Paraquat toxicity is reduced by nitric oxide in rice leaves

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Summary

The role of nitric oxide (NO) in plants has been the object of intensive research. In the present work, we evaluated the protective effect of NO against paraquat (PQ) toxicity of rice (*Oryza sativa*) leaves. PQ toxicity in rice leaves was determined by the decrease of protein content. PQ toxicity of rice leaves was reduced by free radical scavengers such as thiourea, sodium benzoate, ascorbic acid and sodium azide. NO-releasing compounds [N-*tert*-butyl- α -phenylnitrone (PBN), sodium nitroprusside (SNP), 3-morpholiniosydnonimine (SIN-1), and ascorbic acid + NaNO₂] were effective in reducing PQ toxicity in rice leaves. SIN-1 and ascorbic acid + NaNO₂ prevented both the PQ-induced increase in content of malondialdehyde and the PQ-induced decrease in activities of active oxygen species-detoxifying enzymes. The protective effect of SIN-1 or ascorbic acid + NaNO₂ on PQ toxicity, PQ-induced lipid peroxidation and PQ-decreased antioxidative enzyme activities was reversed by 2-(4-carboxy-2-phenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide, a NO-specific scavenger, suggesting that the protective effect of NO-releasing compounds is attributed to NO released. Reduction of PQ toxicity by NO in rice leaves is most likely mediated through increase in antioxidative enzyme activities and decrease in lipid peroxidation.

Key words: lipid peroxidation – nitric oxide – *Oryza sativa* – paraquat

Abbreviations: Asc ascorbate. – APOD ascorbate peroxidase. – CAT catalase. – c-PTIO 2-(4-carboxy-2-phenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide. – GR glutathione reductase. – MDA malondialdehyde. – NO nitric oxide. – PBN N-*tert*-butyl- α -phenylnitrone. – POD peroxidase. – PQ paraquat. – SIN-1 3-morpholiniosydnonimine. – SNP sodium nitroprusside. – SA sodium azide. – SB sodium benzoate. – SOD superoxide dismutase

Introduction

Paraquat (PQ), also known as methyl viologen, is a herbicide widely used in agriculture. It has long been known to exert its toxic effects by catalyzing the transfer of electrons from pho-

tosystem I of chloroplast membrane to molecular oxygen, producing oxygen radicals that cause lipid peroxidation and membrane damage (Calderbank 1968, Farrington et al. 1973, Cha et al. 1982). A normally operated photosynthetic electron transport is usually required for inducing PQ toxicity (Mees 1960, Chang and Kao 1995). Superoxide can serve as a source to generate more active hydroxyl radicals by Haber-

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Weiss and Fenton reactions (Naqui and Chance 1986). Transition metals, such as iron and copper, are able to accelerate Haber-Weiss and Fenton reactions (Gutteridge et al. 1981). It has been demonstrated that iron or copper ions are essential mediators in PQ toxicity in bacterial cells (Kohen and Chevion 1985), in mammalian cells in tissue culture (Sandy et al. 1987) and mice (Kohen and Chevion 1986). It has been shown that PQ toxicity in pea and rice leaves is enhanced by iron and reduced by iron chelators (Zer et al. 1994, Chang and Kao 1997 a). Free radical scavenging properties of polyamines have been documented (Drolet et al. 1986). It was found that polyamines were effective in reducing PQ toxicity of rice and sunflower leaves (Chang and Kao 1997b, Benavides et al. 2000).

Nitric oxide (NO) is a labile free radical and is produced from L-arginine by NO synthase in mammalian cells (Sakuma et al. 1988, Rees et al. 1989). Over the last decade, extensive research about the role of NO in animals has demonstrated that it is a key signal molecule involved in a wide variety of processes including vasorelaxation, neurotransmission, and the innate immune response (Schmidt and Walter 1994). In contrast, the role of NO in plants is less well understood. Evidence is emerging to support that NO may also play some roles in the plant kingdom. Antibodies raised against rabbit NO synthase cross-reacted with proteins from pea embryonic axes and wheat germ (Sen and Cheema 1995). In senescing pea leaves, NO emission was promoted by the addition of ethylene precursors and NO-releasing compounds inhibited leaf expansion (Leshem and Haramaty 1996). Leshem et al. (1998) provided evidence to show that NO is a natural senescence-delaying plant growth regulating agent. NO-releasing compounds were found to promote phytoalexin accumulation in potato tuber tissues (Noritake et al. 1996), inhibit coniferyl alcohol peroxidase activity in the xylem of *Zinnia elegans* (Ferrer and Bacelo 1999), inhibit cytochrome oxidase activity in isolated soybean cotyledon mitochondria (Millar and Day 1996), and inhibit the activity of tobacco aconitase, catalase (CAT), ascorbate peroxidase (APOD) and salicylic acid-induced protein kinase (Clark et al. 2000, Kumar and Klessig 2000, Navarre et al. 2000). The participation of NO as a signal in plant disease resistance has also been reported (Delledonne et al. 1998, Durner et al. 1998, Klessig et al. 2000). Scherer and Holk (2000) also observed that NO could be an intermediate in cytokinin signaling.

Since NO is a toxic free radical, most of the work on NO function in plant cells has focused on its ability to act in the same direction as active oxygen species. In animal systems, several reports convincingly demonstrate a protective role for NO in oxidative stress (Rubbo et al. 1984, Wink et al. 1993, Rauhala et al. 1996, Goss et al. 1997, O'Donnell et al. 1997, d'Ischia et al. 2000). In a series of work conducted by Lamattina's group, it was found that NO protects against cellular damage produced by diquat, PQ and *Phytophthora infestans* in potato leaves (Laxalt et al. 1997, Beligni and Lamattina 1999 a, b). It is not known whether a similar protective role for

NO is also active in other leaf systems. For this reason, we investigated the effect of NO on the toxicity of PQ, a well known free-radical generating chemical, in rice leaves.

Materials and Methods

Materials and chemicals

Rice (*Oryza sativa* L., cv. Taichung Native 1) was cultured as previously described (Lin et al. 1999). The apical 3-cm segments excised from the third leaves of 12-day-old seedlings were used. A group of 10 segments was floated in a Petri dish containing 10 mL of test solution. Incubation was carried out at 27 °C in the light ($40 \mu\text{mol m}^{-2} \text{s}^{-1}$).

Test solutions included PQ, NO-releasing compounds, a NO scavenger (c-PTIO), and antioxidant compounds. PBN, SIN-1 and SNP were used as NO-releasing compounds. We also used a solution containing ascorbic acid (Asc) and NaNO_2 as another NO-releasing compound. Asc, sodium azide (SA), sodium benzoate (SB), and thiourea were used as antioxidants. All chemicals were purchased from Sigma Co. (St. Louis, MO, USA).

Chlorophyll, protein and MDA determinations

Chlorophyll was determined according to Wintermans and De Mots (1965) after extraction in 96 % (v/v) ethanol. For protein extraction, leaf segments were homogenized in 50 mmol L^{-1} sodium phosphate buffer (pH 6.8). The extracts were centrifuged at $17,000 \times g$ for 20 min, and the supernatants were used for determination of both protein by the method of Bradford (1976) and enzyme activities. Malondialdehyde (MDA) was extracted with 5 % (w/v) trichloroacetic acid and determined according to Heath and Packer (1968).

Enzyme assays

Peroxidase (POD) activity was measured using a modification of the procedure of MacAdam et al. (1992). Activity was calculated using the extinction coefficient [$26.6 (\text{mmol L}^{-1})^{-1} \text{cm}^{-1}$ at 470 nm] for tetraguaiacol. 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 [$40 (\text{mmol L}^{-1})^{-1} \text{cm}^{-1}$ at 240 nm] for H_2O_2 (Kato and Shimizu 1987). Superoxide dismutase (SOD) was determined according to Paoletti et al. (1986). APOD was determined according to Nakano and Asada (1981). The decrease in Asc concentration was followed as the decline in optical density at 290 nm and activity was calculated using the extinction coefficient [$2.8 (\text{mmol L}^{-1})^{-1} \text{cm}^{-1}$ at 290 nm] for Asc. Glutathione reductase (GR) was determined by the method of Foster and Hess (1980). One unit of activity for CAT, POD, SOD, APOD, and GR was defined as the amount of enzyme which degraded $1 \mu\text{mol H}_2\text{O}_2$ per min, caused the formation of $1 \mu\text{mol}$ tetraguaiacol per min, inhibited 50 % the rate of NADH oxidation observed in control, degraded $1 \mu\text{mol}$ of Asc per min, and decreased $1 A_{340}$ per min, respectively.

Experimental design

Protein and MDA contents were expressed per g fresh weight (FW). Enzyme activities were expressed as units g^{-1} FW or units mg^{-1} pro-

tein. Absolute contents of each measurement varied among experiments because of seasonal effects. However, the patterns of responses to PQ, PQ plus NO-releasing compounds or PQ plus antioxidants were reproducible. For biochemical analysis, four independent extractions were performed for each treatment. All experiments described here were repeated at least three times. Similar results and identical trends were obtained each time. The data reported here are from a single experiment.

Results and Discussion

Changes in chlorophyll, protein, and MDA contents in detached rice leaves treated with $25 \mu\text{mol L}^{-1}$ PQ in the light are shown in Figure 1. The decrease in protein and increase in

Figure 1. Changes in contents of chlorophyll, protein and MDA in rice leaves treated with PQ. Detached rice leaves were treated with either water or $25 \mu\text{mol L}^{-1}$ PQ in the light. Vertical bars represent standard errors ($n = 4$). Only those standard errors larger than the symbol are shown.

Figure 2. Effect of antioxidants (Asc, SB, SA, and thiourea) on protein content in rice leaves treated with PQ. The concentration of PQ was $25 \mu\text{mol L}^{-1}$. Protein was determined 24 h after treatment in the light. The white columns mean that detached rice leaves were treated with water only. Vertical bars represent standard errors ($n = 4$).

MDA were evident at 4 h after PQ treatment. However, slight decrease in chlorophyll was only observed at 24 h after PQ treatment. Clearly, PQ is more effective in decreasing protein content than chlorophyll content. Thus, PQ toxicity in detached rice leaves was judged by the decrease in protein content in the following experiments. It is generally believed that the primary action of PQ is a result of the deviation of photosynthetic electron transport in chloroplasts (Calderbank 1968). Previously, we demonstrated that considerable reduction of PQ toxicity in rice leaves occurred in the presence of photosynthetic electron transport inhibitors in the light and that PQ was unable to induce toxicity in detached rice leaves under dark condition (Chang and Kao 1995). These results suggest that light is essential for PQ response in rice leaves, although the light intensity ($40 \mu\text{mol m}^{-2} \text{s}^{-1}$) used in the present investigation is quite low compared to sunlight. It appears that detached rice leaves are highly sensitive to PQ.

PQ treatment resulted in a marked increase in MDA content, indicating that PQ brings about lipid peroxidation (Fig. 1). Lipid peroxidation is caused by active oxygen species (Foster and Hess 1980). Free radical scavenging properties of polyamines have been documented (Drolet et al. 1986) and it was found that polyamines were effective in reducing PQ toxicity of rice leaves (Chang and Kao 1997b). Transition met-

als, such as iron and copper, are able to accelerate Haber-Weiss and Fenton reactions (Gutteridge et al. 1981). Evidence was also provided to show that PQ toxicity was reduced by iron chelators in rice leaves (Chang and Kao 1997a). These results support the possibility that PQ-induced toxicity in rice leaves is mediated through oxidative stress. In the present investigation, when free radical scavengers such as thiourea, SB, SA and Asc were used together with PQ, it was found that they partially prevented the toxicity of PQ in rice leaves (Fig. 2). However, Asc, SB, SA, and thiourea alone had no effect on protein content (data not shown). These results support the involvement of free radicals as the chemical species inducing PQ toxicity in rice leaves.

Beligni and Lamattina (1999a, b) reported that NO counteracts cellular damage caused by diquat in potato leaves. Based on their evidence, they propose that NO acts as an antioxidant in reducing cellular damage. Thus, it is of great interest to know whether a similar protective role of NO is also active in the rice leaf system. Consequently, detached rice leaves were treated with PQ in the presence or absence of NO-releasing compounds, such as PBN, SIN-1, SNP and a mixture Asc and NaNO₂ for 24 h in the light. As indicated in Table 1, all NO-releasing compounds used are effective in reducing PQ toxicity in rice leaves. We also observed that 100 $\mu\text{mol L}^{-1}$ SIN-1 and 100 $\mu\text{mol L}^{-1}$ Asc plus 200 $\mu\text{mol L}^{-1}$ NaNO₂ are more effective in reducing PQ toxicity than PBN and SNP.

SIN-1 alone had no effect on protein content (Fig. 3). When applied together with PQ, SIN-1 concentrations between 50 and 200 $\mu\text{mol L}^{-1}$ produced a clear protection against protein loss (Fig. 3). The optimal concentration of SIN-1 in reducing PQ toxicity was observed to be 100 $\mu\text{mol L}^{-1}$. It was also observed that NaNO₂ alone (Fig. 4 A) or Asc alone (Fig. 4 B) had no effect on protein content in detached rice leaves. Both Asc and NaNO₂ are required in reducing PQ toxicity of detached rice leaves (Figs. 4 A and 4 B). To be effective in reducing PQ toxicity, the optimal concentrations of Asc and NaNO₂ were

Figure 3. Effect of SIN-1 concentrations on protein contents in rice leaves treated with either water or PQ. The concentration of PQ was 25 $\mu\text{mol L}^{-1}$. Protein was determined 24 h after treatment in the light. Vertical bars represent standard errors ($n = 4$). Only those standard errors larger than the symbol are shown.

100 and 200 $\mu\text{mol L}^{-1}$, respectively (Figs. 4 A and 4 B). The protective effect of SIN-1 or Asc + NaNO₂ on PQ toxicity was observed to be evident at 4 h after treatment (data not shown).

To investigate whether the protective effect induced by treatment with NO-releasing compounds (SIN-1 and Asc + NaNO₂) was the result of the production of NO, 100 $\mu\text{mol L}^{-1}$ c-PTIO, a NO-specific scavenger, was applied along with 100 $\mu\text{mol L}^{-1}$ SIN-1 or 100 $\mu\text{mol L}^{-1}$ Asc + 200 $\mu\text{mol L}^{-1}$ NaNO₂. The protective effect of SIN-1 or Asc + NaNO₂ on PQ-induced toxicity could be reversed by c-PTIO (Fig. 5). Clearly, the protective effect on PQ toxicity by NO-releasing compounds is due to NO.

The addition of NO to rat brain homogenates caused a decrease in lipid peroxidation (d'Ischia et al. 2000). PQ induces lipid peroxidation in several leaf systems (Calderbank 1968, Farrington et al. 1973, Cha et al. 1982, Chang and Kao 1997a). Until now it was not known whether NO-releasing compounds can confer protection against lipid peroxidation in plant systems. Figure 6 shows the effect of SIN-1 and Asc + NaNO₂ on MDA content of detached rice leaves treated with PQ. PQ alone resulted in a marked increase in MDA content (Figs. 1 and 6). Although SIN-1 or Asc + NaNO₂ alone had no effect on MDA content (data not shown), treatment with SIN-1 or Asc + NaNO₂ caused a reduction of PQ-induced increase in MDA content (Fig. 6). The effect of SIN-1 or Asc + NaNO₂ on PQ-induced MDA content could also be reversed by c-PTIO (Fig. 6).

The protective effect of NO-releasing compounds on PQ toxicity is clearly associated with the decrease in lipid peroxidation (Fig. 6) and protein loss (Fig. 5). Plant cells are equipped with several free radical detoxifying enzymes to protect them against oxidative damage. These enzymes in-

Table 1. Effect of NO-releasing compounds on protein content in rice leaves treated with PQ

Treatment	Protein, mg g ⁻¹ FW
H ₂ O	53.6 ± 1.0
PQ	8.7 ± 0.6
PQ + PBN	17.1 ± 1.5
PQ + SIN-1	20.2 ± 1.7
PQ + SNP	16.1 ± 1.3
PQ + Asc + NaNO ₂	22.9 ± 1.9

The concentrations of PQ, PBN, SIN-1, SNP, Asc, and NaNO₂ were 25, 100, 100, 100, 100, and 200 $\mu\text{mol L}^{-1}$, respectively. Protein content was determined 24 h after treatment in the light, data are the means ± standard errors ($n = 4$).

Figure 4. (A) Effect of NaNO₂ concentrations on protein content of rice leaves treated with water or 25 μmol L⁻¹ PQ + 100 μmol L⁻¹ Asc; (B) Effect of Asc concentrations on protein content in rice leaves treated with water or 25 μmol L⁻¹ PQ + 200 μmol L⁻¹ NaNO₂. Protein was determined 24 h after treatment in the light. Vertical bars represent standard errors (n = 4). Only those standard errors larger than the symbol are shown.

Figure 5. Effect of SIN-1 and Asc + NaNO₂ on PQ toxicity in rice leaves in the presence (+) or absence (-) of c-PTIO. The concentrations of SIN-1, Asc, NaNO₂, PQ, and c-PTIO were 100, 100, 200, 25, and 100 μmol L⁻¹, respectively. Protein was determined 24 h after treatment in the light. The white columns mean that detached rice leaves were treated with water only. Vertical bars represent standard errors (n=4).

clude SOD, APOD, GR, CAT and POD (Foyer et al. 1997, Nakano and Asada 1981). The striking changes in lipid peroxidation seen in leaves treated with PQ alone may be a reflection of the inability of the scavenging enzymes to cope with the increased levels of oxidant species and/or their degradation by these species. Treatment with PQ alone decreases POD, CAT, SOD, APOD and GR activities (Fig. 6). The PQ-induced loss of antioxidative enzyme activities was found to be protected by SIN-1 or Asc + NaNO₂ (Fig. 6). Treatment with c-PTIO alone was found to have no effect on MDA content and antioxidative enzyme activities (data not shown). However, c-PTIO arrested NO-releasing compounds-mediated enzyme protection (Fig. 6). It should be noted that when antioxidative enzymes were expressed as specific activities (units mg⁻¹ protein), PQ increased specific activities of antioxidative enzymes, NO-releasing compounds counteracted PQ-increased specific activities of antioxidative enzymes and c-PTIO reversed the effect of NO-releasing compounds-mediated enzyme protection (Table 2).

Ferrer and Barcelo (1999) found that the NO donor SNP (5 mmol L⁻¹) and NO (55 μmol L⁻¹ s⁻¹) itself were able to inhibit

Table 2. Effect of SIN-1 and Asc + NaNO₂ on the specific activities of antioxidative enzymes in PQ-treated rice leaves in the presence or absence of c-PTIO.

Units mg ⁻¹ protein	Treatment					
	H ₂ O	PQ	PQ + SIN-1	PQ + Asc + NaNO ₂	PQ + SIN-1 + c-PTIO	PQ + Asc + NaNO ₂ + c-PTIO
CAT	0.11±0.01	0.23±0.02	0.16±0.03	0.18±0.01	0.24±0.01	0.24±0.02
POD	0.68±0.02	2.25±0.17	1.71±0.16	1.80±0.22	3.44±0.22	3.60±0.44
SOD	1.01±0.03	4.03±0.16	2.11±0.05	1.28±0.09	4.51±0.19	4.35±0.15
APOD	0.77±0.01	1.56±0.08	1.21±0.16	1.17±0.07	1.95±0.15	2.19±0.23
GR	0.18±0.02	0.60±0.05	0.44±0.07	0.42±0.03	0.94±0.09	1.03±0.15

The concentrations of PQ, SIN-1, Asc, and NaNO₂ were 25, 100, 100, and 200 μmol L⁻¹, respectively. Enzyme specific activities were determined 24 h after treatment in the light, data are the means ± standard errors (n = 4).

Figure 6. Effect of SIN-1 and Asc + NaNO₂ on the content of MDA and the activities of antioxidative enzymes in PQ-treated rice leaves in the presence (+) or absence (-) of c-PTIO. The concentrations of SIN-1, Asc, NaNO₂, PQ, and c-PTIO were 100, 100, 200, 25, and 100 $\mu\text{mol L}^{-1}$, respectively. MDA content and enzyme activities were determined 24 h after treatment in the light. The white columns mean that detached rice leaves were treated with water only. Vertical bars represent standard errors (n=4).

POD activity in the xylem of *Zinnia elegans*. Clark et al. (2000) also demonstrated that NO donor (0.8 mmol L^{-1}) inhibited the activities of tobacco CAT and APOD, heme-containing enzymes. However, our results show that neither SIN-1 nor Asc + NaNO₂ treatment effected the activities of POD, CAT, and APOD in rice leaves (data not shown). Why is SIN-1 or Asc + NaNO₂ able to counteract PQ toxicity, but not able to inhibit the activities of POD, CAT, and APOD in rice leaves? In the present investigation, we used $\mu\text{mol L}^{-1}$ concentration range of SIN-1 and Asc + NaNO₂ and did not measure the concentration of NO released by SIN-1 or Asc + NaNO₂ in rice leaves. We do not know the exact concentration of NO inside the rice leaf tissue. It is possible that the concentration of NO released by SIN-1 or Asc + NaNO₂ is high enough to scavenge free radicals produced by PQ, but not sufficient to inhibit the activities of heme-containing enzymes.

In rice leaves, involvement of active oxygen species in water stress-, methyl jasmonate-, excess Cu-, and excess Cd-

promoted senescence has been demonstrated (Hung and Kao 1998, Chen and Kao 1999, Lin and Kao 2000, Chien et al. 2001). Work is under way to determine whether NO counteracts this promotion of senescence in rice leaves. Preliminary results show that the promotion of rice leaf senescence caused by polyethylene glycol, which induces lipid peroxidation, can be counteracted by NO-releasing compounds, but that caused by sorbitol, which has no effect on lipid peroxidation, cannot be counteracted by NO-releasing compounds (unpublished data).

In conclusion, the results presented in this paper suggest that reduction of PQ toxicity by NO in rice leaves is most likely mediated through increase in antioxidative enzyme activities and decrease in lipid peroxidation.

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