How porphyrinogenic drugs modeling acute porphyria impair the hormonal status that regulates glucose metabolism. Their relevance in the onset of this disease

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A B S T R A C T

This work deals with the study of how porphyrinogenic drugs modeling acute porphyrias interfere with the status of carbohydrate-regulating hormones in relation to key glucose enzymes and to porphyria, considering that glucose modulates the development of the disease. Female Wistar rats were treated with 2-allyl-2-isopropylacetamide (AIA) and 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) using different doses of AIA (100, 250 and 500 mg/kg body weight) and a single dose of DDC (50 mg DDC/kg body weight). Rats were sacrificed 16 h after AIA/DDC administration. In the group treated with the highest dose of AIA (group H), hepatic 5-aminolevulinic acid synthase (ALA-S) increased more than 300%, phosphoenolpyruvate carboxykinase (PEPCK) and glycogen phosphorylase (GP) activities were 43% and 46% lower than the controls, respectively, plasmatic insulin levels exceeded normal values by 617%, and plasmatic glucocorticoids (GC) decreased 20%. GC results are related to a decrease in corticosterone (CORT) adrenal production (33%) and a significant reduction in its metabolism by UDP-glucuronosyltransferase (UGT) (62%). Adrenocorticotropic hormone (ACTH) stimulated adrenal production 3-fold and drugs did not alter this process. Thus, porphyria-inducing drugs AIA and DDC dramatically altered the status of hormones that regulate carbohydrate metabolism increasing insulin levels and reducing GC production, metabolism and plasmatic levels. In this acute porphyria model, glucogenic and glycogenolytic blockages caused by PEPCK and GP depressed activities, respectively, would be mainly a consequence of the negative regulatory action of insulin on these enzymes. GC could also contribute to PEPCK blockage both because they were depressed by the treatment and because they are positive effectors on PEPCK. These disturbances in carbohydrates and their regulation, through ALA-S de-repression, would enhance the porphyria state promoted by the drugs on heme synthesis and destruction. This might be the mechanism underlying the “glucose effect” observed in hepatic porphyrias. The statistical correlation study performed showed association between all the variables studied and reinforce these conclusions.

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1. Introduction

The hepatic heme pathway is closely regulated by its end product. Heme exerts a negative feedback control on 5-aminolevulinic acid (ALA)-synthase (ALA-S), which is the rate-limiting step of the pathway (Fig. 1).

Porphyrias are hereditary disorders in the heme metabolic pathway. They are caused by the de-regulation of its synthesis route due to a deficiency in some of the enzymes of the pathway, leading to lower heme formation. This deficiency triggers the accumulation and excretion of porphyrins and/or their ALA and porphobilinogen (PBG) precursors, and the induction of regulatory enzyme ALA-S (Kappas et al., 1995).

Acute porphyrias are the most dangerous type since they can be fatal. They are also the most susceptible to be triggered and exacerbated by drugs and metabolic factors. Acute porphyrias are hepatic diseases characterized by the accumulation of ALA and PBG precursors and by a neuropsychiatric syndrome (Kappas et al., 1995), which can be promptly relieved by intravenous heme infusion (Kappas et al., 1995; Litman and Correia, 1983).

2-Allyl-2-isopropylacetamide (AIA) increases the destruction of liver heme, particularly that of cytochrome P-450 (Smith and De

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Matteis, 1980). 3,5-Diethoxycarbonyl-1,4-dihydrocollidine (DDC) is a potent depletor of hepatic heme since it is able to both degrade heme and inhibit its synthesis (Marks et al., 1988). The combined treatment of AIA and DDC resulted in acute heme deficiency, marked AIA-S de-repression and, consequently, exacerbated production of ALA and other heme precursors in the liver (Ortiz de Montellano et al., 1981; Lelli et al., 2005) (Fig. 1). This combined treatment has been reported to induce an experimental porphyria resembling quite accurately acute variegate porphyria in rats (Lelli et al., 2005). Moreover, it has been demonstrated that AIA promotes the generation of reactive oxygen species (Monteiro et al., 1989) (Fig. 1).

Glucose administration is known to have beneficial effects on acute porphyria patients, by significantly improving their clinical and biochemical condition (Bonkovsky, 1990; 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 AIA-S, with carbohydrates preventing its induction (Tschudy et al., 1964).

Phosphoenolpyruvate carboxykinase (PEPCK), the rate-limiting enzyme of gluconeogenesis, plays a key role in glucose synthesis both in the liver and the kidney (Hanson and Reshef, 1997). PEPCK gene expression can be increased by several factors such as cyclic AMP, thyroid hormones and glucocorticoids (GC). Conversely, it has been found that it can be inhibited by insulin (Hanson and Reshef, 1997) (Fig. 1). Recently, it has been reported that combined AIA and DDC treatment not only alters the heme pathway but also impairs carbohydrate metabolism by producing, among other disturbances, the gluconeogenic blockage of PEPCK (Lelli et al., 2005).

Glycogen phosphorylase (GP) catalyzes the rate-limiting step in the degradation of glycogen in animals. Glucagon and epinephrine activate GP, whereas insulin decreases its activity. A marked reduction in the gluconeolytic activity of GP has been reported in a rat AIA/DDC acute porphyria model (Lelli et al., 2005).

GC are cholesterol-synthesized steroid hormones that stimulate the expression of gluconeogenesis enzymes, particularly in the liver.

As regards steroid hormone disturbances caused by porphyria-inducing drugs, Lelli et al. (2007) have shown that hexachlorobenzene (HCB) promotes a significant decrease in plasmatic corticosterone (CORT) adrenal synthesis, as well as in their hepatic receptors. It has also been reported that the porphyrinogenic agent AIA alters the hydroxylation of testosterone in rat liver microsomal fraction (Hodgins et al., 1973).

Microsomal UDP-glucuronosyltransferases (UGTs) are a family of isoenzymes which transfer glucuronic acid from UDP-glucuronic acid to endogenous and exogenous compounds and/or their metabolites (Fig. 1), rendering these substances more polar and facilitating their excretion through bile and urine (Burchell and Coughtrie, 1989; Tophly and Burchell, 1990). Two families of UGT (designated UGT1 and UGT2) have been found both in humans and rats. UGT1 isoenzymes are encoded by a single gene (Owens and Ritter, 1995) and are known to conjugate bilirubin and phenols. Protein-encoded UGT2 are steroid-metabolizing enzymes with distinct but overlapping substrate specificities (Mackenzie et al., 1996; Turgeon et al., 2001).

Taking into account that AIA/DDC treatment blocks PEPCK and GP, that PEPCK is positively regulated by GC and negatively regulated by insulin, and that AIA alters the level and synthesis of cholesterol (the biosynthetic precursor of GC) and also impairs the metabolism of testosterone (another steroid hormone), it seemed interesting to study the effect of porphyrinogenic drugs AIA and DDC on the synthesis, metabolism and release of GC, as well as on plasmatic insulin levels, in order to find the potential
causes of PEPCK and GP decreases leading to a lower availability of glucose in hepatocytes. This might be the mechanism underlying the “glucose fault” observed in hepatic porphyrias.

2. Materials and methods

2.1. Materials

AIA was a gift from Roche Co. (Germany). DCC was purchased from Aldrich Chemical Company Inc. (Milwaukee, WI). Triniti CORT (17,21-D,7) corticosterone, specific activity = 70 mCi/mmol and tritiated deoxyCORT (17,21-D,27) deoxycorticosterone, specific activity = 35 Ci/mmol were purchased from New England Nuclear, Life Sciences Products (Boston, MA, USA). AIA, ACTH, 5-adrenoamine, bovine serum albumin, 5-deoxyoxogonine 5’-diphosphate, glucose-1-phosphate, glycerone type III from rabbit, uridine diphospho-glucuronic acid and corticosterone were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Rat insulin RIA kit was purchased from Linco Research Inc. (St. Charles, MO, USA). The antisera against corticotropin was kindly provided by Dr. Celso Gomez-Sanchez from the University of Mississippi Medical Center (MS, USA). Other reagents were of analytical grade.

2.2. Animals and treatment

Animals were treated according to International Guidelines (Guide for Care and Use of Laboratory Animals, National Research Council, USA, 1996, the Council of the European Communities Directive, 86/609/EEC) and also to the guidelines from the Animal Care and Use Committee of the Argentine Association of Specialists in Laboratory Animals (AADALC). Adult female Wistar rats of 200–250 g body weight and purchased from the animal facility of the Facultad de Ciencias Exactas y Naturales (Buenos Aires, Argentina) were used. Animals were kept on rat chow and water ad libitum and housed in stainless steel cages under conditions of controlled temperature (25 °C) and light (12h light–dark cycle from 06:00 am to 06:00 pm). Rats were fasted for 24h before treatment with both drugs and 16h (before sacrifice) and the only food available was water and 0.1% sucrose solution.

Animals were randomly divided into the following groups: group 1 (100 mg AIA + 50 mg DDC/kg body wt.), group 2 (250 mg AIA + 50 mg DDC/kg body wt.), and group 3 (500 mg AIA + 50 mg DDC/kg body wt.). The control group (group 4) only received vehicles; saline solution sc. and corn oil ip.

The same IA and DDC doses have been previously used by other research groups to obtain porphyria models in rats (De Matteis, 1970; De Matteis et al., 1973; Lelli et al., 2005). Three different experiments including three animals from each group were performed.

2.3. Tissue and plasma preparations

Blood samples were collected with a heparinized capillary tube from the ophthalmic venous plexus just before sacrifice and then were centrifuged at 5000 × g for 15 min at 4 °C. Plasma samples were kept at −20 °C until CORT and insulin determinations.

Rats were decapitated 16h after AIA/DDC administration. All assays were performed in rats with a total fasting period of 24h, since this is the optimum way to measure PEPCK and GP activities, as demonstrated by Mazzetti et al. (2004) and Taia et al. (2004). This fasting period enhances the sensitivity of the assays, allowing the standardization of the results obtained with respect to the control.

On the other hand, since the effect of AIA depends on the previous diet (Tschoy et al., 1964), the 8h starvation period prior to the 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 (1:3, v/w) in a solution containing 0.9% NaCl, 0.1 mM Tris–HCl pH 7.4, and 0.5 mM EDTA to determine AIA-5 activity (Marver et al., 1966). The rest of the organ, previously perfused with ice-cold saline solution, was removed and homogenized in a Potter–Elvehjem homogenizer using different solutions. To determine PEPCK activity, a liver fraction was homogenized (1:3, v/w) in 0.25 M sucrose at 0–4 °C. The homogenate was centrifuged for 1h at 100,000 × g, and the resulting supernatant was used for the assay. In the case of GP, livers were homogenized in three volumes of 0.25 M sucrose and 1 mM EDTA. The homogenate was centrifuged for 10 min at 2000 × g and the resulting supernatant was used for the enzymatic determination. To measure UGT activity, a liver sample was homogenized with (1:3, v/w) in 0.15 M KC1 and 1 mM EDTA. The homogenate was centrifuged for 15 min at 9000 × g and the supernatant was centrifuged at 105,000 × g for 60 min to obtain the microsomes. Then the pellet was resuspended in 50 mM Tris–HCl buffer (pH 7.4) containing 0.5 mM MgCl2 and 0.1% sodium cholate, and this solution was used for the enzymatic determination. Adrenals were excised from control and treated animals, quickly isolated and placed on ice-cold saline solution.

2.4. Assays

2.4.1. 5-Aminolevulinic acid synthase

ALA-S hepatic activity was assayd 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 homogenate in a final volume of 2 ml were incubated at 37 °C for 60 min. The product was measured spectrophotometrically at 553 nm as described by Mauzerall and Granick (1956).

2.4.2. Phosphoenoxytroxytrase carboxykinase

PEPCK was measured using deoxyoxygenin 5’-diphosphate as nucleotide substrate. A 50 µl aliquot of 100,000 · g supernatant was used for each assay. The 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).

2.4.3. Glycogen phosphorylase

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, 1 mM 5-AMP, 0.5 M Na2SO4, 0.5 M citrate buffer pH 5.6. Activity was measured as released inorganic phosphate spectrophotometrically determined at 660 nm through the phosphomolybdate complex formed using ascorbic acid as reducing agent (Ayes, 1966).

2.4.4. UDP-glucuronosyltransferase

UGT activity was measured according to Yokota et al. (1999). An aliquot of 200 µl micromsome preparation was assayed in a final volume of 500 µl containing 50 mM Tris–HCl buffer (pH 7.4), 0.5 mM MgCl2, 0.25 mM CORT and 1 mM UDP-glucuronic acid. The mixture was incubated at 37 °C for 60 min. The reaction was stopped by heating in boiled water and then the solution was centrifuged for 15 min at 9000 × g. The supernatant was filtered through a disposable disc and analyzed by HPLC in accordance with the method of Gotelli et al. (1981). Thus, 50 µl filtrated solution was put into a polypropylene eppendorf, mixed with 1.0 ml methylene chloride, shake for 5 min and centrifuged for 1 min. Methylene chloride was carefully decanted into a clean centrifuge tube and 50 ml ethanol/sulfuric acid (70:30, v/v) was added. The solution was shaken for 5 min, centrifuged for 1 min, and then methylene chloride (upper layer) was aspirated and discarded. The remaining layer was immediately placed and kept at −20 °C until it was injected into the liquid chromatograph. A Spectra SERIES (thermo Separation products) Model P200 liquid chromatograph and a FL2000 fluorescence detector set at excitation and emission wavelengths of 366 and 448 nm, respectively, were used in the assays. Separations were carried out on a C18 reverse-phase column. The column was eluted with a mobile phase consisting of 1 vol. of phosphate buffer 0.1 M pH 4.4/8 vol. of tetrahydrofurane/28 vol. of acetonitrile/63 vol. of water.

2.4.5. Plasmatic insulin level

Levels of insulin were measured in plasma with rat insulin RIA kit (Linco Research Inc., St. Charles, MO), as specified by the manufacturer. Glucagon, somatostatin, and pancreatic polypeptide did not show cross-reactivity with this kit. The lowest rat insulin level that could be detected by this assay was 0.1 ng/ml with a 100 µl sample.

2.4.6. Plasmatic corticosterone level

CORT levels were measured in plasma by the method of Gomez-Sanchez by direct radioimmunoassay of this steroid (Gomez-Sanchez et al., 1975). The cross reactivity with aldosterone was less than 0.1%. The sensitivity of the assay was 50 pg/ml.

2.4.7. Corticosterone production by adrenal glands

Each adrenal was cut into four pieces. Tissue was incubated in 2 ml Krebs–Ringer glucose medium containing 10 mM HEPES (pH 7.4) in the presence or the absence of 1 mM ACTH. Incubations were carried out at 37 °C with continuous shaking, and stopped after 1 h by chilling the samples. Media were quickly separated and stored at −20 °C until CORT determination by RIA.

2.4.8. Protein determination

Protein concentration was measured following Lowry et al. (1951) using bovine serum albumin as standard.

2.5. Statistical analysis

Data were subjected to one-way ANOVA using the statistical software BIOMSTAT (BIOMSTAT, 1998).

When the overall F-statistic was significant, multiple comparisons between groups were performed using Tukey–Kramer’s test. The level of significance used was 0.05.

The software was also used to calculate correlations between selected parameters. Values expressed in figures are means ± SEM of nine animals per group since
three different experiments including three animals from each group were performed.

3. Results

3.1. Hepatic ALA-S activity (porphyria marker)

The acute porphyria model was induced and studied using different doses of AIA/DDC. The activity of ALA-S, the key and regulatory enzyme of the porphyrinogen route, showed dose-dependent and statistically significant increases in the three groups studied (Fig. 2a). Activity values were about 190% in group L (100 mg AIA + 50 mg DDC/kg body weight), higher than 300% in group M (250 mg AIA + 50 mg DDC/kg bw) and higher than 400% in group H (500 mg AIA + 50 mg DDC/kg bw) when compared with group C (100%). Mean value ± SEM of group C was 22.0 ± 5.1 nmol ALA/h/g tissue. Enzymatic activities in groups H and M were also statistically different from those of group L.

3.2. Liver PEPCK and GP activities

PEPCK and GP (Fig. 2b) activities were measured in the livers of rats treated with different doses of AIA/DDC to determine the effect of this treatment on gluconeogenesis and glycolysis, respectively.

After 16 h of treatment, PEPCK activity gradually decreased as AIA doses increased (Fig. 2b). The activity of this gluconeogenic enzyme decreased 27%, 35% and 43% with respect to the control in groups L, M and H, respectively. Group C was considered as 100%. Mean value ± SEM of group C was 12.3 ± 0.7 μmole/h/mg protein.

The values observed in groups M and H were also significantly different from those of group L. Fig. 2c shows the response of glycogen phosphorylase to porphyrinogen treatment.

Glycolytic activity expressed as the increase of plasma activities. Thus, GP decreased 23%, 34% and 46% with respect to the control in groups L, M and H, respectively. Group C was considered as 100%. Mean value ± SEM of group C was 22.0 ± 0.5 μmol Pi/min g wet liver. Groups M and H showed statistically significant differences from group C, and values in group H were also statistically different from the group which received the lowest dose (group L).

3.3. Plasmatic insulin level

Since insulin is a key hormone in the regulation of carbohydrates (inhibiting PEPCK gene expression and GP activity), its levels were measured in the plasma of control and treated animals. As shown in Fig. 2d, AIA/DDC treatment promoted a marked dose-dependent increase of plasmatic insulin levels in all the groups. Levels were 316%, 467% and 617% in groups L, M and H, respectively, when compared with group C (100%). Mean value ± SEM of group C was 1.2 ± 0.7 ng/ml. It is worth mentioning that all the groups studied exhibited statistically significant differences between them.

3.4. Plasmatic corticosterone level

Taking into account that glucocorticoids positively regulate gluconeogenic enzyme PEPCK, plasmatic CORT levels were also determined. AIA/DDC-treated rats presented plasmatic CORT values similar to the control, showing a slightly decreasing tendency (Fig. 2e). These values were statistically significant in the group with the highest dose assayed (group H) (20% decrease) when compared with group C (100%). Mean value ± SEM of group C was 126.1 ± 13.3 ng/ml.

3.5. Adrenal corticosterone production

In order to study the metabolism of corticosterone in the present porphyria model, its adrenal synthesis and hepatic metabolism were investigated. CORT adrenal production was studied in the presence and the absence of ACTH. As shown in Table 1, CORT production was substantially lower in the three groups assayed with respect to the control either in the presence or in the absence of ACTH. In both cases (i.e. with and without stimulation), decreases were similar: 25%, 30%, 33% (+ACTH) and 22%, 30%, 31% (−ACTH) in groups L, M and H respectively when compared with the corresponding controls (100%). Mean value ± SEM of group C (+ACTH) was 400.2 ± 20.2 ng CORT/μg protein and (+ACTH) was 100.1 ± 10.2 ng CORT/μg protein for (−ACTH). Multiple comparisons between groups were performed using Tukey-Kramer’s test.

3.6. Liver glucuronidation of corticosterone

Hepatic CORT metabolism was studied measuring UDP-glucuronosyltransferase activity using CORT as substrate and a fluorescent method to follow the reaction. Liver glucuronidation of CORT was markedly and dose-dependently reduced (Fig. 2f). UGT enzyme activity decreased 31%, 47% and 62% in groups L, M and H respectively when compared with group C (100%). Mean value ± SEM of group C was 0.032 ± 0.005 nmol CORT/min mg protein. Values measured in groups M and H also differed significantly from those in group L.

3.7. Correlations among different parameters

When a statistical correlation study was performed between different pairs of the parameters measured, a total of 28 correlations were obtained. Table 2 shows the Pearson correlation coefficients (r) as well as A (intercept) and B (slope) values of the regression lines obtained. This study showed an association between all the variables studied according to the Pearson r values obtained, which ranged from 0.778 to 0.998 (P < 0.0001) for the 28 correlations showed in Table 2.

Fig. 3 illustrates 11 correlations performed between parameters more closely related in the metabolic and physiological aspect. As it can be observed, strong linear positive correlations were obtained in 6 cases: PEPCK vs. stimulated

<table>
<thead>
<tr>
<th>Group</th>
<th>(−ACTH) ng CORT/μg protein</th>
<th>(+ACTH) ng CORT/μg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>100.1 ± 10.2</td>
<td>400.2 ± 20.2</td>
</tr>
<tr>
<td>L</td>
<td>78.1 ± 9.0***</td>
<td>300.5 ± 21.1***</td>
</tr>
<tr>
<td>M</td>
<td>70.3 ± 11.2**</td>
<td>280.2 ± 15.1**</td>
</tr>
<tr>
<td>H</td>
<td>69.2 ± 12.1***</td>
<td>270.3 ± 16.6**</td>
</tr>
</tbody>
</table>

Animals were injected with AIA and DDC doses indicated in Fig. 1. Adrenal tissue was incubated in the presence (+ACTH) or absence (−ACTH) of 1 nM ACTH. Each bar represents the mean ± SEM of nine animals. Mean value ± SEM of group C was 400.2 ± 20.2 ng CORT/μg protein for (+ACTH), and 100.1 ± 10.2 ng CORT/μg protein for (−ACTH).

Multiple comparisons between groups were performed using Tukey-Kramer’s test.

* P < 0.05 is significantly different from (−ACTH) group C.

− P < 0.01 is significantly different from (+ACTH) group C.

# P < 0.05 is significantly different from (−ACTH) group C.

## P < 0.01 is significantly different from (+ACTH) group C.

$ P < 0.01$ is significantly different from (−ACTH) group L.
Fig. 2. Effect of AIA/DDC treatment on the following parameters (a–f) according to AIA dose. Animals were injected subcutaneously (sc) with three different AIA doses and intraperitoneally (ip) with a single DDC dose, and then 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.; group H, 500 mg AIA + 50 mg DDC/kg body wt. The control group (group C) received vehicles: saline solution, sc and corn oil, ip. Each bar represents the mean ± SEM of nine animals. Multiple comparisons between groups were performed using Tukey–Kramer’s test. (a) ALA-S activity. Mean value ± SEM of group C was 22.0 ± 5.1 nmol ALA/h/g tissue. *P < 0.05 is significantly different from group C; †P < 0.05 is significantly different from group L. (b) PEPCK activity. Mean value ± SEM of group C was 12.3 ± 0.7 μmol NADH oxidized/h/mg protein. *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. (c) GP activity. Mean value ± SEM of group C was 2.20 ± 0.25 µmol Pi/min/g wet liver. *P < 0.05 is significantly different from group C; †P < 0.05 is significantly different from group L. (d) Plasmatic insulin level. Mean value ± SEM of group C was 1.2 ± 0.7 ng/ml. *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. (e) Plasmatic CORT levels. Mean value ± SEM of group C was 126.1 ± 13.3 ng/ml. *P < 0.05 is significantly different from group C. (f) UGT enzyme activity. Mean value ± SEM of group C was 0.032 ± 0.005 nmol CORT/min/mg protein. *P < 0.05 is significantly different from group C; †P < 0.05 is significantly different from group L.
Table 2
Correlations between different pairs of parameters.

<table>
<thead>
<tr>
<th>Relationship between</th>
<th>A</th>
<th>B</th>
<th>r ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALA-S vs. PEPC</td>
<td>165.73 ± 10.34</td>
<td>-12.22 ± 1.11</td>
<td>-0.884 ± 13.437</td>
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<tr>
<td>ALA-S vs. GP</td>
<td>155.29 ± 8.20</td>
<td>62.40 ± 4.93</td>
<td>-0.908 ± 12.008</td>
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<td>ALA-S vs. plasmatic insulin</td>
<td>1.91 ± 0.03</td>
<td>11.62 ± 0.78</td>
<td>0.950 ± 10.529</td>
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<tr>
<td>ALA-S vs. plasmatic CORT</td>
<td>297.44 ± 33.74</td>
<td>-2.30 ± 0.31</td>
<td>-0.778 ± 18.020</td>
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<td>ALA-S vs. adrenal stimulated CORT production</td>
<td>188.65 ± 16.50</td>
<td>-0.432 ± 0.05</td>
<td>-0.816 ± 16.578</td>
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<td>ALA-S vs. adrenal CORT production</td>
<td>193.11 ± 19.08</td>
<td>-1.72 ± 0.23</td>
<td>-0.783 ± 17.824</td>
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<td>PEPC vs. GP</td>
<td>150.45 ± 0.45</td>
<td>4.70 ± 0.27</td>
<td>0.946 ± 0.669</td>
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<tr>
<td>PEPC vs. plasmatic insulin</td>
<td>12.95 ± 0.21</td>
<td>-0.87 ± 0.04</td>
<td>-0.981 ± 0.559</td>
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<td>PEPC vs. plasmatic CORT</td>
<td>-10.76 ± 1.84</td>
<td>0.18 ± 0.02</td>
<td>0.880 ± 0.983</td>
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<td>PEPC vs. adrenal stimulated CORT production</td>
<td>-2.41 ± 0.051</td>
<td>0.04 ± 0.002</td>
<td>0.984 ± 0.511</td>
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<tr>
<td>PEPC vs. adrenal CORT production</td>
<td>-2.87 ± 0.78</td>
<td>0.14 ± 0.005</td>
<td>0.994 ± 0.735</td>
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<td>PEPC vs. UGT</td>
<td>3.89 ± 0.34</td>
<td>247.44 ± 15.17</td>
<td>0.942 ± 0.698</td>
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<td>GP vs. plasmatic insulin</td>
<td>2.36 ± 0.06</td>
<td>-0.17 ± 0.01</td>
<td>0.998 ± 0.149</td>
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<td>GP vs. plasmatic CORT</td>
<td>-2.31 ± 0.39</td>
<td>0.03 ± 0.003</td>
<td>0.864 ± 0.209</td>
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<td>GP vs. adrenal stimulated CORT production</td>
<td>-0.52 ± 0.19</td>
<td>0.01 ± 0.006–4</td>
<td>0.891 ± 0.190</td>
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<td>GP vs. adrenal CORT production</td>
<td>-0.634 ± 0.21</td>
<td>0.027 ± 0.002</td>
<td>0.872 ± 0.203</td>
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<td>GP vs. UGT</td>
<td>0.61 ± 0.08</td>
<td>47.65 ± 3.94</td>
<td>0.990 ± 0.181</td>
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<td>Plasmatic insulin vs. plasmatic CORT</td>
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<td>-0.20 ± 0.02</td>
<td>-0.844 ± 1.230</td>
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<td>Plasmatic insulin vs. adrenal stimulated CORT production</td>
<td>16.36 ± 0.95</td>
<td>-0.03 ± 0.003</td>
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<tr>
<td>Plasmatic insulin vs. adrenal CORT production</td>
<td>16.80 ± 1.19</td>
<td>-0.15 ± 0.01</td>
<td>-0.873 ± 1.118</td>
</tr>
<tr>
<td>Plasmatic insulin vs. UGT</td>
<td>10.15 ± 0.39</td>
<td>-272.11 ± 17.63</td>
<td>-0.935 ± 0.811</td>
</tr>
<tr>
<td>Plasmatic CORT vs. adrenal stimulated CORT production</td>
<td>56.71 ± 4.64</td>
<td>0.15 ± 0.01</td>
<td>0.876 ± 4.663</td>
</tr>
<tr>
<td>Plasmatic CORT vs. adrenal CORT production</td>
<td>57.39 ± 6.19</td>
<td>0.59 ± 0.07</td>
<td>0.801 ± 5.785</td>
</tr>
<tr>
<td>Adrenal stimulated CORT production vs. adrenal CORT production</td>
<td>1.56 ± 22.31</td>
<td>3.83 ± 0.27</td>
<td>0.923 ± 20.839</td>
</tr>
<tr>
<td>UGT vs. plasmatic CORT</td>
<td>-0.05 ± 0.001</td>
<td>6.90E ± 0.080–4</td>
<td>0.820 ± 0.005</td>
</tr>
<tr>
<td>UGT vs. adrenal stimulated CORT production</td>
<td>-0.02 ± 0.000</td>
<td>1.34E± 4.009–4</td>
<td>0.920 ± 0.003</td>
</tr>
<tr>
<td>UGT vs. adrenal CORT production</td>
<td>-0.02 ± 0.003</td>
<td>1.34E± 4.978E–6</td>
<td>0.920 ± 0.003</td>
</tr>
</tbody>
</table>

* A, intercept value of the regression line; B, slope value of the regression line; r, Pearson coefficient correlation value, determined from n = 36, SD, standard deviation of the fit. 
* P < 0.0001.

CORT production, UGT vs. stimulated CORT production, UGT vs. plasmatic CORT, GP vs. stimulated CORT production, PEPC vs. UGT, and ALA-S vs. insulin. Likewise, clear linear negative correlations were found in the other 5 cases: ALA-S vs. PEPC, GP vs. insulin, PEPC vs. insulin, ALA-S vs. stimulated CORT production, and ALA-S vs. GP.

The more closely related parameters with the stronger correlations were: GP vs. PEPC, GP vs. insulin, PEPC vs. stimulated CORT production, PEPC vs. UGT, UGT vs. stimulated CORT production, ALA-S vs. insulin and ALA-S vs. GP, and their r values ranged from 0.998 to 0.908 (P < 0.0001).

4. Discussion

The present study shows that the porphyrinogenic treatment combining AIA and DDC caused a noticeable impairment in the adrenal synthesis of GC without modifying adrenal weight. This impairment could be attributed to a failure in one or more steroidogenic enzymes like cytochrome P-450 C21-, P-450 C11-, and P-450 C17-hydroxylases (Fig. 1). In this regard, adrenal cytochrome P-450 C21-hydroxylase has been reported to decrease in guinea pigs treated with polychlorinated biphenyls (Goldman and Yawetz, 1992), drugs that induce an experimental porphyria similar to porphyria cutanea tarda (Smith and De Matteis, 1980). The failure of these steroidogenic enzymes, which work with cytochrome P-450 and are involved in the biosynthesis of GC (Provencher et al., 1992; Matkovic et al., 2001), could mirror the damage caused on this cytochrome by porphyrinogenic drugs AIA and DDC (Fig. 1). In fact, it has been reported that AIA, an allyl-containing acetaldehyde compound, increases the destruction of liver heme, particularly that of cytochrome P-450. This destruction involves the conversion into abnormal green pigments [N-alkyl-protoporphyrin IX], and at the same time the stimulation of ALA-S activity (Smith and De Matteis, 1980). In addition, DDC, a compound containing methyl groups, is a potent depleter of hepatic heme due to its ability to destroy heme (N-methyl-protoporphyrin IX) and inhibit its synthesis at the ferrochelatase level (Marks et al., 1988). As a consequence, there is an excessive accumulation and excretion of protoporphyrin IX and heme pathway intermediates through the increase of rate-limiting enzyme ALA-S, as observed in the present report.

Apparently reduced adrenal GC synthesis could not be the consequence of a lack of cholesterol (its biosynthetic precursor), since its plasmatic level and hepatic synthesis have been reported to increase in mice treated with AIA or other porphyrinogenic drugs like HCB or griseofulvin (Wada et al., 1969). The present results showing a similar decrease in adrenal GC biosynthesis of intoxicated animals in the presence or the absence of ACTH proved that the stimulation process of cortical steroids synthesis elicited by this adrenohypophysis hormone is not altered by AIA/DDC treatment. This observation also suggests that the porphyrinogenic treatment does not impair the production and/or secretion of this tropin from the hypophysis.

Assays on the hepatic metabolization process of GC through UGT activity measured using GC as endogenous substrate show a decrease in the UGT activity as a consequence of AIA/DDC treatment (Fig. 1). This decreased GC metabolization caused by AIA/DDC treatment shown in this work would be in line with the reported depressor effect of AIA on the metabolization of testosterone, another steroid hormone (Hodgins et al., 1973).

Taking into account the effect of AIA/DDC on adrenal synthesis and on hepatic metabolization, the depressor effect observed in both processes might be more or less counterbalanced between them to render plasmatic GC levels in groups L and M slightly lower than in the control. In the case of animals treated with the highest AIA dose (group H), the significant effect on the hepatic metabolization process would exceed (2-fold) that promoted in anabolic adrenal production, thus resulting in a marked decrease of this corticoid hormone in the plasmatic levels. Since GC have a positive effect on PEPC regulation (Hanson and Reshef, 1997) and a permissive effect on GP activation by glucagon and epinephrine (Altuna et al., 2006), the reduced GC levels observed in the present work could contribute to PEPC and GP blockages.
As regards insulin, the other hormone that regulates carbohydrate metabolism (Melloul et al., 2002), it was observed that the porphyria-inducing treatment promotes a net dose-dependent increase in its plasmatic levels. Taking into account that: (1) insulin degradation is a sequential multistep process involving the endocytosis of the insulin–receptor complex, cleavages in the B chain, the reduction of disulfide bonds, multiple proteolysis of the remaining fragments, the insulin-degrading enzyme that is the primary enzymatic mechanism to initiate cellular insulin processing and degradation (Duckworth et al., 1998); (2) the reduction of disulfide bonds is catalyzed by glutathione insulin transhydrogenase, now called protein disulfide isomerase, yielding an intact A chain
and several B chain fragments (Seabright and Smith, 1996); and (3) AIA/DDC treatment promotes an oxidative environment with increases in reactive oxygen species (ROS) (Lelli et al., 2005) and depressed reduced glutathione levels (GSH) (Faut, 2007), we hypothesize that the increase in plasmatic insulin is probably a consequence of AIA/DDC drugs impairing the degradation process, at least at the protein disulfide reduction step, among other targets (Fig. 1). In this regard, the correlation studies (Table 2 and Fig. 3) reinforce this idea, showing a strong correlation between the increase of ALA-S (that generates ROS from ALA) and the increase of insulin.

The decreases of PEPC and GP activities as a function of AIA dose strongly correlate with the increase of plasmatic insulin triggered by AIA/DDC treatment. Similar fit strength linear correlations between the decreases of PEPC and GP activities vs. the decrease of GC adrenal production were obtained when a similar statistical analysis was performed (Table 2 and Fig. 3). This allows us to think that the alterations promoted by drugs treatment on insulin and GC play a role in the gluconeogenic and glycogenolytic blockages of PEPC and GP, respectively (Fig. 1).

The decrease in PEPC activity could mirror a depressed enzyme synthesis as a response to insulin increase via the AF2 site and phospho-tidilinositol-3-kinase, since it has been reported that this protein hormone negatively regulates PEPC gene expression (Hanson and Reshef, 1997), and that insulin cannot act solely blocking the factors present at the AF2 site. Indeed, it has been reported that the insulin signal transduction required for the inhibition of PEPC gene transcription involves phosphotidilinositol-3-kinase (Sutherland et al., 1995).

As mentioned above, the porphyria-inducing treatment promotes glycogenolytic impairment by decreasing GP. This effect on GP activity would be ascribed to insulin increase (Fig. 1). In fact, insulin indirectly activates phosphoprotein phosphatase (PP-1) and phosphodiesterase. As a consequence of these alterations, a decrease in GP activity was achieved: both by direct PP-1 catalyzed dephosphorylation of GPs and by the blockage on cAMP-PKA-dependent phosphorylation cascade that ends with the formation of new GPs (Johnson, 1992).

This hormonal disruption promoted by AIA/DDC on GC and insulin could contribute to the negative control of both glucose synthesis and its release from glycan storage, through PEPC and GP regulation, thus modulating porphyria. In fact, glucose represses heme pathway regulatory enzyme ALA-S (Tschudy et al., 1964), which is induced in porphyrias (Kappas et al., 1995).

In summary, porphyrinogenic drugs AIA and DDC, which induce the heme rate-limiting enzyme ALA-S, significantly disturb the status of hormones that regulate carbohydrate metabolism by increasing insulin levels and decreasing GC synthesis, metabolization and plasmatic levels (Fig. 1). In this acute porphyria model, the gluconeogenic and glycogenolytic blockages caused by PEPC and GP depressed activities, respectively, would be mainly the consequence of the negative regulatory action of insulin on these enzymes (Fig. 1). This hormone acts on PEPC and GP at the gene and protein level, respectively. GC could also contribute to PEPC blockage because their status was found depressed with this drug treatment and because they are positive effectors on PEPC. These disturbances in carbohydrates and their hormonal regulation through ALA-S de-repression would enhance the porphyria state promoted by the drugs on heme synthesis and destruction (Fig. 1). This might be the mechanism underlying the “glucose effect” observed in hepatic porphyrias.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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