress in plant cells.

Nitric oxide (NO) is a bioactive free radical implicated in a number of physiological functions, including intra-cellular mediation of some animal responses (Anbar 1995). In plants, NO is involved in many physiological responses, such as pathogen response, programmed cell death, growth, germination, root organogenesis, phytoalexin production, internal iron availability, and abscisic acid-dependent stomatal closure (Lamattina et al. 2003; Neill et al. 2003).

Several reports convincingly demonstrate that NO is able to counteract the toxicity of paraquat and diquat, which are known to generate AOS, in potato and rice leaves (Beligni and Lamattina 1999; Hung et al. 2002), and block H_2O_2 production induced by jasmonic acid in tomato leaves (Orozco-Cárdenas and Ryan 2002). Thus, a possible participation of NO in antioxidant system in plants, as it does in animals (d'Ischia et al. 2000), is suggested.

In rice leaves, we have shown that NO counteracts oxidative stress induced by paraquat, dehydration, and polyethylene glycol (Cheng et al. 2002; Hung et al. 2002). More recently, we have shown that the promotion of rice leaf senescence caused by abscisic acid, which induces H_2O_2 production and lipid peroxidation, can be counteracted by NO donors (Hung and Kao 2003). In the present investigation, we examined the effect of NO on Cd-induced toxicity of rice leaves.

Material and methods

Plant material and chemicals

Rice (*O. sativa* L., cv. Taichung Native 1) was sterilised with 2.5% sodium hypochlorite for 15 min and washed extensively with distilled water. These seeds were then germinated in Petri dishes with wetted filter paper at 37 °C under

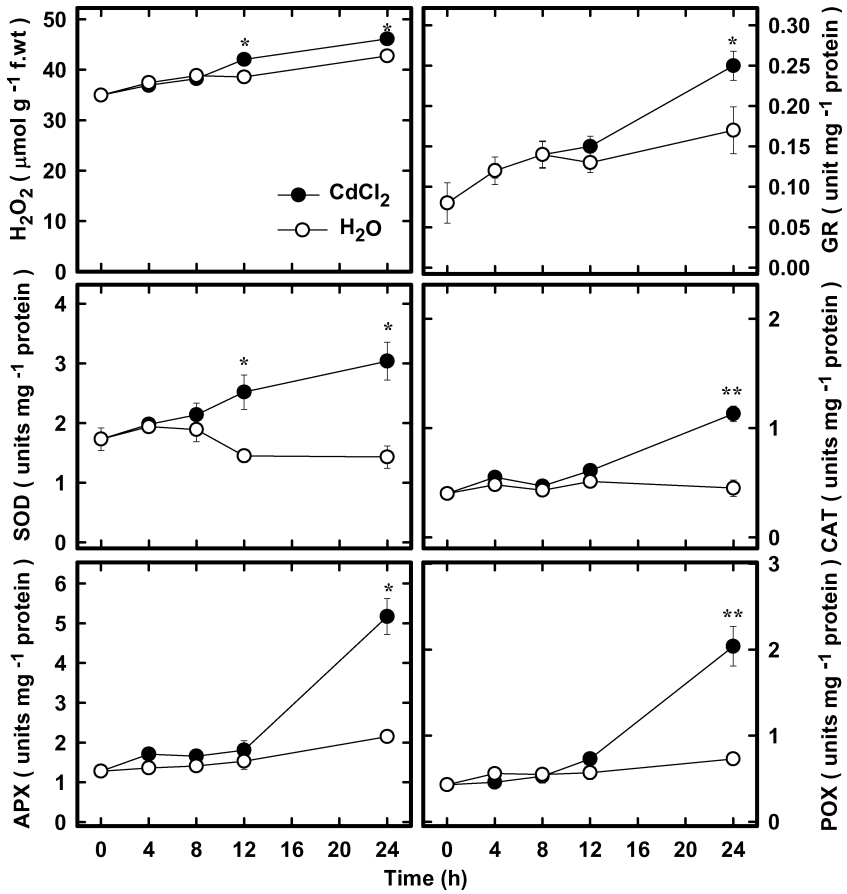

Figure 2. Changes in the contents of H_2O_2 and the specific activities of SOD, APX, GR, CAT, and POX in rice leaves treated with $CdCl_2$. Detached rice leaves were treated with either water or 5 mM $CdCl_2$ in the light. * and ** represent values that are significant at $P < 0.05$ and $P < 0.01$, respectively.

dark condition. After 48 h incubation, uniformly germinated seeds were selected and cultivated in a 500 ml beaker containing half-strength Kimura B solution as described previously (Chu and Lee 1989). The hydroponically cultivated seedlings were grown for 12 days in a Phytotron with natural light 30 °C day (12 h)/25 °C night (12 h) and 90% relative humidity. The apical 3 cm of the third leaf was used in all experiments. Incubation was carried out at 27 °C in the light (40 μmol m^{-2} s^{-1}).

Test solutions included $CdCl_2$, NO donors, and a NO scavenger [2-(4-carboxy-2-phenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (c-PTIO)]. *N-tert*-butyl-α-phenylnitrone (PBN), 3-morpholinosydonimine (SIN-1), and sodium nitroprusside (SNP) were used as NO donors. We also used a solution containing ascorbic acid (ASC) and $NaNO_2$ as another NO donor. All chemicals were purchased from Sigma Co. (St. Louis, MO, USA).

Determination of chlorophyll, protein, H_2O_2, lipid peroxidation, GSH, ASC, NH_4^+, and Cd

Chlorophyll was determined according to Wintermans and De Mots (1965) after extraction in 96% (v/v) ethanol. For protein determination, leaf segments were homogenised in a 50 mM sodium phosphate buffer (pH 6.8). The extracts were centrifuged at 17,600 × *g* for 20 min, and the supernatants used for determination of protein by the method of Bradford (1976) and antioxidant enzyme activities. The H_2O_2 content was measured colorimetrically as described by Jana and

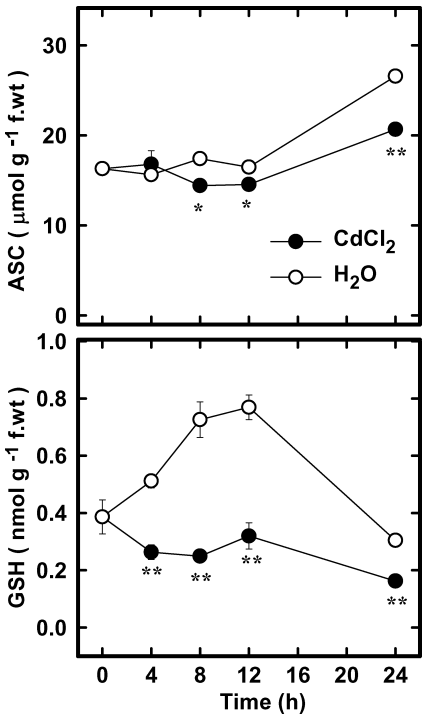

Figure 3. Changes in the contents of ASC and GSH in rice leaves treated with $CdCl_2$. Detached rice leaves were treated with either water or 5 mM $CdCl_2$ in the light. * and ** represent values that are significant at $P < 0.05$ and $P < 0.01$, respectively.

Choudhuri (1981). H_2O_2 was extracted by homogenising leaf tissue with phosphate buffer (50 mM, pH 6.5) containing 1 mM hydroxylamine. The homogenate was centrifuged at 6000 × *g* for 25 min. To determine H_2O_2 content, extracted solution was mixed with 0.1% titanium chloride in 20% (v/v) H_2SO_4. The mixture was then centrifuged at 6000 × *g* for 25 min. The absorbance was measured at 410 nm. The H_2O_2 content was calculated using the extinction coefficient 0.28 μmol^{-1} cm^{-1}. MDA, routinely used as an indicator of lipid peroxidation, was extracted with 5% (w/v) trichloroacetic acid and determined according to Heath and Packer (1968). GSH in 3% sulfosalicylic acid extract and ASC in 5% (w/v) trichloroacetic acid extract were determined as described by Smith (1985) and Laws et al. (1983), respectively. NH_4^+ was extracted and its concentration determined as described previously (Chien and Kao 2000). For determination of Cd, leaves were dried at 65 °C for 48 h and the dried material ashed at 550 °C for 20 h. The ash residue was incubated with 31% HNO_3 and 17.5% H_2O_2 at 72 °C for 2 h, and dissolved in 0.1 N HCl. Cd was then quantified using an atomic absorption spectrophotometer (Model AA-6800, Shimadzu, Kyoto, Japan).

Enzyme assays

Peroxidase (POX) activity was measured using a modification of the procedure of MacAdam et al. (1992). Activity was calculated using the extinction coefficient [26.6 mM^{-1} cm^{-1} at 470 nm] for tetraguaiacol. Catalase (CAT) activity was assayed by measuring the initial rate of disappearance of H_2O_2 (Kato and Shimizu 1987). The decrease in H_2O_2 was followed as the decline in absorbance at 240 nm, and activity was calculated using the extinction coefficient [40 mM^{-1} cm^{-1} at 240 nm] for H_2O_2 (Kato and Shimizu 1987). Superoxide dismutase (SOD) was determined according to Paoletti et al. (1986). Ascorbate peroxidase (APX) was determined according to Nakano and Asada (1981). The decrease in ASC concentration was followed as the decline in absorbance at 290 nm and activity was calculated using the extinction coefficient [2.8 mM^{-1} cm^{-1} at 290 nm] for ASC. Glutathione reductase (GR) was determined by the method of Foster and Hess (1980). One unit of activity for CAT, POX, SOD, APX, and GR was defined as the amount of enzyme which degraded 1 μmol H_2O_2 per min, caused the formation of 1 μmol tetraguaiacol per minute, inhibited 50% the rate of NADH oxidation observed in control, degraded 1 μmol of ASC per minute, and decreased 1 A_{340} per minute, respectively.

For extraction of glutamine synthetase (GS), leaf samples were homogenised with 10 mM Tris–HCl buffer (pH 7.6, containing 1 mM $MgCl_2$, 1 mM EDTA and 1 mM 2-mercaptoethanol) using a chilled pestle and mortar. The homogenate was centrifuged at 15,000 × *g* for 30 min and the resulting supernatant was used for determination of GS activity. The whole extraction procedure was carried out at 4 °C. GS was assayed by the method of Oaks et al. (1980). The reaction mixture contained in a final volume of 1 ml was 80 μmol Tris–HCl buffer, 40 μmol L-glutamic acid, 8 μmol ATP, 24 μmol $MgSO_4$, and 16 μmol NH_2OH; the final pH was 8.0. The reaction was started by addition of the enzyme extract and, after incubation for 30 min

Table 1. Effect of NO donors on chlorophyll, protein, and MDA contents in rice leaves treated with $CdCl_2$.

Treatment	Chlorophyll (mg g^{-1} f.wt)	Protein (mg g^{-1} f.wt)	MDA (nmol g^{-1} f.wt)
H_2O	4.36 ± 0.01 (a)	51.35 ± 1.27 (a)	33.80 ± 2.89 (c)
$CdCl_2$	3.07 ± 0.06 (c)	27.53 ± 0.98 (c)	49.78 ± 1.95 (a)
$CdCl_2$ + PBN	3.52 ± 0.11 (b)	34.72 ± 1.88 (b)	42.55 ± 0.60 (b)
$CdCl_2$ + SIN-1	3.34 ± 0.04 (b)	33.39 ± 0.57 (b)	42.75 ± 1.11 (b)
$CdCl_2$ + SNP	3.35 ± 0.10 (b)	33.71 ± 2.21 (b)	43.15 ± 2.50 (b)
$CdCl_2$ + ASC + $NaNO_2$	3.44 ± 0.09 (b)	33.62 ± 1.75 (b)	44.24 ± 1.07 (b)

The concentrations of $CdCl_2$, PBN, SIN-1, SNP, ASC, and $NaNO_2$ were 5 mM, 100, 100, 100, 100, and 200 μM, respectively. All measurements were determined 24 h after treatment in the light. Values with the same letter are not significantly different at $P < 0.05$, according to Duncan's multiple range test.

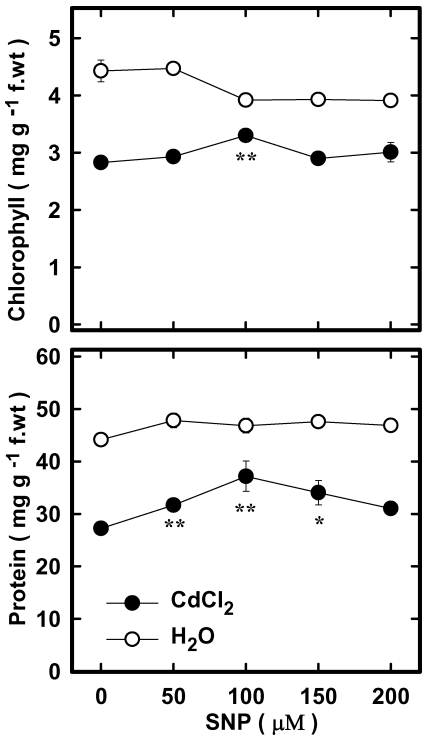

Figure 4. Effect of SNP concentrations on chlorophyll and protein contents in rice leaves treated with $CdCl_2$. The concentration of $CdCl_2$ was 5 mM. Chlorophyll and protein contents were determined 24 h after treatment in the light. * and ** represent values that are significant at $P < 0.05$ and $P < 0.01$, respectively.

at 30 °C, was stopped by adding 2 ml 2.5% (w/v) $FeCl_3$ and 5% (w/v) trichloroacetic acid in 1.5 N HCl. After centrifugation the absorbance of the supernatant was read at 540 nm. One unit of GS activity is defined as 1 μmol L-glutamate γ-monohydroxamate formed per minute. Phenylalanine ammonia-lyase (PAL) was extracted and determined according to Hyodo and Fujinami (1989). The calculation was based on the extinction coefficient (9500 M^{-1} cm^{-1}) for *trans*-cinnamic acid. One unit of activity for PAL was defined as the amounts of enzyme which caused the formation of 1 μmol *trans*-cinnamic acid per hour.

Statistical analysis

Statistical differences between measurements ($n = 4$) on different treatments or on different times were analyzed following the Duncan's multiple range test or Student's *t*-test.

Results

In the present investigation, Cd toxicity in detached rice leaves caused by excess Cd was assessed by a decrease in chlorophyll and protein contents. In previous work, we observed that increasing concentration of $CdCl_2$ from 0.1 to 5 mM progressively decreased chlorophyll and protein contents in detached rice leaves in the light and no further decrease was observed at 10 mM $CdCl_2$ (Chien and Kao 2000). Thus, 5 mM $CdCl_2$ was used in the present investigation. Figure 1 shows the time courses of chlorophyll, protein and Cd contents in detached rice leaves treated with either water or 5 mM $CdCl_2$ in the light. Cd content in control leaves remained unchanged during 24 h of incubation in the light. However, Cd contents in $CdCl_2$-treated detached rice leaves increased with increasing duration of incubation. The increase in Cd content in $CdCl_2$-treated detached rice leaves was evident at 4 h after treatment. The promotion

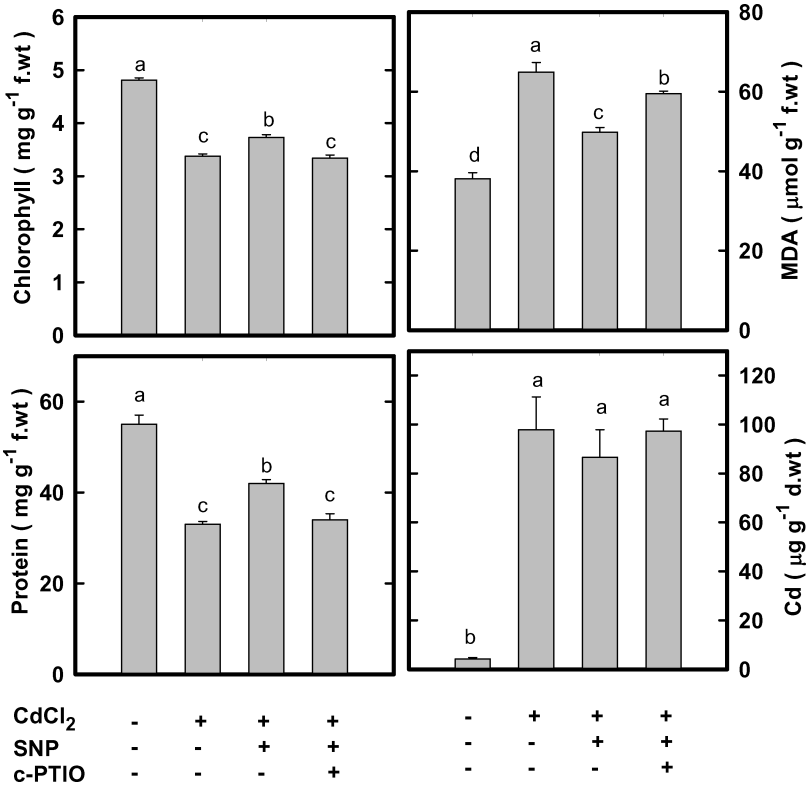

Figure 5. Effect of SNP on the contents of chlorophyll, protein, MDA, and Cd in $CdCl_2$-treated rice leaves in the presence or absence of c-PTIO. The concentrations of $CdCl_2$, SNP, and c-PTIO were 5 mM, 100, and 100 μM, respectively. All measurements of were determined 24 h after treatment in the light. Values with the same letter are not significantly different at $P < 0.05$, according to Duncan's multiple range test.

of the loss of chlorophyll and protein by $CdCl_2$ was evident 8 and 4 h after treatment, respectively.

MDA content in $CdCl_2$-treated detached rice leaves was observed to be greater than that in water-treated controls, throughout the entire duration of incubation (Figure 1). This showed that Cd toxicity in detached rice leaves was linked to lipid peroxidation. Lipid peroxidation is caused by AOS (Thompson et al. 1987). $CdCl_2$ treatment also caused an increase in H_2O_2 content (Figure 2). All these results support the involvement of AOS as the chemical species inducing Cd toxicity in rice leaves.

Plants have evolved a complex antioxidant system to prevent the harmful effects of AOS. The plant antioxidant system is composed of non-enzymatic and enzymatic components. GSH and ASC are the two most important water-soluble non-enzymatic antioxidants (Noctor and Foyer 1998). SOD, APX, GR, CAT, and POX are key antioxidant enzymes (Foyer et al. 1997). The striking increase in lipid peroxidation seen in rice leaves treated with $CdCl_2$ may be a reflection of the changes in the specific activities of antioxidant enzymes and the contents of antioxidants. As shown in Figure 2, $CdCl_2$-treated rice leaves higher specific activities of SOD than the controls at 12 h after treatment. Higher specific activities of APX, GR, POX, and CAT were observed at 24 h after treatment. GSH and ASC contents were observed to be lower than the controls at 4 and 8 h after treatment, respectively (Figure 3). The increased specific activities of antioxidant enzymes and the decreased contents of ASC and GSH in response to $CdCl_2$ are further suggestive of strong induction of oxidative stress.

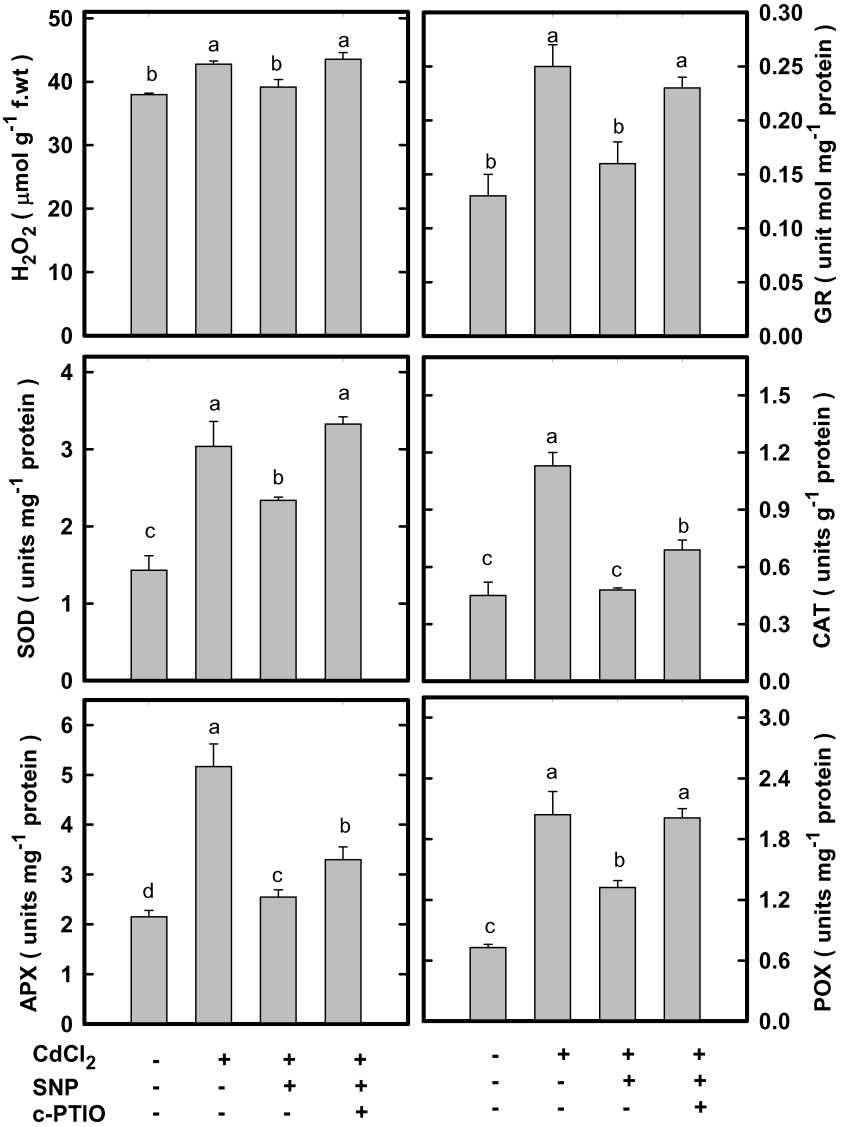

Figure 6. Effect of SNP on the contents of H_2O_2 and the specific activities of SOD, APX, GR, CAT, and POX in $CdCl_2$-treated rice leaves in the presence or absence of c-PTIO. The concentrations of $CdCl_2$, SNP, and c-PTIO were 5 mM, 100, and 100 μM, respectively. All measurements of were determined 24 h after treatment in the light. Values with the same letter are not significantly different at $P < 0.05$, according to Duncan's multiple range test.

There are reports that NO counteracts oxidative stress in plants (Beligni and Lamattina 1999; Beligni et al. 2002; Cheng et al. 2002; Hung et al. 2002; Hung and Kao 2003). Thus, it is of great interest to know whether the protective role of NO is also active in $CdCl_2$-induced toxicity in rice leaves. Consequently, detached rice leaves were treated with $CdCl_2$ in the presence or absence of NO donors, such as PBN, SIN-1, SNP, and a mixture of ASC and $NaNO_2$ for 24 h in the light. As indicated in Table 1, all NO donors used are effective in reducing Cd toxicity and Cd-induced lipid peroxidation in rice leaves.

SNP alone had no effect on protein content and slightly decreased chlorophyll content at 100–200 μM (Figure 4). When applied together with $CdCl_2$, SNP concentration at 100 μM had the highest protective effect on Cd toxicity (Figure 4).

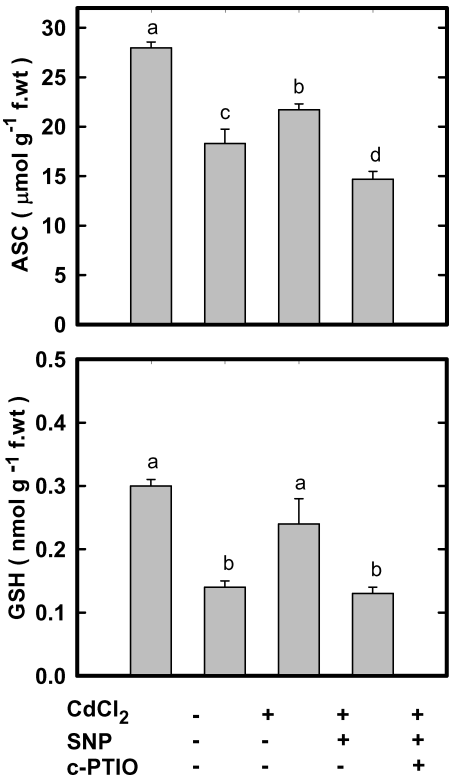

Figure 7. Effect of SNP on the contents of ASC and GSH in $CdCl_2$-treated rice leaves in the presence or absence of c-PTIO. The concentrations of $CdCl_2$, SNP, and c-PTIO were 5 mM, 100, and 100 μM, respectively. All measurements of were determined 24 h after treatment in the light. Values with the same letter are not significantly different at $P < 0.05$, according to Duncan's multiple range test.

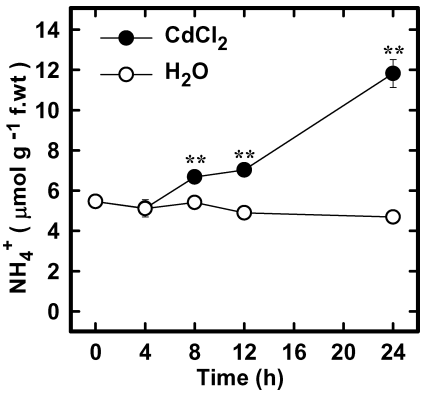

Figure 8. Changes in the contents of NH_4^+ in rice leaves treated with $CdCl_2$. Detached rice leaves were treated with either water or 5 mM $CdCl_2$ in the light. * and ** represent values that are significant at $P < 0.05$ and $P < 0.01$, respectively.

To investigate whether the protective effect induced by SNP treatment was the result of the production of NO, 100 μM c-PTIO, a NO-specific scavenger, was applied along with 100 μM SNP. The effect of SNP on $CdCl_2$-induced toxicity and increase in MDA and H_2O_2 contents could be reversed by c-PTIO (Figures 5 and 6). Figure 5 also shows that SNP treatment was ineffective in reducing $CdCl_2$-induced Cd content in rice leaves, indicating that the effect of SNP on Cd toxicity is unlikely due to the decrease in Cd content in rice leaves. We also observed that SNP counteracted $CdCl_2$-induced increase in the specific activities of antioxidant enzymes (SOD, APX, GR, CAT, and POX) and c-PTIO reversed the effect of SNP-decreased specific activities of antioxidant enzymes (Figure 6). Furthermore, the effect of SNP on $CdCl_2$-decreased ASC and GSH contents could be reversed by c-PTIO (Figure 7). Clearly, the effect of NO donor SNP is attributable to NO released.

Figure 8 shows that the increase in NH_4^+ content by $CdCl_2$ was evident at 8 h after treatment. NH_4^+ accumulation was observed to be correlated with the decrease in activity, rather than the specific activity of GS, a key enzyme in ammonia assimilation (Miflin and Lea 1976). Since NH_4^+ is known to be released through the action of PAL, the first enzyme in the phenylpropanoids (Hahlbrock and Grisebach 1979), it is possible that $CdCl_2$-induced NH_4^+ accumulation is associated with the increase in the activity or the specific activity of PAL in rice leaves. Indeed, we have shown that NH_4^+ accumulation caused by $CdCl_2$ is associated with the increase in specific activity of PAL (Figure 9). Our previous results showed that the decrease in GS activity and the accumulation of NH_4^+ in detached rice leaves are a consequence of oxidative stress caused by excess Cd (Chien and Kao 2002). Furthermore, it has been reported that the specific activity of PAL induced by wounding is related to the ability to produce superoxide radicals in potato tuber (Kumar and Knowles 2003). These results strongly suggest that oxidative stress is involved in NH_4^+ accumulation in $CdCl_2$-treated rice leaves. If this suggestion is correct, then SNP treatment is expected to counteract $CdCl_2$-induced increase in NH_4^+ content, decrease in the activity of GS, and increase in the specific activity of PAL and c-PTIO

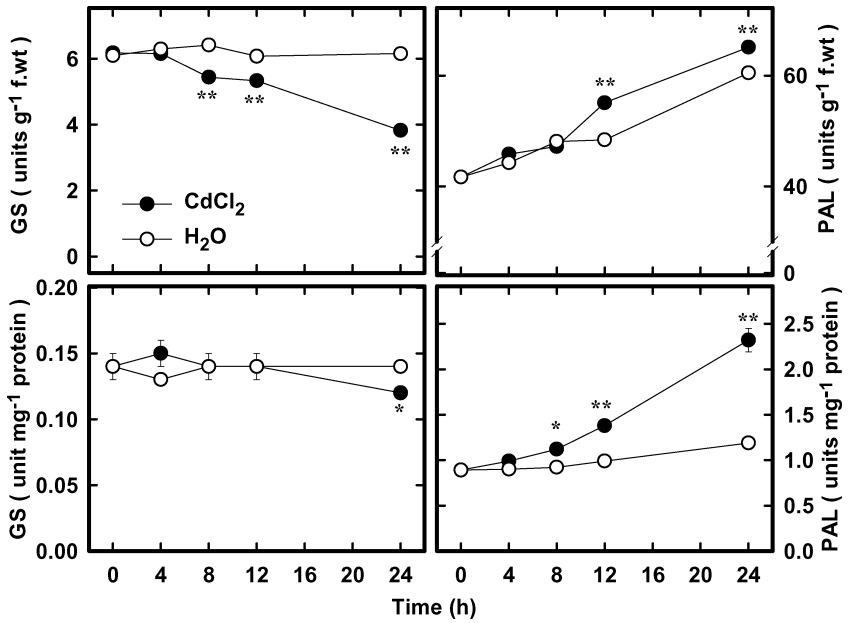

Figure 9. Changes in the activities or specific activities of GS and PAL in rice leaves treated with $CdCl_2$. Detached rice leaves were treated with either water or 5 mM $CdCl_2$ in the light. * and ** represent values that are significant at $P < 0.05$ and $P < 0.01$, respectively.

is expected to reverse these SNP effects. As indicated in Figure 10, this indeed is the case.

Discussion

It has been shown that Cd can cause an increased production of H_2O_2 (Schützendübel et al. 2001; Olmos et al. 2003) and induce lipid peroxidation (Gallego et al. 1996; Chaoui et al. 1997; Lozano-Rodriguez et al. 1997; Chien et al. 2002). These suggest that Cd treatment causes an oxidative stress in plants. Our results not only have shown that $CdCl_2$ increased the content of H_2O_2 (Figure 2) and the specific activities of SOD, APX, GR, CAT, and POX (Figure 2), but also demonstrate that caused a decrease in GSH and ASC contents (Figure 3). Meanwhile, chlorophyll and protein loss and lipid peroxidation were observed in $CdCl_2$-treated rice leaves (Figure 1). All these results suggest that $CdCl_2$ causes an oxidative stress and that $CdCl_2$-induced toxicity in rice leaves in mediated through oxidative stress.

ASC is a major antioxidant in photosynthetic and non-photosynthetic tissues which reacts directly with AOS and is utilised as a substrate for APOD catalysed H_2O_2 detoxification (Noctor and Foyer 1998). GSH is involved in ASC regeneration and functions also as a direct antioxidant of AOS (Noctor and Foyer 1998). In the present investigation, we observed that the decrease in GSH content is one of the earliest steps in oxidative stress induced by $CdCl_2$ in rice leaves, which occurred at 4 h after treatment (Figure 3). It may be suspected that the decrease in GSH may favor the accumulation of AOS in rice leaves. In a recent review, Schützendübel and Polle (2002) also suggest that the depletion of GSH is apparently a critical step in Cd toxicity.

There is only limited information about the mechanism of Cd-induced H_2O_2 production. Olmos et al. (2003) reported that NADPH oxidase-like enzyme was possibly involved in H_2O_2 production in Cd-treated tobacco cells. In the present study, we have not investigated whether H_2O_2 production by $CdCl_2$ in rice leaves is augmented by the stimulation of plasma-bound NADPH oxidases as in tobacco cells. Further research is necessary to clarify this point.

In the present investigation, we found that NO reduced $CdCl_2$-increased the content of MDA and the specific activities of antioxidant enzymes in rice

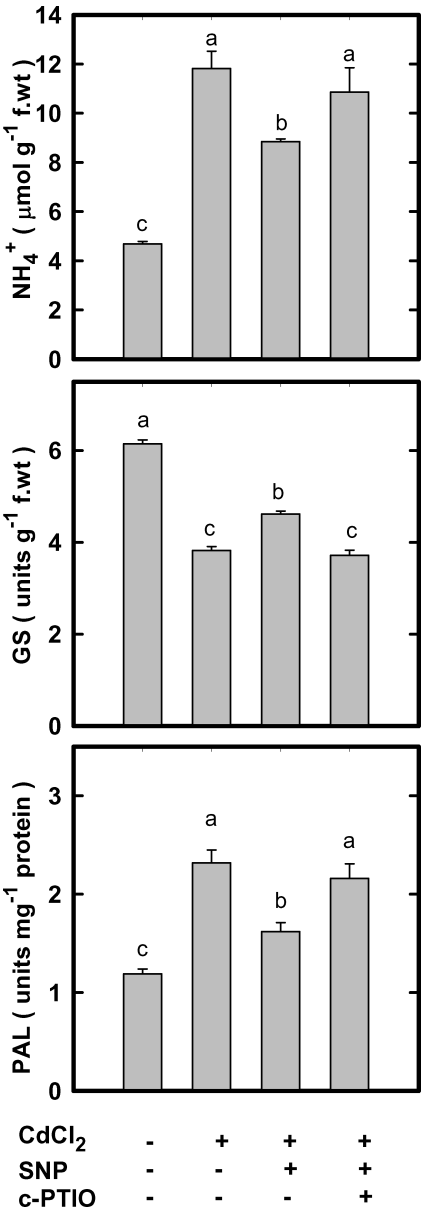

Figure 10. Effect of SNP on the content of NH_4^+, the activity of GS, and the specific activity of PAL in $CdCl_2$-treated rice leaves in the presence or absence of c-PTIO. The concentrations of $CdCl_2$, SNP, and c-PTIO were 5 mM, 100, and 100 μM, respectively. All measurements of were determined 24 h after treatment in the light. Values with the same letter are not significantly different at $P < 0.05$, according to Duncan's multiple range test.

leaves (Figures 5 and 6). These results are in agreement with our previous work, in which we demonstrated that NO counteracted paraquat-increased the content of MDA and the specific activities of antioxidant enzymes (Hung et al. 2002). Because lipid peroxidation and the increase in the specific activities of antioxidant enzymes are the consequence of AOS overproduction (Thompson et al. 1987) and NO acts as an AOS scavenger, therefore, the reduction of the content of MDA and the specific activities of antioxidant enzymes could be a result of low levels of AOS including H_2O_2 in rice leaves treated with NO and $CdCl_2$. The fact that NO counteracted $CdCl_2$-decreased GSH and ASC (Figure 7) may also results in an increase in the capacity of NO to scavenge AOS in rice leaves treated with NO and $CdCl_2$ and might account in part for the lower contents of H_2O_2 observed in rice leaves treated with NO and $CdCl_2$ (Figure 6).

APX, CAT, and POX have been shown to be inhibited by NO (Clark et al. 2000). However, our results show that SNP treatment alone did not affect the specific activities of antioxidant enzymes in rice leaves (data not shown). Thus, the reduction of $CdCl_2$-induced increase in the specific activities of antioxidant enzymes by NO is unlikely due to a direct NO-mediated inhibition of the enzymes.

AOS can react with NO to form peroxynitrite (Kim and Han 2000; Martinez et al. 2000). Peroxynitrite has been shown to react with H_2O_2 to yield nitrite ion and oxygen (Martinez et al. 2000). This reaction has been suggested to be the mechanism of NO cytoprotective actions in animals (Wink et al. 1993). It appears that this mechanism is operating in rice leaves.

Of particular interest in the present investigation is the finding that $CdCl_2$ treatment resulted in an increase in the specific activity of PAL and a decrease in the activity of GS in rice leaves (Figure 9). NH_4^+ released from PAL reaction is known to be trapped in glutamine molecule by the action of GS (Sakurai et al. 2001). It appears that $CdCl_2$-induced NH_4^+ accumulation is mediated through the increase in the specific activity of PAL and the decrease in the activity of GS in rice leaves. It has been shown that the decrease of the activity of GS is caused by oxidative damage (Chien et al. 2002) and the specific activity of PAL induced by wounding is related to the ability to produce superoxide radicals (Kumar and Knowles 2003). The fact that NO counteracts the $CdCl_2$-induced decrease in the activity of GS and increase in the specific activity of PAL in the rice leaves (Figure 10), further strengthens the idea

that antioxidant properties of NO are operating for counteracting oxidative stress in rice leaves.

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