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Involvement of heme oxygenase as antioxidant defense in soybean nodules

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Abstract

Objective: We have previously demonstrated that the inducible form of heme oxygenase plays a critical role in protecting against oxidative stress in mammals. To gain further insight into the functions of this enzyme in plants, we have tested its activity and expression in soybean nodules subjected to cadmium (Cd) stress.

Materials and methods: Four-weeks-old soybean nodulated plants were treated with different cadmium chloride concentrations (0, 50 and 200 μM) during 48 h. Oxidative stress parameters such as TBARS content, GSH levels and antioxidant enzyme activities were measured as well as heme oxygenase activity and expression. Besides, the effect of biliverdin and Zn-protophorphyrin IX were analized.

Results: Treatment with 200 μM Cd during 48 h caused a 67% increase in TBARS content, whereas GSH decreased 44%, and total superoxide dismutase, gluthatione reductase and guaiacol peroxidase were also inhibited 54, 20 and 60%, respectively. A total of 200 μM Cd produced the overexpression of heme oxygenase-1, as well as a 10-fold enhancement of its activity. Co-administration of biliverdin (10 μM) completely prevented the effects caused by Cd. Treatment with Zn protoporphyrin IX, a strong inhibitor of heme oxygenase, expectedly decreased heme oxygenase-1 activity to half. When the inhibitor was given together with Cd, completely prevented the enzyme induction and oxidative stress parameters were significantly enhanced.

Conclusion: Taking together, these results are indicating that heme oxygenase plays a protective role against oxidative cell damage in soybean nodules.

Keywords: *Antioxidant defense; cadmium; heme oxygenase; oxidative stress; plants; soybean*

Abbreviations: *BHT, butylated hydroxytoluene; BV, biliverdin; DTNB, 5,5′ dithio-bis-(2-nitrobenzoic acid); EDTA, ethylenediaminetetraacetic acid (disodium salt); FW, fresh weight; GPOX, guaiacol peroxidase; GR, glutathione reductase; GSH, reduced glutathione; GSSG, oxidized glutathione; HO-1, heme oxygenase-1; HO's, heme oxygenases; NBT, nitroblue tetrazolium; PVP, polyvinylpyrrolidone; ROS, reactive oxygen species; SOD, superoxide dismutase; TBA, thiobarbituric acid; TBARS, thiobarbituric acid reactive substances; TCA, trichloroacetic acid; Tris, tris(hydroxymethyl) aminomethane; ZnPPIX, Zn-protoporphyrin IX*

Introduction

Heme oxygenases (HO's) are ubiquitous enzymes which catalyze the stereo specific cleavage of heme to biliverdin (BV) with the release of free iron and carbon monoxide [1]. Genes encoding HO's have been isolated from a wide variety of organisms including mammals, red algae, cryptophytas, cyano- and pathogenic bacteria [2,3]. HO's perform different cellular functions and exhibit different enzymatic characteristics.

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In mammalians, HO is associated with hemoglobin degradation and heme recycling in senescence red blood cells. The role of HO and its products biliverdin and bilirubin as components of the antioxidant defense system and in protecting against oxidative cell damage is well documented [4–13]. In cyanobacteria and some algae, the function of HO is to provide cofactors for the photosynthetic apparatus. In plants, so far the role of HO's has been investigated in association with the pathway leading to phytochrome chromophores metabolism, functioning in light signaling [14–16].

It has been demonstrated that one of the three known mammalian isoforms, heme oxygenase-1 (HO-1), is induced in animal tissues by many factors including its own substrate heme, several heme-proteins, heavy metals, UVA radiation, hypoxia, hyperoxia and others [17–20]. However, to the best of our knowledge, this effect had not been explored in higher plants.

Soybean is an important crop in the world, offering high-quality protein and increasing the input of combined nitrogen into the soil. However, its yield may be adversely affected by different environmental stresses. One of the major abiotic stresses affecting plant productivity is the heavy metal excess into the soils resulting from industrial and urban activities, sewage sludge and agrochemicals. Cadmium (Cd) is a non-essential element that shows phytotoxicity even at low doses [21,22]. In soybean nodules, Cd produces oxidative damage affecting nitrogen fixation and assimilation [23,24]. Nitrogen fixation is particularly sensitive to oxygen and reactive oxygen species (ROS). The rate of respiration, the high concentration of leghemoglobin (1–3 mM) and the very active iron metabolism in the nodule seems to be closely related to free radical reactions and oxidative stress [25].

Because more detailed studies on the presence of plant HO will be helpful to understand the mechanisms of plant defense, here we investigated the involvement of heme oxygenase as enzymatic antioxidant defense against Cd-induced oxidative stress in nodules of soybean plants.

Materials and methods

Materials

NADPH, reduced glutathione (GSH), oxidized glutathione (GSSG), 5,5′-dithio-bis-(2-nitrobenzoic acid) (DTNB), thiobarbituric acid (TBA) and glutathione reductase, were from Sigma Chemical (Saint Louis, MO), ter-butyl hydroperoxide was from Aldrich Chemical (Phillipsburg, NJ). All other chemicals were of analytical grade.

Plant material and growing conditions

Seeds of soybean (*Glycine max* L.) were surface sterilized with 5% v/v sodium hypochlorite for 10 min and then washed with distilled water four times. The seeds were inoculated with 10^8 cell ml^{-1} of *Bradyrhizobium japonicum* (109, INTA Castelar) and were planted in vermiculite for 5 days. After germination, plants were removed from pots, roots were gently washed and transferred to separated containers for hydroponics. Plants were germinated and grown in a controlled climate room at 24 ± 2°C and 50% relative humidity, with a photoperiod of 16 h and a light intensity of 175 μmol m^{-2} s^{-1}. The hydroponics medium was Hoagland nutrient solution [26]. The medium was continuously aerated and replaced every three days. After 4 weeks, plants were treated with nutrient solution devoid of Cd (control) or containing 50 and 200 μM $CdCl_2$. After 48 h of treatment, nodules were isolated and used for determinations. When the effect of biliverdin (BV, 10 μM) or Zn-protoporphyrin IX (ZnPPIX, 20 μM) was investigated, they were added to Hoagland solution alone, without Cd or conjointly with 50 and 200 μM Cd. Three different experiments were performed, with five replicated measurements for each parameter assayed.

Thiobarbituric acid reactive substances (TBARS) determination

Lipid peroxidation was measured as the amount of TBARS determined by the thiobarbituric acid (TBA) reaction as described by Heath and Packer [27]. Fresh control and treated nodules (0.3 g) were homogenized in 3 ml of 20% (w/v) trichloroacetic acid (TCA). The homogenate was centrifuged at 3500*g* for 20 min. To 1 ml of the aliquot of the supernatant, 1 ml of 20% TCA containing 0.5% (w/v) TBA and 100 μl 4% butylated hydroxytoluene (BHT) in ethanol were added. The mixture was heated at 95°C for 30 min and then quickly cooled on ice. The contents were centrifuged at 10,000*g* for 15 min and the absorbance was measured at 532 nm. Value for non-specific absorption at 600 nm was subtracted. The concentration of TBARS was calculated using an extinction coefficient of 155 mM^{-1} cm^{-1}.

Glutathione determination

Non-protein thiols were extracted by homogenizing 0.3 g of nodules in 3.0 ml of 0.1 N HCl (pH 2.0), 1 g PVP. After centrifugation at 10,000*g* for 30 min at 4°C, the supernatants were used for analysis. Total glutathione (GSH plus GSSG) was determined in the homogenates spectrophotometrically at 412 nm, after precipitation with 0.1 N HCl, using yeast-glutathione reductase, 5,5′ dithio-bis-(2-nitrobenzoic acid) (DTNB) and NADPH. GSSG was determined by the same method in the presence of 2-vinylpyridine and GSH content was calculated from the difference between total glutathione and GSSG [28].

Antioxidant enzymes preparations and assays

Extracts for determination of superoxide dismutase (SOD) and guaiacol peroxidase (GPOX) were prepared from 0.3 g of nodules, homogenized under ice-cold conditions in 2 ml of extraction buffer, containing 50 mM phosphate buffer (pH 7.4), 1 mM EDTA, 1 g PVP, and 0.5% (v/v) Triton X-100 at 4°C. The homogenates were centrifuged at 10,000*g* for 20 min and the supernatant fraction was used for the assays. Total SOD activity was assayed by the inhibition of the photochemical reduction of nitroblue tetrazolium (NBT) [29]. The reaction mixture consisted of 50–150 μl of enzyme extract and 3.5 ml O_2^- generating solution which, contained 14.3 mM methionine, 82.5 μM NBT, and 2.2 μM of riboflavin. Test tubes were shaken and placed 30 cm from a light bank consisting of six 15-W fluorescent lamps. The reaction was allowed to run for 10 min and stopped by switching the light off. The reduction in NBT was followed by reading the absorbance at 560 nm. Blanks and controls were run in the same way but without illumination and enzyme, respectively. GPOX activity was assayed in the homogenates by measuring the increase in absorption at 470 nm due to the tetraguaiacol formation (ε: 26.6 $mM^{-1}\,cm^{-1}$), the reaction mixture contained extract, 50 mM K-phosphate buffer pH 7.0, 0.1 mM EDTA, 10 mM guaiacol and 10 mM H_2O_2. Extracts for determination of glutathione reductase (GR) activity were prepared from 0.6 g of nodules homogenized under ice-cold conditions in 2.5 ml of extraction buffer containing 50 mM Tris-HCl buffer (pH 7.6), and 1 mM EDTA. GR activity was measured by following the decrease in absorbance at 340 nm due to NADPH oxidation. The reaction mixture contained extract, 1 mM EDTA, 0.5 mM GSSG, 0.15 mM NADPH, 50 mM Tris-HCl buffer (pH 7.5) and 3 mM $MgCl_2$ in a final volume of 200 μl [30].

Heme oxygenase preparation and assay

Nodules (0.3 g) were homogenized in a Potter-Elvehejm homogenizer using 4 vol. of ice-cold 0.25 M sucrose solution containing 1 mM phenylmethyl sulfonyl fluoride, 0.2 mM EDTA and 50 mM potassium phosphate buffer (pH 7.4). Homogenates were centrifuged at 20,000*g* for 20 min and supernatant fractions were used for activity determination. Heme oxygenase activity was determined as previously described with minor modifications [5]. The standard incubation mixture in a final volume of 500 μl contained 10 μmol potassium phosphate buffer (pH 7.4), 60 nmol NADPH, 250 μl HO (0.5 mg protein), and 200 nmol hemin. Incubations were carried out at 37°C during 60 min. Activity was determined by measuring biliverdin formation, which was calculated using the absorbance change at 650 nm employing an ε value of 6.25 $mM^{-1}\,cm^{-1}$ (vis_{max} 650 nm) [31].

Western blot analysis for HO-1

Homogenates obtained for HO activity assay were also analyzed by Western immunoblot technique. 40 μg of protein from nodule homogenates were subjected to sodium dodecyl sulfate (SDS)-poliacrylamide gel electrophoresis using a 12% acrylamide resolving gel (Mini Protean II System, BioRad, Hertz, UK), according to Laemmli [32]. Separated proteins were then transferred to nitrocellulose membranes and non-specific binding of antibodies was blocked with 3% non-fat dried milk in PBS, pH 7.4 for 1 h at room temperature. Membranes were then incubated overnight at 4°C in primary antibodies raised against *Arabidopsis thaliana* HY-1 [15] diluted 1:2000 in Tris-NaCl buffer plus 1% non-fat milk. Inmune complexes were detected using alkaline phosphatase-conjugated goat anti-rabbit inmunoglobulin G. The phosfatase-labelled antigens were visualized with the colorigernic substrate 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium.

Protein determination

Protein concentration was evaluated by the method of Bradford, [33] using bovine serum albumin as a standard.

Statistics

Values in the text, figures and tables indicate mean values ± SE. Differences among treatments were analyzed by one-way ANOVA, taking $P < 0.05$ as significant according to Tukey's multiple range test.

Results and discussion

Assessment of oxidative stress parameters

Reactive oxygen species (ROS) are regarded to be initiators of peroxidative cell damage. TBARS formation in plants exposed to adverse environmental conditions is a reliable indicator of tissular free radical generation. TBARS content remained unaltered in nodules treated with 50 μM Cd, while an increase close to 67% was assessed in experiments carried out in the presence of 200 μM Cd (Table I).

GSH is a leading substrate for enzymatic antioxidant functions and it is also a known radical scavenger. It could therefore be expected that if Cd induces the formation of oxidant species it would also affect GSH-nodule levels. Data in Table I show that GSH concentration in nodules treated with 200 μM Cd decreased 44% respect to controls. Again, 50 μM Cd did not affect GSH content.

When the antioxidant enzymes were studied, we observed that treatment with 50 μM Cd did not change the enzyme activities respect to control values. However, the highest Cd concentration diminished

Table I. Effect of cadmium and BV treatments on soybean nodules TBARS and GSH content.

Treatment	TBARS (nmol/g FW)	GSH (μmol/g FW)
Control	94.1 ± 6.2[a]	0.41 ± 0.02[a]
50 μM Cd	100.7 ± 9.4[a]	0.39 ± 0.03[a]
200 μM Cd	157.1 ± 5.3[b]	0.23 ± 0.01[b]
BV	96.6 ± 7.4[a]	0.40 ± 0.04[a]
BV + 50 μM Cd	100.5 ± 8.5[a]	0.38 ± 0.03[a]
BV + 200 μM Cd	100.2 ± 9.1[a]	0.39 ± 0.02[a]

Soybean nodules were treated with 50 or 200 μM cadmium alone or together with 10 μM BV. Data are mean values of three independent experiments ± SE. Each value represents five replicates. Different letters within columns indicate significant differences ($P < 0.05$) according to Tukey's multiple range test.

Table III. Effect of cadmium and BV treatments on soybean nodules heme oxygenase activity.

Treatment	HO[a] (U/mg protein)
Control	4.21 ± 0.41[a]
50 μM Cd	3.90 ± 0.22[a]
200 μM Cd	42.15 ± 4.12[b]
BV	4.12 ± 0.21[a]
BV + 50 μM Cd	3.85 ± 0.30[a]
BV + 200 μM Cd	3.95 ± 0.34[a]

Soybean nodules were treated with 50 or 200 μM cadmium alone or together with 10 μM BV. Enzymatic activity was assayed as described in "Materials and methods" section. Data are mean values of three independent experiments ± SE. Each value represents five replicates. Different letters within columns indicate significant differences ($P < 0.05$) according to Tukey's multiple range test. [a] One unit of the enzyme forms 1 nmol of biliverdin/60 min under assay conditions.

SOD, GR and GPOX activities by 54, 20 and 60%, respectively, compared to controls (Table II).

Biliverdin is an efficient scavenger of ROS. It would therefore be expected that an increase of BV due to HO induction could be a response to the initial oxidative stress. To explore this possibility, BV was added to nutrient solution together with both Cd concentrations. It was found that this treatment completely prevented the increase in TBARS content and the decrease in GSH levels (Table I) as well as the decrease in SOD, GR and GPOX activities (Table II). On the other hand, administration of BV alone affected neither enzyme activities nor oxidative stress parameters (Tables I and II). It is noteworthy that BV does not interact directly with Cd because experiments performed *in vitro* demonstrated that Cd concentration was not altered in the presence of BV (data not shown).

These findings clearly show that the oxidative stress provoked by 200 μM Cd can be prevented by 10 μM BV.

Heme oxygenase activity and expression

We decided to investigate if the generation of oxidative stress by Cd was associated to HO induction, as it occurs in mammals [7,34]. Nodules exposed to 200 μM Cd showed a 10-fold enhancement in HO activity. This impressive increase was totally prevented when samples were treated conjointly with 10 μM BV (Table III). These results corroborated the protection observed in the oxidative stress parameters in nodules treated with BV. On the other hand, this compound alone had not any effect on HO activity. Western blot analysis for HO-1 (Figure 1A) showed only a single band with a molecular mass of 30 kDa, determined by using molecular mass markers (data not shown), a similar value to that reported for the alfalfa root nodules [35]. This assay also demonstrated a positive correlation between enzyme activity (Table III) and expression (Figure 1B).

It has been demonstrated that *HY1* gene of *Arabidopsis* encodes a plastid heme oxygenase (AtHO1) required for the synthesis of the chromophore of the phytochrome family of plant photoreceptors [3,16,31]. Very recently, it has been postulated that HO-1 is involved in leghemoglobin metabolism in the alfalfa mature nodules. However, at variance with our findings, these authors have not found, under their experimental conditions, that HO were induced by some pro-oxidants agents, and they suggest that HO-1 is not regulated by reactive oxygen or nitrogen species, as it occurs in animal tissues [35].

Table II. Effect of cadmium and BV treatments on antioxidant enzyme activities in soybean nodules.

Treatment	Total SOD (U/mg protein)[a]	GR (U/mg protein)[b]	GPOX (U/mg protein)[c]
Control	11.1 ± 0.5[a]	0.063 ± 0.002[a]	0.30 ± 0.02[a]
50 μM Cd	10.7 ± 0.4[a]	0.067 ± 0.003[a]	0.32 ± 0.02[a]
200 μM Cd	5.1 ± 0.5[b]	0.051 ± 0.001[b]	0.12 ± 0.01[b]
BV	10.6 ± 0.7[a]	0.062 ± 0.004[a]	0.31 ± 0.02[a]
BV + 50 μM Cd	10.5 ± 0.5[a]	0.065 ± 0.005[a]	0.35 ± 0.03[a]
BV + 200 μM Cd	11.5 ± 0.9[a]	0.064 ± 0.002[a]	0.33 ± 0.01[a]

Soybean nodules were treated with 50 or 200 μM cadmium alone or together with 10 μM BV. Enzymatic activities were assayed as described in "Materials and methods" section. Data are mean values of three independent experiments ± SE. Each value represents five replicates. Different letters within columns indicate significant differences ($P < 0.05$) according to Tukey's multiple range test. [a] One unit of SOD was defined as amount of enzyme which produced a 50% inhibition of NBT reduction under the assay conditions. [b] One unit of GR oxidizes 1 μmol of NADPH per min under the assay conditions. [c] One unit of GPOX forms 1 μmol of oxidized guaiacol per min under the assay conditions.

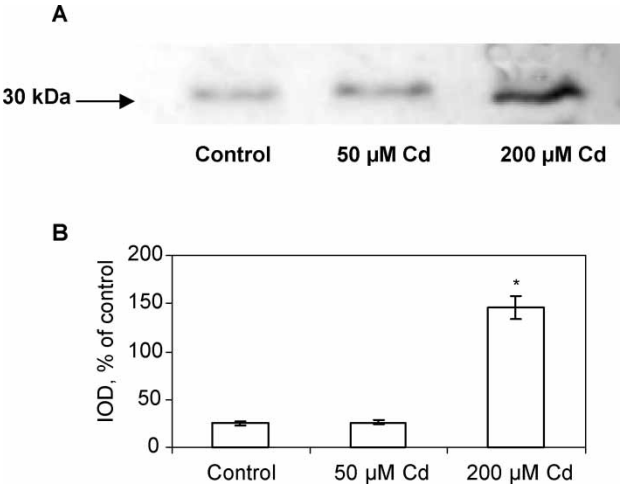

Figure 1. Effect of cadmium on soybean nodules HO expression (A). Densitometry was done by Gel-Pro® analyzer to quantify HO-1 protein expression (B). The blot is representative of three blots with a total of 4–5 samples/group between the three blots.

It has been previously reported that the basic mechanism of heme cleavage has been conserved between plants and other organisms even though the function, subcellular localization and cofactor requirements of HO's differ substantially [3,16,31,36,37]. Mammalian enzymes use NADPH cytochrome P450 reductase as its sole source of electrons, [38] whereas the plant, algal and cyanobacterial HO's use reduced ferredoxin [3,16,31,36,37]. Therefore, several authors have sustained that one factor that is highly variable between different groups of organisms is the source of the reducing equivalents for HO activity [3,14,16,31,36,37]. However, we have found that soybean nodule HO activity can be supported by NADPH as electron donor, which could not be replaced by ferredoxin (data not shown), indicating in this case that the plant redox partner was similar to that in mammals. Accordingly, Muramoto et al., [15] measured HO activity only in the presence of NADPH as reductant agent in *Arabidopsis;* these authors also suggested a plastidic location for this enzyme.

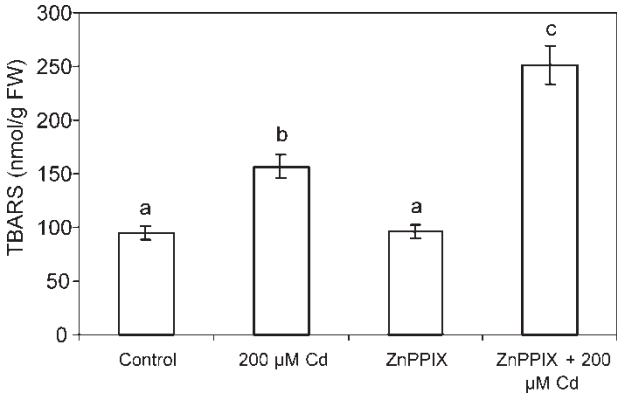

Figure 3. Effect of cadmium and ZnPPIX treatments on soybean nodules TBARS levels. Values are the mean of three different experiments with five replicated measurements, and bars indicate SE. Different letters indicate significant differences ($P < 0.05$) according to Tukey's multiple range test.

Considering the significant increase in HO activity and expression (Table III and Figure 1), we might assume that the amount and activity of HO in the tissues assayed exceeded by far the demands for phytochrome biosynthesis. Therefore, it could play a beneficial antioxidant role through the increased BV levels which may be acting either as phytochrome precursor or as an efficient ROS scavenger.

Effect of Zn-protoporphyrin IX on oxidative stress parameters and HO activity

The behaviour of BV as a protective agent against oxidative stress suggested that inhibition of HO could enhance even more the Cd-induced oxidative damage. Therefore, the effect of ZnPPIX, a well known strong inhibitor of HO, on TBARS, GSH levels and antioxidant enzyme activities was assayed. As expected, treatment

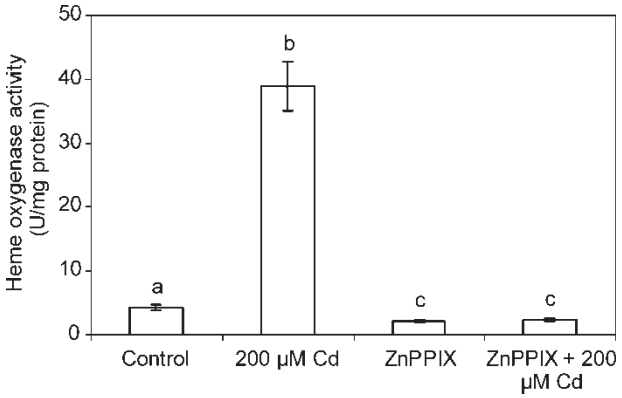

Figure 2. Effect of cadmium and ZnPPIX treatments on soybean nodules HO activity. Values are the mean of three different experiments with five replicated measurements, and bars indicate SE. Different letters indicate significant differences ($P < 0.05$) according to Tukey's multiple range test.

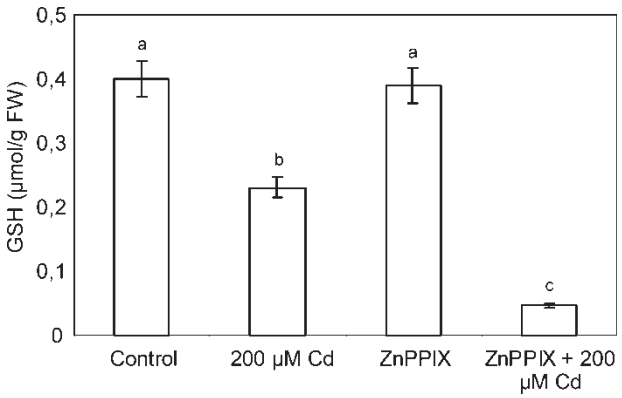

Figure 4. Effect of cadmium and ZnPPIX treatments on soybean nodules GSH content. Values are the mean of three different experiments with five replicated measurements, and bars indicate SE. Different letters indicate significant differences ($P < 0.05$) according to Tukey's multiple range test.

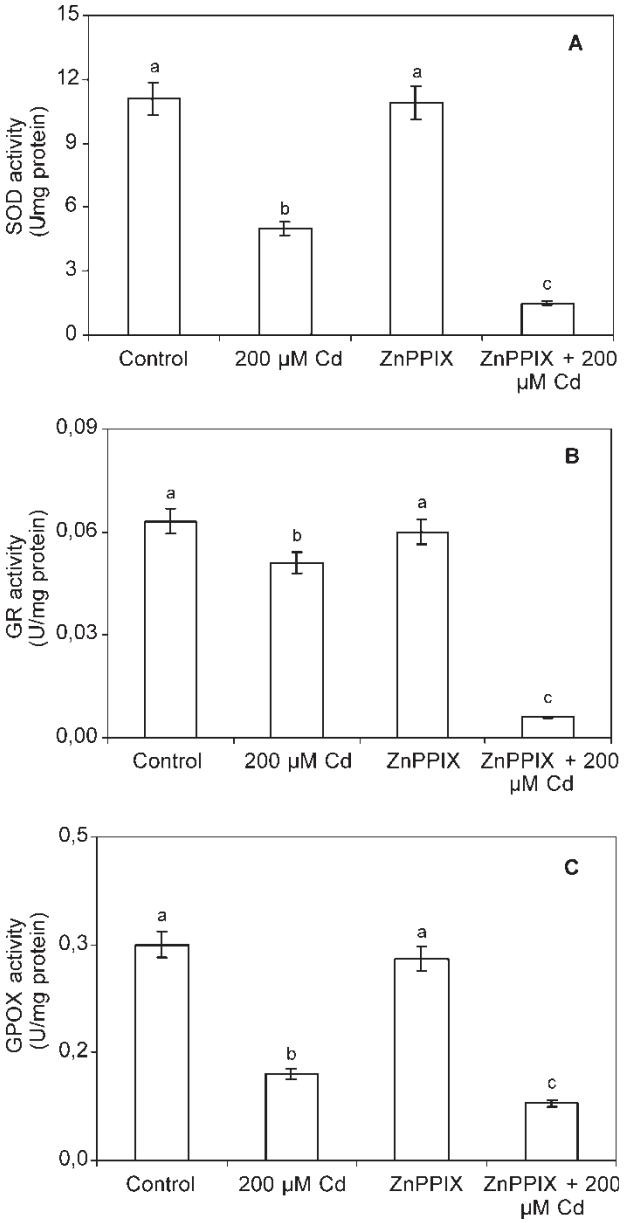

Figure 5. Effect of cadmium and ZnPPIX treatments on soybean nodules SOD (A), GR (B) and GPOX (C) activities. Values are the mean of three different experiments with five replicated measurements, and bars indicate SE. Different letters indicate significant differences ($P < 0.05$) according to Tukey's multiple range test. One unit of SOD was defined as amount of enzyme which produced a 50% inhibition of NBT reduction under the assay conditions. One unit of GR oxidizes 1 µmol of NADPH per min under the assay conditions. One unit of GPOX forms 1 µmol of oxidized guaiacol per min under the assay conditions.

with ZnPPIX produced a decrease in the enzyme activity (Figure 2). Simultaneous treatment with 200 µM Cd and ZnPPIX significantly enhanced TBARS levels (100%) (Figure 3), decreased GSH content (46%) (Figure 4), as well as SOD, GR and GPOX activities (30, 74 and 13%, respectively) (Figure 5A, B and C), when compared with the values obtained when only 200 µM Cd was added. It is noteworthy that in this case HO still remained inhibited (50%) (Figure 2). The effect elicited by the ZnPPIX administration was not due to a pro-oxidant effect because, when it was employed alone, the parameters associated with the oxidative stress, TBARS, GSH content and antioxidant enzyme activities were not modified (Figures 3–5). These results are indicating that the effects of the oxidative stress inductor (200 µM Cd) and the HO inhibitor (ZnPPIX) were additive, showing that HO induction plays a key role against oxidative stress injury in soybean nodules. These results imply, however, that the pro-oxidant effect of ZnPPIX when it was administered together with 200 µM Cd was due to the lack of expression of the HO-1 activity resulting from the presence of the inhibitor. We could still speculate that leghemoglobin breakdown caused by Cd-induced oxidative damage will produce higher heme levels in nodules, which in turn could be enhancing HO activity and expression as it was found in animal tissues [11,12].

Taking together, the results obtained clearly demonstrated the presence in soybean nodules of one HO closely related to HO-1 of mammalian cells, on the basis of its reducing cofactor requirement, its induction by pro-oxidants and its antioxidant response in nodules subjected to Cd stress. Findings here reported showed that an inducible HO might play a key role in the anti-oxidative protection machinery of higher plants.

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