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Anticipatory changes in liver metabolism and entrainment of insulin, glucagon, and corticosterone in food-restricted rats

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Díaz-Muñoz, Mauricio, Olivia Vázquez-Martínez, Raúl Aguilar-Roblero, and Carolina Escobar. Anticipatory changes in liver metabolism and entrainment of insulin, glucagon, and corticosterone in food-restricted rats. *Am J Physiol Regulatory Integrative Comp Physiol* 279: R2048–R2056, 2000.—Restricted feeding schedules entrain behavioral and physiological circadian rhythms, which depend on a food-entrainable oscillator (FEO). The mechanism of the FEO might depend on digestive and endocrine processes regulating energy balance. The present study characterizes the dynamics of circulating corticosterone, insulin, and glucagon and regulatory parameters of liver metabolism in rats under restricted feeding schedules. With respect to ad libitum controls, food-restricted rats showed *1*) an increase in corticosterone and glucagon and a decrease in insulin before food access, indicating a predominant catabolic state; and *2*) a reduction in lactate-to-pyruvate and β-hydroxybutyrate-to-acetoacetate ratios, indicating an oxidized cytoplasmic and mitochondrial redox state in the liver metabolism. All these changes were reversed after feeding. Moreover, liver energy charge in food-restricted rats did not show a significant modification before feeding, despite an increase in adenine nucleotides, but showed an important decrease after food intake. Variations detected in the liver of food-restricted rats are different from those prevailing under 24-h fasting. These observations suggest "anticipatory activity" of the liver metabolism to optimize the processing of nutrients to daily feeding. Data also suggest a possible relationship of the liver and endocrine signals with the FEO.

meal feeding; food-entrained oscillator; hepatic metabolism; endocrine profile

CIRCADIAN TIMING SYSTEMS ARE organized by multiple coupled oscillators (30). In mammals, circadian rhythms are mainly driven by the suprachiasmatic nuclei (SCN) in the hypothalamus, which is entrained by the light-dark cycle. However, the presence of a different oscillator, which is entrained by food (FEO), can be elicited by restricting food access to a few hours daily for a number of consecutive days. Under such conditions, animals show behavioral arousal and increased locomotor activity 2–3 h before food access, which is known as anticipatory activity (AA). Associated with this behavior are several physiological variables that change their circadian phase and shift in relation to the feeding schedule. Such is the case for plasma corticosterone (14), body temperature (16), enteric enzymes (32), and electrical activity of the stomach and duodenum (7). Entrainment to feeding schedules has shown to depend on a circadian timing system independent of the SCN, because it can still be observed after bilateral lesions of these nuclei (35). However, the anatomic and functional characteristics of the FEO have not been identified.

The expression of AA or related metabolic entrainment can only be induced when restriction to food access leads animals toward a catabolic state (14), whereas it is not observed when animals are overfed (28) or obese due to ventromedial hypothalamic lesions (23) or aging (21). Also, to induce AA, the food ingested during the feeding schedule must be the predominant meal for the organism, either by amount or by caloric content (22). Because the expression of FEO is dependent on nutritional properties (34), but not on olfactory (6) or taste cues (20), it is assumed that FEO is related to the animal's energetic metabolism. The relationship of metabolic processes and entrainment by restricted feeding schedules suggests that FEO's mechanism may be closely related to the digestive system, endocrine processes, and their interaction with structures in the nervous system associated with feeding and energy balance (10, 11).

In a previous study, we reported the relationship of metabolic parameters and restricted feeding schedules in rats. For animals during anticipation, we described depletion of liver glycogen and a decrease in triglycerides, followed by an increase in plasma levels of free fatty acids and ketone bodies. Altogether, these findings indicate that entrainment to restricted feeding schedules establishes a temporal cycle of energy balance in which the organisms alternate from an anabolic state after feeding to a catabolic state when anticipating food access (11). In this alternation of energy

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metabolic states, the regulation of liver function by endocrine signals plays a predominant role.

The liver is important for spontaneous feeding, digestion, and metabolic balance in the organism, because it functions as a peripheral integrator of nutrient availability and energetic needs of the organism (18). Most of the nutrients are directed toward the liver from the digestive tract, and the main biomolecules (carbohydrates, lipids, and proteins) are metabolized by the hepatic tissue, depending on the anabolic or catabolic status of the organism. However, the role of liver metabolism during restricted feeding schedules has not been evaluated. An adequate response of the liver to energy requirements is attained through the hormonal profile, which dictates the direction toward anabolic or catabolic processes. Furthermore, hormones play a key role in the communication between peripheral organs and the brain. The relevance of this later process is indicated by evidence that AA is not affected by subdiaphragmatic vagotomy (8) or capsaisin-induced visceral deafferentation (9).

When one considers previous evidence that restricted feeding schedules entrain energy metabolites, it becomes important to identify the influence of feeding schedules on relevant endocrine signals associated with their storage and mobilization, as well as on those factors that control hepatic metabolism. Hence, the present study aimed to *1*) characterize the dynamics of serum corticosterone, insulin, and glucagon and *2*) determine the metabolic condition of the liver by the cytoplasmic and mitochondrial redox state and by the hepatic level of energy charge (EC) in rats maintained under restricted feeding schedules.

MATERIALS AND METHODS

Animals and Housing

Adult male Wistar rats weighing 210–225 g at the beginning of the experiment were maintained in a 12:12-h light-dark cycle (lights on at 0800) and constant temperature (22 ± 1°C). The light intensity at the surface of the cages averaged 400 lux. Rats were kept with free access to Purina Chow and water and were acclimated to environmental conditions for at least 3 wk before the start of the experimental procedures. Rats were then randomly assigned to the control or the restricted feeding condition and were housed in groups of four to six in transparent acrylic cages (40 × 50 × 20 cm). All rats were maintained under the same environmental conditions as described with free access to water. All experimental procedures were approved and conducted according to the institutional guide for care and use of animal experimentation (Universidad Nacional Autónoma de México).

Experimental Design

Rats were randomly assigned to one of two experiments, each consisting of two groups: control rats fed ad libitum and rats exposed to a daily restricted feeding schedule (RFS group), from 1200 to 1400 for 3 wk. To obtain either blood or liver samples, on *day 21* of exposure to their corresponding feeding schedule, rats from each experimental group were randomly killed at 0900, 1000, 1100, 1200, 1400, or 1800.

The first experiment was aimed at characterizing the effect of a restricted feeding schedule on circulating corticosterone, glucagon, and insulin, as well as free fatty acids. The second experiment was aimed at characterizing the effect of a restricted feeding schedule on the liver cytoplasmic and mitochondrial redox states, the hepatic level of EC, and the content of liver glycogen. The effects of a restricted feeding schedule on free fatty acids and liver glycogen were reported previously (11) and were replicated for this study to have an internal control of the experimental procedure. Also, the behavior of some animals from the RFS group was individually monitored during the last 5 days before death with the DiSPAC automatic system for drinking behavior (1) to verify the efficacy of the procedure to induce AA.

Data from the control and the RFS group are reported as means ± SE. Differences between groups were tested by a two-way ANOVA (feeding schedule × time of day), followed by a Tukey's multiple-comparison post hoc test, with α set at $P < 0.01$. Statistical analysis was performed with the statistical package Statistica (version 4.5, 1993).

General and Biochemical Procedures

Blood sampling. Rats ($n = 8$ for each group and time point) were decapitated and 3–4 ml trunk blood were collected in 10-ml silicone-coated test tubes containing a clot-activator gel (Vacutainer), which were centrifuged at 2,500 rpm for 15 min to obtain blood serum. Aliquots of 700 μl were coded and frozen at −70°C for subsequent determination of corticosterone, glucagon, insulin, and free fatty acids.

Liver sampling. Rats ($n = 6$ for each group and time point) were killed by a blow to the back of the head, and three samples of hepatic tissue were removed in <10 s. This procedure is necessary to minimize postmortem degradation of adenine nucleotides. For determination of redox metabolites, one sample (≈300 mg) was extracted in 1.2 ml of chilled 0.6 N $HClO_4$; the second sample (≈1 g) for quantification of adenine nucleotides was similarly extracted (0.8 N $HClO_4$) as indicated previously (12). The extracts were centrifuged at 10,000 *g* for 10 min to separate denatured protein, and the supernatant was preserved in coded aliquots and frozen at −70°C until processing. A third liver sample (≈1 g) was immediately frozen in dry ice and preserved at −70°C for later glycogen determination.

Determinations of serum hormones and metabolites. Aliquots were thawed, and hormonal concentration was determined in duplicate with standard ^{125}I radioimmunoassay kits for insulin (Diagnostic Systems Laboratories), glucagon, and corticosterone (Diagnostic Products) according to Zambrano and Díaz Sánchez (40). Serum-free fatty acids were processed by using colorimetric methods as previously described (11).

Determination of liver metabolites. Perchloric acid extracts were neutralized with 4 M K_2CO_3 and used for metabolite determinations by enzymatic methods: lactate (26), pyruvate (17), β-hydroxybutyrate (37), and acetoacetate (19). Adenine nucleotides (ATP, ADP, and AMP) were quantified by HPLC, according to the method of Hoffman and Liao (13). Glycogen was determined from a 1-g sample as previously described (11).

Estimation of redox state and energy charge. Cytoplasmic and mitochondrial redox states are described by the NAD^+/NADH ratio, which is estimated from the following equation: $NAD^+/NADH$ = [oxidized substrate]/[reduced substrate] × $1/K_{eq}$, where K_{eq} is the equilibration constant. Cytoplasmic redox state was calculated from the lactate-to-pyruvate ratio, considering the $K_{eq} = 1.11 \times 10^{-4}$ M for lactate dehydrogenase (36), and mitochondrial redox state was estimated from the β-hydroxybutyrate-to-acetoacetate ratio, considering the

$K_{eq} = 4.93 \times 10^{-2}$ M for β-hydroxybutyrate dehydrogenase (38). Redox potential (E_h), defined as the ability to dissect an overall electron transfer into two half-reactions (25), was estimated on the basis of the Nerst equation as follows: $E_h = E'o + 0.03 \log NAD^+/NADH$, where $E'o = -314$ mV (25). The difference between cytoplasmic (E_{hc}) and mitochondrial (E_{hm}) redox potentials (ΔE) was calculated from $\Delta E = E_{hm} - E_{hc}$. Liver EC was estimated from the relationship between individual and total adenine nucleotides (TAN) concentration by the method reported by Atkinson (3) as follows: EC = (ATP + 0.5 ADP)/TAN, where TAN = AMP + ADP + ATP.

RESULTS

Behavioral recordings showed clear AA (Fig. 1*A*). Before food ingestion, free fatty acids were increased compared with controls (from 1100 to 1200) and declined after feeding (Fig. 1*B*), whereas liver glycogen was depleted before and recovered to control levels after feeding (Fig. 1*C*). These results are in agreement with previous results (11) and corroborate the efficacy of the procedure to induce the expression of FEO.

Hormonal Profile

Serum levels of corticosterone did not show important changes in the control group, but in the RFS rats showed a significant increase before food presentation (Fig. 2*A*). Insulin was significantly decreased during the hours before food presentation (from 0900 to 1200) and was increased above control levels after feeding (Fig. 2*B*); its lowest values were 50% of control rats. Levels of glucagon in the RFS group showed increased values in all time points but a significant twofold increase compared with controls before food access (Fig. 2*C*). Consequently, the insulin-to-glucagon ratio, which indicates the catabolic or anabolic tendency in the organism, showed a significant reduction in RFS rats during the 3 h before food access and returned to control levels after feeding (Fig. 2*D*). In control animals, this ratio was always above the values from RFS rats. The two-way ANOVA indicated significant effects of feeding condition and its interaction with time points for corticosterone, a significant effect of feeding conditions for glucagon and of time points and their interaction with feeding conditions for insulin (Table 1).

Hepatic Cytoplasmic Redox State

Rats fed ad libitum showed constant concentrations of liver lactate and pyruvate at all time points. Before food presentation, RFS animals showed decreased values of hepatic lactate (reduced metabolite), which reached 20% of control values and recovered to control values after food ingestion (Fig. 3*A*). The opposite was observed for pyruvate levels (oxidized metabolite), which showed a significant increase in RFS rats before food availability and returned to control values immediately after feeding (Fig. 3*B*). As a consequence of the combined decrease in the reduced metabolite and the increase in the oxidized metabolite, the lactate-to-pyruvate ratio showed significant lower values before food access (Fig. 3*C*), whereas at the same time points

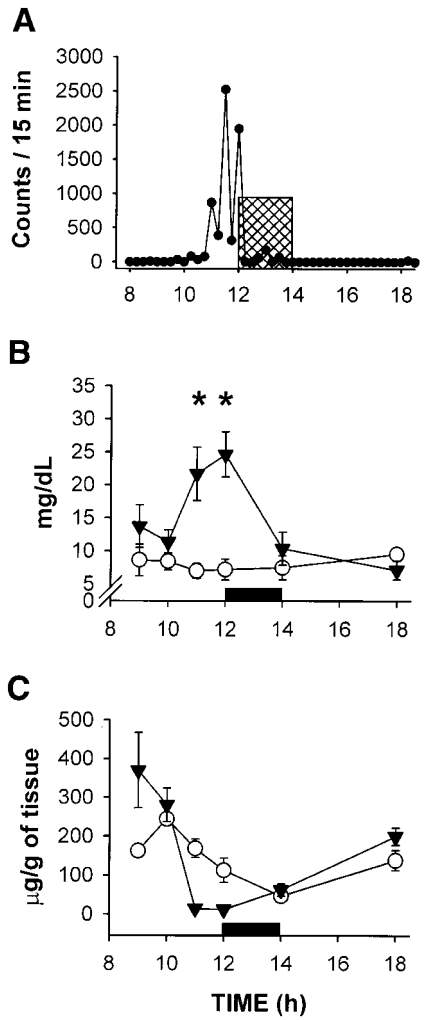

Fig. 1. Activity profile obtained from 5 days of automatic recording of drinking behavior in rats (*A*) exposed to a restricted feeding schedule (access to food, 1200–1400). A clear anticipatory response is observed 1 h before food access (hatched rectangle). Free fatty acid concentration in serum (*B*) and liver glycogen (*C*) of food-entrained rats (▲) and their ad libitum controls (○). Values represent means ± SE. Filled horizontal bar on the abscissa, time of food access. *Significant difference between both groups at the indicated time point (Tukey's post hoc test, $\alpha = 0.01$).

cytoplasmic NAD^+/NADH increased with a peak at 1100 (Fig. 3*D*). After feeding, both ratios returned to control levels. These parameters indicate an oxidized state of hepatocyte cytoplasm during AA. For all parameters, the two-way ANOVA indicated significant effects of feeding conditions, time points, and their interaction (Table 2).

Hepatic Mitochondrial Redox State

Rats fed ad libitum showed constant levels of both hepatic ketone bodies, β-hydroxybutyrate (oxidized metabolite) and acetoacetate (reduced metabolite), at all time points. In contrast, in RFS rats the levels of both ketone bodies were dramatically increased before feeding (0900–1200) and returned to control values in response to food ingestion (Fig. 4, *A* and *B*). Because of

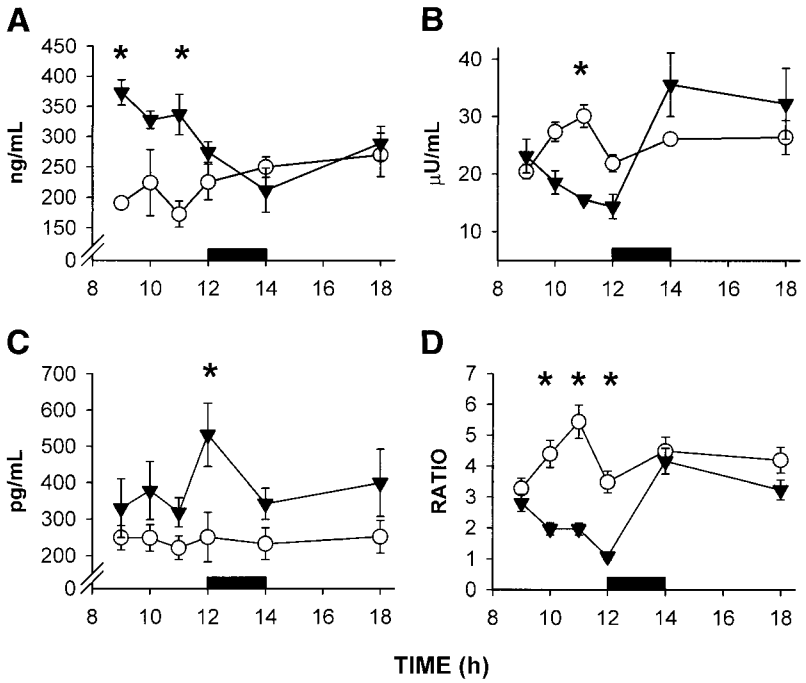

Fig. 2. Hormonal profile for corticosterone (*A*), insulin (*B*), glucagon (*C*), and insulin-to-glucagon ratio (*D*) of rats maintained under a restricted feeding schedule for 3 wk (▲) and their corresponding ad libitum controls (○). Data are means ± SE. Filled horizontal bar, time of food access. *Significant difference between both groups at the indicated time point (Tukey's post hoc test, $\alpha = 0.01$).

a major proportion in the increase of acetoacetate levels, the β-hydroxybutyrate-to-acetoacetate ratio presented a significant diminution before food presentation (Fig. 4*C*). At the same time points, NAD^+/NADH was increased with a peak at 1000, and both parameters returned to control values after feeding (Fig. 4*D*). These parameters indicate an oxidized state of the mitochondria during AA. With the exception of the β-hydroxybutyrate-to-acetoacetate ratio, the two-way ANOVA indicated significant effects of feeding conditions, time points, and their interaction for all parameters (Table 2).

E_{hc} and E_{hm}

In livers of rats fed ad libitum, both E_{hc} and E_{hm} remained constant at all times tested. However, in the cytoplasm of RFS rats, a more positive redox potential was found before food presentation, with a maximum difference of 40 mV at 1100 for cytoplasm (Table 3), whereas in the mitochondrial compartment E_{hm} changed only 6–7 mV at 1000 and 1100 with respect to controls. In rats fed ad libitum, ΔE between both compartments remained constant within a narrow range (44–50 mV). However, in RFS rats there was an increase in ΔE before food ingestion, which returned to control levels after feeding (Table 3). The two-way ANOVA indicated significant effects of feeding conditions, time points, and their interaction for all parameters (Table 4).

Liver EC

Liver adenine nucleotides (AMP, ADP, and ATP) did not vary significantly in control rats for the times tested. In contrast, the three adenine nucleotides changed under restricted feeding schedules. AMP increased only slightly from 0900 to 1200, but it showed a significant increase after food ingestion (Fig. 5*A*). In contrast, ADP and ATP values were increased from 0900 to 1200. ADP showed a trend to decrease with lowest values at 1800 (Fig. 5*C*), whereas ATP showed a pronounced decrease below control values after food intake (Fig. 5*E*). Modifications in the three nucleotides had repercussions in EC values and TAN. EC did not vary with respect to controls before food presentation but decreased to significantly lower values than controls at 1400 and 1800 (Fig. 5*D*), indicating increased metabolic activity in the hepatic tissue after feeding. In contrast, TAN levels were significantly higher before feeding and returned to control values after food ingestion (Fig. 5*B*). This latter parameter may indicate

Table 1. *Values obtained with a 2-way ANOVA for the effects of nutritional schedule (2 levels), time points (6 levels), and their interaction on serum hormones*

	Feeding Schedule		Time Points		Feeding Schedule × Time	
	$F_{(1,84)}$	P	$F_{(5,84)}$	P	$F_{(5,84)}$	P
Corticosterone	19.73	<0.001	0.84	NS	3.76	<0.01
Glucagon	21.71	<0.001	1.36	NS	0.96	NS
Insulin	1.54	NS	5.43	<0.001	5.21	<0.001

NS, not significant.

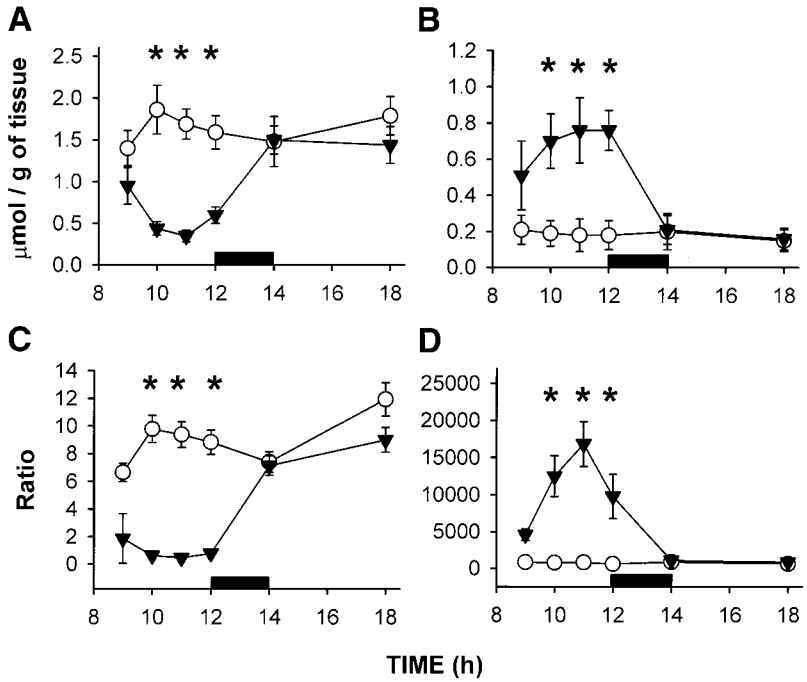

Fig. 3. Liver cytoplasmic parameters for redox state. Lactate (reduced metabolite; *A*), pyruvate (oxidized metabolite; *B*), their relationship (*C*), and global redox state of the cytoplasm (NAD^+/NADH; *D*) in rats maintained under a restricted feeding schedule (▲) and ad libitum controls (○). Other indications are similar to Fig. 2. *Significant difference between both groups at the indicated time point (Tukey's post hoc test, $\alpha = 0.01$).

storage of energy supplies in the liver. The two-way ANOVA indicated main effects of the feeding schedule on all energy parameters and further significant effects of time points and the interaction feeding schedule × time for TAN and EC (Table 4).

DISCUSSION

The present study provides data concerning the dynamics of hormones and liver metabolism with respect to restricted feeding schedules. Most of the studied parameters showed significant changes before food presentation and returned to control values afterward, whereas insulin, liver AMP, ATP, and EC showed important changes, mainly after feeding. The coordinated alternation of metabolic events associated with restricted feeding schedules reflects a singular adaptive process different from mechanisms underlying spontaneous feeding and induced fasting. This is relevant with respect to the mechanisms involved in the expression of FEO and its entrainment.

Under different states of energy demand, hormones such as insulin, glucagon, and corticosterone play an important role, coordinating metabolic activity among organs. Hence, the switch between anabolic and catabolic states in the liver is commanded by the hormonal profile. In the rats anticipating daily meals, changes before feeding or promoted by food intake were observed in the hormonal serum levels of insulin, glucagon, and corticosterone. Changes before feeding were characterized by a sustained elevation of corticosterone and a decrease in the insulin-to-glucagon ratio, due to a drop in insulin and a peak of glucagon levels. This endocrine condition corresponds to a catabolic state in which lipid mobilization, decrease in liver glycogen, and activation of gluconeogenesis are induced to maintain energy balance. After feeding, corticosterone levels returned to the control range and the insulin-to-glucagon ratio returned to values similar to rats fed ad libitum, mainly because of an enhanced insulin secretion by the pancreatic β-cells. This later hormonal

Table 2. *Values obtained with a 2-way ANOVA for the effects of nutritional schedule (2 levels), time points (6 levels), and their interaction on the redox parameters for cytoplasm and mitochondria*

	Feeding Schedule		Time Points		Feeding Schedule × Time	
	$F_{(1,48)}$	P	$F_{(5,48)}$	P	$F_{(5,48)}$	P
Lactate	93.93	<0.001	6.21	<0.001	9.32	<0.001
Pyruvate	58.36	<0.001	7.17	<0.001	6.37	<0.001
Lactate/pyruvate	129.94	<0.001	9.93	<0.001	10.58	<0.001
Cytoplasmic NAD^+/NADH	144.68	<0.001	22.04	<0.001	22.03	<0.001
Acetoacetate	170.6	<0.001	15.05	<0.001	15.02	<0.001
β-Hydroxybutyrate	95.94	<0.001	7.25	<0.001	8.25	<0.001
β-Hydroxybutyrate/acetoacetate	24.42	<0.001	3.34	<0.01	2.04	NS
Mitochondrial NAD^+/NADH	32.57	<0.001	5.36	<0.001	5.16	<0.001

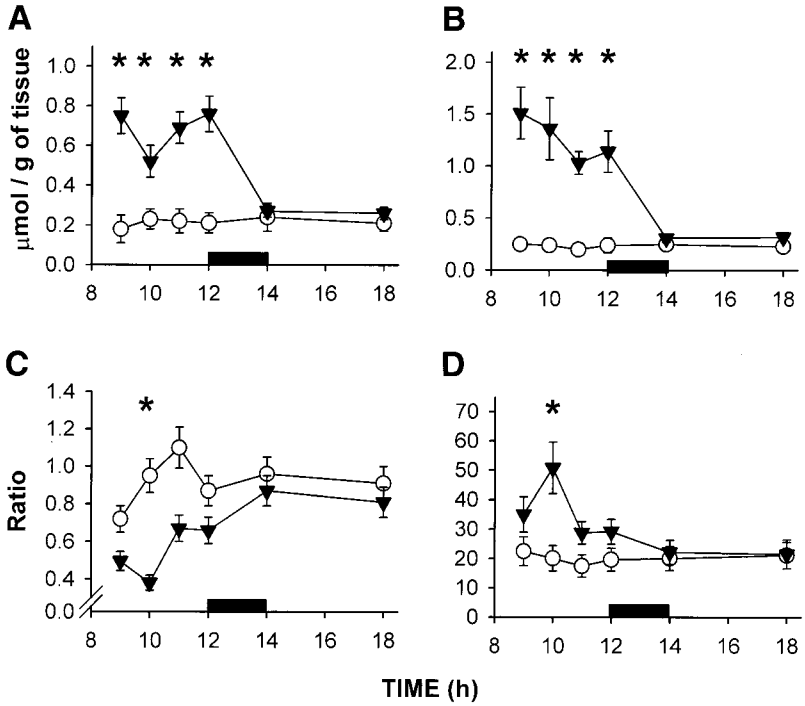

Fig. 4. Liver mitochondrial parameters indicative of the redox state. β-Hydroxybutyrate (oxidized metabolite; *A*), acetoacetate (reduced metabolite; *B*), their relationship (*C*), and global redox state in the mitochondria (NAD^+/NADH; *D*) in rats maintained under a restricted feeding schedule (▲) and ad libitum controls (○). Other indications are similar to Fig. 2. *Significant difference between both groups at the indicated time point (Tukey's post hoc test, $\alpha = 0.01$).

profile is characteristic of an anabolic state. The endocrine alternation between catabolic and anabolic states in RFS animals is consistent with the dynamics of hepatic glycogen and circulating free fatty acids previously reported (11) and replicated in this study.

The present data confirm previous reports describing entrainment of corticosterone rhythm by restricted feeding schedules in intact as well as in SCN-lesioned rats (16). They also are in agreement with previous data (2) obtained from mice maintained by a restricted feeding schedule during 2 wk, which describe a rise in plasma corticosterone and a decrease in insulin before food access. On the other hand, a recent study (10) described a decrease in glucagon and no changes in insulin and corticosterone plasma levels in rats maintained under restricted feeding schedules. The present data differ from these observations in several aspects. However, comparison between both studies is difficult due to a difference in experimental design and the use of anesthetic during the sampling procedure.

The balance between oxidizing and reducing reactions linked to the NAD^+/NADH pair regulates enzyme activities in both cytoplasmic and mitochondrial compartments of the liver (29). Switching between principal anabolic and catabolic pathways in the hepatocyte is dependent on the values of redox state and the proportion of adenine nucleotides (38). The main effect in the liver of RFS rats was the promotion of oxidized cytoplasmic and mitochondrial redox states before food presentation. In the cytoplasm, this effect was enhanced because of an increase in the oxidized metabolite (pyruvate) and a simultaneous decrease in the reduced metabolite (lactate). In the mitochondria, although both ketone bodies were increased, an oxidized state resulted from a higher proportion of acetoacetate (oxidized metabolite) with regard to β-hydroxybutyrate (reduced metabolite). To our knowledge, there are only two other experimental conditions that shift the liver to an oxidizing redox state, a sucrose-casein diet for 5 days, which affects the cytoplasmic redox state, and

Table 3. *Redox potential in cytoplasmic and mitochondrial NAD^+/NADH ratios and the difference in the 2 subcellular compartments from livers of rats entrained to restricted feeding schedules and their ad libitum-fed controls*

	E_{hc}, mV		E_{hm}, mV		ΔE	
Time	AL	RFS	AL	RFS	AL	RFS
0900	−226 ± 3	−204 ± 4	274 ± 5	−268 ± 5	48	64
1000	−227 ± 4	−191 ± 5	−275 ± 5	−263 ± 5	48	72
1100	−227 ± 2	−187 ± 5	−277 ± 3	−270 ± 4	50	83
1200	−230 ± 3	−194 ± 4	−275 ± 4	−270 ± 4	45	76
1400	−226 ± 4	−223 ± 3	−175 ± 6	−274 ± 2	49	51
1800	−230 ± 3	−226 ± 2	−274 ± 3	−274 ± 4	44	48

Values are means ± SE for 5 independent observations. E_{hc} and E_{hm}, cytoplasmic and mitochondrial redox potential, respectively; ΔE, difference between E_{hc} and E_{hm} in 2 subcellular compartments; AL, ad libitum-fed control rats; RFS, restricted feeding schedule group.

Table 4. *Values obtained with a 2-way ANOVA for the effects of nutritional schedule (2 levels), time points (6 levels), and their interaction with redox state, energy nucleotides, and energy charge*

	Feeding Schedule		Time Points		Feeding Schedule × Time	
	$F_{(1,48)}$	P	$F_{(5,48)}$	P	$F_{(5,48)}$	P
E_{hc}	593.46	<0.001	52.04	<0.001	49.70	<0.001
E_{hm}	28.85	<0.001	3.12	<0.01	3.41	<0.01
ΔE	400.66	<0.001	44.66	<0.001	35.17	<0.001
AMP	24.01	<0.001	0.48	NS	2.46	<0.05
ADP	69.58	<0.001	1.49	NS	0.91	NS
ATP	8.27	<0.01	2.77	<0.02	3.84	<0.01
Total nucleotides	174.62	<0.001	8.63	<0.001	7.32	<0.001
Energy charge	20.20	<0.001	2.51	<0.04	9.33	<0.001

diabetes in rats due to alloxan treatment (15), which increases the mitochondrial NAD^+/NADH. The simultaneous shift to a more oxidized cytoplasmic and mitochondrial redox state observed in this study represents a unique condition, because, with those exceptions mentioned, only one compartment was oxidized, excluding oxidation of the other compartment. Furthermore, no changes in redox state have been reported in 24-h-fasted rats (31), and chronic fasting conditions induce only a moderate reducing state. This oxidized condition before food access in RFS rats can be considered as an indicator of high metabolic activity in the hepatocyte (4) and suggests a preparatory process aimed at optimizing the use of nutrients obtained during the 2-h feeding period.

E_{hc} and E_{hm} became less negative before feeding. As observed for the redox state, the absolute change was larger in E_{hc} than E_{hm}. The ΔE indicates the transport of reducing equivalents from the cytoplasm to the mitochondria, which normally maintains a 100-fold dif-

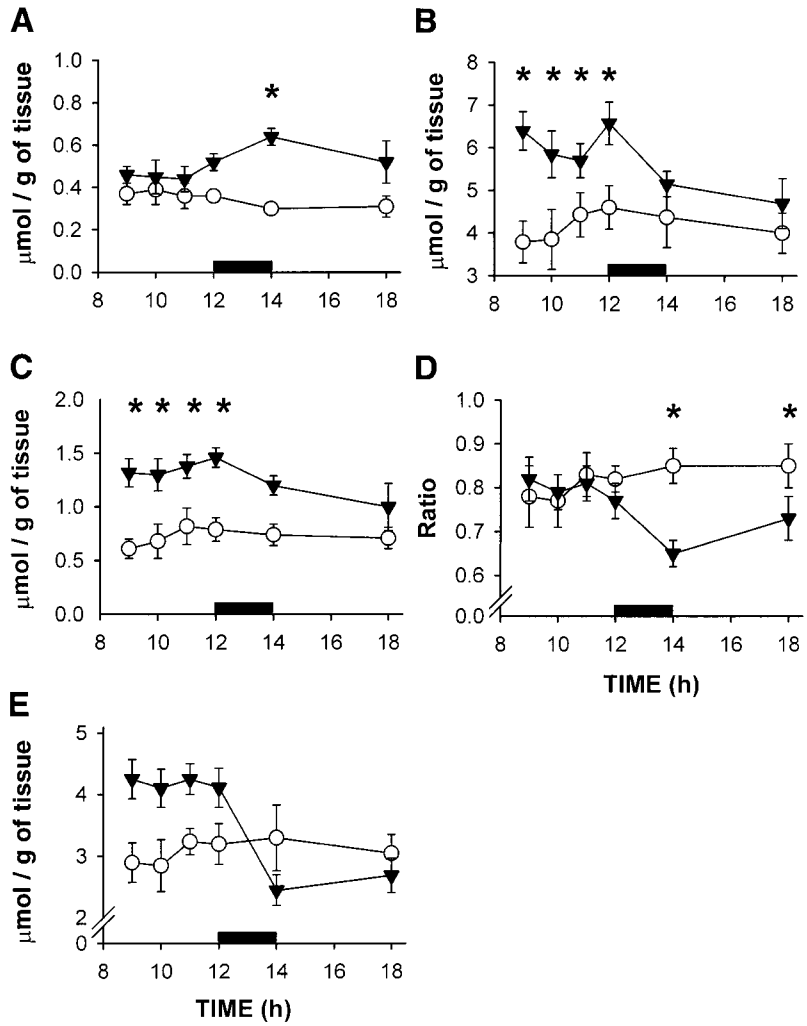

Fig. 5. Liver adenine nucleotides [AMP (*A*), ADP (*C*), and ATP (*E*)], energy charge (EC; *D*), and total adenine nucleotides (TAN; *B*) in rats maintained under a restricted feeding schedule (▲) and ad libitum controls (○). Other indications are similar to Fig. 2. *Significant difference between both groups at the indicated time point (Tukey's post hoc test, $\alpha = 0.01$).

ference in the NAD^+/NADH ratio between both compartments in the hepatocyte (27). The present data indicate that the flux of reducing equivalents toward the mitochondria is increased up to 300-fold before feeding, which would allow in the mitochondria an optimal use of fuels to produce ATP, and support the hypothesis that the liver shows a preparatory process that is coincident with the AA.

The main effects on hepatic adenine nucleotides before feeding were an increase in the TAN, because of an elevation of ATP and ADP, without change in the EC. In contrast, previous studies have reported that rats fasted for 24 h show a decrease in hepatic ATP, TAN, and EC levels (33). The present findings suggest that under a restricted feeding schedule, the liver is undergoing a different metabolic state from that observed under a 24-h prolonged fast. Hence, the increase in adenine nucleotides during AA reflects storage of energy and can be understood as an anticipatory response from the liver getting ready for the challenge of food processing. This is further supported by a decrease in ATP and EC after food intake. Similar adjustments in adenine nucleotides have previously been reported in tissues that are subjected to an increased metabolic challenge, such as the cardiac muscle in the rat and wing muscles of flying insects (24).

The existence of a circadian FEO that is anatomically independent of the SCN is well accepted. However, whether the FEO is located in the central nervous system, in the periphery, or is an interplay between both entities (10, 11) is not clear. The communication between peripheral digestive organs and the central nervous system involves neural and humoral signals, the former through branches of the autonomic system and the later by hormones secreted by digestion-related organs. One strategy for approaching FEO is to characterize its output on several parameters onto which it imposes a temporal order. This can be observed at diverse times of the cycle, specially several hours before feeding. Such is the case for AA, the peak of corticosterone, the elevation of circulating free fatty acids, and the depletion of hepatic glycogen. On the other hand, FEO can be traced from its relationship with feeding schedules, which constitute its main entrainment signal. Hence, an important strategy is to explore metabolic events that occur consequent to feeding, which can be internal time signals that constitute the entrainment pathway. This study provides further information on metabolic parameters that shift their phase under a restricted feeding schedule and therefore may be involved with the FEO, many of them as outputs driven by this oscillator and only a few as possible internal entraining signals phase locked by food ingestion. This could be the case for the switch from catabolism to an anabolic condition, which may be an important time signal for the organism. Also, metabolites like glucose, free fatty acids, or the insulin-to-glucagon ratio may be internal entraining signals to the oscillator.

In conclusion, the present results indicate that hepatic metabolism in RFS rats is modulated with a different pattern from spontaneous feeding or a 22- to 24-h fasted state. During anticipation, the liver of RFS rats should be "metabolically hungry" because of the catabolic state reflected by the hormonal profile, ketogenesis, and breakdown of liver glycogen. However, redox state, TAN, and EC in the liver indicate storage of energy sources that may be relevant for subsequent food processing. This adaptive response by the liver implies an anticipatory function in the control of energy balance. This response may be intrinsic to the hepatocyte or may be signaled by the insulin-to-glucagon ratio or other factors unknown to the present time. The first hypothesis has recently been supported by evidence of a damped oscillation of mPer1 expression in the liver maintained in vitro (39).

Perspectives

In an attempt to identify FEO, recent studies have focused their attention on nutrient properties and metabolic processes associated with restricted feeding schedules. Further studies will have to continue exploring endocrine and neural events associated with this paradigm as well as their temporal interaction. In particular, the possible involvement of some of those events as internal entraining signals for FEO may lead to its identification.

Because of the adaptive response observed in liver metabolism, further studies will have to determine the possible involvement of the liver with the time-keeping mechanisms of FEO. Therefore, it will be necessary to verify whether this anticipation results from intrinsic rhythmic properties of the hepatocyte or is transmitted by hormones. The oxidative state of the hepatocytes, as well as the increase in ΔE, suggests that this process can depend on mitochondrial activity. To further clarify this process, studies should be aimed at exploring the role of "shuttles", malate and oxalacetate, which are enzymes and carriers that regulate the flux of reducing equivalents in the mitochondria.

Another indication of the hepatic AA is the increase in adenine nucleotides before food presentation. Because the liver is the main producer of purine rings for the rest of the organism, it may be predicted that the distribution of purine rings can be part of the global hepatic response after feeding. The role of the liver as generator of purine molecules is intimately linked to diurnal variations of adenosine and its catabolites with a potential function in the sleep-wake cycle (5). The study of adenosine and its catabolites can provide further information to an understanding of this AA in the liver.

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