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Paraquat-generated Oxidative Stress in Rat Liver Induces Heme Oxygenase-1 and Aminolevulinic Acid Synthase

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The *in vivo* effect of the known herbicide, paraquat, on both hepatic oxidative stress and heme metabolism was studied. A marked increase in lipid peroxidation and a decrease in reduced glutathione (GSH) content were observed 1 h after paraquat administration. The activity of liver antioxidant enzymes, superoxide dismutase, catalase and glutathione peroxidase was decreased 3 h after paraquat injection. Heme oxygenase-1 induction started 9 h after treatment, peaking at 15 h. δ-aminolevulinic acid synthase induction occurred once heme oxygenase had been enhanced, reaching its maximum (1.5-fold of control) at 16 h. δ-aminolevulinic acid dehydratase activity was 40% inhibited at 3 h showing a profile similar to that of GSH, while porphobilinogenase activity was not modified along the whole period of the assay. Administration of α-tocopherol (35 mmol/kg body weight) 2 h before paraquat treatment entirely prevented the increase in thiobarbituric acid reactive substances (TBARS) content, the decrease in GSH levels as well as heme oxygenase-1 and δ-aminolevulinic acid synthase induction. This study shows that oxidative stress produced by paraquat leads to an increase in δ-aminolevulinic acid synthase and heme oxygenase-1 activities, indicating that the herbicide affects both heme biosynthesis and degradation.

Keywords: Paraquat; Heme metabolism; δ-aminolevulinic acid synthase; Heme oxygenase-1; Oxidative stress; Antioxidant enzymes

Abbreviations: ALA-D, δ-aminolevulinic acid dehydratase; ALA-S, δ-aminolevulinic acid synthase; CAT, catalase; DTNB, 5,5′ dithiobis-(2-nitrobenzoic acid); GSH-Px, glutathione peroxidase; HO-1, Heme oxygenase isoform 1; HO-2, Heme oxygenase isoform 2; HO-3, Heme oxygenase isoform 3; PBG-ase, porphobilinogenase; ROS, reactive oxygen species; SOD, superoxide dismutase; TBARS, thiobarbituric acid reactive substances

INTRODUCTION

Paraquat (1,1′-dimethyl-4,4′-bipiridylium dichloride), also known as methyl viologen, is one of the most widely used herbicides.

Bipiridyl herbicides, such as paraquat, are extremely valuable for two reasons: their broad toxicity to a wide range of weeds, and because they are completely inactivated on contact with the soil, being tightly bound to clay minerals and destroyed by various microorganisms. However, the benefits of its use are regrettably accompanied by a high toxicity to animals and man. It induces oxidative stress and lipid peroxidation, generating free radicals which lead to acute or chronic lung injury and often death.[1]

The first enzyme in heme biosynthesis is δ-aminolevulinic acid synthase (ALA-S), which by condensation of glycine and succinyl CoA yields δ-aminolevulinic acid (ALA) the first and specific intermediate of this pathway. ALA-S is also the rate-limiting enzyme in the heme pathway, regulated by the intracellular heme pool by feedback and repression mechanisms.[2] When the concentration of heme in the regulatory pool is diminished, ALA-S is induced and its activity increased. Two moles of ALA are condensed to the monopyrrole porphobilinogen (PBG) by the action of δ-aminolevulinic acid dehydratase (ALA-D), then a complex of two enzymes, porphobilinogen-deaminase and uroporphyrinogen III cosynthetase, known as porphobili-

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nogenase (PBGase), forms the octacarboxylic tetrapyrrole uroporphyrinogen III, which is the physiological intermediate. Next, by the action of uroporphyrinogen decarboxylase, uroporphyrinogen III is decarboxylated in a sequential stepwise reaction to the tetracarboxylic coproporphyrinogen III. Coproporhyrinogenase decarboxylates and oxidizes coproporphyrinogen III to protoporphyrinogen IX, then oxidized to protoporphyrin IX by protoporphyrinogen oxidase. Finally heme synthetase, inserts ferrous iron into the ring of protoporphyrin IX to yield heme, the final product of this pathway. In general all of the enzymes after ALA-S in the heme pathway are in excess and non-limiting.

Heme oxygenase (HO) catalyses the rate limiting step in heme degradation producing carbon monoxide, free iron and biliverdin that is subsequently reduced to bilirubin by bilirubin reductase.[3] Both biliverdin and bilirubin are potent antioxidants.[4] Three isoenzymes of HO have been described to date. HO-1 is inducible[5] whereas HO-2 and HO-3 are constitutively expressed. Multiple stimuli including inflammatory agents, heat shock and oxidative stress can markedly enhance HO-1 activity both *in vitro* and *in vivo*.[5] Because unconjugated bilirubin is an efficient antioxidant and scavenger of reactive oxygen species (ROS) its increase is thought to be a cellular defense response to initial oxidative stress.[6–9]

The induction of the HO-1 is entirely prevented by administration of several antioxidants such as α-tocopherol and allopurinol,[10] suggesting that oxidant species play a major role in HO-1 induction either directly or by means of GSH depletion.[7–10]

In this work, we have attempted to asses whether mammalian cells possess inducible pathways of defense against chemically induced oxidative stress. To this end, we have used an *in vivo* experimental model to study the effect of paraquat-induced oxidative stress, on the early enzymes in porphyrin biosynthesis and on heme oxygenase as well as the possible protective role of α-tocopherol against oxidative cell injury.

MATERIALS AND METHODS

Materials

Paraquat dichloride salt (methyl viologen), NADPH, reduced glutathione (GSH), oxidized glutathione (GSSG), 5,5′-dithio-bis-(2-nitrobenzoic acid) (DTNB), thiobarbituric acid (TBA), glutathione reductase, ALA and α-tocopherol were from Sigma Chemical (Saint Louis, MO), ter-butyl hydroperoxide was from Aldrich Chemical (Phillipsburg, NJ); PBG was obtained enzymatically according to Sancovich *et al.*[11] All other chemicals were of analytical grade.

Animals

Female albino Wistar rats (150–180 g) were housed separately, acclimatized before use, maintained on a 12 h light–dark cycle and fed with rat chow (Molinos Río de la Plata) and water *ad libitum*. The rats were fasted for at least 16 h before experiment. Fasted animals were injected i.p. with a single dose of paraquat (45 mg/kg body weight) dissolved in saline solution. α-tocopherol (35 mmol/kg body weight) dissolved in corn oil was injected i.p. 2 h before paraquat treatment. Controls received saline solution. In experiments where the effect of α-tocopherol was tested, controls received corn oil. Animals were treated in accordance with guidelines established by the Animal Care and Use Committee of the Argentine Association of Specialists in Laboratory Animals (AADEALC).

Enzyme Preparations and Assays

Rats were anesthetized with sodium pentobarbital (50 mg/kg body weight, i.p.) immediately before sacrifice and killed 1, 3, 6, 9, 12, 15, 18 and 24 h after the injection of paraquat. Approximately one third of the liver, previously perfused with ice-cold saline solution, was excised and immediately homogenized (1:3, w/v) in a solution containing 0.9% NaCl, 0.1 mM/Tris–HCl pH 7.4, 0.5 mM/EDTA, for ALA-S determination.[12] All homogenates were prepared in a Potter–Elvehejm homogeneizer. The remainder of the organ was removed and homogenized using different solutions. HO activity was performed according to Llesuy and Tomaro.[8] Microsomal HO-1 was obtained as described elsewhere.[13] Catalase (CAT), glutathione peroxidase (GSH-Px) and superoxide dismutase (SOD) activities were determined spectrophotometrically in supernatants of liver homogenates prepared in a medium containing of 140 mM KCl and 25 mM potassium phosphate buffer (pH 7.4), centrifuged at 600*g* for 10 min. CAT activity was determined by measuring the decrease in absorbance at 240 nm,[14] GSH-Px following NADPH oxidation at 340 nm[15] and SOD by inhibition of adenochrome formation rate at 480 nm.[16] ALA-D was determined by measuring the formation of PBG at 555 nm,[17] while porphobilinogenase (PBG-ase) was determined by measuring spectrophotometrically the formation of uroporphyrins[18] and using the expression defined by Rimington.[19]

Lipid Peroxidation

Lipid peroxidation was measured as the amount of thiobarbituric acid reactive substances (TBARS) determined by the thiobarbituric acid (TBA) reaction as described by Buege and Aust.[20] One volume of

homogenate was mixed with 0.5 Vol. TCA (15%, w/v) and centrifuged at 2000*g* for 10 min. Supernatant (1 ml) was mixed with 0.5 ml thiobarbituric acid (0.7%, w/v) and boiled for 10 min. After cooling, sample absorbance was read spectrophotometrically at 535 nm. The concentration of TBARS was calculated using an ϵ of 155 mM^{-1}/cm.

GSH Content

Total glutathione (GSH plus GSSG) was determined in liver homogenates after precipitation with 2% perchloric acid and using yeast-glutathione reductase, DTNB and NADPH, at 340 nm. GSSG was determined by the same method in the presence of 2-vinylpyridine and endogenous GSH calculated from the difference between total glutathione and GSSG.[21]

Western Blot Analysis for HO-1

Samples of homogenate obtained for HO-1 activity assay were also analyzed by Western immunoblot technique as previously described.[22] An amount of protein (100 mg) from homogenates of control and treated rats (15 h of intoxication) was run in sodium dodecyl sulfate (SDS)-poliacrylamide gel electrophoresis using a 12% acrylamide resolving gel (Mini Protean II System, BioRad, Herts, UK). Separated proteins were then transferred to nitrocellulose membranes and the non-specific binding of antibodies was blocked with 3% nonfat dried milk in PBS, pH 7.4 for 1 h at room temperature. Membranes were then probed with a polyclonal rabbit anti HO-1 antibody (Santa Cruz, Bio Tech., California) (1:300 dilution in Tris-buffered saline, pH 7.4) for 1 h at room temperature. Immune complexes were detected using donkey antigoat secondary antibody (1:2000) (Santa Cruz, Bio Tech., California), and were visualized using ECL reagent (Amersham, Pharmacia). Intensity of bands were analyzed with Image Master (Amersham, Pharmacia Biotech).

Protein Determination

Protein concentration was measured by the method of Lowry *el al.*[23] using bovine serum albumin as standard.

Statistics

Figures in the text and tables indicate mean values ± SEM. Differences between control and treated animals were analyzed using Student's *t* test, taking $p < 0.05$ as significant.

RESULTS

Effect of Paraguat on Liver TBARS Generation and GSH Content

Time course studies on the effect of paraquat on lipid peroxidation and hepatic GSH content are shown in Fig. 1. As indicated by TBARS levels, lipid peroxidation rapidly increased peaking (130%) 3 h after intoxication, decreasing thereafter and returning to control levels 15 h after injection.

Hepatic GSH levels reached a minimum (50% of control value) 1 h after injection, increasing thereafter to approach control levels 15 h later (Fig. 1). Control animals did not show any significant changes in either parameter along the 24 h observation period (data not shown).

Enzymatic Antioxidant Defenses

Table I shows that the activities of the antioxidant enzymes, CAT, SOD and GSH-Px, were 22, 48 and 26% diminished, respectively between 6 and 9 h, as compared to controls. Twelve hour after, they began to recover their activity returning to control levels at 15 h.

Heme Oxygenase Induction

Increased TBARS content as well as GSH, SOD, CAT and GSH-Px decreased levels appeared to be closely related events taking place several hours before HO-1 induction. Data given in Fig. 2 show that 40% HO induction was evidenced 9 h after

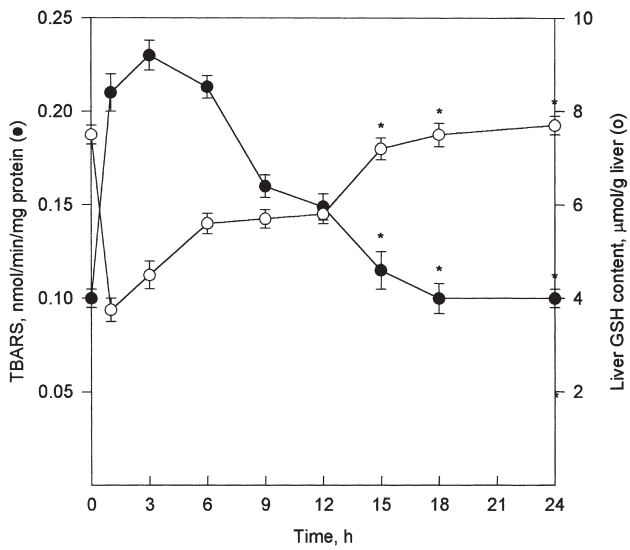

FIGURE 1 Time course of paraquat effect on lipid peroxidation (•) and on GSH liver content (○). Rats were treated as described in "Materials and methods section" and killed at the indicated times. Symbols indicate mean values from 12 rats and bars indicate SEM. *No significant differences ($p > 0.05$) as assessed by Student's *t* test. Values measured in control animals were the same as those at 0 time.

TABLE I Time course of paraquat effect on antioxidant enzyme activities

Time (h)	Catalase (pmol/mg protein)	Total superoxide dismutase (U/mg protein)	Glutathione peroxidase (U/mg protein)*
0	10.5 ± 0.3	9.0 ± 0.2	0.130 ± 0.006
1	10.5 ± 0.4	8.5 ± 0.3	0.121 ± 0.003
3	9.4 ± 0.3*	6.7 ± 0.1*	0.113 ± 0.003*
6	8.2 ± 0.5*	4.7 ± 0.1*	0.096 ± 0.004*
9	8.5 ± 0.4*	5.0 ± 0.1*	0.101 ± 0.004*
12	8.8 ± 0.5*	7.3 ± 0.4*	0.109 ± 0.005*
15	10.3 ± 0.6	9.6 ± 0.5	0.139 ± 0.006
18	10.5 ± 0.5	9.0 ± 0.3	0.132 ± 0.005
24	10.2 ± 0.3	9.2 ± 0.3	0.137 ± 0.004

Paraquat was injected i.p. as indicated in "Materials and methods section" at time 0. Rats were killed at the indicated times. Enzymatic activities were assayed as described in the text. *One unit of the enzyme represents the decrease of 1 mmol of NADPH/min under assay conditions. Data are means ± SEM of two different experiments using six rats each time. *Significant differences ($p < 0.05$) as assessed by Student's t test. Values measured in control animals were the same as those at 0 time.

paraquat administration, peaking at 15 h (150%, with respect to controls), to then rapidly decrease to nearly basal levels 18 h after treatment.

Figure 3 shows that the 150% enhanced HO activity observed 15 h after paraquat injection, was accompanied by a strong increase in the expression of the protein.

Effect of Paraquat on Heme Pathway

On the other hand, when we have a look at the time profile of ALA-S activity (Fig. 2) we observe that after 12 h it is induced by roughly 20% as compared with controls, reaching its maximum induction (45%) at 16 h. Control levels were regained 24 h after paraquat injection.

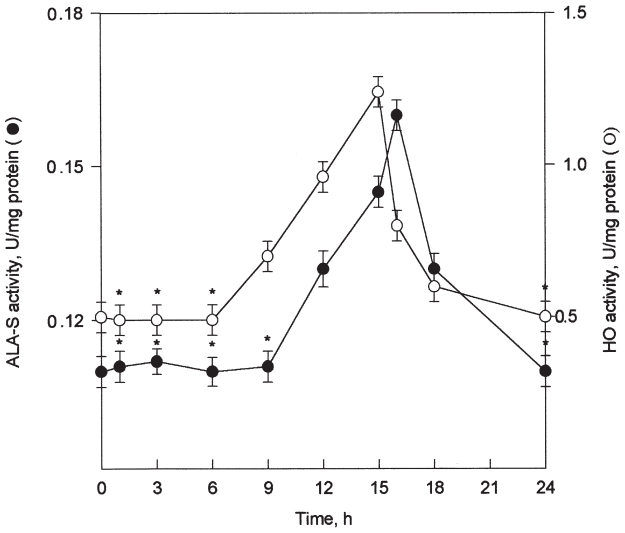

FIGURE 2 Time course of paraquat effect on ALA-S activity (•) and on HO-1 activity (○). Rats were treated as described in "Materials and methods section" and killed at the indicated times. Enzyme units for ALA-S and HO-1 are defined, respectively, as the amount of enzyme producing 1 nmol of ALA/60 min or 1 nmol of bilirubin/30 min under the standard incubation conditions. Symbols indicate mean values from 12 rats and bars indicate SEM. *No significant differences ($p > 0.05$) as assessed by Student's t test. Values measured in control animals were the same as those at 0 time.

Figure 4 shows the effect of paraquat administration on the activity of liver ALA-D and PBG-ase. It can be seen that within 1–3 h, ALA-D is significantly reduced reaching its minimum (40%) at 3 h, increasing thereafter, and regaining control values 12 h after paraquat injection. It is worth to mention that the time profile of ALA-D inhibition greatly resembles that of GSH depletion. PBG-ase activity was not modified by paraquat treatment along the whole period of the assay.

Effect of α-tocopherol Pretreatment

In Table II, we show that administration of α-tocopherol, 2 h before paraquat injection, prevented HO-1 and ALA-S induction as well as the decrease in GSH content and the increase in TBARS levels. Treatment with α-tocopherol affected neither HO-1 and ALA-S activities nor oxidative stress parameters.

DISCUSSION

There is good information regarding the toxic effects of paraquat in the lung,[1] but less is known about its toxicity in liver and much less about its action on heme metabolism.

These results confirm the studies of other reports and our previous observation that induction of HO-1 and ALA-S is linked to oxidative stress.[24] It has been found that a single i.p. dose of paraquat does not induce the formation of H_2O_2 and lipid peroxidation products in the lung but it does increase their concentrations in the liver.[25] We show here that this herbicide provokes a sequence of

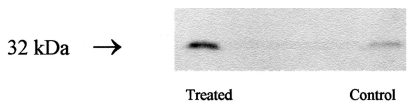

FIGURE 3 Western blot analysis of heme oxygenase-1. Protein expression was analyzed by Western immunoblot technique as described in "Materials and methods section". *Treated*: 15 h after a single dose of paraquat, *Control*: saline injection.

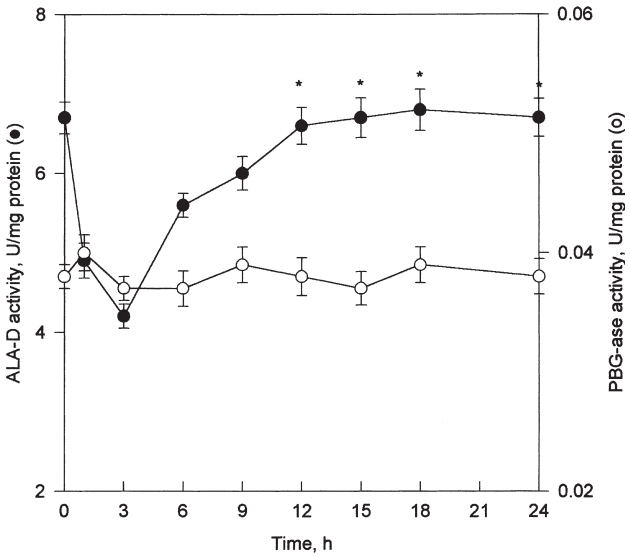

FIGURE 4 Time course of paraquat effect on PBG-ase activity (○) and on ALA-D activity (•). Rats were treated as described in "Materials and methods section" and killed at the indicated times. Enzyme units for PBG-ase and ALA-D are defined as the amount of enzyme producing 1 nmol of uroporphyrin or 1 nmol of PBG per 60 min, respectively, under the standard incubating conditions. Symbols indicate mean values from 12 rats and bars indicate SEM. *No significant differences ($p > 0.05$) as assessed by Student's t test, except for PBG-ase where all the values represented were not significant. Values measured in control animals were the same as those at 0 time.

events leading to lipid peroxidation, depletion of protein thiols, and alterations in both heme synthesis and degradation.

To safeguard normal cellular functions and survival, cells have developed a number of defensive mechanisms including antioxidant molecules containing thiol groups such as GSH and enzymes, such as SOD, CAT and GSH-Px.[26,27]

We have shown here that oxidative stress generated by paraquat, leads to decreased GSH levels and then induction of HO-1 activity (Figs. 1 and 2). Depletion of GSH is in fact an index of oxidative stress and it is also linked with the activation of transcriptional factors and regulation of gene expression.[28] Moreover, induction of HO-1 has been correlated with a decrease of endogenous GSH.[7]

We have also observed that HO-1 induction would appear to occur once lipid peroxidation was increased and the antioxidant defense system (GSH, SOD, CAT, and GSH-Px) was decreased (Fig. 1, Table I). GSH is one of the main antioxidant compounds in the liver,[29] SOD protects cells against oxidative hazards by the dismutation of superoxide radicals; catalase and glutathione peroxidase are two antioxidant enzymes involved in the breakdown of H_2O_2 and therefore, play an essential role in reducing the oxidative damage in mammal tissues and organs. Inhibition of both enzymes could be the consequence of an irreversible autocatalytic process, in which the sustained increase of ROS would finally lead to cellular death.[30] These findings are in agreement with reports of Nakanishi and Yasumoto,[31] who described that dietary paraquat administration diminished the activity of antioxidant enzymes and increased the levels of TBARS and oxidized proteins in blood and liver. They also found a transient increase in HO-1 and their results added further support to the hypothesis that HO-1 plays an important role in the antioxidant response system, although these authors pointed out that induction of HO-1 and its relation to oxidative damage is a process tightly associated with aging.

Of major interest in this work is the result showing that as a consequence of oxidative stimuli, HO-1 expression and activity increase to fulfill its cytoprotective role and this fact is well correlated with a de-regulation in the earlier steps in heme biosynthesis. Several authors have proposed that HO-1 induction by various forms of oxidative stress constitute an antioxidant response operating by decreasing the levels of potential prooxidants and increasing the concentrations of antioxidant active bile pigments. It is already accepted that unconjugated bilirubin, is an efficient scavenger of ROS and its enhancement may be a response to initial oxidative damage.[6,8–9]

It is also widely established that the hepatocellular free heme pool controls the rate limiting enzyme ALA-S through mechanisms involving a negative regulation on the transcription, translation and on its activity.[32] It should be noted that here, ALA-S induction occurs immediately after enhancement of

TABLE II Effect of α-tocopherol administration on TBARS and GSH content, and on HO-1 and ALA-S induction in paraquat-treated rat livers

Groups	TBARS (nmol/min/mg)	GSH content (μmol/g liver)	HO-1 (U/mg)*	ALA-S (U/mg)†
Control	0.10 ± 0.01	7.5 ± 0.2	0.50 ± 0.01	0.11 ± 0.01
Paraquat	0.15 ± 0.01*	5.8 ± 0.2*	0.96 ± 0.06*	0.15 ± 0.01*
α-tocopherol	0.10 ± 0.01	7.3 ± 0.3	0.48 ± 0.01	0.09 ± 0.01
α-tocopherol 2 h before paraquat	0.09 ± 0.01	7.2 ± 0.4	0.53 ± 0.02	0.11 ± 0.01

α-tocopherol (35 mmol/kg body weight) was administered by i.p. injection 2 h before paraquat treatment. Rats were killed 12 h after treatment. Enzymatic activities were assayed as described in the text. *One unit of the enzyme forms 1 nmol of bilirubin/30 min under assay conditions. †One unit of the enzyme forms 1 nmol of ALA/60 min under assay conditions. Data are means ± SEM of two different experiments using six rats each time. *Significant differences ($p < 0.05$) as assessed by Student's t test.

HO-1 activity. ALA-S induction may result from a diminution of the intracellular heme pool, due to either increased heme catabolism or decreased heme synthesis or both. Reduced synthesis of heme would be expected to lead to deficiencies in mitochondrial cytochromes with increased ROS generation.[32] Our results would also show that as a consequence of oxidative stress produced by paraquat at early times, ALA-D activity is inhibited, and this would in turn lead to diminished heme synthesis. After a while, the activity of ALA-D returns to normal values and induction of HO-1 occurs. These findings are indicating that paraquat is both inhibiting heme biosynthesis and then increasing the catabolism of the pre-existing and newly formed heme by enhancing the activity of microsomal HO-1. These findings are suggesting that, when heme degradation is stimulated, ALA-S makes up for the synthesis of bilirubin, by restoring the content of the diminished heme pool. Inhibition of ALA-D activity occurs concomitantly to GSH depletion. It is well known that ALA-D is a thiol enzyme and its activity can be restored or stimulated *in vitro* by GSH and other thiol derivatives.[33] Although PBG-ase is also a thiol enzyme, its sulphydryl groups are situated in not exposed regions,[34] therefore not significant alterations in its activity would be expected by changes in GSH content as here found.

In conclusion, our data show that oxidative stress produced by paraquat affects both heme biosynthesis and degradation, they also demonstrate that the cell possesses at least two inducible pathways against oxidative stress; both of them finally leading to increased bilirubin formation, which then exerts its antioxidant protective action. Vitamin E (α-tocopherol), the primary chain-breaking antioxidant in membranes by reducing peroxil, hydroxyl and superoxide radicals and singlet oxygen showed a strong protection against oxidative damage such as lipid peroxidation *in vivo*.[35] We have found here that administration of α-tocopherol 2 h before paraquat treatment entirely prevented the increase in TBARS content, the decrease in GSH levels as well as HO-1 and ALA-S induction (Table II).

These results shed light on the response of both heme biosynthesis and degradation to paraquat induced oxidative stress.

Acknowledgements

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