Effect of a High-Fat Diet on 24-Hour Pattern in Expression of Prolactin and Redox Pathway Enzymes in the Rat Adenohypophysis

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Abstract: The effect of a high-fat diet (35% fat) on 24-h changes in expression of prolactin and redox pathway enzyme genes was examined in the anterior pituitary of male rats. When body weight of high-fat fed rats attained values about 20-25 % higher than controls (after 66 days of treatment) animals were sacrificed at 6 different time intervals throughout a 24-h cycle. Anterior pituitary mRNA levels were measured by real-time PCR analysis. In control rats expression of the prolactin gene peaked in anterior pituitary at early scotophase, 3 h in advance to the peak in plasma prolactin levels. In high-fat fed rats this correlation was lost. A disruption of 24-h rhythmicity of pituitary gene expression of heme oxygenase (HO)-2, Cu/Zn- superoxide dismutases (SOD), Mn-SOD, catalase, glutathione peroxidase (GPx) and glutathione reductase (GR) was apparent in high-fat fed rats. Anterior pituitary mRNA levels for nitric oxide synthase (NOS)-2 and HO-2, Cu/Zn- and Mn- SOD and catalase augmented in high-fat fed rats, whereas those of GPx and GR decreased. The results indicate a disrupted coordination between prolactin gene expression and prolactin release in high-fat fed rats that comes along with a disturbed expression of redox enzyme genes in the anterior pituitary.

INTRODUCTION

In a recent study we reported the disrupting effect of a high-fat diet (35% fat) on 24-h changes of anterior pituitary hormones including prolactin, luteinizing hormone and thyroid stimulating hormone [1]. A significant alteration of the circadian apparatus in rats fed a high-fat diet may occur, as shown by the disruption of pineal melatonin and plasma corticosterone daily rhythms, two important circadian signals [2].

As a continuation of that study, we undertook the present experiments to analyze the correlation of 24-h expression of prolactin gene in anterior pituitary with the daily secretory profile of prolactin in rats fed a high-fat or a normal diet. Since redox enzymes are involved in prolactin secretory mechanisms [3,4] we wished to correlate the possible changes in prolactin secretion with gene expression of nitric oxide (NO) synthase (NOS), heme oxygenase (HO), super-oxide dismutases (SOD), catalase, glutathione peroxidase (GPx) and glutathione reductase (GR) in the anterior pituitary. Overall we wished to assess whether a disruption in coordination of prolactin gene expression and prolactin release occurs in high-fat fed rats along with a disturbed expression of pituitary redox enzyme genes.

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MATERIALS AND METHODOLOGY

Male Wistar rats (45 days of age) were maintained under standard conditions with controlled light (12:12 h light/dark schedule; lights on at 08:00 h) and temperature (22 ± 2 C). Rats were divided into 2 groups: (a) normal diet ad libitum. (b) high-fat diet ad libitum. Both control (4 % fat) and high-fat (35 % fat) diets were obtained from Harlan Iberica, Barcelona, Spain. The characteristics of the diet employed are summarized in Table **1**. Individual daily food intake was 17 ± 1 g (normal diet) and 19 ± 1 g (high-fat diet).

Table 1. Diet Composition and Consumption

	Control	High-Fat Diet
% Fat	3	35
% Carbohydrates	60	35
% Protein	16	20
% Vitamins and minerals	21	10
Caloric content (Kcal/g)	2.9	5.4
Consumed fat (Kcal/day/animal)	4.86	56.7
Consumed carbohydrates (Kcal/day/animal)	43.2	25.2
Consumed protein (Kcal/day/animal)	11.5	14.4

After 66 days of treatment body weight was 492.5 ± 60.1g (high-fat diet) and 397.5 ± 73.6 g (normal diet). At this time rats were sacrificed by decapitation under conditions of minimal stress at 6 different intervals (8 rats per group), every 4 h throughout a 24-h cycle, starting at 09:00 h. At night intervals animals were killed under red dim light. The brains were rapidly removed and the anterior pituitary was dissected out. Tissues were kept frozen at -70 C until further assayed. The care and use as well as all procedures involving animals were approved by the Institutional Animal Care Committee, Faculty of Medicine, Complutense University, Madrid. The study was in accordance with the guidelines of the Institutional Care and Use Committee of the National Institute on Drug Abuse, National Institutes of Health and the Guide for the Care and Use of Laboratory Animals [5].

Total anterior pituitary RNA extraction was performed using the RNeasy protect mini kit and was analyzed using QuantiTec SYBR green kit (Qiagen, Hielden, Germany). The iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories SA; Madrid) was used to synthesize cDNA from 1 μg of total RNA, according to the manufacturer's protocol. The house keeping gene β-actin was used as a constitutive control for normalization. Reactions were carried out in the presence of 200 nM of specific primers. Primers were designed using Primer3 software (The Whitehead Institute, http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) and are shown in Table **2**.

The specificity of the primers was confirmed by running the products of Polymerase Chain Reaction (PCR) amplification in a 2 % agarose gel against molecular weight markers (product sizes are shown in Table **1**) and by a BLAST software-assisted search of a non-redundant nucleotide sequence database (National Library of Medicine, Bethesda, MD). After each PCR run, melting curves were performed to assure no contamination in the individual wells of PCR plates [6]. Wells showing more than one peak were excluded from subsequent analysis.

To assess whether a high-fat treatment modified the expression of β-actin, PCR with serial dilutions of this housekeeping gene was performed. PCR reactions were carried out in an AbiPrism 7300 (Applied Biosystems, Foster City, CA). The PCR reaction conditions were 15 min at 95 C followed by 40 cycles of 15 sec at 94 C, 30 sec at 60 C and 30 sec at 72 C. Fluorescence data were collected during the 72 C step. Serial dilutions of cDNA from control pituitaries were used to perform calibration curves in order to determine amplification efficiencies. For the primers used there were no differences between transcription efficiencies, the amount of initial cDNA in each sample being calculated by the $2^{-\Delta\Delta Ct}$ method [6]. All samples were analyzed in triplicate and in

Table 2. Sequence of the Primers Used for Real-Time PCR

Gene	Primers		Product Size (bp)
β- Actin	Forward	CTCTCTTCCAGCCTTCCTTC	99
	Backward	GGTCTTTACGGATGTCAACG	
Prolactin	Forward	TTCTTGGGGAAGTGTGGTC	86
	Backward	TCATCAGCAGGAGGAGTGTC	
NOS-1	Forward	ATCGGCGTCCGTGACTACTG	92
	Backward	TCCTCATGTCCAAATCCATCTTCTTG	
NOS-2	Forward	TGGCCTCCCTCTGGAAAGA	93
	Backward	GGTGGTCCATGATGGTCACAT	
HO-1	Forward	TGCTCGCATGAACACTCTG	123
	Backward	TCCTCTGTCAGCAGTGCC	
HO-2	Forward	AGCAAAGTTGGCCTTACCAA	84
	Backward	GTTTGTGCTGCCCTCACTTC	
Cu/Zn-SOD	Forward	GGTGGTCCACGAGAAACAAG	98
	Backward	CAATCACACCACAAGCCAAG	
Mn-SOD	Forward	AAGGAGCAAGGTCGCTTACA	94
	Backward	ACACATCAATCCCCAGCAGT	
Catalase	Forward	GAATGGCTATGGCTCACACA	100
	Backward	CAAGTTTTTGATGCCCTGGT	
GPx	Forward	TGCAATCAGTTCGGACATC	120
	Backward	CACCTCGCACTTCTCAAACA	
GR	Forward	ATCAAGGAGAAGCGGGATG	96
	Backward	GCGTAGCCGTGGATGACTT	

three different measures. Fractional cycle at which the amount of amplified target becomes significant (Ct) was automatically calculated by the PCR device. When tested brain or in muscle tissue no expression of prolactin gene was observed (results not shown).

Statistical analysis of results was performed by a Student´s t test, a one-way analysis of variance (ANOVA) or a two-way factorial ANOVA, as stated. For the factorial ANOVA, the analysis included assessment of the group effect (i.e. the occurrence of differences in mean values between normal and high-fat diet rats), of time-of-day effects (the occurrence of daily changes) and of the interaction between the two factors (diet and time, from which inference about differences in timing and amplitude could be

obtained). Post-hoc Bonferroni´s multiple comparison tests in a one way ANOVA were then employed to show which time points were significantly different within each experimental group to define existence of peaks. P values lower than 0.05 were considered evidence for statistical significance.

RESULTS

The correlation between anterior pituitary prolactin gene expression and plasma prolactin levels in rats (data from [1]) fed a normal or a high-fat diet is depicted in Fig. (**1**). In rats feeding a normal diet the maximum in prolactin gene expression, occurring at early scotophase (2100 h), anticipated by about 3 h the maximum in plasma prolactin
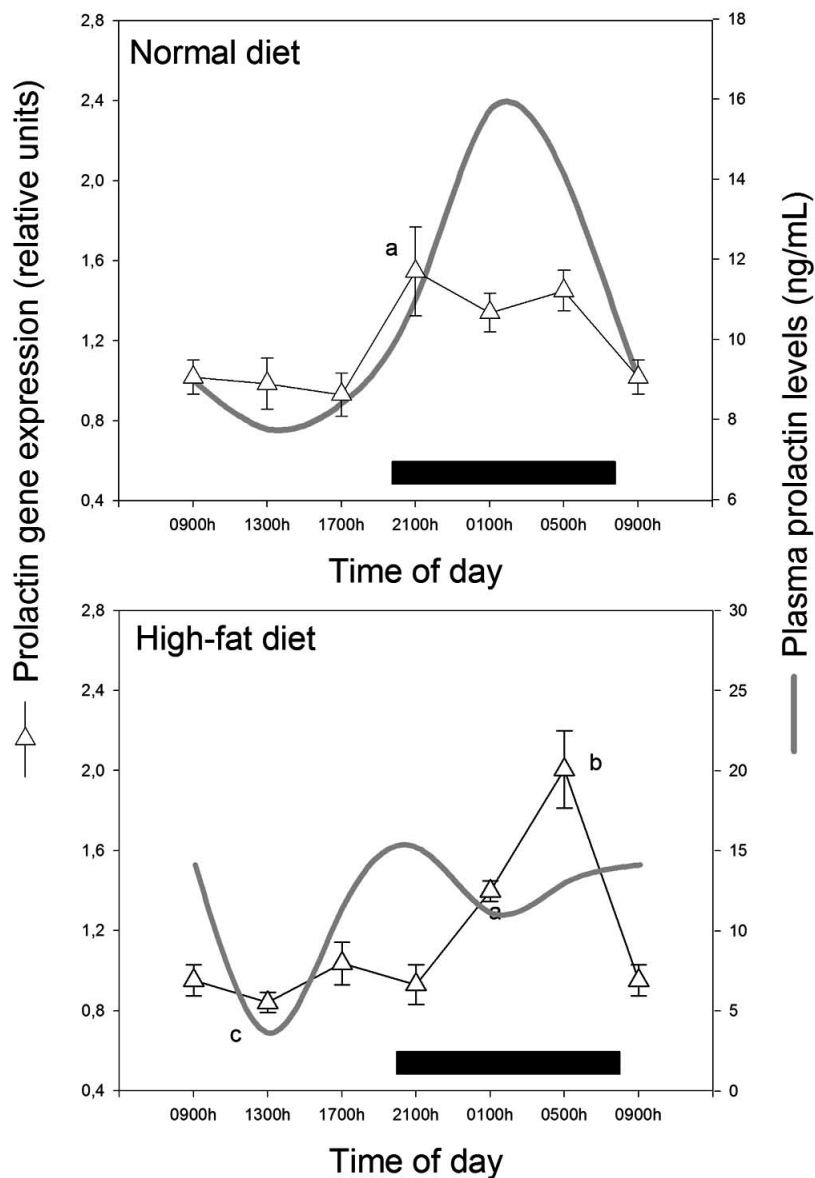

Fig. (1). Effect of a high-fat diet on 24-h changes in plasma prolactin levels and prolactin gene expression in the anterior pituitary of rats. Groups of 6-8 rats were killed by decapitation at 6 different time intervals throughout a 24 h cycle. mRNA levels encoding prolactin were by triplicate real-time PCR analyses of RNA samples as described in the text. Shown are the means ± SEM. Prolactin levels are reproduced from a previous publication [1]. Letters denote significant differences in a one-way ANOVA followed by a Bonferroni´s multiple comparison test, as follows: [a] p< 0.05 *vs.* 1700 h; [b] p< 0.01 *vs.* 0900, 11300, 1700 and 2100 h.

levels (Fig. **1**, upper panel). Such coordination became disrupted in high-fat fed rats, as shown by a delay in prolactin gene expression to the second part of the scotophase as well as by the abolition of peak plasma prolactin levels at night reported previously [1] (Fig. **1**, lower panel). Hence a disruption of prolactin biosynthetic mechanisms at early night appeared to occur in high-fat fed rats.

Fig. (**2**) depicts the relative expression of anterior pituitary NOS-1 and NOS-2 genes in both groups of animals. NOS-1 mRNA levels did not exhibit significant 24-h variations while those of NOS-2 peaked at the early light phase of daily photoperiod (F= 11.5, p< 0.001 for time as a main factor in factorial ANOVA). A high-fat diet augmented NOS-2 mRNA by 37 % (F= 11.8, p< 0.001) without modifying that of NOS-1 significantly (Fig. **2**).
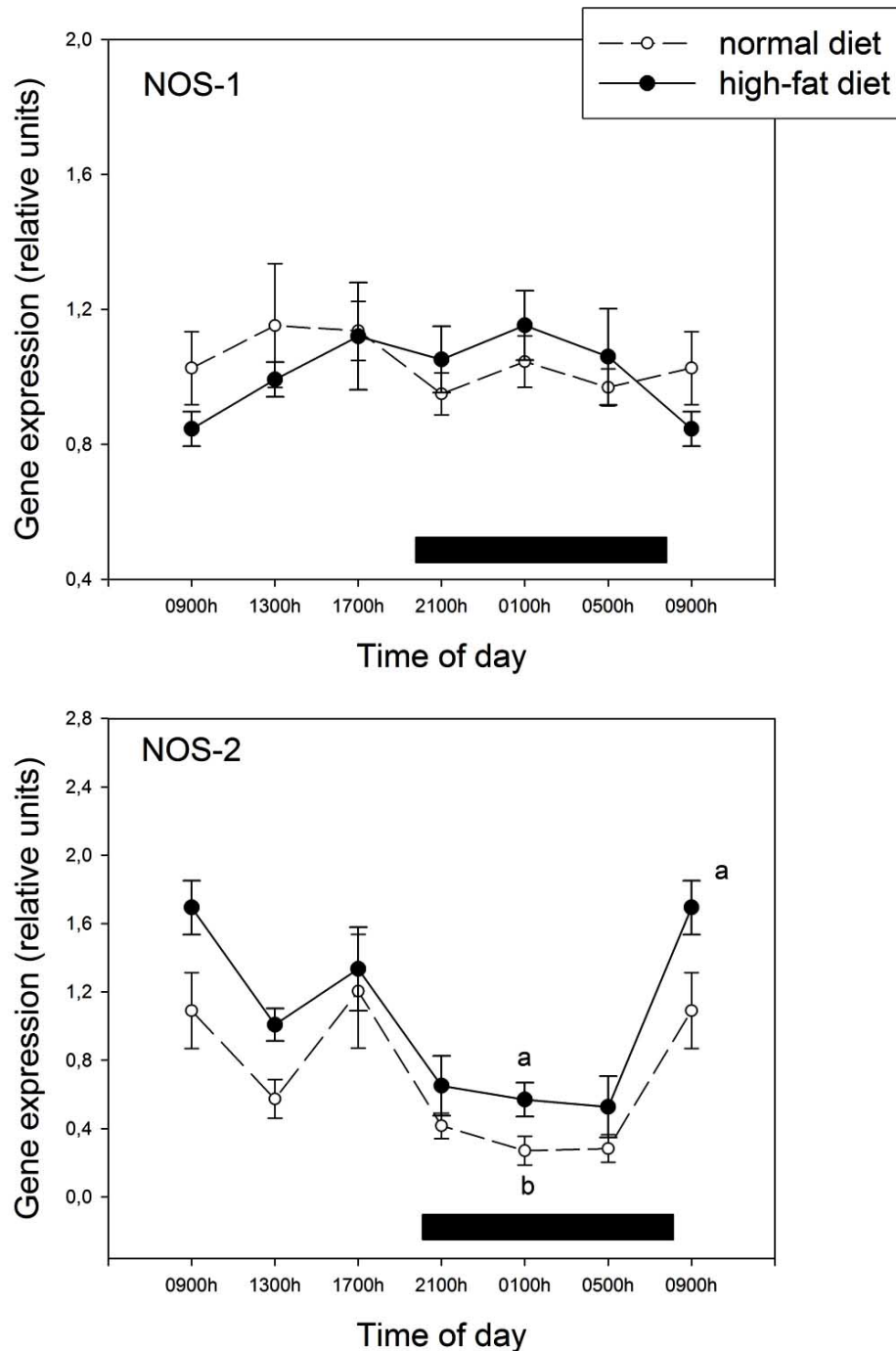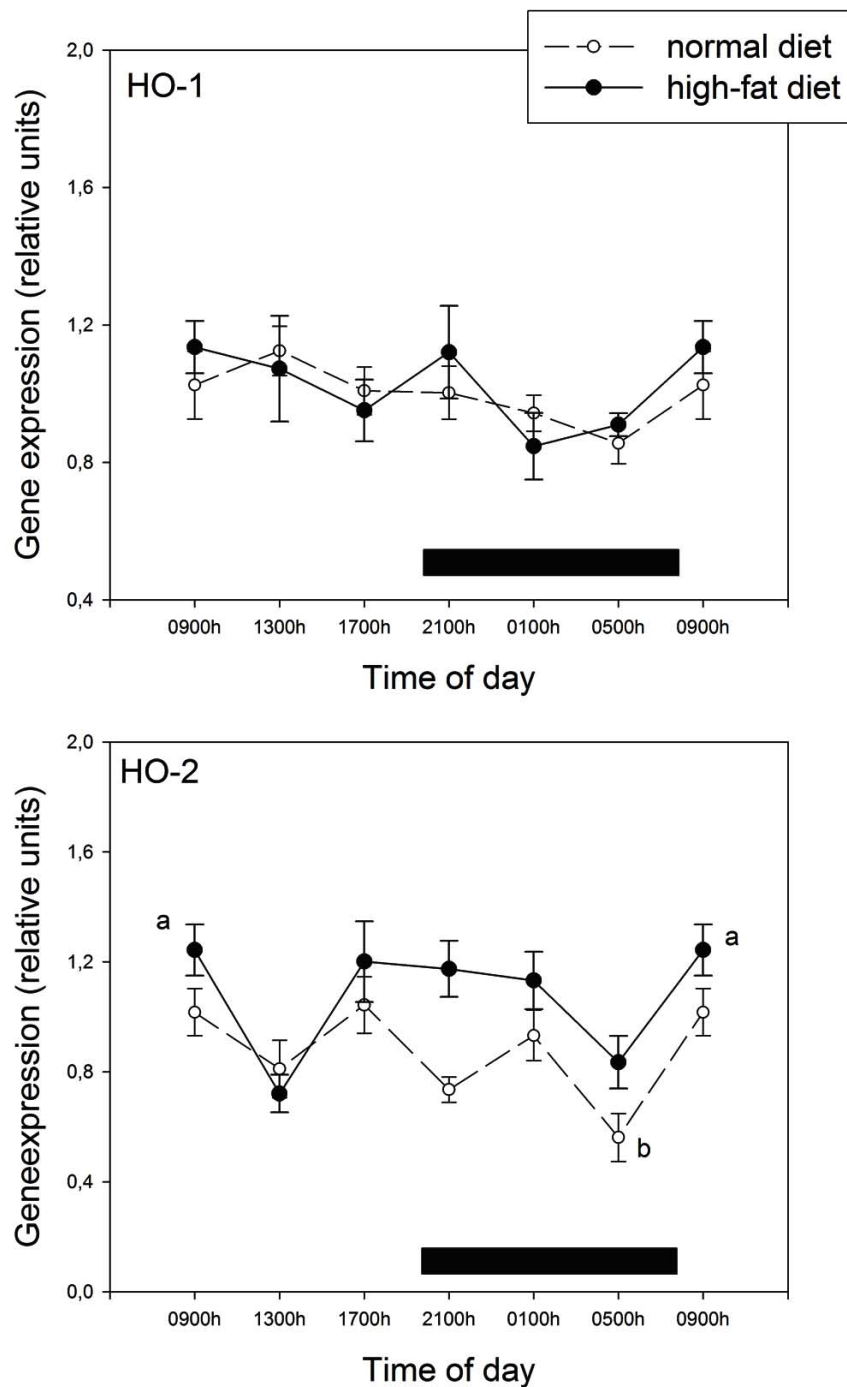

Fig. (2). Effect of a high-fat diet on 24-h changes in expression of mRNA for NOS-1 and NOS-2 in the anterior pituitary of rats. Groups of 6-8 rats were killed by decapitation at 6 different time intervals throughout a 24 h cycle. mRNA levels encoding the NOS-1 and NOS-2 enzymes were measured as described in the text. Shown are the means ± SEM of mRNA determination as measured by triplicate real-time PCR analyses of RNA samples. Letters denote significant differences in a one-way ANOVA followed by a Bonferroni´s multiple comparison test, as follows: [a] p< 0.01 *vs.* 2100, 0100 and 0500 h; [b] p< 0.05 *vs.* 0900 and 1700 h. For further statistical analysis, see text.

Fig. (3). Effect of a high-fat diet on 24-h changes in expression of mRNA for HO-1 and HO-2 in the anterior pituitary of rats. Groups of 6-8 rats were killed by decapitation at 6 different time intervals throughout a 24 h cycle. mRNA levels encoding HO-1 and HO-2 enzymes were measured as described in the text. Shown are the means ± SEM of mRNA determination as measured by triplicate real-time PCR analyses of RNA samples. Letters denote significant differences in a one-way ANOVA followed by a Bonferroni´s multiple comparison test, as follows: [a]$p< 0.05$ *vs.* 1300 h; [b] $p< 0.05$ *vs.* 0900 h and 1700 h. For further statistical analysis, see text.

Anterior pituitary gene expression of HO-1 and HO-2 is depicted in Fig. (**3**). As shown by the analysis of time as a main factor in the factorial ANOVA, HO-1 gene expression did not exhibit 24-h variations whereas that of HO-2 changed significantly as a function of time of day ($F= 7.0$, $p< 0.001$). The high-fat diet augmented mRNA levels for HO-2 ($F= 11.8$, $p< 0.001$) without modifying HO-1 significantly (Fig. **3**). A disruption in 24-h rhythmicity of HO-2 gene

expression was apparent in high-fat fed rats, with a trend to maintain high levels during most of the scotophase.

Anterior pituitary mRNA levels for Cu/Zn-SOD, Mn-SOD and catalase genes are shown in Fig. (**4**). For the three antioxidant enzymes tested, mRNA expression in controls exhibited significant 24-h variations, with maxima at the light phase of daily photoperiod ($p< 0.0001$). In high-fat fed rats these diurnal rhythms were disrupted, with a trend to
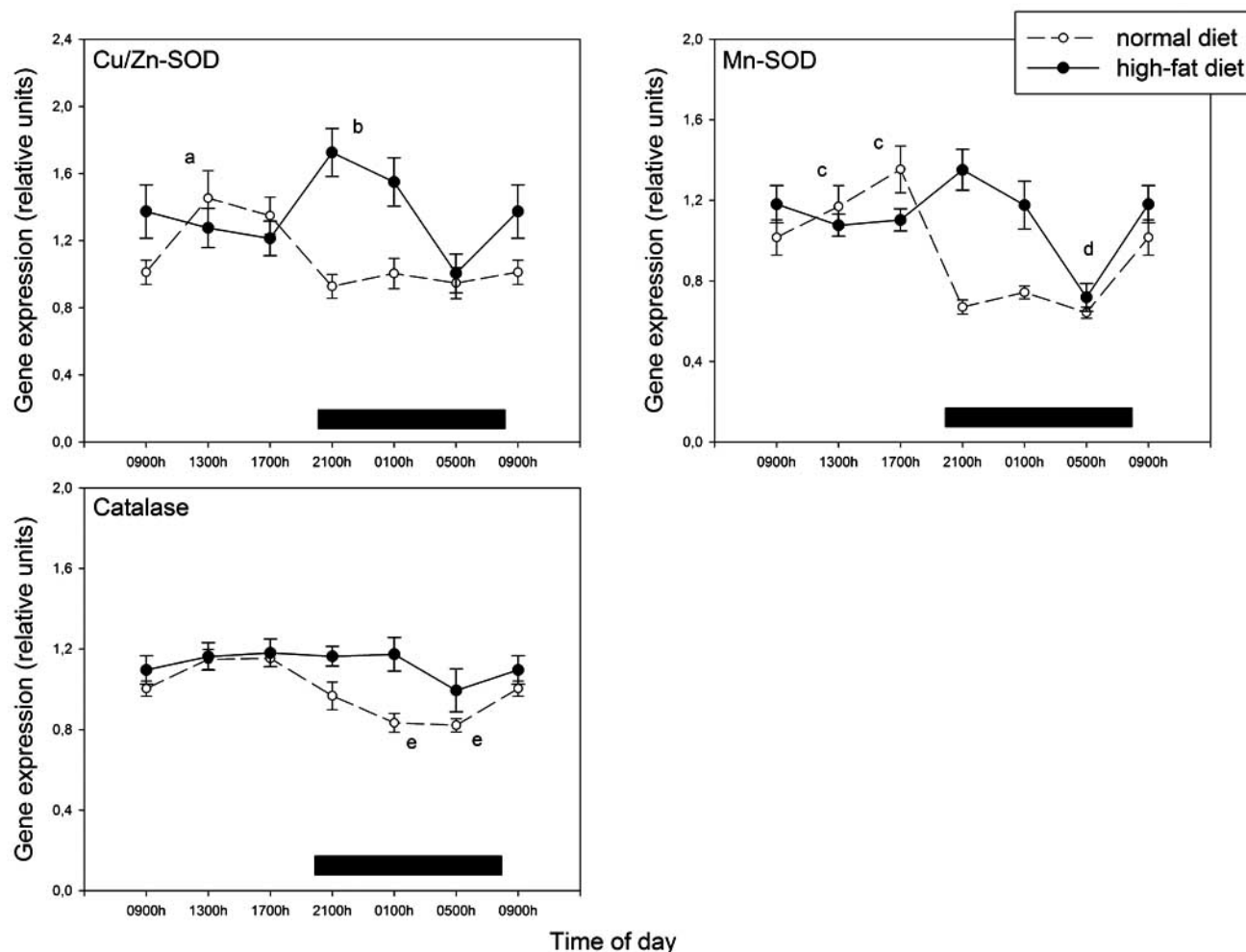
Fig. (4). Effect of a high-fat diet on 24-h changes in expression of mRNA for Cu/Zn-SOD, Mn/SOD and catalase in the anterior pituitary of rats. Groups of 6-8 rats were killed by decapitation at 6 different time intervals throughout a 24 h cycle. mRNA levels encoding Cu/Zn-SOD, Mn/SOD and catalase enzymes were measured as described in the text. Shown are the means ± SEM of mRNA determination as measured by triplicate real-time PCR analyses of RNA samples. Letters denote significant differences in a one-way ANOVA followed by a Bonferroni´s multiple comparison test, as follows: [a] $p < 0.01$ *vs.* 0500 h; [b] $p < 0.05$ *vs.* 2100 and 0500 h; [c] $p < 0.01$ *vs.* 2100, 0100 and 0500 h; [d] $p < 0.02$ *vs.* 0900, 1700, 2100 and 0100 h; [e] $p < 0.05$ *vs.* 1300 and 1700 h. For further statistical analysis, see text.

maintain high levels of expression at early scotophase (Fig. **4**).

As shown in Fig. (**5**), anterior pituitary mRNA levels for the antioxidant enzymes GPx and GR peaked at the first part of scotophase in controls with a significant phase delay in high-fat fed rats. The factorial ANOVA indicated a significant depressive effect of the high-fat diet on GPx and GR gene expression (GPx: F= 5.28, p< 0.03; GR: F= 14.3, p< 0.001), occurring mainly at the first part of scotophase (Fig. **5**).

DISCUSSION

Foregoing results indicate that the disrupted coordination between prolactin gene expression and prolactin release found in high-fat fed rats comes along with a disturbed expression of redox enzyme genes in the anterior pituitary. In particular, adenohypophysial mRNA levels for NOS-2 and HO-2, Cu/Zn- and Mn- SOD and catalase augmented in

high-fat fed rats, whereas those of GPx and GR decreased. Disruption of 24-h rhythmicity of gene expression of HO-2, Cu/Zn-SOD, Mn-SOD, catalase, GPx and GR was apparent in high-fat fed rats.

Obesity is a state of chronic oxidative stress, which is a major mechanism underlying the development of co-morbidities in obesity [7]. Oxidative stress is associated with several indices of adiposity and a low antioxidant defense. In the same animals we previously reported significant changes in 24-h rhythmicity of pituitary hormone release including prolactin [1]. The present results on the disrupted coordination between prolactin gene expression and prolactin release in obese rats can be interpreted in terms of the significant oxidative stress brought about in the anterior pituitary by the high-fat diet.

In this respect, NO presumably plays a major role [3,4]. NO is synthesized by NO-1 (constitutive) and NOS-2 (inducible) NOS, which are expressed in the pituitary gland [8,9]. NOS-1 are responsible for low and persistent
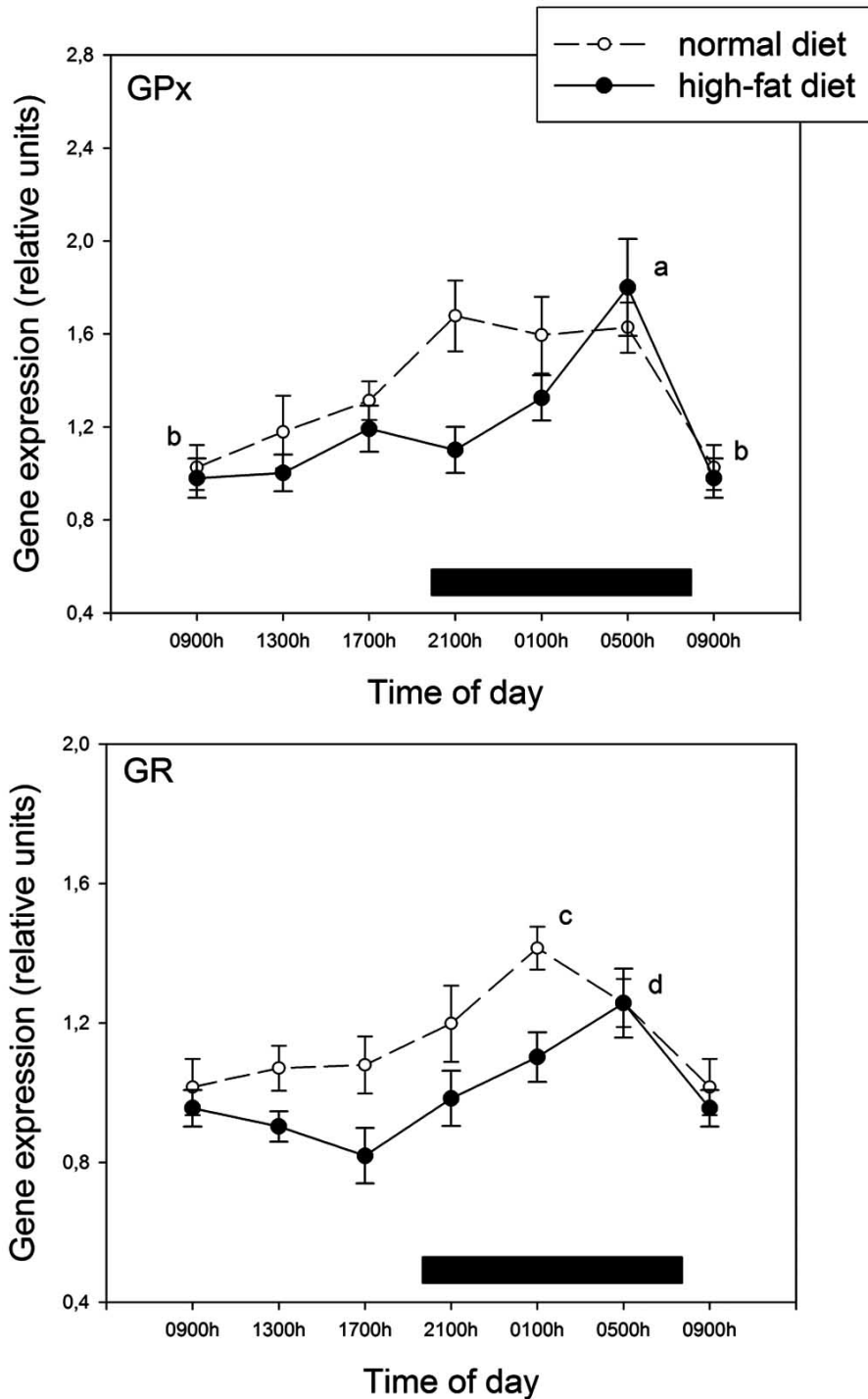
Fig. (5). Effect of a high-fat diet on 24-h changes in expression of mRNA for GPx and GR in the anterior pituitary of rats. Groups of 6-8 rats were killed by decapitation at 6 different time intervals throughout a 24 h cycle. mRNA levels encoding GPx and GR enzymes were measured as described in the text. Shown are the means ± SEM of mRNA determination as measured by triplicate real-time PCR analyses of RNA samples. Letters denote significant differences in a one-way ANOVA followed by a Bonferroni´s multiple comparison test, as follows: [a] $p < 0.02$ *vs.* 0900, 1300, 1700 and 2100 h; [b] $p < 0.05$ *vs.* 2100 and 0500 h; [c] $p < 0.05$ *vs.* 0900 h; [d] $p < 0.05$ *vs.* 1300 and 1700 h. For further statistical analysis, see text.

concentrations of NO, typical of normal situations. The present results indicate that the high-fat diet caused an up-regulation of pituitary NOS-2 expression, presumably resulting in high levels of NO that could be instrumental in the disruption of prolactin release mechanisms herein reported. Indeed, although submicromolar concentrations of

NO can protect anterior pituitary cells from cytotoxic agents, long-term exposure of ptuitary cells to high concentrations of NO induces cellular damage [10].

Because reactive oxygen species (ROS) generation is a continuous and physiological phenomenon, cells possess

efficient antioxidant systems that protect them from oxidative damage (for recent reviews, see [11-13]). These defense systems are thought to prevent free radicals from causing irreparable damage by reacting with lipids, proteins and nucleic acids and are controlled *in vivo* by a wide spectrum of enzymatic [14] and non-enzymatic systems [15]. In the anterior pituitary almost all redox pathway enzymes have been described; however, there is scarce information on the existence of circadian rhythms in enzyme activity or gene expression in this tissue.

HO has an important role in controlling the redox state of the cell by functioning as a rate-limiting enzyme in the heme degradation process [11]. The oxidative degradation of heme, which results in the formation of equimolar amounts of carbon monoxide (CO) and biliverdin, is catalyzed by the microsomal enzyme HO. Biliverdin and bilirubin are known to be potent antioxidants [11]. Three isoforms of HO have been identified. HO-1 is an inducible isoform that is responsive to various stimuli, including oxidative stress. HO-2 is a constitutive isoform that is less inducible by oxidative stress. The remaining isoform, HO-3, has been less well characterized [11]. The anterior pituitary displays both HO-1 and HO-2 mRNA proteins and enzymatic activity, explaining the high CO production rate that the gland has [16-19].

In the present study, and concomitant with the disrupting effect that the high-fat diet has on prolactin secretion, an increase in HO-2 expression was observed, mainly at the first part of scotophase, when the oxidative damage appears to be maximal. The results suggest that an overexpression of HO-2 gene is counteracting the oxidative damage caused by excess NO.

The detoxification of ROS in cells involves the cooperative action of intracellular antioxidant enzymes, among them Cu/Zn-SOD that is cytosolic, Mn-SOD that is mitochondrial, and catalase that is present in peroxisomes [20]. In addition, GPx and GR help to maintain adequate levels of reduced glutathione, a major antioxidant defense of the cells. The changes in redox enzyme mRNA expression brought about by the high-fat diet in the present study can be interpreted in terms of a compensatory increase caused by the augmented oxidative load (i.e., Cu/Zn- and Mn- SOD and catalase) together with a depression of enzyme gene expression caused by excess ROS generation (i.e., GPx and GR). Since ROS play a role in cellular signalling processes, including transcription factors activities such NF-kB and AP-1, the increase of free radicals caused by the high-fat diet would allow modulation of redox-sensitive transcription factors, which could in turn regulate gene transcription [20,21].

There are several limitations to the present study. First, further studies are needed to shed light on the mechanisms that explain high-fat diet effect on redox enzyme gene expression. In particular, Western blotting analysis of enzyme protein levels will be useful in this respect.

Second, whether the altered circadian rhythms found are due to the high fat content of the diet, to elevated caloric intake, or to some indirect result of body weight gain, obesity and fat deposition should be further examined. The study of rats pair-fed the high-fat diet but matched to the caloric input of the rats fed the control diet could yield valuable information on this point.

Third, further examination is needed to assess whether acute overfeeding immediately disrupted diurnal rhythmicity, as shown for cardiovascular parameters in rabbits [22]. An earlier measurement of gene expression at a time before body weights diverged significantly could be useful in this respect.

Another limitation is given by the method of sampling. A pulsatile release exists for prolactin release and such ultradian variations are not measurable with the experimental approach used. Finally, further experiments are needed to assess whether the changes in amplitude as well in timing of 24-h rhythms discussed herein can be attributed to an effect on the SCN or to a masking effect on some output(s) of the clock. The data only draw a correlation and the causality of the changes observed can only be speculated upon. In future studies it will be important to establish actual ROS production in the hypophysis of obese rats and whether by blocking or stimulating ROS alteration in circadian profile of prolactin secretion is obtained.

CONCLUSION

The results support the conclusion that the disrupted coordination between prolactin gene expression and prolactin release in high-fat fed rats comes along with a disturbed expression of redox enzyme genes in the anterior pituitary.

ABBREVIATIONS

ANOVA	=	Analysis of variance
CO	=	Carbon monoxide
GPx	=	Glutathione peroxidase
GR	=	Glutathione reductase
HO	=	Heme oxygenase
PCR	=	Polymerase chain reaction
NOS	=	Nitric oxide synthase
SOD	=	Superoxide dismutase

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