Daytime restricted feeding modifies 24 h rhythmicity and subcellular distribution of liver glucocorticoid receptor and the urea cycle in rat liver

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Abstract

The timing system in mammals is formed by a set of peripheral biological clocks coordinated by a light-entrainable pacemaker located in the suprachiasmatic nucleus. Daytime restricted feeding (DRF) modifies the circadian control and uncouples the light-dependent physiological rhythmicity, food access becoming the principal external time cue. In these conditions, an alternative biological clock is expressed, the food-entrainable oscillator (FEO). Glucocorticoid hormones are an important part of the humoral mechanisms in the daily synchronisation of the metabolic response of peripheral oscillators by the timing system. A peak of circulating corticosterone has been reported before food access in DRF protocols. In the present study we explored in the liver the 24 h variations of: (1) the subcellular distribution of glucocorticoid receptor (GCR), (2) the activities of the corticosterone-forming and NADPH-generating enzymes (11β-hydroxysteroid dehydrogenase type 1 (11β-HSD-1) and hexose-6-phosphate dehydrogenase (H6PDH)), and, (3) parameters related with the urea cycle (circulating urea and activities of carbamoyl phosphate synthetase and ornithine transcarbamylase) elicited by DRF. The results showed that DRF promoted an increase of more than two times of the hepatic GCR, but exclusively in the cytosolic compartment, since the GCR in the nuclear fraction showed a reduction. No changes were observed in the activities of 11β-HSD-1 and H6PDH, but the rhythmicity of all of the urea cycle-related parameters was modified. It is concluded that liver glucocorticoid signalling and the urea cycle are responsive to feeding-restricted schedules and could be part of the FEO.

Key words: Food-entrainable oscillator: Corticosterone: Peripheral oscillators: Carbamoyl phosphate synthetase: Ornithine transcarbamylase

The timing system in mammals is assembled by a set of coordinated oscillators: a pacemaker in the suprachiasmatic nucleus (SCN) and a variety of clocks localised in several brain areas and peripheral organs[1]. Light entrains the SCN via the retino-hypothalamic tract, and then its rhythmicity is communicated to the rest of the organism by means of neural and blood-borne signals[2]. Hence, light coordinates most of the physiological responses by sustaining the daily cycles of rest–activity, fasting–feeding, and hormonal secretion[3]. In this context, daily glucocorticoid secretion in most mammals occurs at the end of the resting period as metabolic preparation for wakefulness, orchestrating the fasting response and promoting the search for food[4].

Daytime restricted feeding (DRF) in nocturnal animals uncouples the SCN from the peripheral oscillators, turning feeding into the principal synchroniser. The daily expression of molecular clock genes from peripheral organs (liver, pancreas, kidney and lung) shifts their phases in response to the imposed mealtime[5]. This protocol does not affect the circadian rhythmicity of the SCN[6]. Restricted feeding is accompanied by metabolic and physiological adaptations in the peripheral organs, including the onset of an arousal behaviour preceding food access known as food anticipatory activity (FAA)[7]. Because these changes remain even in the absence of a functional SCN, the existence of an alternative oscillator known as a food-entrainable oscillator (FEO) has been postulated[8,9]. The peak of serum glucocorticoid is coincident with FAA during restricted feeding at daytime in rats[10,11], and is independent of SCN activity[9].

Glucocorticoid secretion at the day–night transition in rats fed *ad libitum* is under circadian modulation from the SCN, and relies on an adrenocorticotropic hormone-dependent process. However, an alternative pathway involving the autonomic nervous system has also been reported[12]. Cellular

signalling of these hormones involves ubiquitous genomic receptors (with the exception of the adult SCN) known as NR3C1 (nuclear receptor subfamily 3, group 3, member 1), as well as membrane-located receptors associated with second messenger systems[13]. Glucocorticoid action is associated with the induction of metabolic pathways characteristic of fasting, such as gluconeogenesis and the urea cycle, and is dependent on the combination of a variety of co-activators and co-repressors[14].

Another point of regulation of glucocorticoid signalling is the intracellular conversion between the active hormone corticosterone and the inactive metabolite cortisone by the activity of the enzyme 11β-hydroxysteroid dehydrogenase (11β-HSD). In the liver, 11β-HSD type 1 catalyses the NADPH-dependent reduction of cortisone to form corticosterone. The NADPH is supplied by the microsomal enzyme hexose-6-phosphate dehydrogenase (H6PDH)[15].

Glucocorticoids are candidates to link the SCN signalling to the rhythmicity of the peripheral oscillators, based on the ability of dexamethasone to induce circadian gene expression in cultured rat-1 fibroblasts, and transiently change the phase of gene expression in the liver, kidney and heart. It was also postulated that the glucocorticoid response in peripheral oscillators made the daytime food-induced phase shifting slower in the liver and kidney[17]. Reddy et al. reported that glucocorticoids entrain nearly 60 % of the liver circadian transcriptome[18]. This action is accomplished by acting directly in the glucocorticoid response elements of target genes, or indirectly by regulating elements of the molecular clock. Thus, a positive glucocorticoid response element appears to mediate per1 induction by glucocorticoids[19], whereas the promoter of rev-erbα has been proposed to contain a negative glucocorticoid response element that mediates glucocorticoid-induced repression[20].

Glucocorticoids are not the exclusive synchroniser of peripheral tissues, since hepatocytes of mice lacking glucocorticoid receptor (GCR) showed no alteration in circadian gene expression[17]. However, it is highly plausible that glucocorticoids play a strategic role in the coordination between the SCN and peripheral clocks, and in the interplay linking metabolism and circadian oscillators. Nevertheless, still to be determined are the adaptations elicited by DRF in the status of the intracellular GCR, the equilibrium between corticosterone and cortisone, and the activity of the urea cycle.

Hence, in order to gain more understanding regarding the physiological adjustments in the liver of rats under restricted feeding schedules and expressing the FEO, the aim of the present study was to evaluate the 24 h rhythmicity of the cytoplasmic and nuclear localisation of the GCR, the enzymic activity and presence of the 11β-HSD type 1 isoform, and the production of urea, including the activity of two enzymes of the urea cycle: carbamoyl phosphate synthetase-1 (CPS1) and ornithine transcarbamylase (OTC) in the liver.

Experimental methods

Animals and housing

Adult male Wistar rats, weight 120–150 g at the beginning of the experiment, were maintained in a 12 h light–12 h dark cycle (lights on at 08.00 hours) and constant temperature (22 ± 1°C). The light intensity at the surface of the cages averaged 350 lux. Animals were kept in groups of five in transparent acrylic cages ($40 \times 50 \times 20$ cm) with free access to water and food unless stated otherwise. All experimental procedures were approved and conducted according to the institutional guide for the care and use of animals under biomedical experimentation (Universidad Nacional Autónoma de México).

Experimental design

Control and experimental groups were similar to those reported by Luna-Moreno et al.[11]. To determine daily and food-entrained rhythmicity, rats were randomly assigned to one of the following feeding conditions for 3 weeks: (1) control ad libitum feeding with free access to food and water throughout the 24 h period; and (2) restricted feeding (experimental condition), in which food availability was limited manually to 2 h daily, from 12.00 to 14.00 hours.

At the end of the feeding protocol, different subgroups of animals were killed at 3 h intervals over a 24 h period, starting at 08.00 hours.

In addition, to establish a comparison of the fasting and subsequent refeeding responses, two more control groups were included: (1) animals fed ad libitum were maintained with free food access for 3 weeks; on the last day, food was removed at 14.00 hours, and they were killed (at 11.00 hours) after 21 and 45 h (about 1 and 2 d) of deprivation; and (2) a second group of rats was similarly deprived of food for 21 and 45 h, then refed for 2 h (from 12.00 to 14.00 hours), and killed at 14.00 hours before tissue sampling.

Blood and liver sampling

Rats were killed and then beheaded for trunk blood collection. Blood was collected and centrifuged at 5000 rpm for 5 min to obtain serum. A liver section (about 5 g) was processed for homogenate and subcellular fractionation (nucleus, mitochondria, microsomes and cytosol). The subcellular fractionation was done according to the method of Aguilar-Delfín et al.[21]. Briefly, liver was homogenised in 10 mM-2-amino-2-hydroxymethyl-propane-1,3-diol (Tris)–HCl (pH 7·4; 1:10; w/v), the homogenate was centrifuged at 1500 **g** for 15 min, and the pellet was kept for further isolation of the nuclear fraction. The supernatant fraction was centrifuged at 10 000 **g** for 20 min to sediment the mitochondrial fraction. The new supernatant fraction was ultracentrifuged at 100 000 **g** for 60 min, yielding the microsomal (pellet) and the cytosolic fractions (supernatant fraction). The nuclear fraction was prepared from the first pellet using the citric acid method reported by Reiners & Busch[22].

Western blotting for glucocorticoid receptor and 11β-hydroxysteroid dehydrogenase type 1

GCR and 11β-HSD-1 were assayed by Western blotting according to the procedure reported by Chilov et al.[23]. Total protein was measured using the Folin phenol reagent[24]. Equal amounts of proteins were mixed with 2 × Laemmli sample buffer (Bio-Rad) and incubated at 80°C for 10 min. The homogenate, the nuclear and the cytosolic samples were separated on a 10 % polyacrylamide gel, electroblotted onto a nitrocellulose membrane, and then incubated overnight with primary rabbit polyclonal anti-GCR antibody (Sc-1004; Santa Cruz Biotechnology) at 1:250 dilution. The microsomal protein was separated on a 10 % polyacrylamide gel, electroblotted and incubated overnight with primary rabbit polyclonal anti-11β-HSD type 1 antibody (Sc-20 175; Santa Cruz Biotechnology) at 1:500 dilution. Membranes were washed and incubated for 2 h with alkaline phosphatase-conjugated secondary donkey anti-rabbit antibody at 1:5000 dilution (Santa Cruz Biotechnology), and bands were visualised using the alkaline phosphatase conjugate substrate kit (Bio-Rad) according to the manufacturer's instructions. β-Actin and tubulin were used as loading controls, the first for cytoplasmic and microsomal fractions, and the second for the nuclear fraction.

11β-Hydroxysteroid dehydrogenase type 1 activity

This enzymic activity was determined using the assay described by Thurston et al.[25]. Microsomal protein (30 mg) was transferred to glass tubes, containing 700 μl PBS. Blanks contained 100 μl bovine serum albumin solution (1 mg/ml prepared in PBS). Each triplicate set of tubes was pre-incubated for 30 min at 37°C in a water-bath. To initiate the assay, each tube received 4 mM-NADPH (Sigma) and 100 μl PBS containing 3700 Bq (0·1 μCi) [1,2-^{3}H]cortisone (Perkin Elmer) and unlabelled cortisone (Sigma), each to a final steroid concentration of 100 nM. The tubes were then returned to the water-bath for 60 min, and the reactions were terminated by the addition of 2 ml ice-cold chloroform (J. T. Baker) to each tube. To partition the organic and aqueous phases, these tubes were centrifuged at 1000 **g** for 30 min. After aspirating the aqueous supernatant fraction, the organic extracts were evaporated overnight at room temperature. The steroid residues were re-suspended in 20 μl ethyl acetate containing either 1 mM-corticosterone or -cortisone (Sigma) and resolved by TLC, using Silica 60 TLC plates (Merck) in an atmosphere of 92:8 (v/v) chloroform–95 % (v/v) ethanol (Merck). The spots corresponding to corticosterone were scraped off and the [^{3}H]corticosterone was quantified using a Bioscan 200 TLC radiochromatogram scanner (LabLogic). Protein concentration was measured using the Lowry method[24].

Hexose-6-phosphate dehydrogenase activity

H6PDH activity was measured after disrupting the microsomal membranes by preincubation at 4°C with radioimmunoprecipitation assay (RIPA) buffer. The incubation mixture contained 0·3 mM-glucosamine-6-phosphate, 100 mM-glycine–NaOH buffer (pH 10), 1 mM-NADP$^+$, 1% bovine serum albumin, in a total volume of 1 ml. Reactions were started by the addition of tissue homogenate or microsomes (about 10 and 3 mg protein, respectively) to 900 μl of the reaction mixture at room temperature. The increase in absorbance at 340 nm was monitored during the first 5 min of incubation using a spectrophotometer (Ultrospec 3,300; Pharmacia Biotech). The amount of NADPH produced was calculated using 6·22 as the extinction coefficient. Specific activities were expressed as μmol NADPH formed/min per mg protein[26].

Urea cycle parameters

Urea was determined by a standard enzymic method (ELITech) in which urea is first cleaved by urease into CO_2 and NH_3. In a second step, the NH_3 reacts with phenol and hypochlorite under alkaline conditions to produce a blue compound that is determined colorimetrically at 340 nm. CPS1 activity was determined according to the method reported by Pierson[27]. The reaction mixture consisted of 5 mM-NH_4HCO_3, 5 mM-ATP, 10 mM-magnesium acetate, 5 mM-N-acetyl glutamate, 1 mM-dithiothreitol, 50 mM-triethanolamine (pH 8·0) and 200 μg mitochondrial protein in a final volume of 0·6 ml. The reaction was run for 10 min at 37°C and the carbamoyl phosphate was converted to hydroxy-urea by the addition of 2 M-hydroxylamine (30 μl), and incubated for 10 min at 95°C. To quantify the hydroxy-urea, 2·4 ml of chromogenic reagent was added followed by an incubation of 15 min at 95°C. After cooling the samples at room temperature, absorbance was measured at 458 nm in a spectrophotometer. OTC activity was measured following the technique reported by Bagrel et al.[28]. The reaction mixture consisted of 20 mM-ornithine/urease solution (400 μl) and 100 μg mitochondrial protein. The mixture was incubated at 37°C for 5 min and 50 mM-carbamoyl phosphate solution (400 μl) was added, and incubated for another 20 min. The reaction was stopped with 1 ml TCA (100 g/l). Subsequently, the tubes were centrifuged at 5000 rpm for 10 min, and 1 ml of the supernatant fraction was added to diacetyl monoxime solution (100 mM) and 4 ml of phosphoferric–antipyrine reagent (antipyrine 65 mmol, $FeCl_3$ 12·5 mmol, 625 ml H_3PO_4 and 375 ml distilled water) to detect the citrulline formed by the action of the OTC. The mixture was incubated in a boiling water-bath for 20 min followed by 10 min at room temperature. Finally, the colour was read in a spectrophotometer at 460 nm.

Data analysis

Data were grouped for experimental condition and time, and are presented as mean values with their standard errors. They were compared with a two-way ANOVA for independent measures with a factor for group (two levels) and a factor for time (eight levels). In order to determine significant time effects for each curve, a one-way ANOVA was performed for individual groups. The one- and two-way ANOVA were followed by a Tukey post hoc test with significant values set
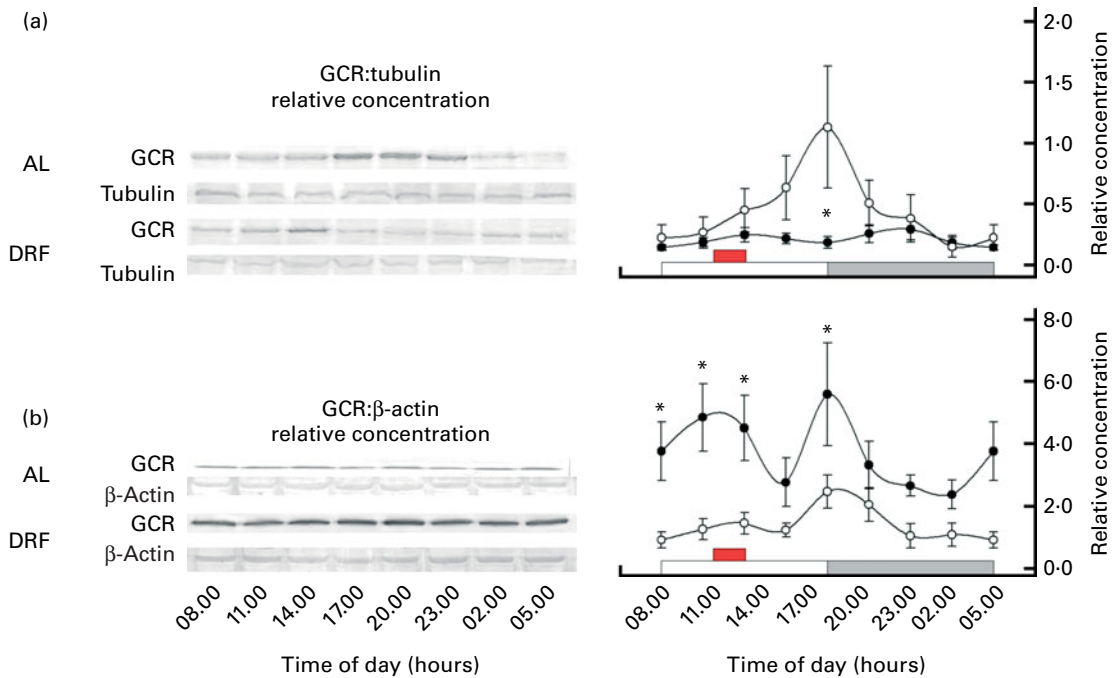
Fig. 1. Daily variations of glucocorticoid receptor (GCR) in the liver during a protocol of daytime restricted feeding (DRF). Western blotting of the 24 h cycle for GCR in rats fed ad libitum (AL; ○) and under DRF (●) in nuclear (a) and cytosolic (b) fractions. Lights were on at 08.00 hours and off at 20.00 hours. The dark red box indicates the mealtime period (12.00 to 14.00 hours). Tubulin and β-actin were used as loading controls for nuclear and cytosolic fractions, respectively. Data are means of at least eight independent observations, with standard errors represented by vertical bars. Representative Western blot experiments are shown. * Mean value was significantly different from that of the AL group ($P < 0.05$; Tukey post hoc test).

at $P < 0.05$. Statistical analysis was performed with the program GraphPad Prism (version 5.0 for Windows; GraphPad Software). All graphs were drawn using the Sigmaplot curve-fitting program (Jandel Scientific).

Results

24 h Rhythmicity of subcellular distribution of liver glucocorticoid receptor

Fig. 1 shows the daily pattern of the liver α subunit of the GCR in rats fed ad libitum and under DRF. The ad libitum group showed a peak in GCR presence in nuclear and cytosolic fractions in the transition between light and dark periods (Fig. 1(a)

and (b), respectively), as was expected in ad libitum-fed conditions. This 24 h pattern is analogous to the pattern of circulating corticosterone reported elsewhere[11]. In contrast, the GCR pattern in the DRF group showed significant differences in both fractions: no fluctuation in the nuclear fraction was observed, and the values were lower than those of the ad libitum group in the light and dark periods (Fig. 1(a) and Table 1). In contrast, DRF promoted a marked increased in the cytosolic presence of the GCR (about 159%), with two peaks, one at the times before and after food access (11.00 and 14.00 hours), and the other in the transition between light and dark periods (Fig. 1(b) and Table 1). This 24 h pattern was similar to the one described for circulating corticosterone in rats under the DRF protocol[11].

Table 1. Liver glucocorticoid receptor in liver homogenate, nucleus and cytosol in rats under a protocol of daytime restricted feeding (DRF)†

(Mean values of at least eight independent observations with their standard errors)

	Glucocorticoid receptor:β-actin or tubulin (relative concentration)											
	Homogenate				Nucleus				Cytosol			
	AL		DRF		AL		DRF		AL		DRF	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Average	0·61	0·12	1·49*	0·28	0·46	0·20	0·21	0·05	1·43	0·37	3·70*	0·89
Light	0·63	0·11	1·54*	0·21	0·39	0·17	0·20	0·04	1·21	0·29	3·90*	0·96
Dark	0·59	0·12	1·43*	0·35	0·54	0·24	0·22	0·06	1·65	0·45	3·48*	0·80

AL, ad libitum (control).

* Mean value was significantly different from that of the AL group ($P < 0.05$; Tukey post hoc test).

† Comparison between groups fed AL and under DRF of average values of the 24 h cycle, the light period (08.00, 11.00, 14.00 and 17.00 hours) and the dark period (20.00, 23.00, 02.00 and 05.00 hours). Values were calculated from data in Fig. 1.

No differences were observed in the effect of DRF promoting the higher levels of liver GCR when light and dark periods were compared (Table 1).

As to the control of feeding conditions, the effect of fasting–refeeding on the presence of liver GCR is shown in Fig. 2. A period of 1 d of fasting followed by 2 h of food access did not affect the presence of GCR in liver homogenate. However, 2 d of fasting promoted a significant increase that reverted after mealtime. In comparison, DRF showed higher levels of GCR than the groups of 1 d fasting–refeeding, but without differences from the rats with fasting–refeeding of 2 d (Fig. 2(a)). In the nuclear fraction an effect of refeeding decreasing the levels of GCR after 1 d of fasting was evident,

an effect that was not observed in the groups of 2 d fasting–refeeding. A significant reduction of GCR was shown in the rats under DRF (about 85 %), in comparison with both controls of feeding condition (Fig. 2(b)). In contrast, in the cytoplasmic fraction the presence of GCR in the DRF groups was increased about four times in comparison with both controls of feeding condition. A discrete but significant diminution was observed after 2 d of fasting that was partially reverted by meal access (Fig. 2(c)).

The occurrence of corticosterone, the active glucocorticoid in rodents, within the liver depends on the activity of a system formed by two enzymes: the NADPH-dependent 11β-HSD-1 and the NADPH-regenerating H6PDH. Fig. 3 shows the effect of DRF on the presence and activity of 11β-HSD-1 (Fig. 3(a) and (b), respectively), as well as the activity of H6PDH (Fig. 3(c)). In spite of some tendencies, no significant changes were observed: None of these parameters showed a rhythmic pattern, and neither was there any significant difference between the temporal patterns of the control *ad libitum* group and the DRF-treated rats. In contrast, acute fasting–refeeding of 1 and 2 d showed significant changes in the presence and activity of 11β-HSD-1 in comparison with the DRF group (Fig. 4). It can be seen in Fig. 4(a) that the presence of 11β-HSD-1 was responsive to the feeding condition, since there was a discrete but significant reduction caused by refeeding in the group of 1 d of acute treatment as well as in the DRF rats. However, the most conspicuous difference was in the higher levels of this enzyme promoted by the DRF protocol: it increased about 95 % in fasting and about 70 % in the fed state (including 1 and 2 d of acute treatment). The activity of 11β-HSD-1 was also sensitive to the fasting–refeeding protocol (Fig. 4(b)). This time the activity was higher after refeeding in both acute treatments (1 and 2 d), but not in the DRF groups. In accordance with the Western blot results, the activity of 11β-HSD-1 was two to four times significantly higher in the rats under the DRF protocol. Hence, the data indicated an up-regulation of 11β-HSD-1 promoted by restricted food access. The fasting–refeeding condition did not affect the activity of H6PDH in any of the experimental groups (Fig. 4(c)).

Glucocorticoid hormones are some of the principal endocrine regulators of the induction and activity of the enzymes underlying the urea cycle[29]. Fig. 5 shows the effect of the DRF protocol on the 24 h rhythmicity of circulating urea (Fig. 5(a)) and the activities of the liver mitochondrial enzymes of the urea cycle: CPS1 (Fig. 5(b)) and OTC (Fig. 5(c)). In agreement with the temporal pattern of circulating corticosterone[11] and nuclear and cytoplasmic liver GCR (Fig. 1), circulating urea in the *ad libitum* rats depicted a peak in the transition between the light and the dark periods (Fig. 5(a)). Differently, DRF promoted high levels of urea preceding food access (at 11.00 hours), which decreased to very low levels in response to mealtime (14.00 hours). After 17.00 hours, the circulating urea was enhanced to reach a peak (08.00 and 11.00 hours) just before feeding (Fig. 5(a)). In spite of the dissimilar rhythmicity, there was no change in the average values of blood urea between the *ad libitum* and DRF groups, or in the values measured in the light and
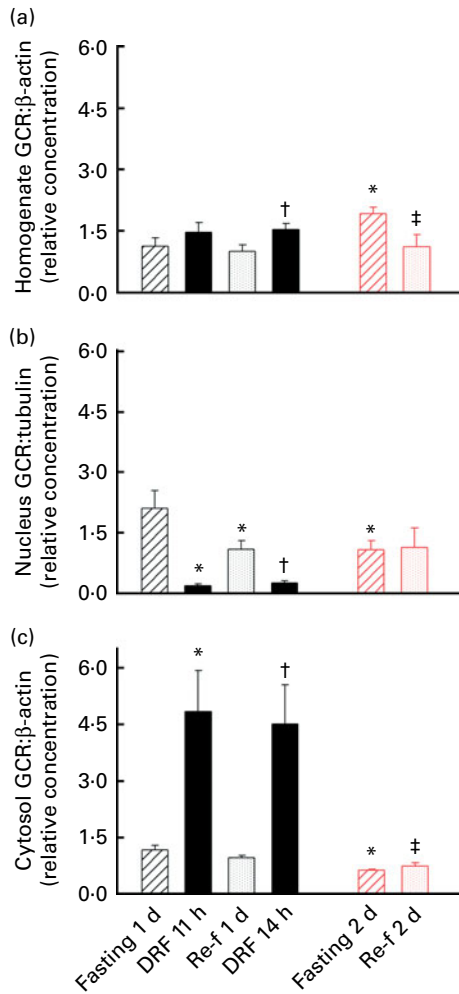

Fig. 2. Comparison between a daytime restricted feeding (DRF) protocol and fasting and refeeding (about 1 and 2 d) on the levels of glucocorticoid receptor (GCR) in the liver. Western blotting of GCR in groups of fasting and refeeding (Re-f) for about 1 and 2 d, as well as rats under DRF. Experiments were done in liver homogenate (a), nuclear fraction (b) and cytosol (c). (▨), Fasting about 1 and 2 d; (▧), fasting about 1 and 2 d plus 2 h refeeding; (■), DRF before (at 11.00 hours) and after (14.00 hours) food access. Tubulin (for nucleus) and β-actin (for homogenate and cytosol) were used as loading controls. Data are means of at least eight independent observations, with standard errors represented by vertical bars. * Mean value was significantly different from that for fasting 1 d (*P* < 0·05; Tukey *post hoc* test). † Mean value was significantly different from that for Re-f 1 d (*P* < 0·05; Tukey *post hoc* test). ‡ Mean value was significantly different from that for fasting 2 d (*P* < 0·05; Tukey *post hoc* test).

in the dark periods (Table 2). CPS1 is the rate-limiting enzyme of the urea cycle. It had a rhythm in the *ad libitum* group, with higher values (about 44%) in the darkness (Fig. 5(b) and Table 2). CPS1 rhythm was lost in the DRF group, but food restriction caused significant higher CPS1 activity during the light period (about 72%) (Fig. 5(b) and Table 2). Diurnal variation of OTC showed constant values in the *ad libitum* rats, with the exception of a significant diminution at 05.00 hours (Fig. 5(c)). In contrast, DRF promoted a very different rhythmicity in OTC activity, with low values in the light period and a significant increment in the darkness (about 80%) (Fig. 5(c) and Table 2).

Fig. 6 shows the effects of fasting–refeeding on the levels of circulating urea and the activities of CPS1 and OTC. Urea in blood was responsive to the feeding condition, decreasing by about 28% and by about 44% by refeeding after 1 and 2 d of fasting, respectively. The effect associated with food access showed a more accentuated response in the groups under the DRF protocol (reduction of about 62%) (Fig. 6(a)). The activity of CPS1 did not show any change comparing the fasted and the fed states in all conditions (1 and 2 d of fasting–refeeding, and DRF at 11.00 hours and 14.00 hours). However, it was evident that the activity of CPS1 in the groups under the DRF protocol decreased by more than 50% in comparison with their control groups of feeding condition (Fig. 6(b)). In contrast, OTC activity was sensitive to fasting–refeeding in a complex way: after 1 d of fasting, refeeding increased OTC activity by about 50%, but after 2 d of fasting, refeeding reduced OTC activity by about 40%. Under the protocol of DRF, OTC activity was more similar to
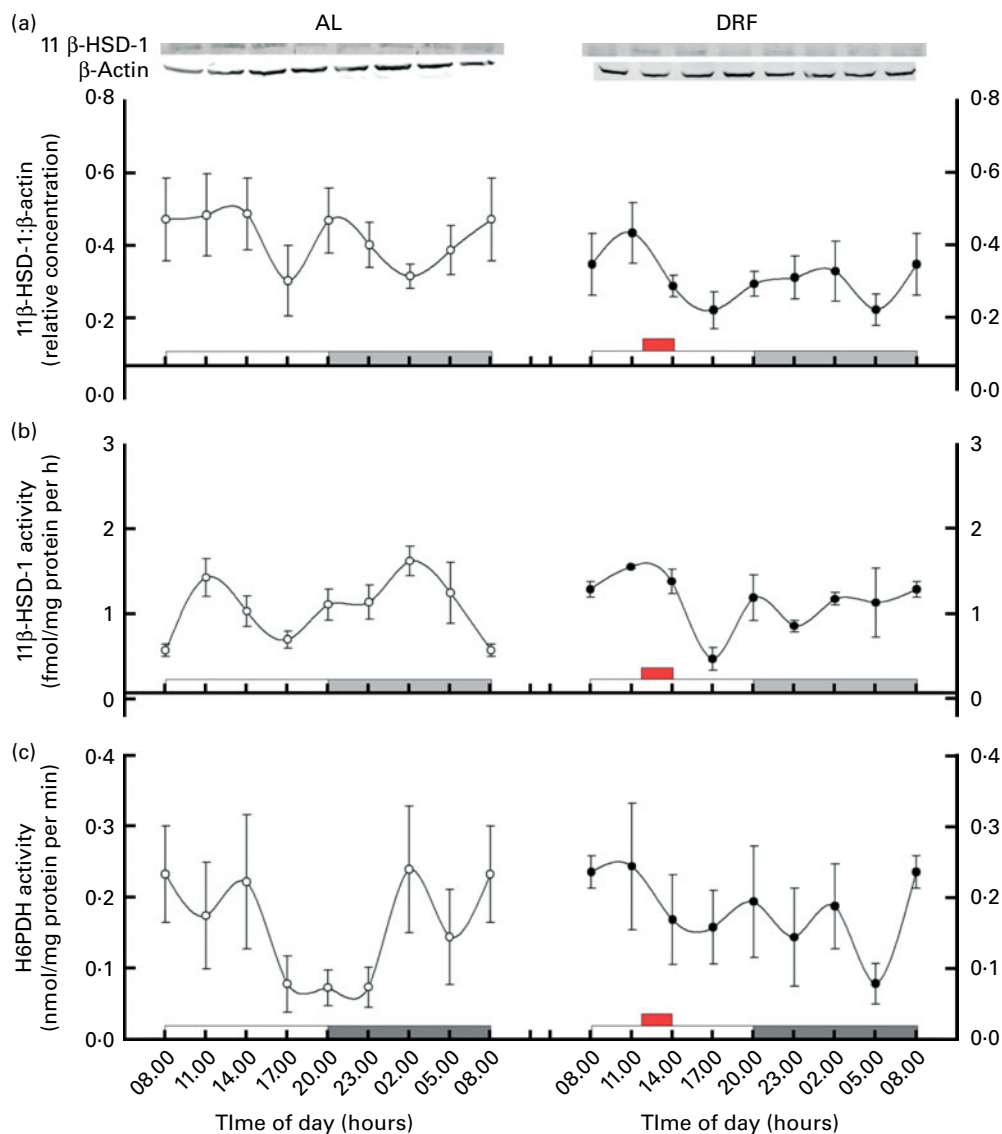

Fig. 3. Daily variations of 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD-1) (presence and activity) and hexose-6-phosphate dehydrogenase (H6PDH) (activity) in the liver during the protocol of daytime restricted feeding (DRF) in rats fed *ad libitum* (AL; ○) and under DRF (●). (a) Western blotting of the 24 h cycle for 11β-HSD-1; β-actin was used as the loading control; (b) 24 h cycle of 11β-HSD-1 activity (fraction); (c) 24 h cycle of H6PDH activity. Experiments were done in the liver microsomal fraction. Lights were on at 08.00 hours and off at 20.00 hours. The dark red box indicates the mealtime period (12.00–14.00 hours). Data are means of at least eight independent observations, with standard errors represented by vertical bars.

the 2 d of fasting–refeeding response since it was decreased after mealtime by about 29 % (Fig. 6(c)).

Discussion

Restricted feeding during daytime for nocturnal animals is an efficient Zeitgeber capable of influencing most of the aspects of the circadian rhythmicity[30]. Glucocorticoids are endocrine messengers that are secreted rhythmically and contribute to the diurnal entrainment of peripheral oscillators[12], including a diverse range of functionally important circadian genes[18]. Because the 24 h rhythmicity of circulating corticosterone changes in association with DRF[11], and the metabolic activity of the liver is notoriously modified[10,31], the aim of the present study was to explore features of glucocorticoid signalling: (1) the subcellular distribution of GCR; (2) the availability of corticosterone within the liver; and (3) parameters related to the urea cycle (considered as one of the liver metabolic outputs of glucocorticoid action).

Glucocorticoid signalling and restricted feeding schedules

An accepted marker in the DRF protocol is the appearance of a peak of circulating corticosterone during the time of FAA[10,11]. Additional information is obtained when the 24 h rhythm is considered: rats with restricted food access show an increase of about 23 % in the daily level of blood corticosterone, and a second peak is seen in the transition between the light and dark periods[11]. The second peak of circulating glucocorticoid is similar to the acrophase shown by the control group fed *ad libitum*, in amplitude and in time[11]. Interestingly, the temporal pattern of GCR in Fig. 1 is analogous to the diurnal variations of circulating glucocorticoids: two peaks in the DRF group (only in the cytosolic fraction) and one peak in the group fed *ad libitum* (in both the cytoplasmic and nuclear fractions). These data indicate that the 24 h rhythmicity of GCR under DRF is controlled by an oscillatory mechanism different from the one regulating diurnal variations of GCR in *ad libitum* conditions. One possibility is that this oscillator could be the FEO, but more experiments are needed to test the feasibility of this notion.

An outstanding result of the present study is the significant increase (about 150 %) in the presence of liver GCR in the DRF protocol, mainly in the cytoplasmic fraction (Fig. 1 and Table 1). Dutta & Sharma[32] also reported an enhanced level (about 41 %) of GCR in the hepatic cytosolic fraction of Balb/c mice fed on alternate days for a period of 3 months. The feeding protocol and the rodent species used by these authors were different from the experimental conditions in the present study, providing possible explanations of the evident difference in the magnitude in the increment of GCR observed in the two reports. It has been reported that GCR are up-regulated by the presence of glucocorticoid ligands, and in response to the transcriptional activity of factors such as cellular myeloblastosis (c-myb), cellular-E twenty-six (c-Ets) and hepatocyte nuclear factor (HNF)-1α[33,34]. In our experimental protocol, the first condition is accomplished:
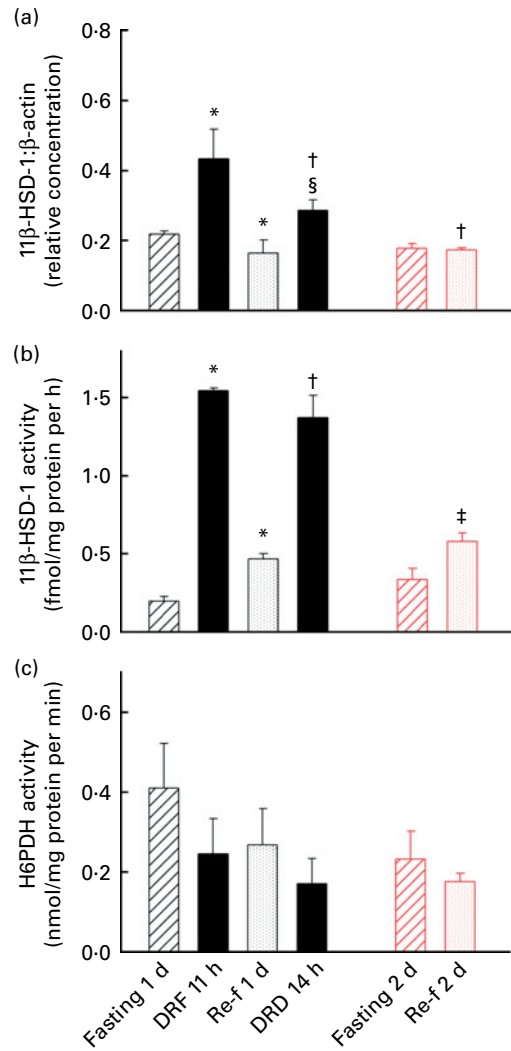

Fig. 4. Comparison between a daytime restricted feeding (DRF) protocol and fasting and refeeding (about 1 and 2 d) on the presence and activity of 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD-1), and the activity of hexose-6-phosphate dehydrogenase (H6PDH) in the liver. Western blotting (a) and activity (b) of 11β-HSD-1 and activity of H6PDH (c) in groups of fasting and refeeding (Re-f) for about 1 and 2 d, as well as rats under DRF. Experiments were done in the liver microsomal fraction; for Western blot experiments; β-actin was used as the loading control. (▨), Fasting about 1 and 2 d; (▧), fasting about 1 and 2 d plus 2 h refeeding; (■), DRF before (at 11.00 hours) and after (14.00 hours) food access. Data are means of at least eight independent observations, with standard errors represented by vertical bars. * Mean value was significantly different from that for fasting 1 d ($P < 0.05$; Tukey *post hoc* test). † Mean value was significantly different from that for Re-f 1 d ($P < 0.05$; Tukey *post hoc* test). ‡ Mean value was significantly different from that for fasting 2 d ($P < 0.05$; Tukey *post hoc* test). § Mean value was significantly different from that for DRF 11 h ($P < 0.05$; Tukey *post hoc* test).

corticosterone is increased about 23 % under DRF[11], but the participation of the other factors remains to be explored.

Besides the increment of liver GCR, another aspect that changed noticeably in the rats under DRF was their subcellular distribution: most of the receptors were located in the cytosol, and only a small proportion within the nucleus (Fig. 1). It is unlikely that this finding is due to modifications in the sedimentary properties of the hepatic cellular fractions, since we have observed no changes in the yield and the characteristics

of the mitochondrial[35] and microsomal (A Báez-Ruiz and M Díaz-Muñoz, unpublished results) fractions under the DRF protocol. In the cytoplasm, the GCR interact with heat shock proteins forming dynamic complexes that are subject to circadian regulation[36]. Upon ligand contact, the heat shock proteins dissociate and the activated GCR is translocated into the nucleus to exert a transcriptional role. However, recent reports also indicate a signalling activity of the glucocorticoid–GCR complex in the cytosol, in complement to the nuclear transcriptional activity. These actions may involve the modulation of ligand- and voltage-dependent ion channels, and the activation of G proteins and extracellular signal-regulated kinase-1/2 (ERK1/2)-related pathways[13]. The possibility that these actions could take place in the liver of rats under DRF is high because of the elevated ratio of cytosolic: nuclear GCR, but additional experiments should be done.

Similarly to the 24 h fluctuations of blood corticosterone, the rhythm shown by the cytoplasmic GCR in DRF rats also showed a bimodal pattern. The existence of two peaks associated with DRF (one at a time of food access and the second in the day–night transition) has been observed in other parameters: in the liver, presence of Per1, PPARα and γ[11,37], phosphoenolpyruvate carboxykinase (PEPCK) and acylCoA oxidase activities (data not shown) as well as in FAA[38]. A tentative explanation for this bimodal pattern is a dual control exerted by different oscillatory mechanisms (i.e. the SCN and FEO).

Within the liver of rodents, the active ligand corticosterone is in redox equilibrium with the inactive metabolite cortisone. The formation of corticosterone requires the activity of the NADPH-dependent enzyme 11β-HSD. The NADPH is supplied by the microsomal enzyme H6PDH. Both enzymes have been considered as diabetogenic factors and are involved in obesity as well as in the generation of the metabolic syndrome[39]. The present results did not show changes in the rhythmicity of both enzymes, or an effect by feeding
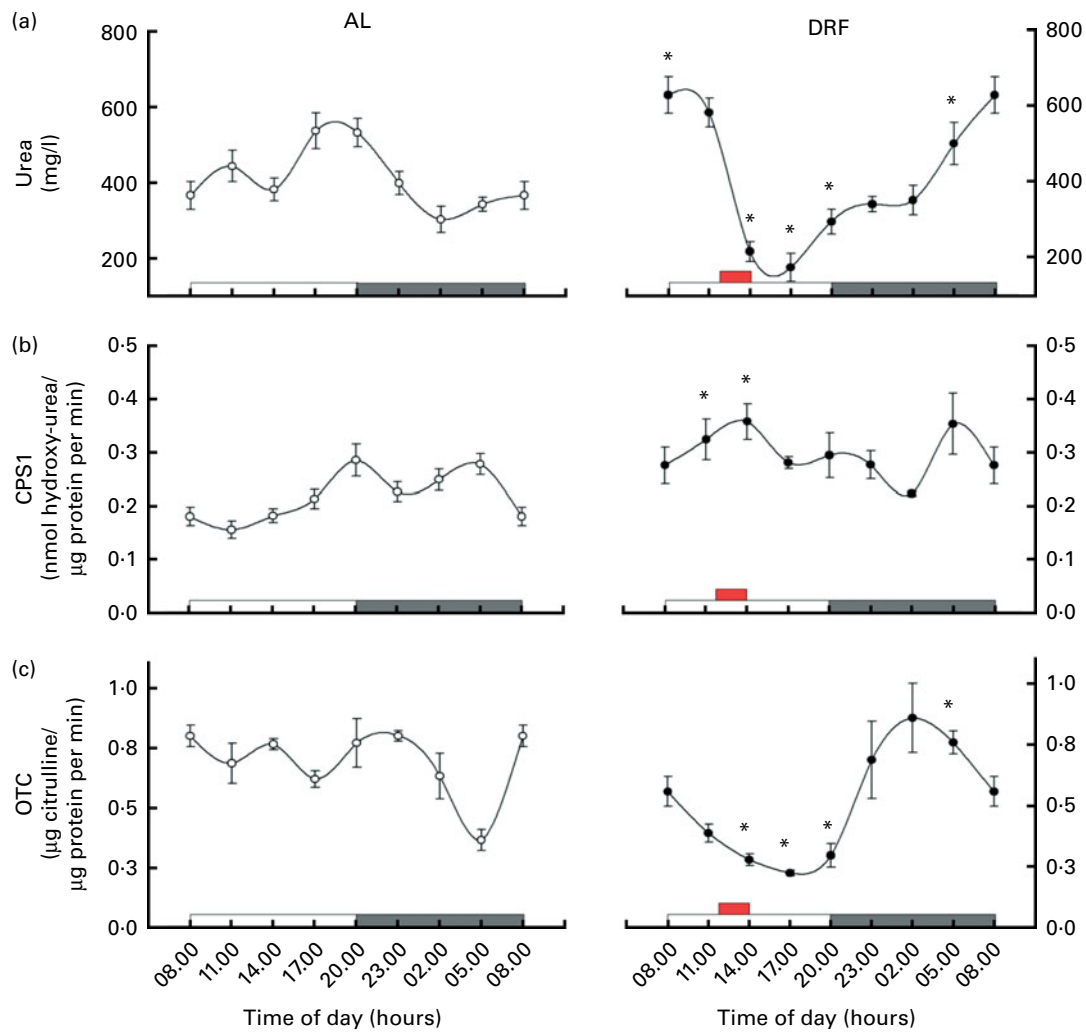

Fig. 5. Daily variations of circulating urea and activities of carbamoyl phosphate synthetase-1 (CPS1) and ornithine transcarbamylase (OTC) in the liver during a protocol of daytime restricted feeding (DRF). Rats were fed *ad libitum* (AL; ○) or were under DRF (●). (a) The 24 h cycle of circulating urea; (b) 24 h cycle of CPS1 activity; (c) 24 h cycle of OTC activity. Enzymic activities were measured in the liver mitochondrial fraction. Lights were on at 08.00 hours and off at 20.00 hours. The dark red box indicates the mealtime period (12.00–14.00 hours). Data are means of at least eight independent observations, with standard errors represented by vertical bars. *Mean value at a time point was significantly different from that of the AL group ($P < 0.05$; Tukey *post hoc* test).

Table 2. Urea cycle-related parameters in rats under a protocol of daytime restricted feeding (DRF)‡

(Mean values of at least eight independent observations with their standard errors)

| | Urea (mg/l) | | | | CPS1 (mmol hydroxy-urea/µg protein per min) | | | | OTC (µg citrulline/µg protein per min) | | | |
| | AL | | DRF | | AL | | DRF | | AL | | DRF | |
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Mean	412·0	34·0	386·0	38·0	0·22	0·02	0·29*	0·03	0·68	0·05	0·50*	0·06
Light	431·0	39·0	400·0	37·0	0·18	0·02	0·31*	0·03	0·72	0·04	0·36*	0·03
Dark	393·0	30·3	371·0	38·0	0·26†	0·02	0·28	0·03	0·64	0·06	0·65†	0·09

CPS1, carbamoyl phosphate synthetase-1; OTC, ornithine transcarbamylase; AL, *ad libitum* (control).

* Mean value was significantly different from that of the AL group ($P < 0.05$; Tukey *post hoc* test).

† Mean value was significantly different from that in the light period ($P < 0.05$; Tukey *post hoc* test).

‡ Comparison between groups fed AL and under DRF of average values of the 24 h cycle, the light period (08.00, 11.00, 14.00 and 17.00 hours) and the dark period (20.00, 23.00, 02.00 and 05.00 hours). Values were calculated from data in Fig. 5.

condition. However, it should be considered that the enzymic assays were done in *in vitro* conditions and possibly far from the biochemical circumstances of the intracellular milieu.

Urea cycle parameters and restricted feeding schedules

GCR output varies according to the metabolic and physiological condition of each tissue, depending on a complex set of transcriptional co-activators and co-inhibitors. Not only the presence but also covalent modifications can modulate the activity of GCR[40]. In addition, GCR can initiate a cascade of gene activation and act through intermediate factors to finally modulate the output of metabolic pathways. Reddy *et al.*[18] reported that only about 24 % of the circadian genes regulated by dexamethasone contain glucocorticoid response elements. Glucocorticoids fulfill many different functions in body homeostasis and stress responses[41]. Among the principal targets of these hormones is the regulation of N metabolism, which in ureotelic organisms is accomplished by the urea–ornithine cycle, an enzymic system that converts ammonia into urea in the periportal section of the liver[42]. The urea cycle is controlled by both nutritional (fasting and high-protein diet) and hormonal (glucagon and glucocorticoids) factors, whereas insulin acts as an inhibitory signal[43].

So far, no reports have been published exploring the rhythmicity of urea cycle-related parameters in the DRF protocols. Our findings showed an evident change in the 24 h rhythmicity of circulating urea and in two of the most important enzymes in the urea cycle, CPS1 and OTC (Fig. 5). The temporal pattern of urea and corticosterone in blood, as well as liver GCR, showed a good coincidence in the control and experimental groups: in the *ad libitum* rats a single peak in the transition between the light and dark periods, and two peaks (during FAA and also in the transition between light and dark) in the rats under the DRF protocol. A distinctive feature is the marked decrease in circulating urea and corticosterone after feeding[11], maybe a response to an intense insulin signalling. Micro-array data indicated a down-regulation of some genes related to the urea cycle at this time: at 11.00 hours, CPS1 and argininosuccinate synthetase-1; at 14.00 hours, arginosuccinate synthetase-1 and arginosuccinate lyase (data not shown). In spite of the significant changes in the rhythmicity of plasma urea, the average value

in the *ad libitum* and DRF groups was very similar (Table 2), suggesting that 2 h restricted feeding did not promote a negative energy balance. Transcriptional regulation of the urea cycle involves a variety of factors such as CCAAT-enhancer-binding proteins (C/EBP), specificity protein 1 (Sp1), neurofibromin 1 (NF-1), nuclear factor Y (NF-Y) and HNF-4α. The action exerted by glucocorticoid signalling on the urea cycle is mediated mainly by HNF-4α[18,44]. It remains to be explored if HNF-4α is also modulated during DRF.

CPS1 is an abundant mitochondrial enzyme that initiates a set of reactions in the urea cycle. Its activity is allosterically dependent on N-acetylglutamate[45]. The average of the 24 h levels was higher in the DRF group, mainly because of an increment in CPS1 activity during the light period (Fig. 5 and Table 2). This difference is most probably due to an augmentation in the presence of the enzyme. It remains to be explored if the availability of N-acetylglutamate is regulated by food restriction. It has been reported that sirtuin 5, a deacetylase mitochondrial enzyme, recognises CPS1 as a substrate, resulting in an enhancement of its activity[46]. This fact relates CPS1 to the nutritional state of the organism, since sirtuins are a family of NAD$^+$-dependent proteins that are responsive to the metabolic status of the cell[47].

OTC is the second enzyme of the urea cycle and also located within the liver mitochondria. Its activity is only slightly responsive to hormonal stimulation (glucagon and glucocorticoids), but it shows a very clear diurnal rhythmicity[18,48]. The DRF protocol promoted a substantial change in the 24 h rhythmicity of OTC in comparison with the control group fed *ad libitum* (Fig. 5(c)). OTC is not an allosteric enzyme, but recently it was demonstrated that is also regulated by acetylation–deacetylation, not by sirtuin 5, but by sirtuin 3. As with CPS1, deacetylation of OTC increases its activity[49].

Circadian adaptations of liver metabolism during restricted feeding

After 3 weeks, daily rhythmicity is greatly modified by 2 h of daytime restricted food access. The adaptations occur at several levels: (1) a great hyperphagia after mealtime alters the process of nutrient assimilation[50]; in consequence, the
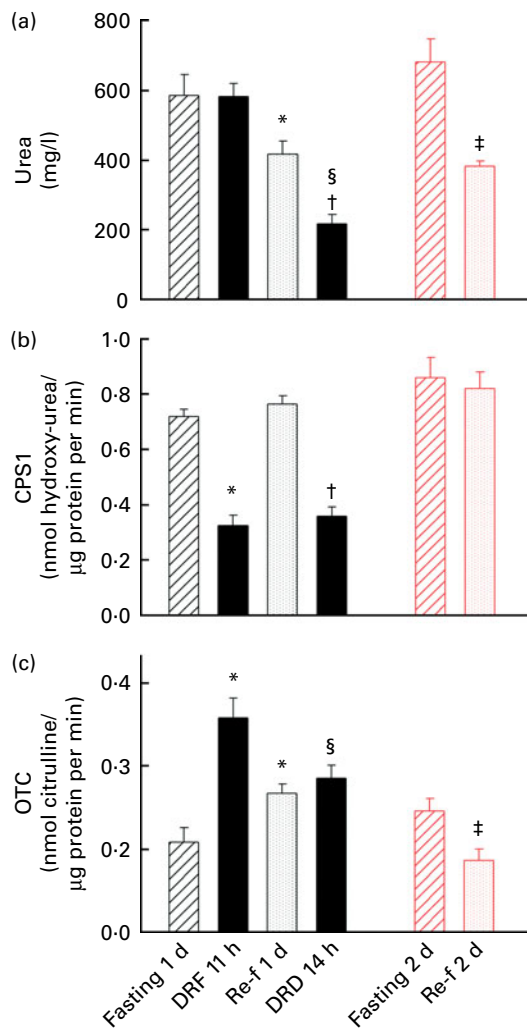
Fig. 6. Comparison between a daytime restricted feeding (DRF) protocol and fasting and refeeding (about 1 and 2 d) on the levels of circulating urea and activities of carbamoyl phosphate synthetase-1 (CPS1) and ornithine transcarbamylase (OTC) in the liver. Circulating urea levels (a), activity of CPS1 (b) and activity of OTC (c) in groups of fasting and refeeding (Re-f) for about 1 and 2 d, as well as rats under DRF. Data are means of at least eight independent observations, with standard errors represented by vertical bars. * Mean value was significantly different from that for fasting 1 d ($P < 0.05$; Tukey *post hoc* test). † Mean value was significantly different from that for Re-f 1 d ($P < 0.05$; Tukey *post hoc* test). ‡ Mean value was significantly different from that for fasting 2 d ($P < 0.05$; Tukey *post hoc* test). § Mean value was significantly different from that for DRF 11 h ($P < 0.05$; Tukey *post hoc* test).

content of food in the gastric chamber last about 20 h and promotes changes in the rhythm of circulating ghrelin (data not shown); (2) 24 h fluctuations of a number of endocrine signals are largely modified, including thyroid hormones[51], growth hormone and insulin-like growth factor-1 (data not shown), and cytokines[11]; (3) an enhancement in lipid mobilisation from the adipose tissue and reduction of hepatic glycogen degradation[31,52]; and (4) change in the set point of regulatory parameters in the metabolic control of the liver such as cytoplasmic and mitochondrial redox state as well as adenine nucleotide-related energy charge[10]. The modifications include not only phase shifts of 24 h rhythmicity, for example,

expression of liver PER1[11], but also significant increment (for example, liver peroxisomal markers[37]) or reduction (for example, activity of liver phosphoenolpyruvate carboxy-kinase (PEPCK), data not shown) in the amplitude of diurnal rhythms. In addition, some parameters show a significant increment (for example, mitochondrial proton-motive electro-chemical force[35]), whereas others are reduced (for example, leptin[50]). One possible interpretation of all these modifications underlying circadian entrainment to food is the emergence of an alternative distributed oscillator different from the SCN, known as the FEO that integrates metabolic, physiological and behavioural responses[53,54].

It is in this context in which the findings of the present research should be considered: upon the DRF protocol or expression of the FEO, glucocorticoid signalling adopts a new category of rheostatic equilibrium in which there is an up-regulation of GCR as well as a modification in the rhythmicity of the urea cycle.

Acknowledgements

References

1. Yamazaki S, Numano R, Abe M, *et al.* (2000) Resetting central and peripheral oscillators in transgenic mice. *Science* **288**, 682–685.
2. Stratmann M & Schibler U (2006) Properties, entrainment and physiological functions of mammalian peripheral oscillators. *J Biol Rhythms* **21**, 494–506.
3. Laposky AD, Bass J, Kohsaka A, *et al.* (2008) Sleep and circadian rhythms: key components in the regulation of energy metabolism. *FEBS Lett* **582**, 142–151.
4. Chung S, Son GH & Kim K (2011) Circadian rhythm of adrenal glucocorticoid: its regulation and clinical implications. *Biochim Biophys Acta* **1812**, 581–591.
5. Damiola F, Le Minh N, Preitner N, *et al.* (2000) Restricted feeding uncouples circadian oscillators in peripheral tissues from central pacemaker in the suprachiasmatic nucleus. *Genes Dev* **14**, 2950–2961.
6. Stokkan KA, Yamazaki S, Tei H, *et al.* (2001) Entrainment of the circadian clock in the liver by feeding. *Science* **291**, 490–493.
7. Mistlberger RE (2011) Neurobiology of food anticipatory circadian rhythms. *Physiol Behav* **104**, 535–545.

8. Stephan FK (2002) The "other" circadian system: food as a Zeitgeber. *J Biol Rhythms* **17**, 284–292.

9. Mendoza J (2007) Circadian clocks: setting time by food. *J Neuroendocrinol* **19**, 127–137.

10. Díaz-Muñoz M, Vázquez-Martínez O, Aguilar-Roblero R, *et al.* (2000) Anticipatory changes in liver metabolism and entrainment of insulin, glucagon, and corticosterone in food-restricted rats. *Am J Physiol Regul Integr Comp Physiol* **279**, R2048–R2059.

11. Luna-Moreno D, Aguilar-Roblero R & Díaz-Muñoz M (2009) Restricted feeding entrains rhythms of inflammation-related factors without promoting an acute-phase response. *Chronobiol Int* **26**, 1409–1429.

12. Dickmeis T (2009) Glucocorticoids and the circadian clock. *J Endocrinol* **200**, 3–22.

13. Prager EM & Johnson LR (2009) Stress at the synapse: signal transduction mechanisms of adrenal steroids at neuronal membranes. *Sci Signal* **86**, re5.

14. Desvergne B, Michalik L & Wahli W (2006) Transcriptional regulation of metabolism. *Physiol Rev* **86**, 465–514.

15. Seckl JR & Walker BR (2001) Minireview: 11β-hydroxysteroid dehydrogenase type 1 – a tissue-specific amplifier of glucocorticoid action. *Endocrinology* **142**, 1371–1376.

16. Balsalobre A, Brown SA, Marcacci L, *et al.* (2000) Resetting of circadian time in peripheral tissues by glucocorticoid signaling. *Science* **289**, 2344–2347.

17. Le Minh N, Damiola F, Tronche F, *et al.* (2001) Glucocorticoid hormones inhibit food-induced phase-shifting of peripheral circadian oscillators. *EMBO J* **20**, 7128–7136.

18. Reddy AB, Maywood ES, Karp NA, *et al.* (2007) Glucocorticoid signaling synchronizes the liver circadian transcriptome. *Hepatology* **45**, 1478–1488.

19. Yamamoto T, Nakahata Y, Tanaka M, *et al.* (2005) Acute physical stress elevates mouse *period1* mRNA expression in mouse peripheral tissues via a glucocorticoid-responsive element. *J Biol Chem* **280**, 42036–42043.

20. Torra IP, Tsibulsky V, Delaunay F, *et al.* (2000) Circadian and glucocorticoid regulation of Rev-erbα expression in liver. *Endocrinology* **141**, 3799–3806.

21. Aguilar-Delfín I, López-Barrera F & Hernández-Muñoz R (1996) Selective enhancement of lipid peroxidation in plasma membrane in two experimental models of liver regeneration: partial hepatectomy and acute CC14 administration. *Hepatology* **24**, 657–662.

22. Reiners JJ & Busch H (1980) Transcriptional and post-transcriptional modulation of cytoplasmic ribonucleic acids in regenerating liver and Novikoff hepatoma. *Biochemistry* **19**, 833–841.

23. Chilov D, Hofer T, Bauer C, *et al.* (2001) Hypoxia affects expression of circadian genes *PER1* and *CLOCK* in mouse brain. *FASEB J* **15**, 2613–2622.

24. Lowry OH, Rosebrough NJ, Farr AL, *et al.* (1951) Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**, 265–275.

25. Thurston LM, Abayasekara DR & Michael AE (2007) 11β-Hydroxysteroid dehydrogenase expression and activities in bovine granulosa cells and corpora lutea implicate corticosteroids in bovine ovarian physiology. *J Endocrinol* **193**, 299–310.

26. Nammi S, Dembele K & Nyomba BL (2007) Increased 11β-hydroxysteroid dehydrogenase type-1 and hexose-6-phosphate dehydrogenase in liver and adipose tissue of rat offspring exposed to alcohol *in utero*. *Am J Physiol Regul Integr Comp Physiol* **292**, R1101–R1109.

27. Pierson DL (1980) A rapid colorimetric assay for carbamyl phosphate synthetase I. *J Biochem Biophys Methods* **3**, 31–37.

28. Bagrel A, Museur G & Siest G (1975) Colorimetric measurement of ornithine carbamoyl transferase activity in plasma, and results for a supposedly healthy population. *Clin Chem* **21**, 1716–1720.

29. Morris SM, Moncman CL, Rand KD, *et al.* (1987) Regulation of mRNA levels for five urea cycle enzymes in rat liver by diet, cyclic AMP, and glucocorticoids. *Arch Biochem Biophys* **256**, 343–353.

30. Schibler U, Ripperger J & Brown SA (2003) Peripheral circadian oscillators in mammals: time and food. *J Biol Rhythms* **18**, 250–260.

31. Escobar C, Díaz-Muñoz M, Encinas F, *et al.* (1998) Persistence of metabolic rhythmicity during fasting and its entrainment by restricted feeding schedules in rats. *Am J Physiol* **274**, R1309–R1316.

32. Dutta D & Sharma R (2003) Regulation of hepatic glucocorticoid receptors. *Horm Metab Res* **35**, 415–420.

33. Geng CD, Schwartz JR & Vedeckis WV (2008) A conserved molecular mechanism is responsible for the auto-up-regulation of glucocorticoid receptor gene promoters. *Mol Endocrinol* **22**, 2624–2642.

34. Lin WY, Hu YJ & Lee YH (2008) Hepatocyte nuclear factor-1α regulates glucocorticoid receptor expression to control postnatal body growth. *Am J Physiol Gastrointest Liver Physiol* **295**, G542–G551.

35. Báez-Ruiz A, Escobar C, Aguilar-Roblero R, *et al.* (2005) Metabolic adaptations of liver mitochondria during restricted feeding schedules. *Am J Physiol Gastrointest Liver Physiol* **289**, G1015–G1023.

36. Reinke H, Saini C, Fleury-Olela F, *et al.* (2008) Differential display of DNA-binding proteins reveals heat-shock factor 1 as a circadian transcription factor. *Genes Dev* **22**, 331–345.

37. Rivera-Zavala J, Báez-Ruiz A & Díaz-Muñoz M (2011) Changes in the 24-h rhythmicity of liver PPARs and peroxisomal markers when feeding is restricted to two daytime hours. *PPAR Res* (epublication ahead of print version 5 April 2011).

38. Báez-Ruiz A & Díaz-Muñoz M (2011) Chronic inhibition of endoplasmic reticulum calcium-release channels and calcium-ATPase lengthens the period of hepatic clock gene *Per1*. *J Circadian Rhythms* **9**, 6.

39. Morton NM (2010) Obesity and corticosteroids: 11β-hydroxysteroid type 1 as a cause and therapeutic target in metabolic disease. *Mol Cell Endocrinol* **316**, 154–164.

40. Nader N, Ng SS, Lambrou GI, *et al.* (2010) AMPK regulates metabolic actions of glucocorticoids by phosphorylating the glucocorticoid receptor through p38 MAPK. *Mol Endocrinol* **24**, 1748–1764.

41. Norris DO (2007) *Vertebrate Endocrinology*, 4th ed. San Diego: Elsevier Academic Press.

42. Takiguchi M & Mori M (1995) Transcriptional regulation of genes for ornithine cycle enzymes. *Biochem J* **312**, 649–659.

43. Christowitz D, Mattheyse FJ & Balinsky JB (1981) Dietary and hormonal regulation of urea cycle enzymes in rat liver. *Enzyme* **26**, 113–121.

44. Bailly A, Briançon N & Weiss MC (2009) Characterization of glucocorticoid receptor and hepatocyte nuclear factor 4α (HNF4α) binding to the *hnf4α* gene in the liver. *Biochimie* **91**, 1095–1103.

45. Lusty CJ (1978) Carbamoylphosphate synthetase I of rat-liver mitochondria. Purification, properties, and polypeptide molecular weight. *Eur J Biochem* **85**, 373–383.

46. Nakagawa T & Guarente L (2009) Urea cycle regulation by mitochondrial sirtuin, SIRT5. *Aging* **1**, 578–581.

47. Asher G & Schibler U (2011) Crosstalk between components of circadian and metabolic cycles in mammals. *Cell Metab* **13**, 125–137.

48. Lin RC, Snodgrass PJ & Rabier D (1982) Induction of urea cycle enzymes by glucagon and dexamethasone in monolayer cultures of adult rat hepatocytes. *J Biol Chem* **257**, 5061–5067.

49. Hallows WC, Yu W, Smith BC, *et al.* (2011) Sirt3 promotes the urea cycle and fatty acid oxidation during dietary restriction. *Mol Cell* **41**, 139–149.

50. Martínez-Merlos MT, Angeles-Castellanos M, Díaz-Muñoz M, *et al.* (2004) Dissociation between adipose tissue signals, behavior and the food-entrained oscillator. *J Endocrinol* **181**, 53–63.

51. Aceves C, Escobar C, Rojas-Huidobro R, *et al.* (2003) Liver 5'-deiodinase activity is modified in rats under restricted feeding schedules: evidence for post-translational regulation. *J Endocrinol* **179**, 91–96.

52. Díaz-Muñoz M, Vázquez-Martínez O, Báez-Ruiz A, *et al.* (2010) Daytime food restriction alters liver glycogen, triacylglycerols, and cell size. A histochemical, morphometric, and ultrastructural study. *Comp Hepatol* **9**, 5.

53. Aguilar-Roblero R & Díaz-Muñoz M (2010) Chronostatic adaptations in the liver to restricted feeding: the FEO as an emergent oscillator. *Sleep Biol Rhythms* **8**, 9–17.

54. Blum ID, Lamont EW & Abizaid A (2012) Competing clocks: metabolic status moderates signals from the master circadian pacemaker. *Neurosci Biobehav Rev* **36**, 254–270.