Response of glucose metabolism enzymes in an acute porphyria model
Role of reactive oxygen species

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Abstract
Acute hepatic porphyrias are human metabolic diseases characterized by the accumulation of heme precursors, such as 5-aminolevulinic acid (ALA). The administration of glucose can prevent the symptomatology of these diseases. The aim of this work was to study the relationship between glucose metabolism disturbances and the development of experimental acute hepatic porphyria, as well as the role of reactive oxygen species (ROS) through assays on hepatic key gluconeogenic and glycogenolytic enzymes; phosphoenolpyruvate carboxykinase (PEPCK) and glycogen phosphorylase (GP), respectively. Female Wistar rats were treated with three different doses of the porphyrinogenic drug 2-allyl-2-isopropylacetamide (AIA) and with a single dose of 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC). Thus, rats were divided into the following groups: group L (100 mg AIA + 50 mg DDC/kg body wt.); group M (250 mg AIA + 50 mg DDC/kg body wt.) and group H (500 mg AIA + 50 mg DDC/kg body wt.). The control group (group C) only received vehicles (saline solution and corn oil). Acute hepatic porphyria markers ALA-synthase (ALA-S) and ferrochelatase, heme precursors ALA and porphobilinogen (PBG), and oxidative stress markers superoxide dismutase (SOD) and catalase (CAT) were also measured in hepatic tissue. On the other hand, hepatic cytosolic protein carbonyl content, lipid peroxidation and urinary chemiluminescence were determined as in vivo oxidative damage markers. All these parameters were studied in relation to the different doses of AIA/DDC. Results showed that enzymes were affected in a drug–dose-dependent way. PEPCK activity decreased about 30% in group H with respect to groups C and L, whereas GP activity decreased 53 and 38% in group H when compared to groups C and L, respectively. On the other hand, cytosolic protein carbonyl content increased three-fold in group H with respect to group C. A marked increase in urinary chemiluminescence and a definite increase in lipid peroxidation were also detected. The activity of liver antioxidant enzyme SOD showed an induction of about 235% in group H when compared to group C, whereas CAT activity diminished due to heme depletion caused by both drugs. Based on these results, we can speculate that the alterations

Abbreviations: AIA, 2-allyl-2-isopropylacetamide; ALA, 5-aminolevulinic acid; ALA-S, ALA-synthase; CAT, catalase; DDC, 3,5-diethoxycarbonyl-1,4-dihydrocollidine; DNPH, 2,4-dinitrophenylhydrazine; GP, glycogen phosphorylase; HCB, hexachlorobenzene; H2O2, hydrogen peroxide; HO•, hydroxyl radical; LDH, lactic dehydrogenase; MDA, malondialdehyde; NADH, β-nicotinamide adenine dinucleotide; PBG, porphobilinogen; PBG-D, porphobilinogen deaminase; PCT, porphyria cutanea tarda; PEPCK, phosphoenolpyruvate carboxykinase; ROS, reactive oxygen species; O2•−, superoxide anion; SOD, superoxide dismutase; TBARS, thiobarbituric acid reactive substances
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observed in glucose metabolism enzymes could be partly related to the damage caused by ROS on their enzymatic protein structures, suggesting that they could be also linked to the beneficial role of glucose administration in acute hepatic porphyria cases.

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1. Introduction

The hepatic heme biosynthetic pathway is closely regulated, and intermediates 5-aminolevulinic acid (ALA) and porphobilinogen (PBG), as well as porphyrins, the oxidized forms of porphyrinogen, are excreted only in trace amounts. However, this regulation is altered in acute hepatic porphyrias, and the activity of ALA-synthase (ALA-S), the rate limiting step of the pathway, feedback-regulated by intracellular heme concentration, is increased, causing the overproduction and subsequent accumulation and excretion of porphyrins, their precursors, or both (Kappas et al., 1995). The induction response of hepatic ALA-S elicited by certain chemicals can be enhanced by concurrent heme depletion and heme synthesis inhibition (Granick, 1966).

ALA has been associated with iron-mediated oxidative damage to biomolecules and cell structures (Rocha et al., 2003). Moreover, this compound is susceptible to metal catalyzed oxidation, producing reactive oxygen species (ROS) such as superoxide anion (O$_2^{-}$), hydrogen peroxide (H$_2$O$_2$), hydroxyl radical (•OH), and ALA enoyl-radical (•ALA) (Monteiro et al., 1989), which act as pro-oxidants both in vivo and in vitro (Bechara, 1995; Demasi et al., 1996). ROS are able to oxidize nucleic acids, proteins, lipids, or carbohydrates, inactivating key cellular functions (Halliwell and Gutteridge, 1999).

Through the years, the administration of glucose to porphyric patients has been reported to produce significant clinical progress (Doss et al., 1985; Kappas et al., 1995). The prevention of acute experimental porphyria through high carbohydrate and/or protein intake is an example of the effect of glucose on ALA-S, in which carbohydrates prevent its induction (Granick, 1966).

Schoenfeld et al. (1985) observed that the impairment of ALA-S induction by 2-allyl-2-isopropylacetamide (AIA) occurred in the presence of glucose. Likewise, Rose et al. (1961) found that high glucose intake prevented the development of experimental porphyria induced by AIA, whereas fat intake had no effects whatsoever.

On the other hand, Correia and Lunetta (1989) reported that gluconeogenesis was altered in rats with porphyria induced by phenobarbital and 4-ethylidihydroxyphenylalanine, which are compounds that jointly produced significant heme depletion. They also caused gluconeogenic blockage, which – according to these authors – might be the underlying mechanism of the “glucose effect” in hepatic porphyria.

AIA and 3,5-diethoxycarbonyl-1,4-dihydocolcholide (DDC) produce significant heme depletion and increase ALA-S activity leading to ALA accumulation. Whereas DDC inhibits ferrochelatase, the heme synthesizing enzyme, causing protoporphyrin IX accumulation, AIA destroys cytochrome P-450 heme (Smith and De Matteis, 1980; Marks et al., 1988). Both effects significantly decrease the regulatory heme pool. Heme exerts a negative feedback control on ALA-S activity, and in order to meet the need for cytochrome P-450 synthesis, ALA-S induction occurs as a consequence of this heme depletion condition (Whiting and Granick, 1976).

Taking into account the reports already mentioned on the effects of porphyrinogenic drugs on glucose metabolism, and due to the fact that AIA/DDC triggers an experimental porphyria similar to acute human porphyria, we considered it important to analyze the relation between the porphyria and the alterations caused by the drugs in different glucose metabolism enzymes. The aim of this study was to investigate the effect of oxidative damage induced by acute intoxication with AIA and DDC on the glucose metabolism of rats and its relation to the degree of porphyria developed.

2. Materials and methods

2.1. Materials

AIA was a gift from Roche Co. (Germany). DDC was purchased from Aldrich Chemical Company Inc. (Milwaukee, WI). ALA, 5′-adenosine monophosphate, ascorbic acid, bovine serum albumin, deoxyguanosine 5′-diphosphate, epinephrine, glucose-1-phosphate, glycogen type III from rabbit liver, LDH, MoNH$_4$, malic dehydrogenase, MDA, NADH, pyruvate, diosbarbituric acid, Dowex 1 and Dowex 50W were obtained from Sigma Chemical Co. (St. Louis, MO). Guanidine hydrochloride was obtained from Invitrogen (Carlsbad, CA). All other chemicals were of analytical grade.
2.2. Animals and treatment

Female Wistar rats (180–200 g) were purchased from the National Committee of Atomic Energy (CONEA-Argentina). They were maintained on food and water ad libitum and housed under conditions of controlled temperature (25 °C) and light (12 h light-dark cycle, light from 06:00 to 18:00 h). Rats were fasted 24 h (8 h before treatment and 16 h after treatment) before sacrifice. Total urine was collected during 16 h after intoxication using metabolic cages. Animals were treated in accordance with guidelines from the Animal Care and Use Committee of the Argentine Association of Specialists in Laboratory Animals (AADEALC), and the Guide to the Care and Use of Experimental Animals issued by the Argentine Council on Animal Care.

AIA was dissolved in saline solution (0.15%, w/v) (De Matteis, 1970) and DDC was dissolved in 2.5 ml corn oil (De Matteis et al., 1973). Both solutions were prepared just before administration. Rats were fasted 8 h before treatment with both drugs. AIA was injected subcutaneously (sc) in three different doses and DDC, intraperitoneally (ip) in a single dose. Thus, animals were divided into the following groups: group L (100 mg AIA + 50 mg DDC/kg body wt.), group M (250 mg AIA + 50 mg DDC/kg body wt.), and group H (500 mg AIA + 50 mg DDC/kg body wt.). Control group (group C) only received vehicles: saline solution, sc and corn oil, ip. These AIA and DDC doses were previously used by other research groups (De Matteis, 1970; De Matteis et al., 1973) in order to obtain porphyria models in rats.

2.3. Liver preparation and assays

Rats were decapitated 16 h after AIA/DDC administration. All assays were performed in rats with a total fasted period of 24 h, since this is the optimum way to measure PEPCK and GP activities, as demonstrated by Mazzetti et al. (2004) and Taira et al. (2004). This fasting period enhances the sensitivity of the assays, permitting the standardization of results obtained respect to the control. On the other hand, since the effect of AIA depends on the previous diet (Tschudy et al., 1964), the 8 h starvation period prior to intoxication guaranteed that the effect of the drug was not altered by the diet. A fraction of liver (1 g) was excised and immediately homogenized in a solution containing 0.9% NaCl, 0.1 mM Tris–HCl, pH 7.4, and 0.5 mM EDTA. The resulting supernatant was used for enzymatic determinations.

For hepatic ferrochelatase determination, homogenates were prepared with 0.154 M KCl (1:5 w/v).

2.3.1. 5-Aminolevulinic acid synthase

ALA-S hepatic activity was assayed in whole homogenates by the method of Marver et al. (1966). Incubation mixtures containing 0.1 M glycine, 0.01 M EDTA, 0.08 M Tris–HCl buffer (pH 7.2), and 0.5 ml of homogenate (group C) only received vehicles: saline solution, sc and corn oil, ip. These AIA and DDC doses were previously used by other research groups (De Matteis, 1970; De Matteis et al., 1973) in order to obtain porphyria models in rats.

2.3.2. 5-Aminolevulinic acid and porphobilinogen hepatic contents

To determine ALA and PBG hepatic content, portions of whole liver homogenates (Marver et al., 1966) were deproteinised with 0.6 M TCA. Supernatants (0.3–1 ml) were adjusted to pH 4.5–6.0. Contents were essentially determined by the method of Piper et al. (1973). Two separate columns were used; PBG was eluted from a Dowex 1 column with 1 M acetic acid, whereas ALA was eluted from a Dowex 50W column with 1 M sodium acetate after removing urea. ALA pyrrole and PBG were measured colorimetrically as described by Mauzerall and Granick (1956).

2.3.3. Ferrochelatase activity

Ferrochelatase activity was measured in a mitochondrial fraction. Pellets were frozen at −20 °C for 24–48 h. Enzymatic activity was measured using protoporphyrin IX as substrate, as described by Porra and Jones (1963).
2.3.4. Phosphoenolpyruvate carboxykinase activity

PEPCK was measured using deoxyguanosine 5′-diphosphate as nucleotide substrate. A 50 μl aliquot of 100,000 × g supernatant was used in each assay. Oxaloacetate formed during the reverse enzyme reaction was determined by reduction with malic dehydrogenase in the presence of NADH. Changes in absorbance were measured spectrophotometrically at 340 nm. The reaction was allowed to proceed for 2 min at room temperature (Petrescu et al., 1979).

2.3.5. Glycogen phosphorylase activity

GP activity was assayed according to Newman and Armstrong (1978). Final reagent concentrations were 50 mM glucose-1-phosphate, 1% rabbit liver glycogen III, 0.15 M NaF, 0.5 mM 5′-AMP, 0.5 M Na2SO4, 0.5 M citrate buffer pH 6.5. Activity was measured as released inorganic phosphate spectrophotometrically determined at 660 nm through the phosphomolybdate complex formed using ascorbic acid as reducer (Ayes, 1966).

2.3.6. Protein carbonyl content

Carbonyl groups formed on cytosol proteins were quantified adding 1 ml 10 mM DNPH in 2 M HCl to a cytosol volume containing 2 mg protein (200 μl approximately). They were allowed to stand in the dark at room temperature for 1 h, with vortex mixing every 10 min. Samples were precipitated with TCA (20% final concentration) and centrifuged in a tabletop micro-centrifuge for 5 min. Supernatants were discarded and protein pellets were washed twice with TCA and then three times with 1 ml of ethanol/ethyl acetate (1:1) to remove free DNPH. Protein samples were resuspended in 1 ml 6 M guanidine hydrochloride, pH 2.3, at 37 °C for 15 min with vortex mixing. Carbonyl contents were determined measuring absorbance at 390 nm with a molar absorption coefficient = 22,000 M−1 cm−1 (Levine et al., 1994).

2.3.7. Lipid peroxidation

Lipid peroxidation in liver was determined measuring TBARS production rate. The red MDA-thiobarbituric acid complex produced was extracted with butanol to avoid the interference of endogenous porphyrins. TBARS content was determined measuring absorbance at 532 nm with a molar absorption coefficient = 156,000 M−1 cm−1 (Ohkawa et al., 1979).

2.3.8. Liver antioxidant defense system enzymes

CAT activity was measured recording the decrease in H2O2 absorbance at 240 nm (Chance et al., 1979). SOD activity, on the other hand, was determined by the rate of autoxidation of epinephrine to adrenochrome (Misra and Fridovich, 1972). In this assay, a unit is defined as the amount of SOD necessary to inhibit 50% epinephrine autoxidation.

2.4. Rat urinary chemiluminescence

Total urine was collected for 16 h after intoxication using metabolic cages. It was homogenized and kept frozen until evaluation. Then, 1 ml urine was centrifuged at 300 × g for 10 min (Ríos de Molina et al., 1998). Chemiluminescence was quantified using a Beckman LS-3150P liquid scintillation counter operated in the out-of-coincidence mode.

2.5. Protein determination

Protein concentration was measured following Lowry et al. (1951) using bovine serum albumin as standard. Protein concentration in carbonyl content assay was determined in guanidine-dissolved samples measuring absorption at 280 nm. The amount of protein was calculated from a bovine serum albumin standard curve (0.25–2 mg/ml) dissolved in guanidine hydrochloride and read at 280 nm (Reznick and Packer, 1994).

2.6. Statistical analysis

Values expressed in figures and tables are the means of three different experiments performed in duplicate, and involving two animals each. They indicate mean values ± S.E.M. Data were submitted to one-way ANOVA. When the overall F-statistic was significant, multiple comparisons among groups were performed using Tukey–Kramer’s test. The level of significance used was P < 0.05.

3. Results

3.1. Heme pathway biosynthesis and precursors

Changes in porphyrin biosynthesis were studied using different AIA/DDC doses to evaluate their effects on some parameters of the acute intoxication model. Thus, the activities of ALA-S and ferrochelatase (key enzymes of the porphyrin pathway), and the contents of ALA and PBG in liver were analyzed (Table 1). AIA/DDC administration enhanced ALA-S activity up to 300% in group H (500 mg AIA + 50 mg DDC/kg body wt.), and this enhancement statistically differed from groups C (control), L (100 mg AIA + 50 mg DDC/kg body wt.), and M (250 mg AIA + 50 mg DDC/kg body wt.). ALA autoxidation of epinephrine to adrenochrome (Misra and Fridovich, 1972). In this assay, a unit is defined as the amount of SOD necessary to inhibit 50% epinephrine autoxidation.
Table 1
Dose-response of hepatic ALA-S and ferrochelatase activities, and ALA and PBG hepatic contents to AIA/DDC treatment

<table>
<thead>
<tr>
<th></th>
<th>Control group</th>
<th>AIA + DDC treated</th>
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<tr>
<td></td>
<td></td>
<td>Group L</td>
</tr>
<tr>
<td>ALA-synthase (nmol ALA/g h)</td>
<td>25.5 ± 2.7</td>
<td>33.90 ± 4.9*</td>
</tr>
<tr>
<td>Ferrochelatase (nmol heme/mg h)</td>
<td>1.37 ± 0.23</td>
<td>0.62 ± 0.19*</td>
</tr>
<tr>
<td>ALA hepatic content (nmol ALA/g)</td>
<td>7.5 ± 0.7</td>
<td>9.30 ± 0.90*</td>
</tr>
<tr>
<td>PBG hepatic content (nmol PBG/g)</td>
<td>0.32 ± 0.02</td>
<td>2.43 ± 0.03*</td>
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Animals were injected subcutaneously (sc) with three different doses of AIA and intraperitoneally (ip) with a single dose of DDC, divided into the following groups: group L (100 mg AIA + 50 mg DDC/kg body wt.), group M (250 mg AIA + 50 mg DDC/kg body wt.), and group H (500 mg AIA + 50 mg DDC/kg body wt.). Control group (group C) only received vehicles: saline solution, sc and corn oil, ip. Each value represents the mean ± S.E.M. of six animals. Activity is expressed as nmol ALA/g wet liver and hepatic contents are expressed as nmol/g wet liver.

* P < 0.05 is significantly different from group C. Multiple comparisons among treatment groups were performed using Tukey–Kramer’s test.

# P < 0.05 is significantly different from group L. Multiple comparisons among treatment groups were performed using Tukey–Kramer’s test.

§ P < 0.05 is significantly different from group M. Multiple comparisons among treatment groups were performed using Tukey–Kramer’s test.

and PBG hepatic contents in group H increased 276 and 5025%, respectively, when compared to C, and were statistically different from groups C and L. In addition, PBG content in group H was statistically different from group M. On the other hand, ferrochelatase activity decreased about 50% in all the treated groups with respect to the control.

3.2. PEPCK and GP activities

Figs. 1 and 2 show PEPCK and GP activities, respectively, measured in the liver of animals treated with different AIA/DDC doses, as well as in controls. After 16 h of treatment, PEPCK suffered 30 and 24% inhibition in groups H and M, respectively, when compared to controls and are statistically different from groups C and L. GP activity also showed statistically significant decreases of about 53 and 20% in group H with respect to group C and group L, respectively. In group M, GP suffered 36% inhibition when compared to group C.

3.3. Oxidative stress parameters

ROS are responsible for protein carbonylation and membrane peroxidation damage. After the 16 h treatment, cytosolic carbonylated proteins suffered statistically significant 2.3- and 3.0-fold increases in groups M and H, respectively, when compared to the control, and were statistically higher than group L (Fig. 3).

Lipid peroxidation measured as TBARS exhibited a statistically significant increase in group L ((116.8 ± 38) × 10^3 cpm) and 205% in group M ((156.2 ± 60) × 10^3 cpm) with respect to the control group ((51.7 ± 25) × 10^3 cpm); P < 0.05. On the other hand, animals treated with the highest dose (group H) fell deeply asleep due to the treatment and, therefore, the urinary excretion volume was not enough for chemiluminescence determination to be performed.

As regards urinary chemiluminescence, values showed a statistically significant increase; 127% in group L ((116.8 ± 38) × 10^3 cpm) and 205% in group M ((156.2 ± 60) × 10^3 cpm) with respect to control (51.7 ± 25) × 10^3 cpm); P < 0.05. On the other hand, animals treated with the highest dose (group H) fell deeply asleep due to the treatment and, therefore, the urinary excretion volume was not enough for chemiluminescence determination to be performed.

Fig. 1. Dose-response of hepatic PEPCK activity to AIA/DDC treatment. Animals were injected subcutaneously (sc) with three different AIA doses and intraperitoneally (ip) with a single dose of DDC, divided into the following groups: group L (100 mg AIA + 50 mg DDC/kg body wt.), group M (250 mg AIA + 50 mg DDC/kg body wt.), and group H (500 mg AIA + 50 mg DDC/kg body wt.). Control group (group C) only received vehicles: saline solution, sc and corn oil, ip. Each bar represents the mean ± S.E.M. of six animals. Activity is expressed as nmol NADH oxidized/mg protein/min.

* P < 0.05 is significantly different from group C. Multiple comparisons among groups were performed using Tukey–Kramer’s test.
3.4. Enzymatic defense systems

Fig. 5A and B shows SOD and CAT activities, respectively, measured in livers from animals treated with AIA/DDC and in controls. SOD activity increased 235% in group H, 41.6% in group M, and 19% in group L with respect to the control group. On the contrary, CAT activity suffered 24, 11 and 9% inhibition in the same groups, respectively. Both SOD and CAT showed a drug–dose-dependent behavior in the multiple comparisons between groups statistically analyzed.

4. Discussion

The hepatic acute porphyria model presented in this study proved to be useful since it allows us to observe, among other parameters indicative of porphyria, the significant increase in hepatic ALA concentration caused by ALA-S de-repression. This de-repression is the result of the depletion of the regulatory hepatic heme pool produced by the two drugs used in this study; AIA and DDC. These porphyrinogenic drugs depleted the free regulatory heme pool, causing an imbalance. AIA acts through heme alkylation, yielding “green pigments” that lead to the suicidal and irreversible process of cytochrome P-450 inactivation. In this process, the heme moiety of cytochrome P-450 is destroyed and, consequently, the free heme pool decreases (Ortiz de Montellano and Mico, 1981; Smith and De Matteis, 1980). In addition, DDC leads to the formation of N-alkylporphyrins, which act as inhibitors of ferrochelatase activity, thus reducing the heme pool (Marks et al., 1988). Whiting and Granick (1976) have demonstrated that, after 18 h, a combination of these drugs may result in a 600-fold increase of ALA-S activity in chick embryo liver.

Although the animal model herein presented does not exactly mimic any human hepatic porphyria, from the biochemical point of view, it resembled quite accurately the variegate porphyria. In fact, early reports on this
Fig. 5. Dose–response of (A) SOD and (B) CAT activities to AIA/DDC treatment. Animals were injected with the same doses of AIA and DDC as indicated in Fig. 1. Each bar represents the mean ± S.E.M. of six animals. SOD activity is expressed as U/mg protein (in SOD assay, a unit is defined as the amount of enzyme necessary to inhibit 50% epinephrine autoxidation), and CAT activity as μmol H₂O₂ consumed/mg protein min. *P < 0.05 is significantly different from group C; #P < 0.05 is significantly different from group L; §P < 0.05 is significantly different from group M. Multiple comparisons among treatment groups were performed using Tukey–Kramer’s test.

human disease described an inhibition of ferrochelatase activity (Cole and Marks, 1984), and later studies reported an inhibition of hepatic protoporphyrinogen oxidase activity (Kappas et al., 1995).

The liver plays a major role in blood glucose homeostasis by maintaining a balance between the uptake and storage of glucose via glycogenogenesis, and the release of glucose via glycogenolysis and gluconeogenesis (Hers, 1990; Nordlie et al., 1999). In our study, the activities of two enzymes were measured; one involved in glucose synthesis (PEPCK) and the other in glycogen degradation (GP). Both activities appear particularly diminished. PEPCK showed deficient activity in the model used by Correia and Lunetta (1989), in which the administration of phenobarbital (a cytochrome P-450 hepatic inducer) and 3,5-dicarbethoxy-2,6-dimethy-1,4-ethyl-1,4-dihydro-pyridine (a P-450 suicide inhibitor) caused the abrupt depletion of hepatic heme. This model presented a distinct pattern of accumulation of gluconeogenic intermediates proximal to PEPCK, such as malate and aspartate, which increased significantly, while all distal intermediates were definitely depleted (Correia and Lunetta, 1989). PEPCK also exhibited a lower activity in chronic intoxication with the porphyrinogenic drug HCB (Mazzetti et al., 2004). In this case, the time-course effect on enzyme activity with this xenobiotic showed an early decrease, which was intensified during the treatment. Stahl et al. (1993) also reported a hepatic PEPCK decrease in rats treated with 2,3,7,8-tetrachlorodibenzo-p-dioxin (another porphyrinogenic drug) during 4 and 8 days due to a decrease in mRNA levels. As regards GP, there are not previous reports on the determination of this activity on acute porphyria models. Taira et al. (2004) found that in a model of chronic porphyria caused by hexachlorobenzene (HCB) administration to Wistar rats, this glycogenolytic enzyme also exhibited a diminished activity during the 5, 7 and 9th weeks of treatment when compared to the control. Moreover, ultrastructural studies with HCB-treated rats showed that glycogen replaced smooth endoplasmic reticulum proliferation (Mollenhauer et al., 1975).

It was also observed that numerous glycogen particles appeared in the extensive cytoplasm area of hepatocytes located in the intermediary zone, while only few monoparticles of glycogen were deposited in the centrilobular area (Boiger et al., 1979). These reports prove that in the HCB model there is an increase of hepatic glycogen due to a decrease in GP activity.

PEPCK and GP decreases observed in the AIA/DDC model herein presented agree with those reported for other porphyrinogenic drugs, such as HCB and 2,3,7,8-tetrachlorodibenzo-p-dioxin (Taira et al., 2004; Stahl et al., 1993). According to these results, these decreases observed in our model would lead to insufficient glucose availability within the hepatocyte.

The increases in oxidative stress markers herein reported – protein carbonyl content, TBARS, urinary chemiluminescence and SOD antioxidant response – agree with the oxidative damage caused by hepatic ALA accumulation. In this respect, Bechara (1996) reported that oxidative stress might be triggered by ALA in acute intermittent porphyria and lead poisoning. Our work showed that CAT suffered a slight but statistically significant decrease due to a reduction in the heme pool elicited by both drugs. This was expected, considering the heme-protein nature of the enzyme (Price et al., 1962).
It is worth mentioning that both enzymatic responses (PEPCK and GP), as well as the behavior of oxidative stress markers already mentioned exhibited a similar pattern against the different AIA/DDC doses, suggesting a link between ROS and enzyme decreases. However, we can not discard other causes that could be concomitant and that could lead to a reduction in PEPCK and GP activities as it was proposed for PEPCK decrease in HCB-induced experimental porphyria. Such causes are the effect of tryptophan (Mazzetti et al., 2004) and glucocorticoids (Lelli et al., 2004). In fact, it has already been demonstrated that tryptophan administration resulted in hypoglycaemia, that tryptophan and its metabolites alter hepatic gluconeogenesis (Smith and Pogson, 1977), and that hepatic tryptophan levels significantly increase in HCB porphyric rats (Llambías et al., 2003). According to Mazzetti et al. (2004), tryptophan would be responsible for the decrease in gluconeogenic PEPCK activity at the higher stages of fungicide intoxication, thus adding its effect to that exerted by the free radicals already mentioned.

It is worth mentioning that in an acute porphyria rat model, in which heme was depleted by phenobarbital and 3,5-dicarboxyloxy-2,6-dimethoxy-4-ethyl-1,4-dihydrolpyridine treatment, Correia and Lunetta (1989) reported a blockage in tryptophan degradation with the consequent increase in its hepatic level, concomitantly with a blockage in gluconeogenesis.

On the other hand, and considering that glucocorticoids are important regulators of glucose synthesis, Lelli et al. (2004) reported that HCB triggers an early hormonal disruption at the plasmatic glucocorticoids level, as well as an alteration on the number and affinity of its hepatic receptors in relation to PEPCK activity. They postulate that this hormonal disturbance could be one of the causes of the gluconeogenic blockage elicited by HCB at the enzymatic level.

It is important to note the dose–response relationships observed both in protein carbonylation and in PEPCK and GP enzymatic activities as a consequence of AIA/DDC treatment. Based on these results it could be speculated that the decrease of enzymatic activities could be due, in part, to the oxidative damage suffered by their protein structures during carbonylation. In addition, we speculate that both enzymes (PEPCK and GP) would be susceptible to carbonylation due to the presence of target amino acids in their sequences and especially in key sites of their enzymatic functions. Thus, amino acids sensitive to this kind of oxidative modification such as Arg, Lys, and Thr, highly conserved in several species, can be found in the active site of PEPCK (Aich et al., 2003).

On the other hand, carbonyl groups may be introduced into proteins through a secondary reaction of the nucleophilic side chains of Cys, His, and Lys residues, with aldehydes produced during lipid peroxidation (Dalle-Donne et al., 2003). The carbonylation of these critical amino acids of the active site would lead to a decrease in enzymatic activity, in line with the results obtained in our work. Assays on rat hepatic GP showed that one Lys was responsible for enzymatic activity, and two Cys were involved in the association of subunits that allow an active protein conformation (Schiebel et al., 1992). On the other hand, the lipid peroxidation increase observed in this model also contributed to protein carbonylation through ROS produced during that event.

Our results demonstrate for the first time that alterations caused by AIA/DDC on two key enzymes related to glucose, one involved in its synthesis and the other in its liberation, would lead to a deficit in the availability of hepatic glucose in treated animals. Therefore, we can speculate that these results could contribute to explain, through the determination of gluconeogenic PEPCK and glycogenolytic GP activities, the beneficial role of carbohydrate administration to patients suffering acute hepatic porphyrias. In this type of diseases, the oxidative stress caused by high ALA accumulation would act on the protein structure of key enzymes of the carbohydrate metabolism, diminishing the amount of hepatic glucose available.

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