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# Protoporphyrin IX and Oxidative Stress

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The short- and long-term pro-oxidant effect of protoporphyrin IX (PROTO) administration to mice was studied in liver. A peak of liver porphyrin accumulation was found 2 h after the injection of PROTO (3.5 mg/kg, i.p.); then the amount of porphyrins diminished due to biliar excretion. After several doses of PROTO (1 dose every 24 h up to 5 doses) a sustained enhancement of liver porphyrins was observed. The activity of δ-aminolevulinic acid synthetase was induced 70–90% over the control values 4 h after the first injection of PROTO and stayed at these high levels throughout the period of the assay. Administration of PROTO induced rapid liver damage, involving lipid peroxidation. Hepatic GSH content was increased 2 h after the first injection of PROTO, but then decreased below the control values which were maintained after several doses of porphyrin. After a single dose of PROTO, Cu–Zn superoxide dismutase (SOD) was rapidly induced, suggesting that superoxide radicals had been generated. Increased levels of hydrogen peroxide coming from the reaction catalyzed by SOD and lipid peroxides as a consequence of membrane peroxidation, induced the activity of catalase and glutathione peroxidase (GPx), while decreased GSH levels induced glutathione reductase (GRed) activity. However after 5 doses of PROTO, the activity of SOD was reduced reaching control values. GPx and catalase activities slowly went down, while GRed continued increasing as long as the levels of GSH were kept very low. TBARS values, although lower than those observed after a single dose of PROTO, remained above control values; Glutathione S-transferase activity was instead greatly diminished, indicating sustained liver damage.

Our findings would indicate that accumulation of PROTO in liver induces oxidative stress, leading to rapid increase in the activity of the antioxidant enzymes to avoid or revert liver damage. However, constant accumulation of porphyrins provokes a liver damage so severe that the antioxidant system is compromised.

*Keywords:* Antioxidant enzymes, liver damage, oxidative stress, porphyrias, protoporphyrin

## INTRODUCTION

Erythropoietic protoporphyria (EPP) is an inherited disorder of heme metabolism, in which protoporphyrin (PROTO) accumulates. Since PROTO is a natural photosensitizer, EPP is characterized by a typical visible light cutaneous photosensitivity. The porphyrin has also toxic dark effects; therefore many patients may actually have hepatocellular abnormalities and develop overt liver disease, with recurrent cholestatic hepatitis,

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fibrosis and cirrhosis, progressing to terminal liver failure.[1] Liver damage may be caused by excess PROTO coming from hepatic or extrahepatic heme synthesis, from erythrocytes caught by liver macrophages or serum PROTO taken up by hepatocytes.[2] It has been postulated that the toxic dark effects of PROTO, and other porphyrins, are due to their interference with cellular redox systems.[3–5] *In vitro* studies have demonstrated that porphyrins can rise the formation of reactive oxygen species (ROS) in the dark, and lipid peroxidation has been proposed as a prime mechanism for cellular membrane dysfunction and tissue injury associated with free radical-initiated processes.[6,7]

In view of its central role in metabolism, the liver should rely on its antioxidant potential; however, a sustained disturbance of the redox state could exceed its antioxidant capacity, thus leading to oxidative stress.

## MATERIALS AND METHODS

### Materials

Protoporphyrin was from Porphyrin Products. Other chemicals were reagent grade and were purchased from Sigma Chemical Co. (St Louis, MO).

### Animals

Male *CF1* mice weighing 25–30 g were maintained in controlled conditions and allowed free access to food (Purina 3, Asociación de Cooperativas Argentinas, San Nicolás, Buenos Aires, Argentine) and water. Animals were treated at the same time of the day. All animals received humane care as outlined in the Guide for the Care and Use of Laboratory Animals.

### Experimental Design

One group of 36 animals received a single dose of 3.50 mg/kg, i.p. of PROTO and were sacrificed at different times after the injection. A second group (24 mice) received a daily dose of 3.50 mg/kg, i.p. of PROTO and were sacrificed 24 h after the last injection. Control animals received the vehicle 0.1 M borate buffer (pH 7.5), and they were sacrificed at the same time as the treated animals. Mice were starved 16 h prior to sacrifice and were killed under ether anesthesia. Liver and blood were processed immediately.

### Homogenates Preparation

A fraction of non-perfused liver was excised and homogenized in NaCl (0.9%) containing EDTA (0.5 mM) and Tris-HCl buffer (pH 7.4; 10 mM) (1 : 3, w/v) and was used to measure ALA-S activity. Afterwards, the remainder liver was perfused with sterile ice cold saline and removed. A fraction was homogenized (1 : 3, w/v) in ice cold 0.25 M sucrose. After differential centrifugation of the homogenate, the supernatant of 18,000 $\times g$ was employed to determine ALA-D and PBG-D activities, and to measure the content of glutathione (GSH), protein bound glutathione (GSSP) and porphyrins; and the supernatant obtained after centrifugation at 105,000 $\times g$ for 90 min was used for measuring Cu–Zn superoxide dismutase (SOD) and glutathione peroxidase (GPx) activities. Other fraction of the perfused liver was homogenized (1 : 10, w/v) in ice cod 50 mM sodium phosphate buffer (pH 7.4) to analyse lipid peroxidation, and the supernatant obtained after centrifugation at 18,000 $\times g$ for 20 min was used for measuring catalase and glutathione reductase (GRed) activities. To determine the activity of glutathione S-transferase (GST) the homogenate was prepared from perfused liver (5% w/v) in Tris-HCl buffer (pH 8.1; 2 mM) containing 230 mM mannitol and 70 mM sucrose and centrifuged at 18,000 $\times g$ for 20 min.

For blood porphyrin determinations, whole blood was hemolyzed with Triton X-100 (5%) and diluted in Tris-HCl buffer (pH 7.4; 0.05 M) (1 : 2, v/v).

### Assays

ALA-S activity was measured by the method of Marver *et al.*;[8] ALA-D according to Batlle *et al.*[9] and PBG-D by the method of Batlle *et al.*[10] Catalase activity was measured as described by Chance and Maehly,[11] GRed according to Pinto and Bartley,[12] Gpx following Paglia and Valentine[13] and SOD by the method of Paoletti *et al.*[14] GST activity was determined by the method of Habig *et al.*[15]

GSH and GSSP were quantified according to Rossi *et al.*[16] Lipid peroxidation was estimated as TBARS according to Niehaus and Samuelson.[17]

Liver and blood porphyrins were determined fluorometrically as described by Polo *et al.*[18] Fecal porphyrins were esterified and extracted following the method of Polo *et al.*,[19] and the quantification was carried out as described by Rimington and Sveinson.[20]

Protein concentration was determined by the procedure of Lowry *et al.*[21]

Enzyme units were defined as the amount of enzyme forming 1 nmol of product under the standard incubation conditions. Specific activity was expressed as units/mg protein. One unit of SOD is defined as the amount of SOD able to cause 50% inhibition of the rate of NADH oxidation measured in the control. Results are expressed as percentage of the corresponding control animals taken as 100%.

### Control Values

ALA-S activity: $22.95 \pm 3.42$ nmol ALA/mg prot. ($n = 15$); ALA-D activity: $28.78 \pm 2.72$ nmol PBG/mg prot. ($n = 15$); PBG-D activity: $0.650 \pm 0.054$ nmol porphyrins/mg prot. ($n = 13$); GST activity: $80.84 \pm 5.14$ µmol complex/mg prot. ($n = 15$); GRed activity: $34.14 \pm 2.96$ nmol NADP/mg prot. × min ($n = 12$); Gpx activity: $646.24 \pm 37.48$ nmol NADPH/mg prot. × min ($n = 15$); catalase activity: $72.82 \pm 5.37$ µmol $H_2O_2$/mg prot. × min ($n = 14$); SOD activity: $65.41 \pm 5.62$ U/mg prot. ($n = 15$).

Liver porphyrins: $110.31 \pm 7.71$ ng/mg prot. ($n = 16$); blood porphyrins: $8.98 \pm 0.71$ ng/mg prot. ($n = 15$); TBARS: $0.135 \pm 0.012$ nmol MDN/mg prot. ($n = 15$); GSH: $0.965 \pm 0.084$ nmol/mg prot. ($n = 14$); GSSP: $(15.26 \pm 0.76) \times 10^{-3}$ nmol GSH/mg prot. ($n = 15$).

### Statistical Analysis

All data point represent the mean value ± SD of at least three experiments run in duplicate. Newman–Keuls test was used to assess the degree of significance. A probability level of 0.01 and 0.05 was used in testing for significant differences between experimental groups.

## RESULTS

### Effect of Protoporphyrin on the Heme Pathway

Two hours after the administration of a single dose of PROTO, a significant increase of liver porphyrins was observed, which then progressively diminished returning to control values 24 h after (Figure 1A). This diminution would be due to the biliar excretion of the porphyrins since the highest amounts of fecal porphyrins were found between 4 and 16 h after PROTO administration (Table I). Blood porphyrin levels were high throughout the whole period studied, when a single dose of PROTO was given (Figure 1A).

The administration of several doses of PROTO caused a time-progressive increase in liver porphyrins and a slight time-decrease of blood porphyrins (Figure 1B).

ALA-S activity was greatly induced 4 h after the administration of a single dose of PROTO, and induction was sustained along the whole period of study (Figure 2A). ALA-D and PBG-D activities were induced 24 h after one injection of porphyrin (Figure 2A). With several doses of PROTO, the enzyme activities maintained their high levels nearly without variations (Figure 2B).

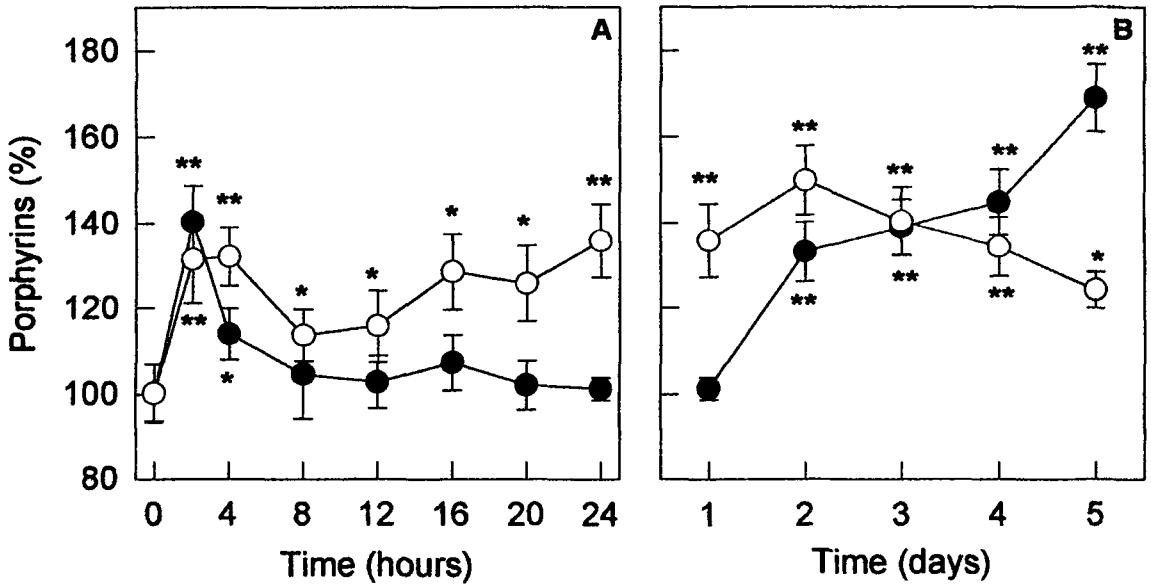


FIGURE 1 Effect of PROTO on liver and blood porphyrin. Animals received a single dose (A) or a daily dose (B) of PROTO (3.5 mg/kg, i.p.) and were sacrificed at the indicated times. Porphyrins from liver (●) and blood (○) were determined spectrofluorometrically and are expressed as percentage of the corresponding control values taken as 100%. (*): $p < 0.05$; (**): $p < 0.01$.

TABLE I Fecal porphyrins

| | Time (h) | | | | | |
|---|---|---|---|---|---|---|
| | 0 | 2 | 4 | 16 | 20 | 24 |
| Porphyrins (ng/mg) | 0.81 ± 0.08 ($n = 5$) | 5.37 ± 0.08 ($n = 5$) | 353.80 ± 0.16 ($n = 4$) | 22.95 ± 0.12 ($n = 4$) | 7.90 ± 0.12 ($n = 4$) | 4.50 ± 0.08 ($n = 5$) |

Porphyrins were measured in feces, at different times after the administration of a single dose of PROTO.

### Liver Damage Induced by Protoporphyrin

Lipid peroxidation occurred during the whole period studied after both a single dose and several injections of PROTO (Figure 3).

GST activity was induced immediately after the administration of a single dose of the porphyrin up to 4 h but then it significantly reduced its activity, reaching values below the controls 16 h after (Figure 3A), which remained low after administration of several doses of PROTO (Figure 3B).

### Free and Protein Bound Glutathione

Liver GSH content was strikingly increased 2 h after administration of a single dose of PROTO, followed by a progressive decrease to reach levels below control values at 24 h (Figure 4A) and also after administration of several doses of PROTO (Figure 4B).

Liver GSSP content was around the control values up to 16 h after the administration of a single dose of porphyrin (Figure 4A), then it showed a progressive increase which was sustained after administration of several doses of PROTO (Figure 4B).

### Glutathione Cycle Enzymes

GRed showed a peak of activity 4 h after the administration of a single dose of PROTO, and then decreased to slightly above the control

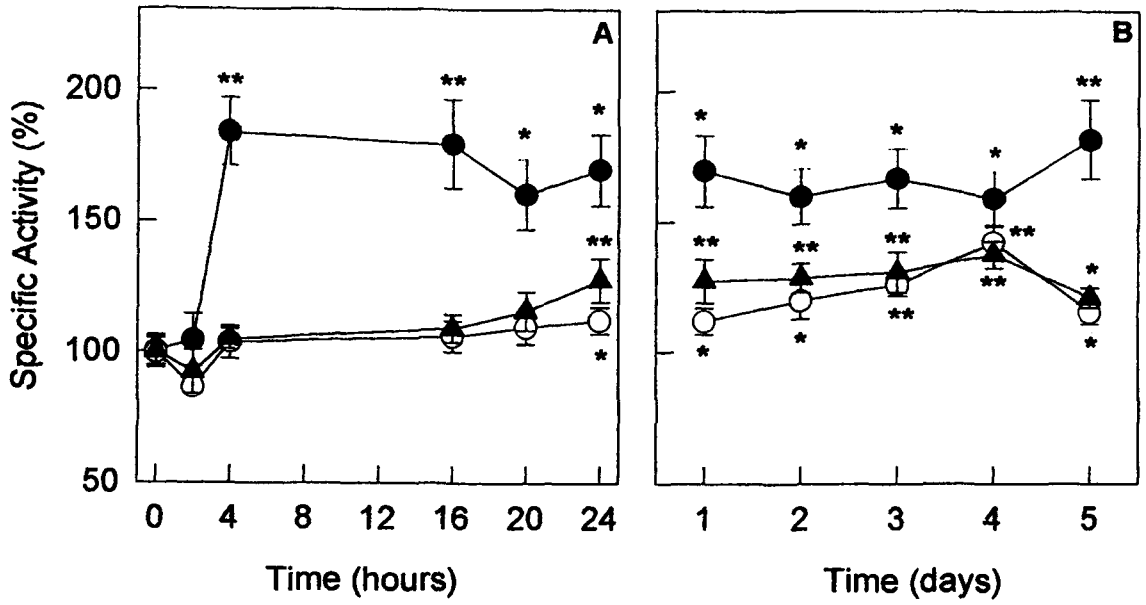


FIGURE 2 Effect of PROTO on the activity of some heme-enzymes. Animals received a single dose (A) or a daily dose (B) of PROTO (3.5 mg/kg, i.p.) and were sacrificed at the indicated times. The liver enzymic activities of ALA-S (●), ALA-D (○) and PBG-D (▲) are expressed as percentage of the corresponding control values taken as 100%. (*): $p < 0.05$; (**): $p < 0.01$.

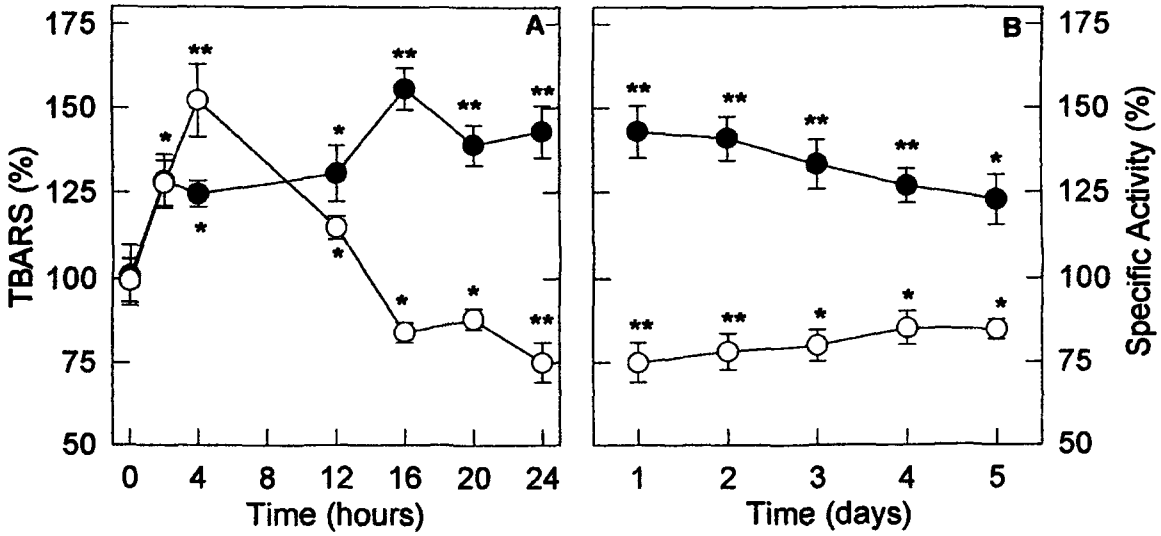


FIGURE 3 Induction of liver damage by PROTO. Animals received a single dose (A) or a daily dose (B) of PROTO (3.5 mg/kg, i.p.) and were sacrificed at the indicated times. TBARS formation (●) and GST activity (○) are expressed as percentage of the corresponding control values taken as 100%. (*): $p < 0.05$; (**): $p < 0.01$.

values up to 24 h (Figure 5A). After administration of several doses of PROTO a constant and significant increase in the GRed activity was observed (Figure 5B).

GPx activity was kept around control values up to 16 h after a single dose of the porphyrin, then it was induced up to 50% above the control activity at 24 h (Figure 5A), and the administration of

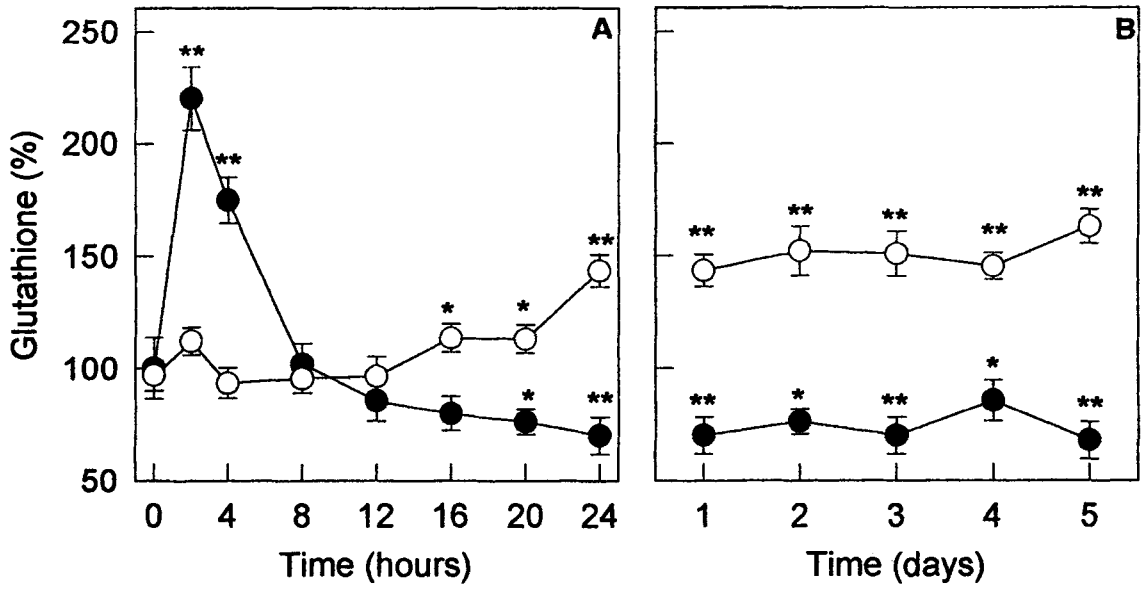


FIGURE 4 Effect of PROTO on the hepatic levels of tree (GSH) and protein bound (GSSP) glutathione. Animals received a single dose (A) or a daily dose (B) of PROTO (3.5 mg/kg, i.p.) and were sacrificed at the indicated times. Hepatic levels of GSH (●) and GSP (○) are expressed as percentage of the corresponding control values taken as 100%. (*): $p < 0.05$; (**): $p < 0.01$.

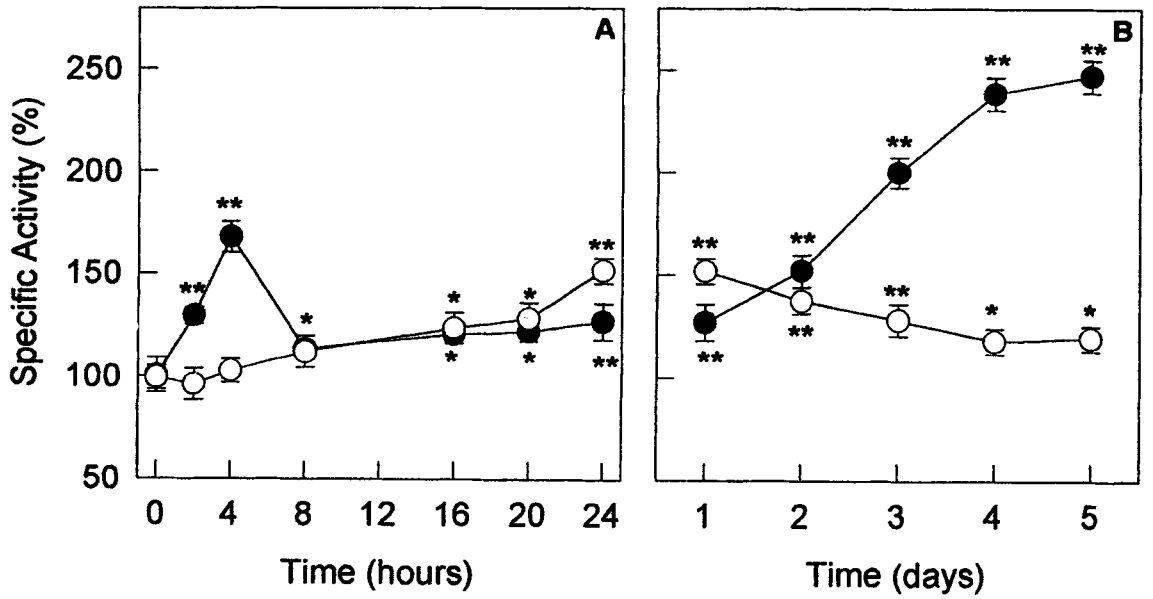


FIGURE 5 Effect of PROTO on the activity of glutathione reductase (GRed) and glutathione peroxidase (GPx). Animals received a single dose (A) or a daily dose (B) of PROTO (3.5 mg/kg, i.p.) and were sacrificed at the indicated times. The activity of GRed (●) and GPx (○) are expressed as percentage of the corresponding control values taken as 100%. (*): $p < 0.05$; (**): $p < 0.01$.

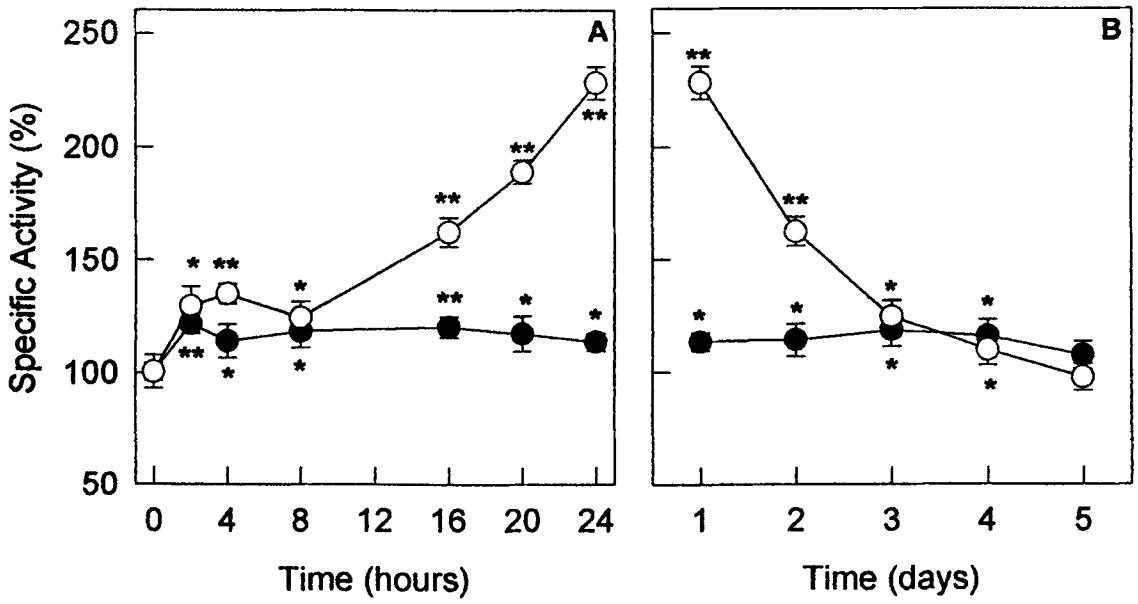


FIGURE 6 Effect of PROTO on the activity of catalase and superoxide dismutase (SOD). Animals received a single dose (A) or a daily dose (B) of PROTO (3.5 mg/kg, i.p.) and were sacrificed at the indicated times. The activity of catalase (●) and SOD (○) are expressed as percentage of the corresponding control values taken as 100%. (*): $p < 0.05$; (**): $p < 0.01$.

several doses of PROTO did not cause a further increase of Gpx activity (Figure 5B).

### Catalase and Superoxide Dismutase

Catalase activity increased about 30% 2 h after the administration of a single dose of PROTO and stayed at that level throughout the period of the assay (Figure 6A) and even after administration of several doses of the porphyrin (Figure 6B).

SOD activity steadily increased from the beginning up to 24 h after a single dose of PROTO (Figure 6A), but then decreased after several injections of the porphyrin (Figure 6B).

## DISCUSSION

PROTO intraperitoneally administered to mice soon reaches the blood and bound to albumin and hemopexin is carried to the liver.[22–25] This rapid transport of PROTO would explain the enhancement of liver and blood porphyrins only 2 h after its administration (Figure 1A). Hepatic porphyrins are rapidly excreted via biliar (Table I). However, it appears that the injection of several doses of PROTO would overpass the liver excretory ability, leading to the accumulation of porphyrins (Figure 1B).

PROTO exogenously administered was not completely released in the liver from the blood since the blood levels of porphyrins were still high 24 h after the first injection of PROTO (Figure 1A), probably due to the great affinity of porphyrins for albumin.[23,24]

Under normal conditions, ALA-S activity is very low, but this enzyme rapidly respond to cellular requirements of heme.[26] The heme molecule is the prostetic group of several hemoproteins such as cytochromes, catalase and peroxidases, some of these proteins are involved in the antioxidant cellular defense system.[27,28] If excessive amounts of PROTO induced oxidative stress in the cells, then heme biosynthesis would be accelerated to cope with the requirements of antioxidant enzymes, and consequently ALA-S

activity should increase. This fact would be the reason why ALA-S activity was constantly high throughout the whole period of the assay (Figure 2).

ALA-D and PBG-D can maintain the flow of heme intermediates without significant variations in their activities, even though the level of their substrates were high.[22,25] However, when the heme requirements were too great the activity of both enzymes should increase. This would explain the rise in ALA-D and PBG-D activities 24 h after administration of PROTO (Figure 2).

The administration of PROTO also induced rapid liver damage, as indicated by alteration of GST activity (Figure 3). This damage would involve lipid peroxidation of liver membranes as suggested by the high levels of TBARS from the beginning of the treatment (Figure 3). Liver damage persisted even though the hepatic porphyrin levels diminished after the first injection of PROTO (Figures 1A and 3A).

GSH is consumed by the antioxidant defense system; however, an initial increase in its hepatic concentration has been reported[29,30] being beneficial for the rapid detoxication and antioxidant capability of the hepatocytes.[30] This is consistent with our findings of a rapid rise followed by a pronounced decrease in the liver GSH content (Figure 4). The lowering of the GSH levels could be due to its use by the antioxidant enzymes, and to its binding to proteins for protection of their essential SH groups, which is consistent with the observed increase in the levels of liver GSSP (Figure 4).

GPx needs reduced glutathione to detoxify peroxides. A high concentration of GSH is maintained within the cell; when oxidized, GSSG is reduced back to GSH by GRed.[28] Under physiological conditions, GRed is able to reduce the GSSG generated by GPx. Under oxidative stress conditions, both enzymes should increase their activities to reinforce the cellular antioxidant systems. The early peak of GRed (Figure 5A) would be consistent with the large increase of GSH (Figure 4A). The induction of GPx, consuming GSH and generating GSSG would lead to the further enhancement of GRed activity (Figure 5A). Nevertheless, although the GRed activity continued increasing (Figure 5B) and the GSH levels were constantly depressed (Figure 4B), GPx activity did not increase after several doses of PROTO (Figure 5B).

Catalase and SOD are induced under oxidative stress conditions.[28] The strong specificity for the substrate[31] and the enhancement of SOD activity suggest the generation of superoxide radicals above the physiological values. The reaction catalyzed by SOD yields $H_2O_2$. This would lead to a rise in catalase activity. Moreover, the high activity of SOD would also produce an increase of lipid peroxidation, because of the decrease of the termination reaction of hydroperoxides.[28,32]

$$ROO^{\bullet} + O_2^{\bullet -} \rightarrow ROOH + O_2$$

thus leading to an enhancement of TBARS (Figure 3A).

Because GPx reduces lipid peroxides and detoxifies $H_2O_2$ with higher efficiency than catalase,[28] induction of GPx (Figure 5A) should be higher than induction of catalase (Figure 6A).

After several injections of PROTO, the activity of SOD was rapidly reduced, reaching the control values upon the administration of 5 doses (Figure 6B). Strong inhibition of SOD by its product has been reported.[27,28] It is also known that the recovering capacity of cells exposed to oxidative stress depends on its intensity; the higher the stress, the lower the recovery.[33–35] Both facts could explain the decrease in SOD activity (Figure 6B), which would be accompanied by reduced formation of $H_2O_2$, diminishing the requirements of catalase (Figure 6B) and Gpx (Figure 5B). Since the termination reaction of hydroperoxides would not be decreased due to the lower activity of SOD, a rise in lipid peroxidation would not be expected although the level of liver porphyrins is high (Figure 1B). Nevertheless, the level of TBARS, even lower than that observed after a single dose of PROTO, stayed above the control

values (Figure 3B), while GST activity and GSH levels (Figures 3B and 4B) are very reduced, indicating sustained liver damage.

These findings would indicate that accumulation of PROTO in liver induces oxidative stress, leading to rapid increase in the activity of the antioxidant enzymes to avoid or revert liver damage. However, constant accumulation of porphyrins provokes a liver damage so severe that the antioxidant system is unable of reversing.

Although great amounts of PROTO are accumulated in the EPP liver, the first clinical sign of the disease is its skin photosensitivity. Therefore, our next approach towards the understanding of the mechanism underlying cutaneous reaction to light, will be to investigate the effects of exogenously administrating PROTO, on the skin of animals exposed and protected from UV light.



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