Original Research

Restricted feeding modulates the daily variations of liver glutamate dehydrogenase activity, expression, and histological location

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Impact statement

For the first time, we are reporting the changes in daily rhythmicity of glutamate dehydrogenase (GDH) mRNA, protein and activity that occur in the liver during the expression of the food entrained oscillator (FEO). These results are part of the metabolic adaptations that modulate the hepatic timing system when the protocol of daytime restricted feeding is applied. As highlight, it was demonstrated higher GDH protein and activity in the mitochondrial fraction. These results contribute to a better understanding of the influence of the FEO in the energy and nitrogen handling in the liver. They could also be significant in the pathophysiology of hepatic diseases related with circadian abnormalities.

Abstract

Glutamate dehydrogenase is an important enzyme in the hepatic regulation of nitrogen and energy metabolism. It catalyzes one of the most relevant anaplerotic reactions. Although its relevance in liver homeostasis has been widely described, its daily pattern and responsiveness to restricted feeding protocols has not been studied. We explored the daily variations of liver glutamate dehydrogenase transcription, protein, activity, and histochemical and subcellular location in a protocol of daytime food synchronization in rats. Restricted feeding involved food access for 2 h each day for three weeks. Control groups included food *ad libitum* as well as acute fasting (21 h fasting) and refeeding (22 h fasting followed by 2 h of food access). Glutamate dehydrogenase mRNA, protein, activity, and histological location were measured every 3 h by qPCR, Western blot, spectrophotometry, and immunohistochemistry, respectively, to generate 24-h profiles. Restricted feeding promoted higher levels of mitochondrial glutamate dehydrogenase protein and activity, as well as a loss of 24-h rhythmicity, in comparison to ad libitum conditions. The rhythmicity of glutamate dehydrogenase activity detected in serum was changed. The data demonstrated that daytime restricted feeding enhanced glutamate dehydrogenase protein and activity levels in liver mitochondria, changed the rhythmicity of its mRNA and serum activity, but without effect in its expression in hepatocytes surrounding central and portal veins. These results could be related to the adaptation in nitrogen and energy metabolism that occurs in the liver during restricted feeding and the concomitant expression of the food entrainable oscillator.

Keywords: Glutamate dehydrogenase, mitochondria, food synchronization, daily variations

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Introduction

L-Glutamate dehydrogenases (GDHs) (EC 1.4.1.2-4) are a family of enzymes that catalyze the reversible conversion of glutamate and α-ketoglutarate, which is important in the handling of cellular ammonium.[1] In this reaction, a primary amine, glutamate, acts as a nitrogen donor, whereas a ketoacid, α-ketoglutarate, is the acceptor. GDHs are present in bacteria and are ubiquitous in eukaryotes.[2] GDH is a homohexameric protein that mostly resides within the mitochondria, where it catalyzes one of the most important anaplerotic reactions, replenishing the intermediates of the Krebs cycle.[3] However, it has been reported that during liver regeneration after two-thirds of partial hepatectomy, GDH and other mitochondrial enzymes are released into the serum independently of any tissue damage.[4]

The *Glud1* gene promoter contains the following transcription binding sites: ZIC2/Zic2, Pax-2, Pax-2a, Elk-1, ER-alpha, HOXA9, HOXA9B, Meis-1, Meis-1a, and FOXJ2 (long isoform). In the liver, gender-specific differences were reported in the cellular distribution of the *Glud1* transcript.[5] Hepatic GDH expression can also be positively regulated by signaling molecules and hormones such as cAMP,

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cortisone and ACTH, and it is repressed by insulin and corticosterone.[6]

GDH in animals, but not in other organisms, gained regulatory properties that allow a more finely tuned metabolic modulation: (1) it is controlled by a redox reaction, responding to both $NAD(P)^+$ and NAD(P)H; (2) it is allosterically regulated by a wide array of ligands such as ADP and leucine (activators), and GTP and ATP (inhibitors); (3) its catalytic activity is inhibited by sirtuin 4-dependent ADP-ribosylation, and activated by sirtuin 3-dependent deacetylation.[7,8]

In the liver, GDH constitutes about 1% of the protein content and plays an important role in the biochemical transformation of glutamate[9]; therefore, it is part of the metabolic response that, in the liver, coordinates the Krebs and urea cycles, gluconeogenesis, the ratios glutamate/α-ketoglutarate and glutamine/glutamate, and fatty acid oxidation.[9] Besides its important biochemical role, GDH has been implicated in pathological conditions such as cancer, hyperinsulinism, and ethanol-induced cell necrosis.[10–12]

Using a food restriction protocol (2 h of daytime food access for three weeks), we have reported a set of biochemical adaptations in liver mitochondrial activity and metabolic nitrogen-handling responses that could influence GDH functional properties: (1) enhanced mitochondrial synthesis of ATP,[13] (2) reduced mitochondrial lipid peroxidative activity,[14] (3) increased gluconeogenesis and ureagenesis,[15,16] and (4) modified activity and expression of glutamine synthetase and GABA transaminase.[17,18] This protocol has also been used to study an alternative circadian clock, different from the suprachiasmatic nucleus, which is entrained by food access (known as the food entrainable oscillator, FEO).[19] FEO expression promoted by repeated cycles of fasting and re-feeding over at least three weeks elicits a chronostatic/rheostatic adaptation in the liver, including a behavioral anticipatory activity without a detrimental impact on metabolic networks and cellular energy status,[20] which is not present after a single cycle of fasting and re-feeding.[21]

In this study, we have further investigated the effect of the chronobiological protocol of 2 h daytime restricted feeding on the daily profile of rat liver GDH at different levels of regulation such as mRNA expression, GDH protein content of subcellular fractions by Western blot and immuno-histochemistry as well as mitochondrial, cytosolic and serum enzymatic activity. The results showed significant changes in the 24-h rhythmicity of liver GDH in response to the restricted feeding schedule and suggest the possibility that liver GDH adaptations could be part of the FEO.

Methods

Animals and housing

Experimental animals and conditions were previously reported.[22,23]

Experimental design

Experimental protocol was similar to the one reported previously.[23]

Quantitative real time polymerase chain reaction

The relative mRNA expression of *Glud1* in the liver was analyzed by quantitative real time polymerase chain reaction (RT-qPCR) using a protocol previously reported.[17] Primer sequences were synthesized by Sigma-Aldrich Co. (St. Louis, MO, USA), and the corresponding sequences were: for *Glud1*, forward 5′-ACAGCAGAGTTCCAGG ACAG -3′, reverse 5′-GTCTATGTGAAGGTCACGCC-3′ (GenBank number NM_012570.2) and for ribosomal protein S18 (rps18), forward 5′-TTCAGCACATCCTGCGAGTA-3′, reverse 5′-TTGGTGAGGTCAATGTCTGC-3′ (GenBank number BC126072.1). Amplification for *Glud1* and housekeeping rps18 was performed in SYBR Green Master Mix, according to the following protocol: DNA denaturation at 95°C for 10 min, followed by 40 amplification cycles consisting of 10 s at 95°C, 30 s at 63°C for Glud1 or 60°C for rps18 and 30 s at 72°C.[17]

Western-blot

Tissue sampling and subcellular fractionation were done according to the report of Aguilar-Delfín et al.[24] Liver homogenates and mitochondrial fractions were subjected to denaturing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions. Equal amounts of proteins (40 μg of homogenates, 80 μg of cytoplasmic fraction and 25 μg of mitochondrial fraction, as measured by Bradford method) were separated by 10% SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes (Immobilon-P, Millipore, Darmstadt, Germany). Western blot was done as previously reported using the following antibodies:[23] mouse anti-GDH antibody (clone GS-6, MAB302; Millipore, Darmstadt, Germany) diluted 1/1000 in Tris-Buffered Saline and Tween 20 (TBST), and mouse anti-glyceraldehyde 3-phosphate dehydrogenase antibody (ab56676; Abcam, Cambridge, UK) diluted 1/1000, as control. For mitochondrial fractions, rabbit anti-VDAC1/Porin antibody (ab15895; Abcam) diluted 1/1000 was used.

GDH activity

GDH activity was measured by an enzyme-coupled reaction according to Schmidt, 1974.[25] The redox reaction was followed spectrophotometrically by the oxidation of NADH to NAD^+ at 340 nm coupled to the conversion of α-ketoglutarate to glutamate in the presence of ammonium. Results were calculated using the extinction coefficient of $6220\ M^{-1}cm^{-1}$. GDH activity was quantified in serum as well as in liver mitochondrial and cytosolic fractions.

Immunohistochemistry

Immunohistochemical processing and analysis were done following a previous report, using an antibody against GLUD-1 (ab168352) diluted 1:100 in TBST buffer.[16]

Data analysis

Data display and statistical analysis were done as reported previously.[15]

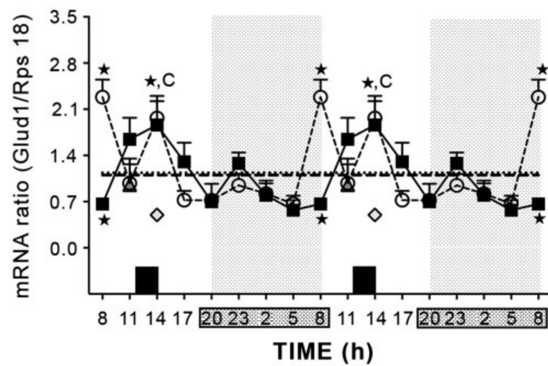

Figure 1 Daily variations of mRNA of liver glutamate dehydrogenase under the protocol of daytime restricted feeding. Data are depicted as a double-plotted 24-h profile. Group fed ad-libitum, AL (filled squares); group under restricted feeding, RF (white circles); group with acute fasting (Fasted; gray triangles); group with acute fasting followed with refeeding, Refed (gray rhombus). Light on at 08:00 h and off at 20:00 h. Hatched bars indicate mealtime (12:00 h–14:00 h). Data are expressed as mean ± SEM of at least four independent observations. Note that the symbols of the Fasted and RF groups (at 11:00 h) are coincident. ⋆Time points with a significant difference within the group detected by one-way ANOVA (Tukey *post hoc* test, $P < 0.05$); [c]significant difference between Refed group and AL and RF groups detected by Student's *t*-test ($P < 0.05$)

Results

RF promoted a change in the GDH mRNA rhythmicity

Glud1 gene expression was determined by RT-qPCR. The 24-h time course is shown in Figure 1. The ad libitum (AL) group showed increased levels of GDH mRNA during the light period (63%) with a peak at 11:00 h and 14:00 h. The restricted feeding (RF) group also showed the peak at 14:00 h, but an additional peak at 08:00 h implicating a changed rhythmicity, and higher proportion in diurnal values (89%). On average, GDH mRNA did not differ between AL and RF rats. Values for the acute control of fasting condition group (Fa) were similar to those of the AL and RF rats (at 11:00 h). The acute refed group showed no differences from the Fa group, i.e. it did not show the elevated values observed in the AL and RF groups (at 14:00 h).

Presence of GDH protein shows diurnal fluctuations

Figure 2 shows the 24-h pattern of GDH detected by Western blot assay in liver homogenate (panel A), mitochondrial (panel B), and cytosolic fractions (panel C). Similar to the *Glud1* gene expression pattern, the liver homogenates of both the AL and RF groups showed clear oscillations with the peak or acrophase at the beginning of the light period (08:00 and 11:00 h) and a valley that spanned the rest of the light period (from 14:00 to 20:00 h). Diurnal values were 25% higher than nocturnal. During the dark period, the relative amount of GDH increased gradually, again reaching the peak at 08:00 h. There was a clear tendency for the acrophase of RF rats to be higher than the one of the AL group. In addition, the diurnal values were still higher (41%) than the AL rats. Interestingly, the acute fasting condition significantly reduced the GDH protein in comparison to AL and RF in liver samples collected at 11:00 h, while the Refed group showed a similar low level of GDH at 14:00 h.

In the mitochondrial fraction, the time course of GDH protein showed a well-defined rhythmic pattern in the AL group. Clearly, the concentration of the enzyme was higher during the light period (49%) than in the dark period. The daily profile in RF group did not show rhythmicity, but the value of the 24-h average was significantly higher (~40%) in comparison to the AL group. In fact, the 24-h average level of mitochondrial GDH for the RF group was similar to the diurnal peak of the AL group. The GDH levels for the acute controls of feeding conditions were similar to each other and to those of the AL and RF groups at 11:00 and 14:00 h, respectively.

GDH protein in the cytosolic fraction of AL and RF groups varied rhythmically according to analysis of one-way ANOVA. The AL group showed a profile similar to that observed in the mitochondrial fraction: higher values (47%) in the light period in comparison to the dark period. The 24-h pattern of cytosolic GDH in the RF group showed a peak at 11:00 h (before food access), with low, almost constant values during the rest of the 24-h cycle. The 24-average in the RF group was significantly higher than the one shown by the AL group; however, the diurnal values were not so increased (only 22%). The concentration of cytosolic GDH was highest in the Fa group (at 11:00 h), and it decreased significantly after feeding (at 14:00 h).

RF promoted changes in rhythmicity of GDH activity

GDH activity was measured by an enzyme-coupled redox reaction in mitochondrial and cytosolic fractions of liver, as well as in serum (Figure 3). Panel A shows the results obtained in the mitochondrial fraction. GDH being a mitochondrial matrix enzyme, the GDH specific activity was highest in this fraction. The AL group showed a rhythmic profile with a significant reduction (~55%) at 17:00 h. Constant values in GDH activity were observed from 23:00 to 14:00 h. In contrast, the RF group showed no rhythmicity with a mean value significantly higher (~36%) than the AL group during the 24-h cycle. Acute fasting promoted a low GDH activity at 11:00 h, similar to the valley value in the RF group. The Refed group showed ~80% greater GDH activity at 14:00 h in comparison to the Fasted group.

GDH activity measured in the cytosolic fraction (Panel B) was clearly lower than that in the mitochondrial fraction. No differences were observed between the AL and RF groups; both of these two groups showed linear profiles implying a lack of 24-h rhythmicity. As to the control of feeding conditions, GDH activity in the Fasted group (at 11:00 h) was similar to that of the AL and RF groups. However, Refed rats had markedly higher (~450%) GDH activity at 14:00 h than the Fasted group.

Detectable GDH activity could be measured in serum (Panel C). AL group showed a 24 h-rhythm with a peak in the light period (17:00 h). RF group also showed a rhythmic pattern but with the peak extended to the 20:00 h. Controls of feeding condition showed clear differences: Fasted rats depicted lower GDH activity (at 11:00 h) whereas the Refed group showed even minor levels at 14:00 h.

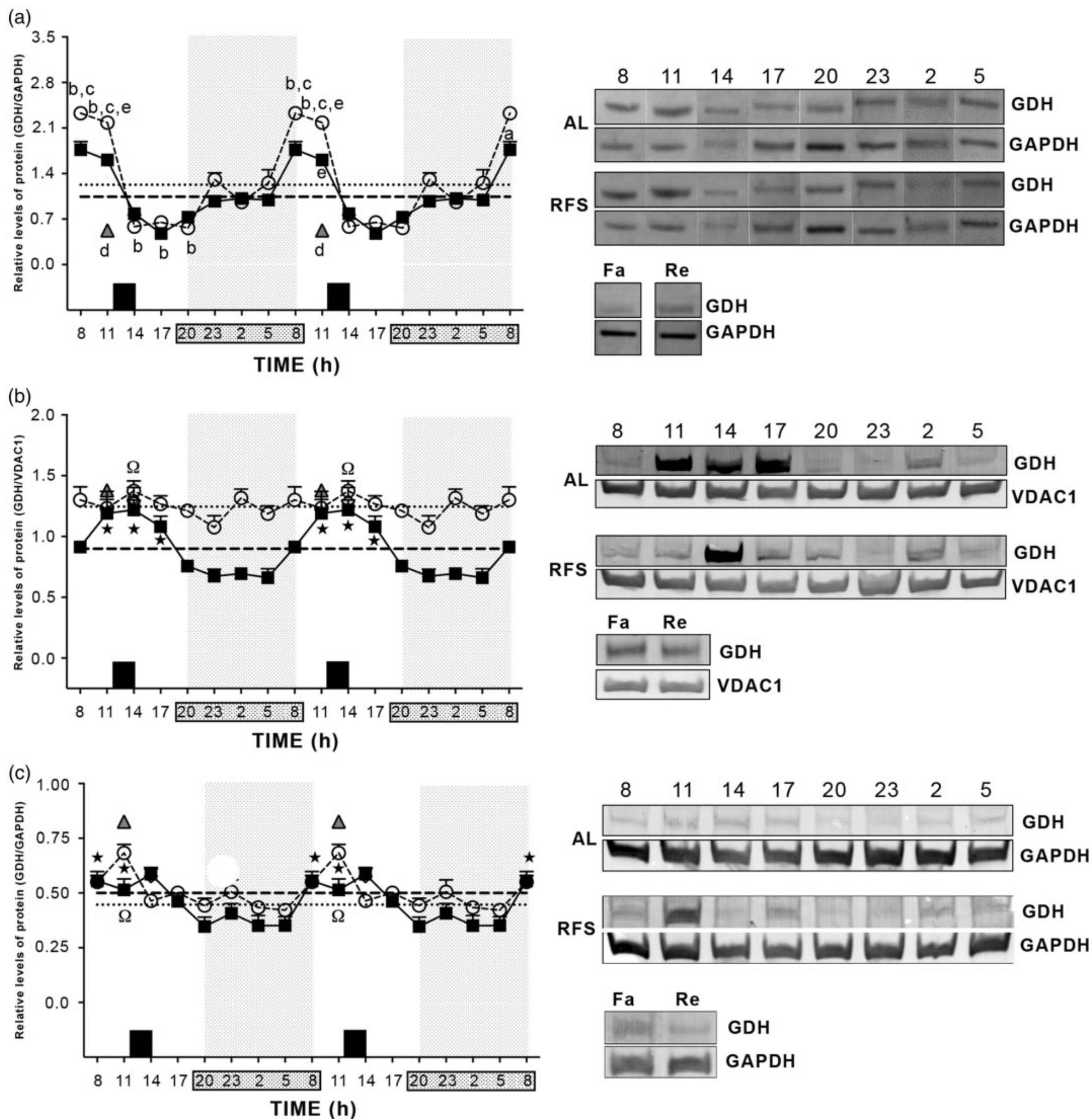

Figure 2 Daily variations of liver GDH protein under the daytime restricted feeding protocol. Data are depicted as a double-plotted 24-h profile. Panel A, homogenate; panel B, mitochondrial fraction; panel C, cytosolic fraction. Group fed *ad-libitum*, AL (filled squares); group under restricted feeding, RF (white circles); group with acute fasting (Fasted; gray triangles); group with acute fasting followed with refeeding, Refed (gray rhombus). Light on at 08:00 h and off at 20:00 h. Hatched bars indicate mealtime (12:00 h–14:00 h). Data are expressed as mean ± SEM of at least four independent observations. Note the coincidence of symbols in panel A (Refed and AL groups, at 14:00 h), in panel B (Fasted and AL and RF groups at 11:00 h, as well as Refed and AL and RF groups at 14:00 h), and in panel C (Refed and AL groups at 14:00 h). ⋆Time points with a significant difference within the group detected by one-way ANOVA (Tukey *post hoc* test, $P < 0.05$); $^{\Omega}$significant difference between AL and RF groups detected by two-way ANOVA (Sidak *post hoc* test, $P < 0.05$); ^{a}significant difference between Fasted and Refed groups detected by Student's *t*-test ($P < 0.05$); ^{b}significant difference between Fasted group and AL and RF groups detected by Student's *t*-test ($P < 0.05$)

Expression of GDH in hepatocytes

Immunohistochemical detection of liver GDH at 08:00 h (at peak of protein level, Figure 2, panel A) and at 17:00 h (at valley of protein level, Figure 2, panel A) is shown in Figure 4, panel A. Consistent with the Western blot result, the GDH signal was more intense at 08:00 h than at 17:00 h. No differences were detected between the AL and RF groups. As it can be observed in panel B, a striking observation was that not all hepatocytes expressed GDH equally; most of the signal was detected in hepatocytes around central (CV) and portal veins (PV). This distribution was similar in AL and RF groups. Only a few hepatocytes were GDH-positive in the acute fasted and refed groups.

Discussion

In the present study, we described for the first time the daily variations of hepatic GDH and its responsiveness to a protocol of RF that it is known to synchronize circadian rhythmicity at different levels of regulation: transcriptional, post-transcriptional and enzymatic activity.

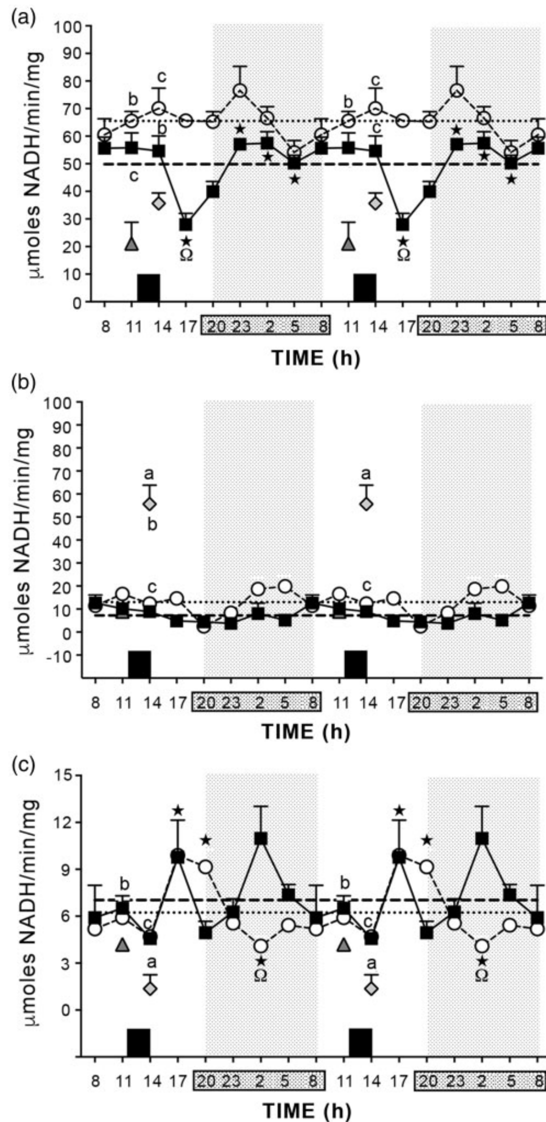

Figure 3 Daily variations of the enzymatic activity of liver glutamate dehydrogenase under the daytime restricted feeding protocol. Data are depicted as a double-plotted 24-h profile. Panel A, mitochondrial fraction; panel B, cytosolic fraction; panel C, serum. Group fed *ad-libitum*, AL (filled squares); group under restricted feeding, RF (white circles); group with acute fasting, Fa (gray triangles); group with acute fasting followed with refeeding, Refed (gray rhombus). Light on at 08:00 h and off at 20:00 h. Hatched bars indicate mealtime (12:00 h–14:00 h). Data are expressed as mean ± SEM of at least four independent observations. Note that the symbols of the Fasted and AL and RF groups (at 11:00 h) are coincident (panel B). ⋆Time points with a significant difference within the group detected by one-way ANOVA (Tukey *post hoc* test, $P < 0.05$); $^{\Omega}$significant difference between AL and RF groups detected by two-way ANOVA (Sidak *post hoc* test, $P < 0.05$); ^{a}significant difference between Fasted and Refed groups detected by Student's *t*-test ($P < 0.05$); ^{b}significant difference between Fasted group and AL and RF groups detected by Student's *t*-test ($P < 0.05$); ^{c}significant difference between Refed group and AL and RF groups detected by Student's *t*-test ($P < 0.05$)

Correlation among expression, presence, activity and location of liver GDH under restricted feeding

Each step in the synthesis of a functional enzyme is susceptible to regulation. For example, transcription depends on: the equilibrium between factors that relax or tighten chromatin structure, the presence of positive and negative factors that recognize gene promotors, processing of introns, etc. For translation to occur, a mature and stable mRNA, lengthening or shortening of its poly (A) tail, and formation of the initiation and elongation complexes are required; once formed, the enzyme is subjected to the actions of post-translational mechanisms that modulate its conformation and activity. The approach taken in this study has been used to characterize the daily variations of glutamine synthetase, GABA transaminase and liver serotonin metabolism. In all these cases, no clear correlation was observed among the 24-h profiles of the mRNA, the protein, and the activity of the enzyme.[17,18,26]

The RF protocol promoted a distinctive result: a specific enhancement of liver GDH protein and activity in the mitochondrial fraction (Figure 2, panel B, and Figure 3, panel A). The increased GDH within mitochondria in RF rats was not directly associated with an elevation of its transcriptional activity (Figure 1). The possibility of a higher mitochondrial yield is also unlikely, since the RF protocol did not modify the specific activity of mitochondrial markers.[13] The simplest explanation is that RF favored the translocation of GDH into the mitochondria by a mechanism that remains to be explored; hence, the increased GDH protein within this organelle (Figure 2, panel B) was responsible for the higher enzymatic activity recorded in this cellular fraction (Figure 3, panel A).

As to liver GDH temporal profiles, a correlation was detected between the daily patterns of mRNA and the presence of GDH protein in the mitochondrial fraction and tissue homogenate (Figures 1 and 2). This correlation was present in both experimental groups, AL and RF, with their acrophases at the beginning of the light period (08:00 and 11:000 h). In the AL group, the level of GDH mRNA also correlated with GDH activity, since the reduction in mRNA and protein coincided with a clear valley in activity (14:00–17:00 h) (Figure 3, panel A). In contrast, the RF group showed higher levels of mitochondrial GDH protein (without rhythmicity) and higher values of GDH activity (17:00 and 20:00 h) (Figure 2, panel B and Figure 3, panel A).

Not very much is known regarding transcriptional regulation of liver GDH: (1) its expression is activated by dexamethasone in ovine liver,[27] (2) transcriptional activity is lower in male than in female rats,[5] and (3) its expression is enhanced in the liver of triploid fishes.[28] Indeed the factors involved in the daily rhythmicity of GDH activity, mRNA and protein, seem to be similar in the AL and FR groups. This fact suggests that for GDH mRNA and protein, the timing clue has more influence than the AL and RF feeding protocols.

Regarding the sustained time course of GDH activity in the mitochondrial fraction of RF rats, the following should be considered: (1) the assay was done *in vitro* under optimal supply of substrates and coenzyme; (2) it is improbable that allosteric factors could remain attached to the GDH protein during the mitochondrial isolation procedure; (3) in both the AL and RF groups, there was a good correlation between the GDH protein and GDH activity (rhythmic in AL rats and persistent in RF rats); and (4) it is unlikely that sirtuin 3 and/or the mitochondrial Redox state played a role in the daily GDH activity profile.[8,21]

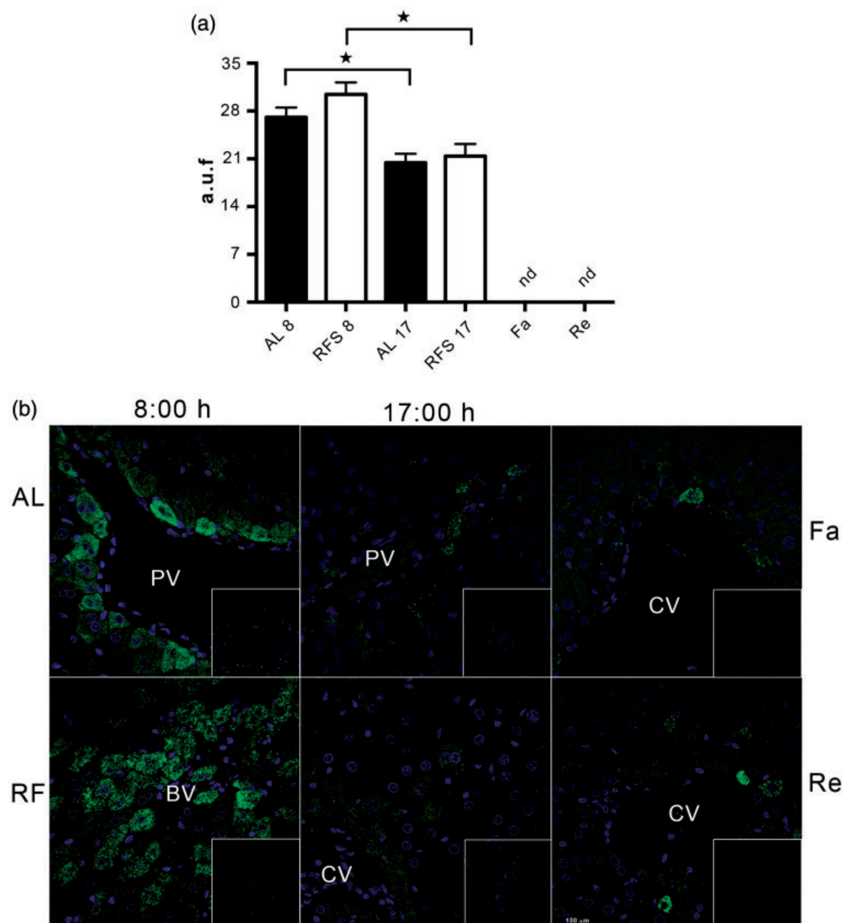

Figure 4 Immunohistochemical location of liver glutamate dehydrogenase at 08:00 h and 17:00 h under the protocol of daytime restricted feeding. Panel A, black bars, AL group; white bars, RF group; acute fasted and refed groups, not detectable (nd). Data are expressed as mean ± SEM of at least 4 independent observations. a.u.f. arbitrary units of fluorescence. ⋆Time points that differed significantly by Student's *t*-test ($P < 0.05$). Panel B, representative immunohistological stains for GDH in liver sections illustrating GDH expression at 08:00 h (left), 17:00 h (center) and in acute Fasting and Refed (right); AL and acute Fasted groups are at top, whereas RF and Refed groups are at bottom. Inlets in each image represent negative controls (absence of primary antibody). CV = central vein, PC = portal vein and BV = blood vessel. Scale bar = 100 µm

The presence of GDH and other "mitochondrial" enzymes in serum does not necessarily reflect liver necrosis. On the contrary, it has been shown to depend largely on hemodynamic changes in the liver.[4] Our data showed a minor change in the rhythmicity of serum GDH activity (Figure 3, panel C).

Our immunohistochemical results for the location of GDH in the hepatic acinus did not show a preference between pericentral and periportal hepatocytes, and they confirmed a previous report regarding the presence of GDH in hepatocytes around veins.[29] No differences in the histological location of GDH were observed between the AL and RF groups (Figure 4).

Daytime restricted feeding protocol

Daytime restricted feeding is an experimental protocol that has been used to study the circadian timing system associated with an alternative daily oscillatory profile in the liver, which is not necessarily dependent on SCN function.[30] Food access limited to 2 h per day over three weeks results in a new type of interaction between the circadian molecular clock and the biochemical networks.[20] These novel metabolic and physiological outcomes are supported by two conceptual frameworks: a circadian entrainment that shifts the phase of the clock proteins and regulatory metabolic mechanism to mealtime,[31] and a hypocaloric condition that is implicit in the ~30% lower food intake in the RF group.[32,33] It has been reported that three key regulatory parameters controlling liver metabolism are modified by the daytime restricted feeding schedule: redox state (becomes oxidized before food access), energy charge (ATP increases during food anticipatory activity), and mitochondrial activity (elevation of mitochondrial membrane potential) (in Mendoza[34] and references within).

There are two ways to interpret the concept of FEO as a system to predict the availability of food: one is to consider the FEO as a variety of oscillators, responding to mealtime, located in the brain and periphery that act in coordination to modulate the behavioral, endocrine and metabolic responses when food-related cues alter the circadian timing system.[34] The other is to consider the FEO as a manifestation of a change in the physiological set point control of the circadian timing system (rheostatic adaptation). In this

case, the FEO would be an emergent property of a distributed oscillator encompassing brain areas and peripheral organs that constitute the intersection of circadian time, eating behavior, central nervous system (CNS) arousal, and metabolic state.[20,35]

In conclusion, our data confirm and extend previous reports showing daily variations of liver GDH activity,[36] and they demonstrated a modulatory action of the RF protocol, especially its enhancement of GDH protein and activity in mitochondria. It is very probable that these adaptations in hepatic GDH metabolism are associated with the physiology of the FEO, but additional experiments are needed to support this notion. In a more general perspective, the daily transcriptional and biochemical regulation of GDH in the liver opens the possibility of temporal regulation of glutamate metabolism in other physio-pathological circumstances, such as the control of energy homeostasis as well as the onset of hepatic cancerogenic disorders.[37,38]

Authors' contributions: OV-M and IM performed experiments, analyzed data, and helped writing the paper; IT, HV-G and MP-M did and interpreted experiments; MD-M designed the study and wrote the paper.

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DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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