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Hepatic 11 beta-hydroxysteroid dehydrogenase 1 involvement in alterations of glucose metabolism produced by acidotic stress in rat

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11 beta-hydroxysteroid dehydrogenase (HSDs) enzymes regulate the activity of glucocorticoids in target organs. HSD1, one of the two existing isoforms, locates mainly in CNS, liver and adipose tissue. HSD1 is involved in the pathogenesis of diseases such as obesity, insulin resistance, arterial hypertension and the Metabolic Syndrome. The stress produced by HCl overload triggers metabolic acidosis and increases liver HSD1 activity associated with increased phosphoenolpyruvate carboxykinase, a regulatory enzyme of gluconeogenesis that is activated by glucocorticoids, with increased glycaemia and glycogen breakdown. The aim of this study was to analyze whether the metabolic modifications triggered by HCl stress are due to increased liver HSD1 activity. Glycyrrhetinic acid, a potent HDS inhibitor, was administered subcutaneously (20 mg/ml) to stressed and unstressed four months old male *Sprague Dawley* rats to investigate changes in liver HSD1, phosphoenolpyruvate carboxykinase (PECPK) and glycogen phosphorylase activities and plasma glucose levels. It was observed that all these parameters increased in stressed animals, but that treatment with glycyrrhetinic acid significantly reduced their levels. In conclusion, our results demonstrate the involvement of HSD1 in stress induced carbohydrate disturbances and could contribute to the impact of HSD1 inhibitors on carbohydrate metabolism and its relevance in the study of Metabolic Syndrome Disorder and non insulin-dependent diabetes mellitus.

Key words: HSD1, Stress, Glycyrrhetinic acid, Glucose, PEPCK.

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11 beta-hydroxysteroid dehydrogenase (HSDs) enzymes regulate the activity of glucocorticoids (GCs) in target organs (28, 10). Their two existing isoforms are HSD1 and HSD2. HSD1, distributed mainly in CNS, liver and adipose tissue (30), is bidirectional, catalyzing *in vivo* the conversion of inactive GC into active GC, and *in vitro* the inverse reaction using corticosterone as substrate. Although it was the first isoform discovered, its biological action has been studied in depth only recently (27).

Previous reports demonstrate that the stress produced by HCl increases HSD1 activity in liver and adrenal gland in a rapid and highly reproducible way (34, 1). The changes caused by HCl on this enzyme activity would contribute to the development of the Metabolic Syndrome, characterized by hyperglycaemia, dyslipemia, diabetes, arterial hypertension and hypercortisolemia. The effects of HCl-induced stress observed were: a) over ten-fold increase in circulating GC levels (35), and b) stimulation of HSD1 adrenal activity, which increased the adrenal GC supply, thus activating phenylethanolamine N-methyl transferase, and improving the synthesis of epinephrine as part of the stress response (34).

Studies carried out by our research group showed that the stress exerted by HCl increased liver HSD1 activity, which is associated with increases in liver phosphoenolpyruvate carboxykinase (PEPCK) activity and glycaemia, and with a decrease in glycogen level (1). PEPCK, the rate-limiting enzyme of gluconeogenesis, plays a key role in the synthesis of glucose in liver and kidney. PEPCK gene expression can be increased by several factors such as cyclic AMP, thyroid hormones and GC. Conversely, insulin inhibits its expression (9).

Glycogen is the storage form of carbohydrates for virtually every organism from yeast to primates. Glycogen phosphorylase (GP) isozymes have been found in liver and muscle. In fact, two GP conformers, T (inactive) and R (active), with different ligands have been described (5). Thus, GP activity is modulated by the allosteric effectors glucose or caffeine to promote the less active T state and by the activator (AMP) to promote the more active R state (8). Phosphorylase is regulated by a number of small-molecular-weight effectors that bind to three sites on the enzyme. Recently, a fourth site referred to as the indole inhibitor site was identified. The indole derivative GP inhibitors (GPIs) tested were all potent inhibitors. However, endogenous inhibitors (glucose, ADP, ATP, fructose 1-phosphate, glucose 6-phosphate, UDP-glucose) and AMP significantly reduced the inhibitory effect of GPIs (7). Inhibition of hepatic GP is a promising and useful treatment strategy for attenuating hyperglycaemia in type 2 diabetes (8, 3). Glucogenolysis is a key contributor to hepatic glucose output, and GP is the enzyme that catalyzes this process (11).

Hepatic glucose production is increased in people with type 2 diabetes. Glucose released from storage in liver glycogen by phosphorylase accounts for approximately 50% of the glucose produced after an overnight fast. Glucose is the only intracellular effector that changes greatly *in vivo*. Thus, it may be the major regulator of human liver GP activity (6).

The aim of the current study was to observe whether the metabolic modifications produced by HCl stress, previously described by us, are due to increased liver HDS1 activity. For this purpose, we

administered glycyrrhetinic acid (GA, Fig. 1), a potent HSD inhibitor (19, 21), to HCl stressed and unstressed animals and studied the modifications in the activities of HSD1, PEPCK and GP liver enzymes and plasma glucose levels.

Material and Methods

Chemicals.– Tritiated corticosterone [1,2,6,7-^{3}H]corticosterone (76.5 µCi/nmol) was purchased from New England Nuclear, Life Sciences Products, Boston, MA., USA. Reagents were obtained from Merck-Darmstadt (Federal Republic of Germany), Sigma (St. Louis, MO, USA) or Aldrich (Milwaukee, WI, USA), unless otherwise specified. Liquid enzymatic glycaemia AA was generously provided by Wiener Lab. Liquid scintillation fluid used was Opti Phase "Hi Safe" 3 (Wallac, Finland). 18B-Glycyrrhetinic acid was from Sigma Chemical Co.

Animals and treatment.– Animals used in this study were treated in accordance with the principles and guidelines of the Guide for the Care and Use of Laboratory Animals, National Research Council, (National Academy Press, Washington, D.C. 1996). Four months old male *Sprague Dawley* rats (300-350 g) were used. They were kept on rat chow and water *ad libitum*. All *in vivo* experiments were carried out between 9:00 am and 11:00 am. Three animals were used per group in each experiment and six to eight experiments were performed by duplicated for each measured parameter. Animals were anesthetized with ether and blood was withdrawn from the abdominal aorta. Then, 0.9% NaCl was administered in the same way, in order to wash livers before removal. Food was withdrawn 12 h before the experiment.

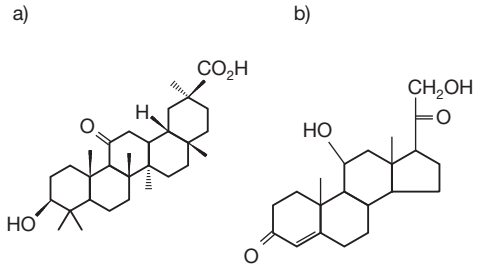

Fig. 1. *(a) Structure of glycyrrhetinic acid GA, a HSD1 inhibitor and of corticosterone (b), substrate of HSD1.*

Animals were subjected to stress already tested in our laboratory (34, 35, 15). They were divided into four groups:

- *Control group:* Unstressed animals whose blood was withdrawn from the aorta before the liver was removed. They received previously 2 doses of 0.5 ml olive oil administered subcutaneously 18 h and 4 h before sacrifice.

- *Control + GA group (C + GA)*: Unstressed animals previously injected with 2 doses of 20 mg/ml GA dissolved in olive oil, 0.5 ml each time. The first dose was administered subcutaneously 18 h before sacrifice and the second intramuscularly 4 h before liver removal, since GA exerts maximum inhibition of *in vivo* activity 3-4 h after administration and absorption (13).

- *Acidotic group (HCl)*: Animals subjected to an overload of 10 ml 200 mM HCl by oropharyngeal gastric intubation 2 h before liver removal. This is a standard procedure used by the National Toxicology and Environmental Program.

- *Acidotic + GA group (HCl + GA)*: Animals subjected to an overload of 10 ml 200 mM HCl by oropharyngeal gastric intubation 2 h before liver removal, and previously injected with 2 doses of 20 mg/ml GA dissolved in olive oil, 0.5 ml each time. The first dose was administered subcutaneously 18 h before sacrifice and

the second intramuscularly 4 h before liver removal.

Glycaemia determination.– Two hours after stress was induced, blood was withdrawn using 5 ml heparinized syringes immediately before the liver was removed, and plasma was separated by 1,000 x *g* centrifugation for 15 min. Glycaemia was measured using *Liquid Enzymatic Glycaemia AA* (Wiener Lab). Data from spectrophotometric determination at 505 nm were transformed by multiplying the reading of the unknown sample by 1/standard, in mg/dL glucose concentration.

Preparation of liver microsomes for HSD1 determination.– Livers were weighed and homogenized with a Teflon homogenizer, using 25 ml Krebs/Ringer phosphate buffer (1.2 mM $MgSO_4$, 128 mM NaCl, 5 mM KCl, 2.8 mM $CaCl_2$, 10 mM sodium phosphate buffer, pH 7.4; KRB) per *g* of tissue. Then, they were centrifuged at 12,000 x *g* for 30 min at 4 ºC. Pellets were discarded and supernatants were centrifuged at 105,000 x *g* for 60 min at 4 ºC. Pellets obtained from 1 g original tissue were resuspended in 250 µl KRB. Protein concentrations of the microsomes obtained were measured using Bradford's method (4).

Preparation of liver cytosol and determination of phosphoenolpyruvate carboxykinase (PEPCK).– Livers were homogenized (1:3 w/v) in 0.25 M sucrose and centrifuged at 12,000 x *g* at 0-4 ºC for 20 min. Supernatants were centrifuged at 105,000 x *g* at 0-4 ºC for 80 min and the resulting supernatants were used for PEPCK determination (22).

Preparation of liver fraction for glycogen phosphorylase (GP) determination.– Livers were homogenized (1:3 w/v) in 0.25 M sucrose and 1 mM EDTA. The homogenate was centrifuged for 10 min at 2,000 x *g* at 0–4 ºC and the resulting was supernatant used for GP determination (18).

Enzymatic activity determinations.– *HSD1 w*as determined by measuring the conversion rate of [^{3}H1,2,6,7] corticosterone into [^{3}H1,2,6,7] 11-dehydrocorticosterone. Microsomal suspensions (15 µg protein/ml), previously determined by Bradford's method, were incubated in 250 µl KRB, 2 nM NADP, 50 nM [^{3}H1,2,6,7] corticosterone, and 0 to 10 µM radio inert corticosterone for 10 min at 37 ºC (20). The reaction was terminated with ice and two 500-µl samples were extracted with ethyl acetate, evaporated with nitrogen at 37 ºC, resuspended in methylene chloride/methanol 95:5, and sown on a Silica Gel 60 F 254 plate (Merck-Darmstadt, Germany) using 10 µg 11-dehydrocorticosterone and 10 µg corticosterone as standards. Steroids were separated by thin layer chromatography (TLC) in a container saturated with 96% chloroform/ethanol 92:8. Silica areas corresponding to each corticoid studied were scraped and 1 ml scintillation fluid was added to them. The following day, radioactivity was measured with a liquid scintillation counter (34). Activity was expressed as nmol/min/mg protein.

PEPCK activity was measured using deoxyguanosine 5´-diphosphate as nucleotide substrate. Aliquots of 50 µl 105,000 x *g* supernatant were used in each assay. Oxaloacetate formed during the reverse enzyme reaction was determined by reduction with malic dehydrogenase in the presence of NADH. The reaction was allowed to proceed for 2 min at room temperature (26). Changes in absorbance were measured spectrophotometrically at 340 nm. Activity was expressed as µmol/min/mg protein.

GP activity was assayed according to Newman and Armstrong (23). Final reagent concentrations were 50 nM glucose-1-phosphate, 1% rabbit liver glycogen III 0.15 M NaF, 1mM 5`-AMP, 0.5 M Na_2SO4, 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 (2). Activity was expressed as µmol/min/mg protein.

Statistical analysis.– Values were expressed as means ± standard error (SEM). Differences between groups were evaluated by analysis of variance (ANOVA) using the statistical software BIOMSTAT (BIOMSTAT, 1998), followed by the Tukey-Kramer test. Statistical significance was set at $p < 0.05$.

Results

HSD1 activity.– An important increase (38%) was observed in HSD1 activity of stressed animals from the HCl group when compared with the Control group. Upon GA treatment, HSD1 activity decreased by 57% in animals from the Control + GA group and by 26% in the HCl + GA group, when compared with unstressed animals (Control group), and by 53% when compared with the HCl group. It is noteworthy that animals treated with GA showed similar HSD1 activity values, even when enzymatic activity had increased with stress (Fig. 2.a).

PEPCK activity.– The activity of gluconeogenic enzyme PEPCK was measured in all groups studied (Fig. 2). As it can be seen a significant increase (86%) in enzyme activity of stressed animals (HCl) was observed when compared with unstressed animals (Control). The administration of GA to animals stressed with HCl decreased PEPCK activity by 35% when compared with the stressed group (HCl). A slight but significant 20% difference was found between control and stressed animals treated with GA (Fig. 2.b).

GP activity.– The glucogenolytic activity of the GP enzyme was studied in all the groups. Figure 3.a shows 80% increase of this activity in stressed animals (HCl) when compared with the Control group. This increase was significantly lower (31%) in stressed animals previously treated with GA (HCl + GA) when compared with the Control. No significant differences were found between Control + GA and Control animals.

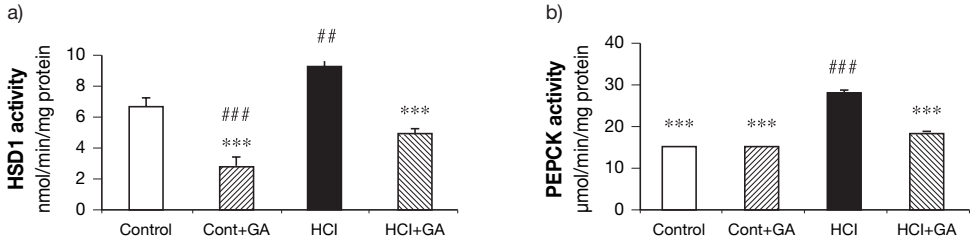

Fig. 2. *Effect of GA on HSD1 (a) and PEPCK activity (b) in control and stressed animals.* Results represent mean ± SEM of 8 different experiments performed in duplicate. ###p<0.001; ##p<0.01 *vs.* Control group. ***p<0.001 *vs.* HCl group. GA, glycyrrhetinic acid; HSD1, 11,-hydroxysteroid dehydrogenase 1; PEPCK, phosphoenolpyruvate carboxykinase.

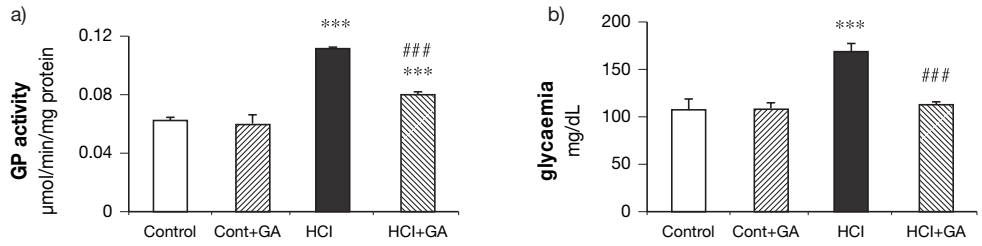

Fig. 3. *Effect of GA on GP activity (a) and on glycaemia (b) in control and stressed animals.* Each bar represents mean ± SEM of 6 different experiments performed in duplicate. ***p<0.001 *vs.* control group or control + GA group; ###p<0.001 *vs.* HCl group. GA, glycyrrhetinic acid; GP, glycogen phosphorylase.

Glycaemia.– Results show that glycaemia increased by 56% in stressed (HCl) animals, but that this was reverted when they were treated previously with GA (HCl + GA). On the other hand, no differences were found between the Control groups when GA was administered (Fig. 3.b).

Discussion

Since stress has a multifactorial influence on metabolic changes and the numerous mechanisms that trigger them, we administered GA, a known HSD1 inhibitor, both to stressed and unstressed animals and studied hepatic metabolism under HCl-induced stress.

The stress caused by HCl overload triggers metabolic acidosis and produces an increase in liver HSD1 activity associated with increased PEPCK, a regulatory enzyme of gluconeogenesis shown to be activated by GCs (1). Data strongly suggest that the increase in HSD1 liver activity caused by increasing the supply of active GCs in this organ is responsible for the metabolic changes observed.

The administration of GA significantly reduced liver HSD1 activity. This was reflected in PEPCK, GP and glycaemia activities of stressed animals, which dramatically decreased. In control animals, however, the decrease observed was not significant.

The fact that GP increases with stress could be attributed to the effect of the higher level of active GCs generated by increased HSD1 activity. GCs are known to have a permissive or potentiating effect on the stress-induced glucagon and epinephrine glucogenolytic action (1). In fact, glucagon and epinephrine activate GP through AMPc, increasing glucogenolysis and glucose, an effect that could mask the inhibitory effect exerted on GP by the glycaemia. Considering that there are two isoforms, T (inactive) and R (active), and that AMPc promotes the active form (5, 8), the R isoform would predominate in HCl-induced stress situations. The reduced GP activity observed in stressed animals treated with GA would respond to a lower hormone stimulus of GP via glucagon, epinephrine and AMPc. In this case, the balance between T and R would be more displaced towards the T form.

The increase in PEPCK activity observed in stressed animals would be due to an increase in the protein synthesis of the enzyme at the transcription and post transcription levels, promoted by the

higher level of active GCs generated by HSD1, since PEPCK activity is known to be regulated positively by GCs (1) and a synthetic GC such as methylprednisolone was recently reported to exert a dual regulatory (transcriptional and post transcriptional) effect on PEPCK (16). The reduction in PEPCK activity in stressed animals caused by the administration of the HSD1 inhibitor confirms that the modulation of this activity is regulated by active GCs.

The significant decrease in glycaemia observed upon GA administration in stressed animals can be attributed to the inhibitory effect exerted by GA on HSD1. The isoform decreased in active GCs that revert, as demonstrated in this study, the increased activity of liver enzymes PEPCK and GP, which are closely involved in the increase of glycaemia during stress situations.

The most probable explanation of the results obtained with unstressed groups is that animals were fasting for 12 h and, therefore, gluconeogenic and glucogenolytic enzyme activities remained more or less constant for the needs of the organism, even in the presence of HSD1. On the other hand, in the stressed group, the enzymes studied depended both on GC circulating levels and, to a large extent, on the presence of active GCs that exert a paracrine effect on the specific organ, increasing glycaemia either directly or indirectly.

Considering that: 1) GA (3 beta-hydroxy-11-oxo-18 beta,20 beta,olean-12-en-29-oic acid) shares some structural similarity with corticosterone as shown in Fig. 1; 2) GA is a competitive inhibitor of HSD1 (14), whose inhibition *in vivo* was demonstrated in the current study; and 3) HSD1 is a protein bound to the endoplasmic reticulum (ER) through its N-terminal transmembrane domains (30, 25) whose active site is located in the lumenal compartment of the ER, we can speculate that GA would be funnelled to the HSD1 active site cleft from the hydrophobic membrane environment in a similar way to that proposed by Ogg (24) for corticosterone, its natural substrate, thus exerting its inhibitory ability on the dehydrogenase.

However, an effect at the protein synthesis level cannot be discarded since GA was reported to have a "pretranslational" effect on HSDs in addition to its competitive inhibitory effect (33, 17).

Results showing that both HSD1 activity and its corresponding stimulatory effect are blocked by GA administration agree with reports from clinical studies and basic research which observed that inhibitors of HSD1 activity produced a better adaptation of the organism in patients with Cushing syndrome (19, 12, 29, 32).

They also agree with recent studies using the transgenic method and showing that mice that overexpress HSD1 in adipose as well as in liver tissue present Metabolic Syndrome symptoms, whereas those having a transgenic deletion of HSD1 avoid the metabolic complications of obesity (31).

The cellular functions of 11 beta-HSD1 as cortisol-producing catalyst in insulin resistance and metabolic disorders establish this enzyme as a novel pharmacological agent, not only in non insulin-dependent diabetes mellitus but also in the Metabolic Syndrome Disorder. In conclusion, our results involves HSD1 in stress induced carbohydrate disturbances and provide further knowledge of one of HSD1 inhibitors, whose effects and impact on carbohydrate metabolism are relevant for the treatment of the above mentioned diseases.

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