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Food restricted schedules promote differential lipoperoxidative activity in rat hepatic subcellular fractions☆

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Abstract

Restricted access to food (from 12:00 to 14:00 h) produces a behavioral activation known as food anticipatory activity (FAA), which is a manifestation of the food entrained oscillator (FEO). Peripheral oscillators, especially in the liver, are thought to be part of the FEO. A variety of metabolic adaptations have been detected in the liver during the expression of this oscillator, including activation of mitochondrial respiration and changes in the cytoplasmic and mitochondrial redox states. Biological clocks are regulated by redox-sensitive factors. The present study explored the lipoperoxidative activity (LP) in the liver during the activity of the FEO. Conjugated dienes (CD) and thiobarbituric acid reactive substances (TBARS), with and without Fe^{2+}-supplementation, were quantified in six subcellular fractions: whole homogenate, plasma membrane, mitochondria, microsomes, nucleus, and cytosol. The experimental protocol involved control groups of ad libitum fed and 24-h fasted rats, and groups under the restricted food schedule (RFS) which were sampled before FAA (08:00 h), during FAA (11:00 h) and after feeding (14:00 h). Clear differences in pro-oxidant activity was observed between ad libitum fed and 24-h fasted rats in almost all the subcellular fractions studied. RFS rats presented: CD levels more similar to the fasted rats, even at 14:00 h, after food presentation, and basal and Fe^{2+}-supplemented TBARS levels tended to be lower than both controls, suggesting an increased antioxidant capacity associated with food restriction. In addition, a microarray analysis showed that several isoforms of peroxiredoxins, a family of antioxidant and hydrogen peroxide-catabolizing enzymes, were consistently up-regulated in each and every condition in which RFS was applied. Together, these data indicate a rheostatic adaptation of the liver in the handling of pro-oxidant reactions during the activity of the FEO.

Keywords: Food entrained oscillator; Biological rhythm; Food intake; Liver; Pro-oxidant reactions; Lipid peroxidation; Conjugated dienes; Microarrays; Rheostasis

1. Introduction

Pro-oxidant reactions are ordinary outcomes of the environmental, physiological and metabolic networks in which most contemporary organisms survive. These reactions are the result of a delicate balance between generation of reactive species of oxygen and nitrogen (ROS and RNS, respectively), and the presence and activity of a variety of antioxidant systems (Hermes-Lima, 2004). Variation from this equilibrium may have two main consequences: 1) Under regulated conditions, cells may display a redox signaling which requires activation of specific redox-sensitive receptors and transcriptional factors (Rhee et al., 2003); and 2) During excessive generation of ROS and/or RNS, or an imbalance between oxidants and antioxidants, a pathophysiological condition termed oxidative stress can be produced (Sies, 1991; Maxwell and Greig, 2001).

Pro-oxidant reactions can be quantified by several methodologies. One of the most common and successful approaches is

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to follow the oxidation of membrane phospholipids by the process known as lipid peroxidation (LP). LP can occur by enzymatic or non-enzymatic reactions, usually involving activated chemical species in the form of ROS and RNS. LP is a chain reaction, in most cases catalyzed by transition metals, where active oxidants cause the breakdown of polyunsaturated fatty acids in membrane phospholipids (De Zwart et al., 1999). There are several assays for the determination of LP, but two of the more frequent techniques to measure this process are to quantify the presence of CD, and to determine thiobarbituric acid-reactive substances (TBARS). Conjugated dienes are formed by the rearrangement of double bonds of the PUFAs during the peroxidative process, and they are considered an estimation of "in vivo" LP, whereas the TBARS assay relies on the adducts formed between thiobarbituric acid and the carbonyl end products of lipid peroxidation progression, mainly malondialdehyde (Abuja and Albertini, 2001).

Pro-oxidant reactions are strongly linked to the metabolic handling of oxygen, hence, intensive feeding behavior has been associated with high rates of respiration and consequently with ROS formation (Faine et al., 2002). In contrast, intake of a limited amount of calories per meal, has been associated with low levels of lipid peroxidation and with an enhancement of life span (Gredilla et al., 2001). However, some reports have indicated opposite results, indicating that the relation between food intake and ROS formation is complex and so far unresolved (Boss et al., 1998; Selman et al., 2005).

Many animals have evolved the ability to anticipate periodic food availability. In mammals, this ability is due to a circadian clock that still functions after ablation of the suprachiasmatic nucleus (SCN), although such lesions abolish (or severely disrupt) virtually all circadian rhythms in behavior and physiology when food is available ad-libitum. Restriction of meals to a certain time of day elicits anticipatory behavior known as food anticipatory activity (FAA). FAA is concurrent with major and dramatic adjustments in metabolic and physiological parameters, and accumulated evidence indicates that all these adaptive phenomena are clearly under the control of a circadian oscillator (Reppert and Weaver, 2002). Despite many attempts to find the locus of the feeding entrainable oscillator (FEO) by central nervous system (CNS) lesions, endocrine gland extirpation, etc., the locus of the FEO is still unknown (Stephan, 2002). Recently, it was shown that it is possible to induce FAA independently from the activation of the FEO, by offering palatable food (chocolate) in particular hours to ad libitum fed rats (Mendoza et al., 2005). However, in conditions of FRS, the onset and the establishment of the FAA is linked to the activation of the FEO.

The working hypotheses of our group is that the FEO is a distributed system that results from the interplay between brain areas controlled by signals derived from the energetic metabolism, and peripheral organs which metabolizes nutrients such as carbohydrates, lipids and proteins. The communication between these two entities involves neuronal and humoral signals (Escobar et al., 1998; Ángeles-Castellanos et al., 2005). A crucial feature of this model is the role played by the liver.

Previously, we reported a "biochemical anticipation" that corresponds to the behavioral anticipation and that takes place in the liver of rats expressing the FEO (Díaz-Muñoz et al., 2000). Extensive changes coincide with the onset of the FAA in crucial biochemical parameters that control the equilibrium between anabolic and catabolic reactions in the liver metabolism, such as energy, and the cytoplasmic and mitochondrial redox state (NAD^+/NADH ratio) (Díaz-Muñoz et al., 2000). More recently, we reported that metabolism in liver mitochondria is severely modified during the activity of the FEO, giving further support to the notion that schedule limiting access to food promote rheostatic adaptations in the oxidative metabolism of that organ. Interestingly, the redox status has been postulated as a metabolic parameter that could regulate the activity of clock genes (Rutter et al., 2001).

To further characterize the rheostatic adaptations in the liver during RFS, in this study we report the changes in another parameter that is related to oxygen handling and the redox state in the liver during the activity of the FEO, namely the lipid peroxidative activity. We measured this parameter in hepatic subcellular fractions: plasma membrane, mitochondria, microsomes, nuclei, and cytosol. In addition, we detected by DNA microarrays assay the changes in expression of the main genes involved in the oxidative stress response.

2. Materials and methods

2.1. Animals

Adult male Wistar rats (*Rattus norvegicus*) weighing 180–220 g at the beginning of the experiment were maintained in a 12:12 h light–dark cycle (lights on at 08:00 h) and constant temperature (22 ± 1 °C). The light intensity at the surface of the cages averaged 350 lx. Animals were kept in groups of five in transparent acrylic cages (40 × 50 × 20 cm) with free access to water. All experimental procedures were approved and conducted according to the institutional guide for care and use of animals for biomedical experimentation (Universidad Nacional Autónoma de México).

2.2. Experimental design

The experimental procedure reported by Davidson and Stephan (1999) was followed with some modifications. Rats were randomly assigned to one of three experimental groups: 1) control rats fed ad libitum, 2) rats exposed to a restricted feeding schedule (RFS group) had access to food only from 12:00 to 14:00 h for three weeks, and 3) rats fasted 24 h. To obtain liver samples, rats from groups 1 and 2 were randomly sacrificed at 08:00 (before FAA), 11:00 (during FAA), and 14:00 h (after feeding). Rats fasted 24-h were killed, and a sample of their liver was removed at 11:00 h.

2.3. Liver sampling and subcellular fractionation

For each temporal point and each group, six rats were first anesthetized with sodium pentobarbital (Anestesal from Pfizer,

MX), and then beheaded for trunk blood collection. A sample of approximately 3 g was taken from the liver and homogenized in 30 mL of 10 mM Tris–HCl (pH 7.4). Cellular fractionation was done as previously reported (Aguilar-Delfín et al., 1996). Briefly, the homogenate was centrifuged at 1500 g for 15 min, and the resulting pellet was resuspended and divided into halves for further isolation of plasma membrane and nuclear fractions. The supernatant was spun at 10,000 g for 15 min to sediment the mitochondrial fraction. The supernatant was recentrifuged at 100,000 g for 60 min, resulting in a pellet designated the microsomal fraction and a supernatant, which was the cytosolic fraction. Both the mitochondrial and microsomal fractions were resuspended in the Tris–HCl buffer. All centrifugations were performed at 4 °C. The plasma membrane fraction was obtained by centrifuging the first pellet through a Percoll gradient, as described by Loten and Redshaw-Loten (1986). Nuclei were prepared using the citric acid method reported by Reiners and Busch (1980).

2.4. Lipid peroxidation

LP measured in vitro was quantified by the 2-thiobarbituric acid method (Ottolenghi, 1959) using liver homogenate and subcellular fractions as pro-oxidative sources. Some modifications to the original method were introduced (Hernández-Muñoz et al., 1984); briefly: a sample of the homogenate (approximately 1 mg protein) was incubated for 30 min at 37 °C in 1 mL of 0.15 M Tris, pH 7.4; incubation was ended by adding 1.5 mL of 20% acetic acid (adjusted to pH 2.5 with KOH) and 1.5 mL of 0.8% of thiobarbituric acid. The samples were kept for 45 min in a boiling water bath, and then 1 mL of 2% KCl was added to each sample. The colored complex formed was extracted with butanol–pyridine (15:1, v/v) and quantified at 532 nm. Malondialdehyde was used as standard (extinction coefficient: 1.56×10^5 cm^{-1} M^{-1}). LP in vivo was determined by measuring the UV absorption at 233 nm of CD in lipid extracts (chloroform–methanol 2:1, v/v) of liver homogenate and subcellular fractions (Klaassen and Plaa, 1969).

2.5. Calculations and statistics

The results are expressed as mean±SEM of at least six individual experimental observations. The statistical significance of differences between RFS and ad libitum groups was assessed with a two-way ANOVA (feeding condition x time), followed by a Tukey multiple-comparisons post hoc test with α set at 0.05. The differences between RFS at 11:00 h, ad libitum at 11:00 h, and fasted groups were assessed with a one-way ANOVA (feeding condition), followed by a Tukey multiple-comparisons post hoc test with α set at 0.05.

2.6. Total RNA preparation

Total RNA was extracted with Trizol reagent from rat liver following the method of Chomczynski and Sacchi, 1987. For each phase of the experimental protocol (08:00 h before, 11:00 h during and 14:00 after the activity of FEO), 1 g of liver from each of six different animals was used to prepare total RNA. The total RNA from all six animals was pooled and kept in aliquots for later determination of its purity and integrity.

2.7. Printing of arrays

Rattus norvegicus five thousand 70-mer oligo library sets from Operon Oligo (http://www.operon.com/arrays/oligosets_overview.php) were resuspended (50 μM) in Micro Spotting solution (ArrayIt Brand Products). SuperAmine coated slides 25×75 mm (TeleChem International, INC) were printed in duplicate and fixed at 80 °C for 4 h. For pre-hybridization, the slides were rehydrated with water vapor at 60 °C, and fixed with two cycles of UV light (1200J). After boiling for two min at 92 °C, slides were washed with 95% ethanol for one min and prehybridized in 5X SSC, 0.1% SDS, and 1% BSA for one h at 42 °C. The slides were washed and dried for further hybridization.

2.8. Probe preparation and hybridization to arrays

Aliquots (10 μg) of total RNA were reversed transcribed into cDNA incorporating dUTP-Cy3 or dUTP-Cy5 and employing the CyScribe First-Strand cDNA labeling kit (Amersham). Incorporation of fluorophore was analyzed by using the absorbance at 555 nm for Cy3 and 655 nm for Cy5. Using hybridization solution UniHyb (TeleChem International INC), equal quantities of labeled cDNA were hybridized to the five thousand oligo Rat arrays for 14 h at 42 °C. The experiments used RNA from independent groups of animals, and the fluorophore used was dUTP-Cy3 for Co8 and dUTP-Cy5 for AA8, dUTP-Cy3 for Co11 and dUTP-Cy5 for AA11, dUTP-Cy3 for Co14 and dUTP-Cy5 for AA14, dUTP-Cy3 for Co11 and dUTP-Cy5 for AY, and dUTP-Cy3 for AA11 and dUTP-Cy5 for AY.

2.9. Data acquisition and analysis of array images

Acquisition and quantification of array images were performed in ScanArray 4000 with its accompanying software ScanArray 4000 from Packard BioChips. All images were captured using 65% PMT gain, 70% to 75% laser power, and 10 μm resolution at 50% scan rate. For each spot the Cy3 and Cy5 density mean value and the Cy3 and Cy5 background mean value were calculated with software ArrayPro Analyzer from Media Cybernetics.

2.10. Data analysis

Microarray data analysis was performed with free software genArise, developed in the Computing Unit of Cellular Physiology Institute of UNAM (http://www.ifc.unam.mx/genarise/). GenArise carries out a number of transformations: background correction, lowest normalization, intensity filter, *s* analysis of replicates, and selection of differentially expressed genes. The goal of genArise is to identify which of the genes show good evidence of being differentially expressed. The

software identifies differential expressed genes by calculating an intensity-dependent z-score. It uses a sliding window algorithm to calculate the mean and standard deviation within a window surrounding each data point, and it defines a z-score where z measures the number of standard deviations a data point is from the mean:

$$z_i = \left(R_i - \text{mean}(R)\right) / \text{sd}(R)$$

where z_i is the z-score for each element, R_i is the log-ratio for each element, and sd(R) is the standard deviation of the log-ratio. Elements with a z-score >2 standard deviations are considered to be the significantly and differentially expressed genes.

3. Results

3.1. Oxidative reactions in subcellular fractions of the liver

In animals under RFS, CD and the levels of TBARS, as parameters to estimate peroxidative events, were measured in subcellular fractions isolated from liver. Samples were obtained from experimental animals before (08:00 h), during (11:00 h), and after the FAA (14:00 h), and on liver homogenates, as well as the following subcellular fractions were assayed: plasma membrane, mitochondria, microsomes, nucleus and cytosol. Animals fed ad libitum and fasted for 24 h, were used as controls.

Homogenate (Fig. 1). CD from rats fed ad libitum showed a significant increase at 11:00 h (≈25%), probably of circadian nature, compared to 08:00 and 14:00 h (panel A). As expected, the CD levels in 24-h fasted rats were 72% lower than in the ad libitum fed group (panel A). RFS rats from all experimental groups had CD values similar to the 24-h fasted group, even the rats already fed (14:00 h) (panel A). In contrast to these CD results, production of TBARS was the same in ad libitum fed and 24-h fasted groups (panel B). However, RFS rats at 11:00 h (during FAA) presented a significantly lower TBARS levels than the control groups. This reduced value was also observed in the RFS rats after feeding (14:00 h) (panel B). TBARS generation in the presence of Fe^{2+} ions promoted an increased lipid peroxidation (panel C). In this condition, ad libitum fed rats showed no changes at the 3 times studied. On the other hand, the 24-h fasted group showed 100% higher values than the rats fed ad libitum. In contrast, TBARS generation in all RFS groups was 70% less than in their ad libitum fed counterparts and 90% less than in the 24-h fasted group (panel C).

Plasma Membrane (Fig. 2). CD in this fraction showed no changes in the ad libitum fed group, but the CD levels in the 24-h fasted rats were about 60% less than their ad libitum fed counterpart (panel A). All RFS rats had similar CD values, however the group evaluated after food intake (14:00 h) had lower levels than the ad libitum fed group (panel A). The CD levels in the RFS group displaying FAA (11:00 h) were significantly higher (≈50%) than the 24-h fasted rats (panel A). TBARS production in rats fed ad libitum was unchanged, but

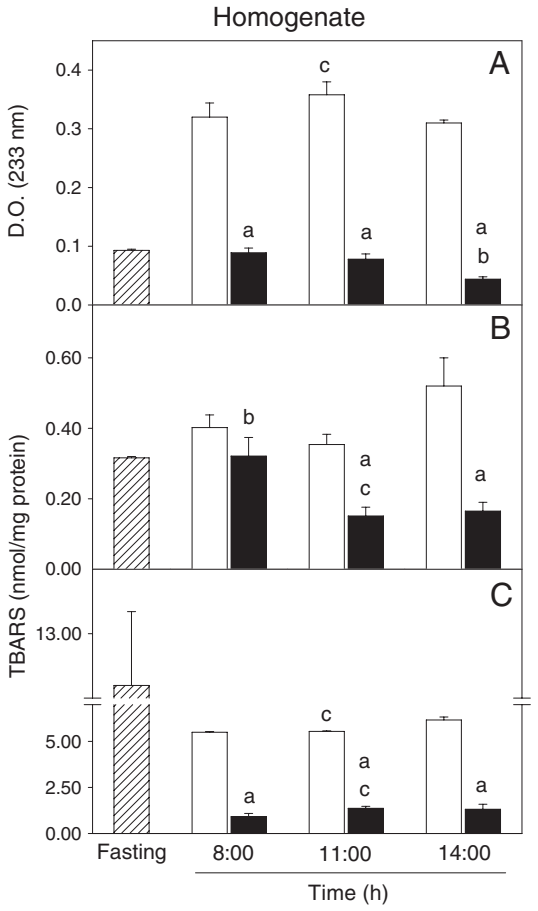

Fig. 1. Lipid peroxidation in liver homogenates of rats exposed for 3 weeks to restricted feeding schedules (food presentation from 12:00 to 14:00 h; black bars) and their respective ad libitum-fed controls (white bars). Experimental groups were sacrificed before (08:00 h), during the food anticipatory activity (11:00 h), and 2 h at the end of the meal (14:00 h). The control group with 24-h fasted was sacrificed at 11:00 h (hatched bars). Panel A, conjugated dienes. Panel B, basal TBARS. Panel C, Fe^{2+}-supplemented TBARS. Values represent means±SE of 5 independent experimental observations, $P<0.05$. Significant difference between ad libitum fed and food anticipatory activity rats at the indicated time point is represented by a. Significant difference between points at different times in rats with the same feeding schedule is indicated by b. Significant difference against 24-h fasted rats is indicated by c.

the value at 11:00 h was significantly higher (210%) than the value reported for the rats after 24-h fasted. Coincident with the FAA (11:00 h), RFS promoted a significant increase in TBARS compared to before FAA (08:00 h) and after feeding (14:00 h). In both cases, the augmentation in TBARS was approximately 220%. This peak in TBARS during FAA was significantly higher than the value in the 24-h fasted rats. At the three times tested, the generation of TBARS in the plasma membrane was lower than in the control rats fed ad libitum (panel B). Lipid peroxidation supplemented with Fe^{2+} caused, as expected, a marked increment in TBARS in all experimental groups. Interestingly, the peroxidative pattern in this condition was very similar to that in the non-Fe^{2+}-supplemented groups: higher lipid peroxidation in the groups fed ad libitum compared to the rats under RFS at the three times tested and to the control group after 24-h fasted; a significant increase of TBARS in the RFS

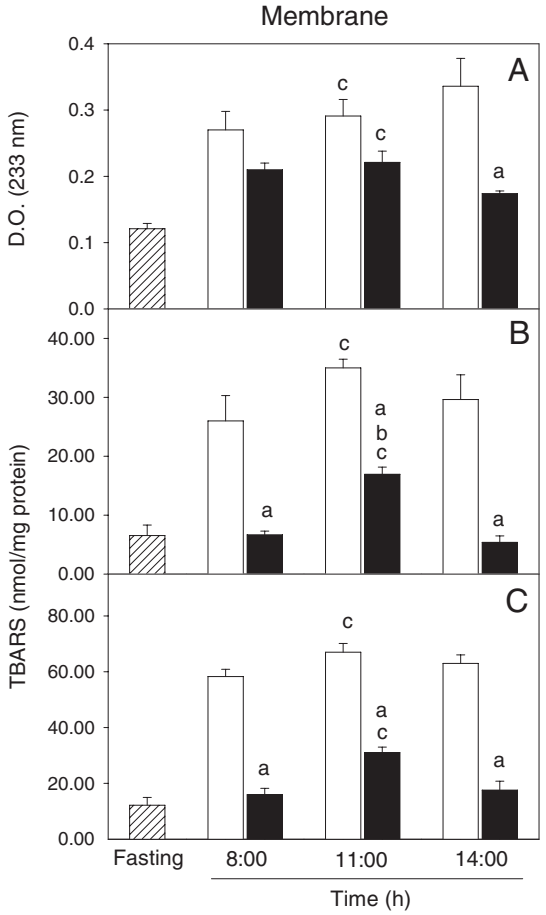

Fig. 2. Lipid peroxidation in liver plasma membrane fraction of rats exposed for 3 weeks to restricted feeding schedules (food presentation from 12:00 to 14:00 h; black bars) and their respective ad libitum-fed controls (white bars). Experimental groups were sacrificed before (08:00 h), during the food anticipatory activity (11:00 h) and 2 h at the end of meal (14:00 h). The control group with 24-h fasted was sacrificed at 11:00 h (hatched bars). Panel A, conjugated dienes. Panel B, basal TBARS. Panel C, Fe^{2+}-supplemented TBARS. Values represent means ± SE of 5 independent experimental observations, $P<0.05$. Significant difference between ad libitum fed and food anticipatory activity rats at the indicated time point is represented by a. Significant difference between points at different times in rats with the same feeding schedule is indicated by b. Significant difference against 24-h fasted rats is indicated by c.

group at 11:00 h (during FAA) compared to the other two groups with food restrictions, and to the 24-h fasted rats (panel C).

Microsomes (Fig. 3). CD levels in this subcellular fraction did not show differences within the groups fed ad libitum and the groups under RFS; furthermore, no differences were detected when the two treatments were compared. However, the presence of CD in the microsomes of 24-h fasted rats was significantly higher than the ad libitum fed and RFS groups (panel A). Endogenous TBARS synthesis presented no changes among the three groups fed ad libitum and the 24-h fasted rats. In contrast, the rats under RFS at the three times tested showed a significant reduction (≈35%) of TBARS in comparison to the control groups of rats with food ad libitum, and to the 24-h fasted group (panel B). Microsomal TBARS in presence of the Fe^{2+} showed no changes in the groups fed ad libitum. However, the rats with 24-h of fasting presented a 100% augmentation in comparison with the ad libitum fed rats. Distinctively, in the groups under RFS, a peak of TBARS was displayed (≈33%) after the rats were fed (14:00 h). At 11:00, the RFS group was similar to the rats fed ad libitum, and in consequence, the TBARS produced were much lower than in the 24-fasted rats (panel C).

Mitochondria (Fig. 4). The groups of rats fed ad libitum did not exhibit any changes in the levels of CD. However, comparison between rats fed ad libitum and 24-h fasted rats showed a significant reduction (≈55%) in the group without food. CD in the RFS groups presented, in general, levels similar to those in the 24-h fasted rats; the rats with FAA (11:00 h) depicted statistically lower values. In consequence, the three RFS groups showed significantly lower mitochondrial CD

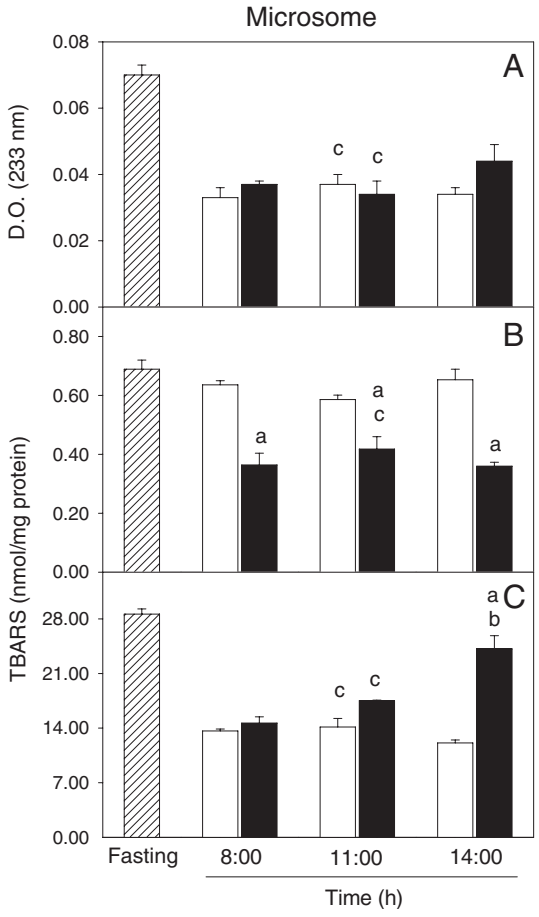

Fig. 3. Lipid peroxidation in liver microsomal fraction of rats exposed for 3 weeks to restricted feeding schedules (food presentation from 12:00 to 14:00 h; black bars) and their respective ad libitum-fed controls (white bars). Experimental groups were sacrificed before (08:00 h), during the food anticipatory activity (11:00 h) and 2 h at the end of meal (14:00 h). The control group with 24-h fasted was sacrificed at 11:00 h (hatched bars). Panel A, conjugated dienes. Panel B, basal TBARS. Panel C, Fe^{2+}-supplemented TBARS. Values represent means ± SE of 5 independent experimental observations, $P<0.05$. Significant difference between ad libitum fed and food anticipatory activity rats at the indicated time point is represented by a. Significant difference between points at different times in rats with the same feeding schedule is indicated by b. Significant difference against 24-h fasted rats is indicated by c.

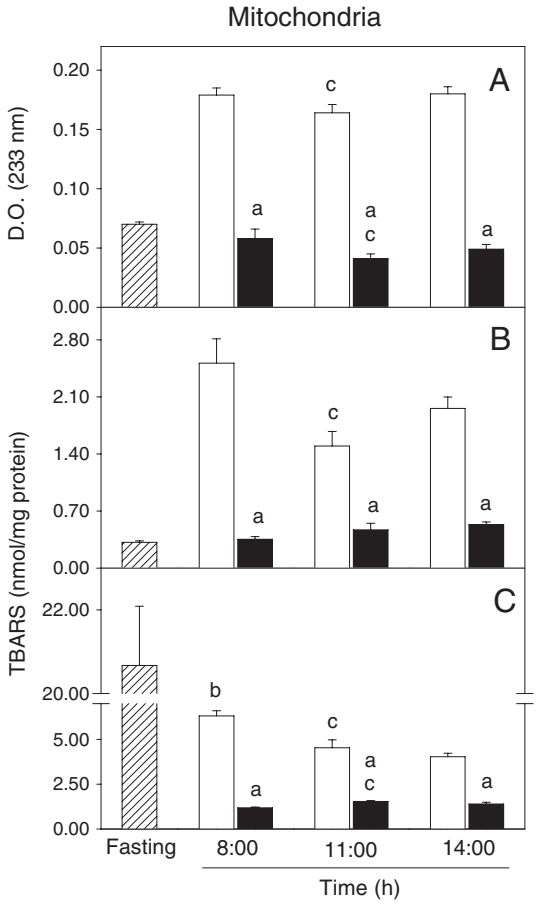

Fig. 4. Lipid peroxidation in liver mitochondrial fraction of rats exposed for 3 weeks to restricted feeding schedules (food presentation from 12:00 to 14:00 h; black bars) and their respective ad libitum-fed controls (white bars). Experimental groups were sacrificed before (08:00 h), during the food anticipatory activity (11:00 h) and 2 h at the end of meal (14:00 h). The control group with 24-h fasted was sacrificed at 11:00 h (hatched bars). Panel A, conjugated dienes. Panel B, basal TBARS. Panel C, Fe^{2+}-supplemented TBARS. Values represent means ± SE of 5 independent experimental observations, $P<0.05$. Significant difference between ad libitum fed and food anticipatory activity rats at the indicated time point is represented by a. Significant difference between points at different times in rats with the same feeding schedule is indicated by b. Significant difference against 24-h fasted rats is indicated by c.

levels (≈65%) than their ad libitum-fed controls (panel A). Basal TBARS production showed a similar pattern to the one described in the CD quantification, i.e. ad libitum fed rats presented much higher values than the 24-h fasted and the RFS groups at all times tested. There were no differences between the two treatments, 24-h fasted and RFS groups, although the RFS rats at 14:00 h were already fed (panel B). Mitochondrial TBARS supplemented with Fe^{2+} demonstrated a pattern similar to the one presented in TBARS supplemented with Fe^{2+} in whole homogenate. This pattern is characterized by a considerable lipid peroxidation in the 24-h fasted group in comparison with the rats fed ad libitum and the rats under RFS. On the other hand, the three groups fed ad libitum were significantly higher than their counterparts under RFS (panel C).

Nucleus (Fig. 5). CD in the nuclear fraction from rats fed ad libitum varied within the group: rats at 08:00 h depicted lower CD levels (≈25%) than the rats at 11:00 and 14:00 h, representing possible day–night variations of "in vivo" lipoperoxidative activity in this fraction. Rats under RFS and with 24-h fasted presented a significant reduction (≈50–60%) in CD levels in comparison to ad libitum fed groups. No differences were observed in CD content in the groups of 24-h fasted and RFS (panel A). Non-supplemented TBARS production presented again significant variations within the ad libitum fed groups. In this subcellular fraction, the TBARS levels were maximum at 14:00 h, intermediate at 11:00 h and minimum at 08:00 h. 24-h fasted rats presented very low TBARS in comparison to the ad libitum fed rats (≈15%). The effect of RFS in relation to their ad libitum fed counterparts varied according to the time tested: At 08:00 h TBARS were ≈100% higher, at 11:00 h they were ≈70% lower, and at 14:00 h 90% lower. TBARS in the 24-h fasted group was once more lower than the TBARS quantified in RFS rats at 11:00 h (≈40%) (panel B).

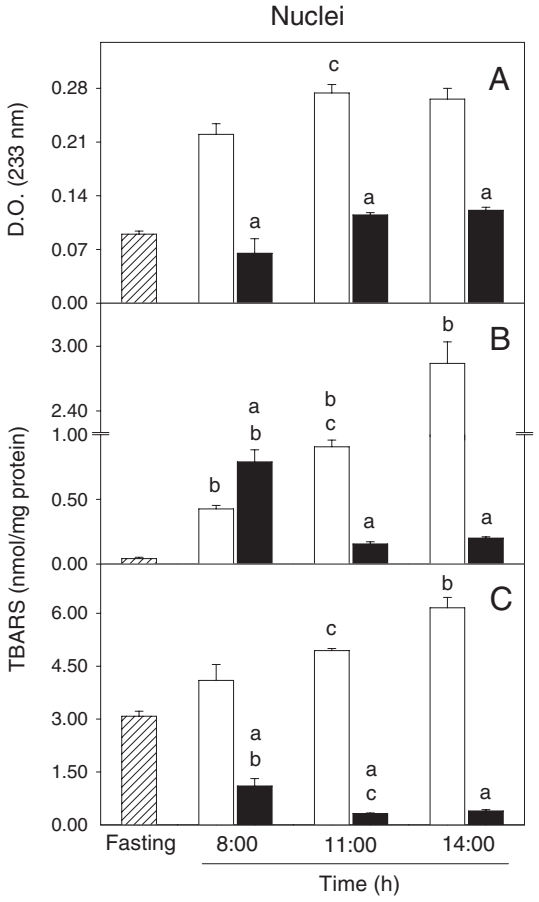

Fig. 5. Lipid peroxidation in liver nuclear fraction of rats exposed for 3 weeks to restricted feeding schedules (food presentation from 12:00 to 14:00 h; black bars) and their respective ad libitum-fed controls (white bars). Experimental groups were sacrificed before (08:00 h), during the food anticipatory activity (11:00 h) and 2 h at the end of meal (14:00 h). The control group with 24-h fasted was sacrificed at 11:00 h (hatched bars). Panel A, conjugated dienes. Panel B, basal TBARS. Panel C, Fe^{2+}-supplemented TBARS. Values represent means ± SE of 5 independent experimental observations, $P<0.05$. Significant difference between ad libitum fed and food anticipatory activity rats at the indicated time point is represented by a. Significant difference between points at different times in rats with the same feeding schedule is indicated by b. Significant difference against 24-h fasted rats is indicated by c.

TBARS generation in the presence Fe^{2+} was significantly different between the ad libitum fed rats at 08:00 and 14:00 h. The ad libitum fed group at 11:00 h was ≈50% higher than the group with 24-h fasted. The rats under RFS at the three times tested presented lower TBARS levels (≈60–90%) than the rats fed ad libitum (panel C).

Cytosol (Fig. 6). CD levels in this fraction did not show any differences among the experimental groups studied (panel A). In contrast, TBARS production presented significant changes in both basal and Fe^{2+}-supplemented TBARS production. Rats fed ad libitum generated ≈10 times more TBARS under basal conditions than the group with 24-h fasted. RFS groups presented similar levels of TBARS as the rats fed ad libitum, with the exception of the group showing FAA (11:00 h). At this time, there was a significant reduction (≈50%) in the rats under RFS in comparison to the control group fed ad libitum (panel B). With

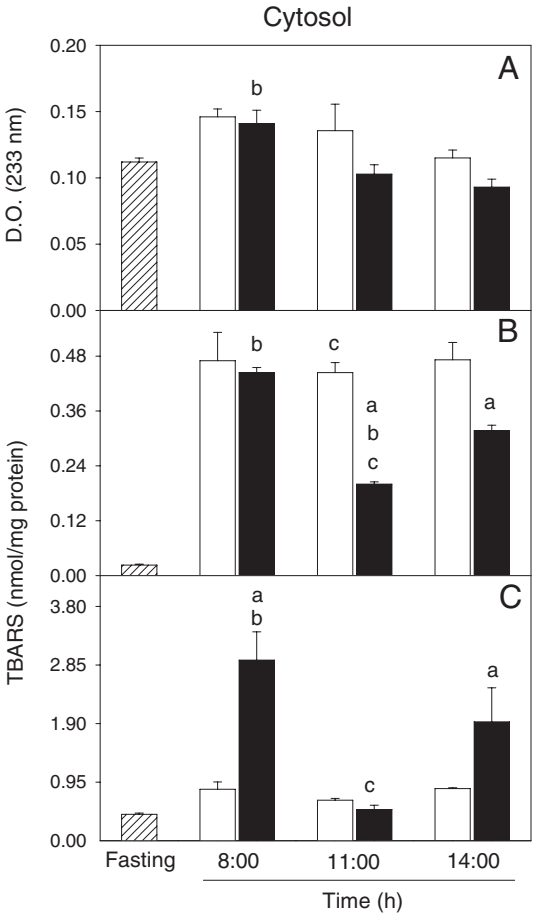

Fig. 6. Lipid peroxidation in liver cytosolic fraction of rats exposed for 3 weeks to restricted feeding schedules (food presentation from 12:00 to 14:00 h; black bars) and their respective ad libitum-fed controls (white bars). Experimental groups were sacrificed before (08:00 h), during the food anticipatory activity (11:00 h) and 2 h at the end of meal (14:00 h). The control group with 24-h fasted was sacrificed at 11:00 h (hatched bars). Panel A, conjugated dienes. Panel B, basal TBARS. Panel C, Fe^{2+}-supplemented TBARS. Values represent means ± SE of 5 independent experimental observations, $P<0.05$. Significant difference between ad libitum fed and food anticipatory activity rats at the indicated time point is represented by a. Significant difference between points at different times in rats with the same feeding schedule is indicated by b. Significant difference against 24-h fasted rats is indicated by c.

Fe^{2+} supplementation, no changes were observed in the TBARS produced in the groups fed ad libitum and with 24-h fasted. In contrast, rats under RFS showed significant increases in comparison to their ad libitum fed counterparts before FAA (08:00 h) (200%) and after feeding (14:00 h) (100%). However, the RFS group during the FAA (11:00 h) was similar to the other two RFS groups (panel C).

3.2. Expression of genes related to oxidative stress

Our experimental design enabled us to compare the differences in hepatic gene expression by microarray analysis of control groups of ad libitum fed and 24-h fasted rats, in rats expressing the FEO, and allowed us to compare the liver genetic activity in the experimental groups before FAA (08:00 h), during FAA (11:00 h), and after feeding (14:00 h) (Fig. 7). Of the 6145 unique genes on the array, RFS caused significant changes in the expression of 1416 genes (754 were up-regulated and 662 were down-regulated, data not shown). Table 1 shows the genes related to pro-oxidant reactions and oxidative stress, whose expression varied significantly. At 08:00 h (before FAA), 3 genes related with oxidative stress were up-regulated and 8 were down-regulated. The 3 genes with augmented transcriptional activity in the rats under RFS encoded for enzymes that break down hydrogen peroxide (H_2O_2): peroxiredoxins type 3 and 4, and catalase. Among the genes that were down-regulated by the RFS were enzymes related to glutathione synthesis (γ-glutamylcysteine synthetase light chain and γ-glutamylcysteine synthetase regulatory protein) and glutathione metabolism (several isoforms of glutathione *S*-transferases). The genes that presented the most dramatic reduction (−26.49) at this time were the ones for a protein tyrosine phosphatase, which is an enzyme modulated by H_2O_2 and is a negative feedback regulator of the cell cycle, and the gene for a copper chaperone for superoxide dismutase (−19.14). At 11:00 h (during FAA), 10 genes showed significant changes, 7 were up-regulated and 3 were down-regulated. The genes with increased transcriptional activity in the rats showing FAA encoded enzymes with a well recognized antioxidant role (glutathione peroxidase, several isoforms of glutathione *S*-transferases, hemo oxygenase type 2, and peroxiredoxin 1). Among the genes with decreased activity at this time, the one that presented the largest change was again the copper chaperone for superoxide dismutase (−12.93). After feeding (14:00 h), only 3 genes related to oxidative stress changed, 1 was up-regulated (peroxiredoxin 3), and 2 were down-regulated (γ-glutamylcysteine synthetase light chain and the copper chaperone for superoxide dismutase). When comparing liver gene activity of rats under RFS against the control group with 24-h fasted, 7 genes related to oxidative stress were found to change: 2 up-regulated and 5 down-regulated. The 2 up-regulated genes were peroxiredoxin 1 and glutathione peroxidase 1, whereas the genes with decreased activity were isoforms of glutathione *S*-transferases, hemo oxygenase and glutathione peroxidase type 4. In contrast, only 2 genes related to oxidative stress changed in a significant way between the control groups of rats fed ad libitum and those with 24-h fasted: glutathione peroxidase type

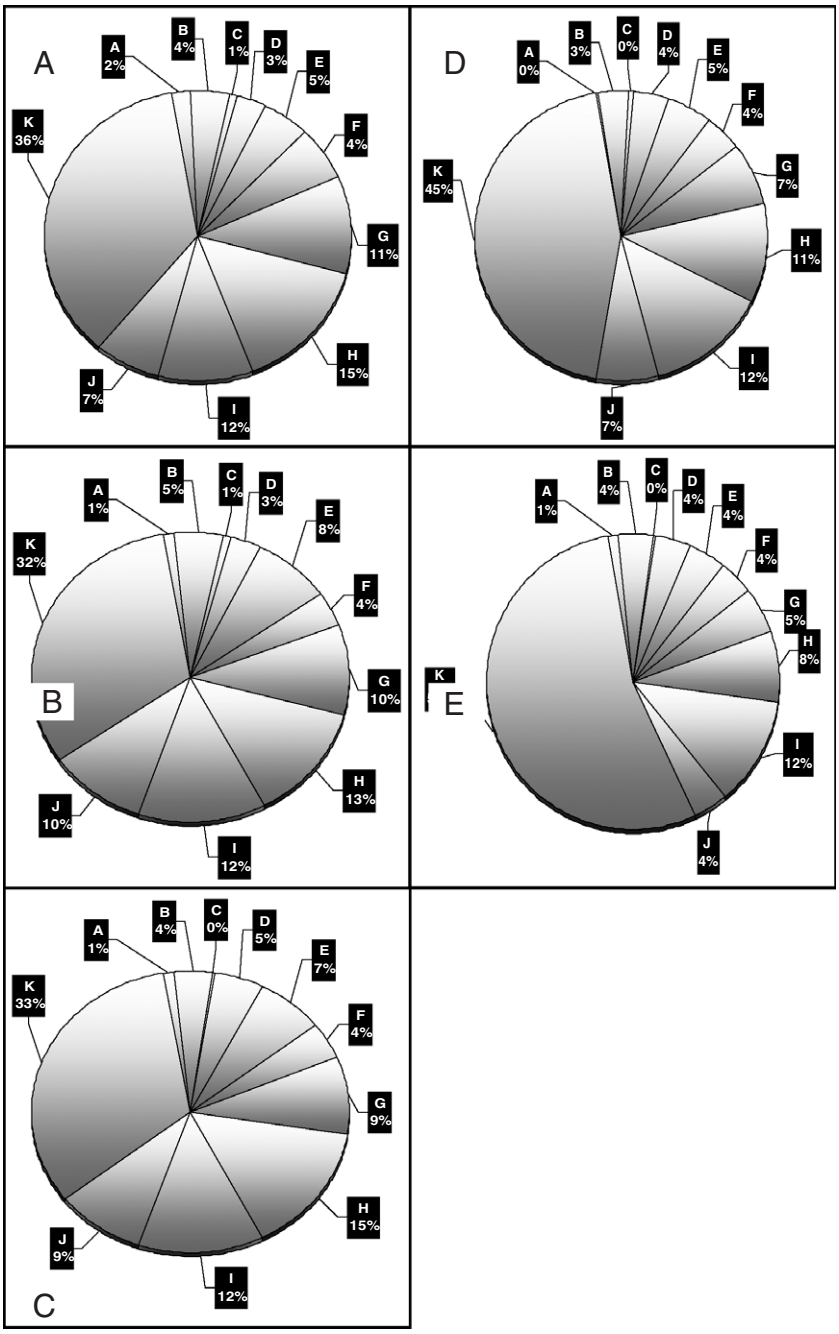

Fig. 7. Pie chart representation of the liver genes analyzed by cDNA microarrays that presented changes in the rats exposed for 3 weeks to restricted feeding schedules. A) Ad libitum fed vs FRS groups at 08:00 h; B) Ad libitum fed vs FRS groups at 11:00 h; C) Ad libitum fed vs FRS groups at 14:00 h; D) Ad libitum fed vs 24-h fasted groups at 11:00 h; E) 24-h fasted vs FRS groups at 11:00 h. Type of genes: A) Oxidative stress, B) Transcription, C) Circadian rhythms, D) Cell proliferation, cell cycle and programmed cell death, E) Protein turnover, maturation and synthesis, F) Cell structure, cell adhesion and biogenesis, G) Transport and vesicular traffic, H) Signaling, I) Metabolism and energy production, J) Defense and inflammatory response and K) Unknown function. To avoid a more complicated representation, percentages with lower value than 1 (usually between 0.3 and 0.7%) are depicted by zero.

4 was up-regulated and glutathione *S*-transferase type α2 was down-regulated.

4. Discussion

The present study provides data concerning the liver handling of oxygen in the context of pro-oxidant reactions related to the activity of the FEO. Supporting this aim, previous work by our group had shown that there are important adaptations in the main metabolic parameters of the liver as well as in mitochondrial physiology associated with RFS (Díaz-Muñoz et al., 2000; Báez-Ruiz et al., 2005). We tested the hypothesis that during the FEO activity, pro-oxidant activity would be altered before and after food presentation, and these changes would be different from that accompany spontaneous feeding and induced fasting. Indeed, we have demonstrated in

Table 1
DNA microarray analysis of gene expression in liver in food-restricted rats

Gene symbol	Access to GeneBank	Fold change	Description
08:00 h FAA vs ad libitum			
Prdx4	AF106945	3.1	Peroxiredoxin 4, defense response, oxidative stress, antioxidant protein.
Prdx3	NM_022540	3.4	Peroxiredoxin 3, mitochondrial peroxidase.
Cat	NM_012520	3.2	Catalase, peroxisome protein.
Gstm2	NM_017014	−2.3	Glutathione-*S*-transferase, mu type 2 (Yb2), subunit of glutathione *S*-transferase.
Abcc1a	AJ277881	−13.8	ATP-binding cassette, sub-family C (CFTR/MRP), member 1a.
Gstp2	X02904	−2.51	Glutathione *S*-transferase, pi 2, response to oxidative stress.
Glclr	NM_017305	−2.99	Glutamate-cysteine ligase (gamma-glutamylcysteine synthetase), regulatory
Glclc	J05181	−2.72	Glutamylcysteine gamma synthetase light chain.
Ptpn16	X84004	−26.49	Protein tyrosine phosphatase, non-receptor type 16. Act as a negative feedback regulator of mitogen.
Ccs	AF255305	−19.14	Copper chaperone for superoxide dismutase, superoxide-ions regulation.
11:00 h FAA vs ad libitum			
Gpx1	NM_030826	4.6	Glutathione peroxidase 1
Hmox2	NM_024387	4.6	Heme oxygenase 2, porphyrin metabolism, heme metabolism.
Gsta1	NM_031509	3.4	Glutathione-*S*-transferase, alpha type (Ya)
Gsta2	NM_017013	5.8	Glutathione-*S*-transferase, alpha type2
Prdx1	D30035	16.7	Peroxiredoxin 1, binds heme.
Abcc1a	AJ277881	5.65	ATP-binding cassette, sub-family C (CFTR/MRP), member 1a. Release of glutathione disulfide
Gstp2	L29427	5.7	Glutathione *S*-transferase, pi 2
Nac-1	AF015911	−2.35	NAC-1 protein. Enhance reduced glutathione.
GstYb4	NM_020540	−2.65	Glutathione *S*-transferase Yb4 gene
Ccs	AF255305	−12.93	Copper chaperone for superoxide dismutase
14:00 h FAA vs ad libitum			
Prdx3	NM_022540	8.0	Peroxiredoxin 3, mitochondrial peroxidase.
Glclc	J05181	−7.84	Glutamylcysteine gamma synthetase light chain.
Ccs	AF255305	−2.73	Copper chaperone for superoxide dismutase, superoxide-ions regulation.
11:00 h FAA vs 24-h fasted			
Prdx1	D30035	12.39	Peroxiredoxin 1,binds heme, may act as a thiol peroxides.
Gpx1	NM_030826	4.6	Glutathione peroxidase 1
Hmox2	NM_024387	−6.4	Heme oxygenase 2. Porphyrin metabolism, heme metabolism.
Mgst1	J03752	−6.7	Microsomal glutathione *S*-transferase 1
Mgst1	NM_017165	−6.4	Glutathione peroxidase 4, binds selenium.
Gstm5	U86635	−2.4	Glutathione *S*-transferase, mu 5.
Gstt2	NM_012796	−2.7	Glutathione *S*-transferase, theta 2.
11:00 h 24-h fasted vs ad libitum			
Gpx1	NM_030826	2.59	Glutathione peroxidase 4, oxidative stress response.
Gsta2	NM_017013	−2.3	Glutathione-*S*-transferase, alpha type2, oxidative stress response.

A positive number indicates up-regulated genes; negative numbers indicate down-regulated genes. Total RNA was pooled from six rats from each experimental group. Microarray methodology and statistical analysis to detect the genes that change significantly are explained in the Materials and methods section.

this study that RFS induces: 1) differential lipoperoxidative activity in subcellular fractions of the liver; 2) changes in the hepatic genes related to oxidative stress and 3) a physiological reaction in the liver that is unlike the responses observed during the ad libitum feeding and the 24-h fasted, and which could be considered as rheostatic in its nature.

4.1. Lipid peroxidation

Most of the tissues present oxidative metabolism, and the liver is no exception. Oxygen is needed for proper energetic metabolism and correct mitochondrial function, but at the same time, it promotes the formation of ROS and, in consequence, oxidation of biomolecules. Lipid peroxidation is a suitable assay to estimate pro-oxidant reactions. By means of this technique, it is possible to infer the rate of peroxidative activity under "in vivo" conditions (measuring conjugated dienes), and it is also feasible to deduce the balance between pro-oxidant reactions and antioxidant defenses using the determination of TBARS (Hermes-Lima, 2004). When the TBARS assay is done with Fe^{2+} supplementation, it offers another set of information: Because it enhances the breakdown of hydroperoxides, the Fe^{2+}-induced lipid peroxidation is maximum and gives an idea about the total antioxidant mechanisms in the system as well as the occurrence of unsaturated fatty acids present in the membrane studied (Fenton, 1984).

There is a link between pro-oxidant reactions in the liver and the nutritional status of the organism, mainly in situations when the food intake is controlled, i.e., fasted animals are prone to develop oxidative stress whereas caloric restriction has been

correlated with limited pro-oxidation and an extended life span (Katsuki et al., 2004; Domenicali et al., 2005). Activity of the FEO in our experimental protocol does not involve loss of weight by the experimental animals (their weight increased ≈15%, data not published). However, RFS entails alternating periods of fasting (22 h) and very intense food intake (2 h); as a matter of fact, during the 2 h period of the meal, the animals ingest up to 25–30 g of food (the maximum amount of food within the stomachs of rats fed ad libitum is 4–5 g) (Martínez-Merlos et al., 2003). The marked hyperphagia coupled to the activity of the FEO could be an important synchronization signal, and at the same time it imposes on the digestive tract, especially the liver, the necessity to handle and process a great amount of food. In consequence, animals under RFS fluctuate every day between 2 different stages of the hunger–satiety cycle: 1) after feeding, a vast stomach distention is followed by a very gradual emptying of this organ that lasts ≈20–21 h; 2) the stage when the stomach is almost empty corresponds to the onset of the FAA and the expectation for the food presentation. It is in this context that the FEO is installed as the main biological clock associated with restricted feeding.

Our experimental protocol allows us to study the 3 phases that are relevant in the FEO activity, the metabolic adaptations in the liver before (08:00 h) and during FAA (11:00 h), as well as after feeding (14:00 h). As was expected based on our hypothesis, the manifestation of pro-oxidant reactions was very different in the control groups of rats fed ad libitum and with 24-h fasted from the experimental animals under RFS. Indeed, when the results of "in vivo" lipid peroxidation are considered, the levels of CD were lower in almost all subcellular fractions in the 24-h fasted group than in the group fed ad libitum, with the exception of cytosol (no changes) and microsomes (CD values were higher in fasting than in ad libitum fed rats). The interpretation of this result is that without food, the fasted animals deal with a less oxygen-demanding medium and then the redox equilibrium is shifted towards a mild pro-oxidant state (Marczuk-Krynicka et al., 2003). In some circumstances such as liver injury by halogenated compounds, the microsomal fraction is more prone to present pro-oxidant reactions than other cellular endomembranes (Hernández-Muñoz et al., 1997). More experiments are needed to clarify the odd peroxidative activity of this fraction during the fasting state. The RFS group during FAA (at 11:00 h) is directly comparable to control ad libitum fed and fasted rats. At this time, the food-restricted animals have completed almost 24-h without food access. Hence, it would be expected that the "in vivo" pro-oxidative response of this experimental group would be more similar to the one present in the 24-h fasted rats than to the CD levels shown by the rats fed ad libitum. However, the rats under RFS presented a CD pattern analogous to the 24-h fasted group in just two of the subcellular fractions studied: cytosol and nucleus. In contrast, in the plasma membrane and microsomal fractions, the RFS group showed values similar to the CD quantified in rats fed ad libitum. In mitochondria and the whole homogenate, the CD concentrations in the RFS group were even lower than both controls. Taken together, these results indicate that the hepatic tissue in rats under food restriction exhibits distinctive pro-oxidant activity, as measured by the presence of CD, in comparison to the control ad libitum fed and fasted groups. In previous reports, we observed significant changes in the rats under RFS when they were fed (Díaz-Muñoz et al., 2000). However, the CD levels in the RFS rats changed only in the whole homogenate and in the plasma membrane fraction after feeding (in both cases, they decreased), whereas no variations were observed in the rest of the subcellular fractions.

The TBARS assay involves a 30-min incubation period in which the lipid peroxidation results from the balance between pro- and antioxidant factors. This balance is characteristic of each subcellular fraction. When 24-h fasted and ad libitum fed groups are compared, again, there is generally less peroxidative activity in the fasted rats. The exception was in the whole homogenate and the microsomal fraction. In just 2 subcellular fractions studied from rats from RFS, mitochondria and nucleus, TBARS production was analogous to the 24-h fasted group. In the plasma membrane and cytosol fractions, the RFS group showed intermediate values between the ad libitum fed and the 24-h fasted rats, whereas in microsomes and the whole liver homogenate the TBARS concentrations in the RFS group were even lower than in both controls. Food intake in the RFS groups had a minor effect on TBARS generation: only 2 subcellular fractions changed in the RFS rats after food presentation: cytosol presented an increment and the plasma membrane a reduction of TBARS. The other fractions remained without changes after feeding.

When the TBARS assay is performed in the presence of Fe^{2+}, the hydroperoxides suffer a decomposition process equivalent to the Fenton reaction (Nagasawa et al., 2001). As a result, lipid peroxidation is maximized such that the entire progression of the pro-oxidant reactions depends on the total antioxidant capacity and the availability of unsaturated fatty acids in the system (Hermes-Lima, 2004). In this condition, liver homogenate from the 24-h fasted group presented a two-fold increase in TBARS in comparison to the rats fed ad libitum. This increment was also observed in the microsomal and mitochondrial fractions. In contrast, cytosol, nucleus, and especially plasma membrane from 24-h fasted rats, showed lower TBARS than the group fed ad libitum. A very different pattern was present in the RFS group, since the whole homogenate showed a very low Fe^{2+}-supplemented lipoperoxidative activity in comparison to both, rats fed ad libitum and with 24-h fasted. This pattern is paradoxical since at 11:00 h, the RFS rats had fasted 23 h, and their lipoperoxidative activity was completely different from that in the rats with simple 24-h fasted. This extremely low TBARS production was observed again in the nucleus and mitochondria. In the microsomal fraction the TBARS levels associated with RFS was lower than the 24-h fasted group, but not different form the ad libitum fed rats. In the plasma membrane lipid peroxidation was higher than in the 24-h fasted group, but lower than in the rats fed ad libitum. No changes were observed in cytosol. Feeding the rats under RFS (at 14:00 h) had a modest effect on the Fe^{2+}-supplemented TBARS values, since no changes were detected in any of the subcellular fractions tested, except an augmentation in cytosol and microsomes.

Taking the three different forms for measuring lipoperoxidative activity together, the results strengthen the notion of a complex oxygen handling by the liver in the rats under RFS. Even though RFS involves an experimental situation that resembles fasting — fed cycles, the patterns of lipid peroxidation in the RFS groups measured as CD and TBARS, usually differed from the groups with 24-h fasted and normal intake of food. This observation agrees with the suggestion that RFS and the concomitant activity of the FEO, promote a rheostatic adaptation in the organism that is characterized, at least in the liver, by a complete transformation of the metabolic and physiological status.

4.2. Expression of oxidative stress-related genes

A great proportion of the significant changes in the oxidative stress-related genes, occurred before (08:00 h) and during (11:00 h) the FAA. At 08:00 h most of the genes were down-regulated, especially those related to glutathione transformation (isoforms of GSH-*S*-transferases, biosynthetic enzymes for glutathione). In contrast, the genes that varied at 11:00, during the FAA, were up-regulated, and again they were primarily the genes associated with the antioxidant role of glutathione (isoforms of GSH-*S*-transferases and GSH peroxidase). Given the relevant role of glutathione as an antioxidant factor (Schafer and Buettner, 2001), a preliminary interpretation of these results is that before FAA, the role of glutathione as a protector agent against oxidative reactions in the liver is constrained; on the contrary, during the expression of the FAA, antioxidant reactions by glutathione are enhanced.

Another set of genes related to the antioxidant defense of the liver showed an interesting pattern of transcriptional activity: the peroxiredoxins (Prxs). Prxs are a family of antioxidant thioredoxin-dependent peroxidases that have been identified in a large variety of organisms. The major functions of Prxs comprise cellular protection against oxidative stress, modulation of intracellular signaling cascades that involve hydrogen peroxide (H_2O_2) as a second messenger molecule, and regulation of cell proliferation (Immenschuh and Baumgart-Vogt, 2005). Our results demonstrated that at all times tested (08:00, 11:00, and 14:00 h), RFS groups up-regulated one or two isoforms of Prxs when compared with rats fed ad libitum or with 24-h of fasting. It is unusual that the transcriptional activity of this family of genes is increased by food restriction at all experimental times and conditions tested. More often, the variation of a gene associated with RFS, is confined to a certain time, for example, just prior to the FAA, or during the FAA, or after feeding. It is also common to detect genes that change in response to the feeding status of the animal, i.e., there are genes that are regulated before feeding and others whose transcription is altered after feeding. Hence, it is noteworthy that Prdx behave as a "state variable": Its expression seems to be closely associated with all the phases in which the FEO is expressed, independently of the times tested and feeding status. This finding gives further support to the experimental evidence of metabolic modulation of pro-oxidant reaction in the liver as a consequence of restricted feeding and the concomitant activity of the FEO. In addition, it strongly suggests a negative control in the liver of the H_2O_2-associated cellular signaling. This metabolite is produced by the action of G protein-, growth factors, and cytokines-associated receptors, and its mechanism of action is a redox regulation of cysteine-containing proteins such as tyrosine phosphatases (Rhee et al., 2003).

The present data further support the notion that the activity of the FEO involves a rheostatic adaptation of the metabolic and physiological condition of the liver, including the control of the pro-oxidant reactions in this organ. In addition, they open the possibility that the hepatic oscillator could be also controlled by a redox-sensitive mechanism, similarly to other pacemakers. However, further studies are needed to determine the role of other parameters related to the redox regulation of the hepatocyte, in particular the possibility of a redox signaling associated with the endocrine-promoted synthesis of H_2O_2, and the role played by antioxidant systems such as glutathione and peroxiredoxins.

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