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Daily oscillation of glutathione redox cycle is dampened in the nutritional vitamin A deficiency

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Abstract

Examples of hormonal phase-shifting of circadian gene expression began to emerge a few years ago. Vitamin A fulfills a hormonal function by binding of retinoic acid to its nuclear receptors, RARs and RXRs. We found retinoid- as well as clock-responsive sites on regulatory regions of Glutathione reductase (GR) and Glutathione peroxidase (GPx) genes. Interestingly, we observed retinoid receptors, as well as GSH, GR and GPx, display daily oscillating patterns in the rat liver. We also found that feeding animals with a vitamin A-free diet, dampened daily rhythms of RAR α and RXR β mRNA, GR expression and activity, GSH, BMAL1 protein levels and locomotor activity. Differently, day-night oscillations of RXR α , GPx mRNA levels and activity and PER1 protein levels, were phase-shifted in the liver of vitamin A-deficient rats. These observations would emphasize the importance of micronutrient vitamin A in the modulation of biological rhythms of GSH and cellular redox state in liver.

Keywords

glutathione; glutathione peroxidase; glutathione reductase; vitamin A; biological rhythm; retinoid nuclear receptor

Introduction

Day-night cycles are known as the main *zeitgeber* (from German, for “time giver”, synchronizer) for a wide number of living beings, including mammals. Most mammal’s tissues show circadian oscillations and have their own cellular clock machinery which controls and synchronizes local 24-h oscillating gene expression (Balsalobre et al., 2000; Yamazaki et al., 2000; Panda and Hogenesch, 2004; Brewer et al., 2005). The heterodimeric basic helix-loop-helix-Per Arnt Sim (bHLH-PAS) transcription factor: BMAL1:CLOCK (from Brain and Muscle ARNT Like protein 1: Circadian Locomotor Output Cycles Kaput protein) drives the expression of clock (Per1, 2 and 3 and Cry1 and 2) and clock-controlled genes by its binding to E-box enhancers on target promoters. RevErba and ROR

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transcription factors, members of the retinoic acid-related orphan receptor (ROR) family, complete the molecular clock machinery. They bind to Rev-erb/ROR elements (RORE) on the promoter of target genes, with Rev-erb α and Rev-erb β acting as transcriptional repressors and ROR α or ROR γ as transcriptional activators (Emery and Reppert, 2004).

Feeding cycles can also entrain peripheral clocks such as the liver, independently of light entrainment (Stokkan et al., 2001; Selmaoui and Thibault, 2002; Brewer et al., 2005). While the mechanism could remain unknown, examples of hormonal phase-shifting of circadian gene expression in peripheral organs begun to emerge with Balsalobre et al. (2000). Indeed, there is some evidence that in a ligand-dependent manner, retinoid (vitamin A) nuclear receptors can interact with the cellular clock machinery partners, CLOCK, or its homolog MOP4, and inhibit BMAL1:CLOCK and/or BMAL1:MOP4 heterodimer-mediated expression of circadian responsive genes (McNamara et al., 2001). Additionally, it has been reported that transcription factors such as albumin promoter D site binding protein (DBP) and Rev-Erba, both involved in circadian regulation, heterodimerize with retinoid receptors. Recently, McClintick et al. (2006) showed vitamin A deficiency (VAD) increased DBP and Rev-Erba mRNA levels in the rat liver.

VAD is the most common micronutrient deficiency worldwide. Vitamin A and its derivatives, the retinoids regulate developmental, physiological and cellular processes by activating retinoid nuclear receptors. Retinoids bind to two distinct families of ligand-activated transcription factors, namely retinoic acid receptors (RAR α , β and γ) and retinoid X receptors (RXR α , β and γ). Retinoic acid (RA)-depending transcriptional regulation is mediated either by RAR:RXR heterodimers or by RXR:RXR homodimers which bind to RARE and RXRE sites, respectively, on the target genes promoters (Soprano et al., 2004). RARE sites are usually composed of direct repeats of the consensus AGGTCA half site sequence separated by five nucleotides while RXREs are usually direct repeats of the same consensus sequence separated by only one nucleotide. In the absence of RA, RAR:RXR heterodimers have been shown to be associated with corepressor molecules such as N-CoR and SMRT. Upon binding RA, corepressor association is disrupted and interaction with coactivator molecules occurs (Klein et al., 2000). It is well known, liver stores 80% of vitamin A in the whole body as retinyl palmitate and a significant expression of retinoid receptors, predominantly, RAR α , RAR β , RXR α and RXR γ , has been observed in this organ in the adult rat (Hellemans et al., 2004; Ulven et al., 1998).

Vitamin A also functions as antioxidant and radical scavenger (Ciaccio et al., 1993; Palacios et al., 1996). It has been demonstrated that VAD produces oxidative stress, increasing the levels of lipid peroxidation and affecting the antioxidant enzymes activities (Anzulovich et al., 2000; Sohlenius-Sternbeck et al., 2000; Arruda et al., 2009). On the other hand, Rutter and his collaborators (2001) have shown that the DNA-binding activity of BMAL1:CLOCK, as well as its analog BMAL1:NPAS2, heterodimer is regulated by the cellular redox state in a purified system. Cellular redox state is the product of a coordinated balance between metabolic pathways and antioxidant enzymatic and non-enzymatic systems, and it is determined by the levels of several redox couples such as NADH/NAD⁺, NADPH/NADP⁺ and 2GSH/GSSG (Schafer and Buettner, 2001).

Glutathione (GSH) has a relevant role in the cellular antioxidant defense system and fulfills important functions in cells and tissues. For example, GSH prevents cellular membranes' oxidative damage by reducing lipid peroxides and keeping protein sulphhydryl groups in their reduced state. The couple 2GSH/GSSG is a key component in the maintenance of the best redox state for cellular functioning and viability. An optimal 100/1 up to 500/1 GSH/GSSG ratio, depending on cell and tissue, is the result, among others, of the antioxidant glutathione reductase (GR) and glutathione peroxidase (GPx) enzymatic activities (Schafer and

Buettner, 2001). GPx, one of the well characterized selenoproteins, is involved in redox regulation of intracellular signaling and redox homeostasis (Papp et al., 2007). Previously, we and others observed that nutritional VAD increases GPx, and catalase activity in the rat liver (Anzulovich et al., 2000; Sohlenius-Sternbeck et al., 2000). Daily rhythms of antioxidant enzymes activity, such as superoxide dismutase (SOD), GPx and GR, as well as of GSH levels, have been demonstrated in several tissues and under different physiological conditions (Baydas et al., 2002; Hardeland et al., 2003; Pablos et al., 1998; Subramanian et al., 2008). However, until now, we have found no published studies on circadian, GR or GPx, gene expression in mammals.

Above observations raise the possibility that nutritional factors might modulate the circadian expression of target genes, for example, by modifying cellular redox state, BMAL1 expression and/or BMAL1:CLOCK DNA-binding activity.

Considering: 1) VAD produces oxidative stress, increasing the levels of lipid peroxidation and affecting the antioxidant enzymes activity, 2) a retinoid receptor-mediated effect has been observed on cellular clock activity, and 3) VAD increases clock-related Rev-Erba and DBP expression, our specific goals were, first, to evaluate whether RARa, RXRa and RXRb expression displayed a day-night oscillation in the rat liver; second, to verify whether GSH and GSSG levels as well as GR and GPx expression and activity exhibited a daily rhythm; and third, to assess to which extent VAD could modify the temporal patterns of retinoid receptors expression as well as GSH cycle and cellular redox state in the liver, a peripheral oscillator with a relevant function in metabolism and maintaining of physiological homeostasis.

Materials and Methods

Animals and Diet

Male Holtzman rats were bred in our animal facilities (LABIR, National University of San Luis, Argentina), and maintained in a 21–23°C controlled environment with artificial 12-h light (7:00 am–7:00 pm):12-h dark (7:00 pm–7:00 am) cycles. They were weaned at 21 days old and immediately assigned randomly to either the experimental diet, devoid of vitamin A [vitamin A-deficient (VAd) group] or the same diet with 4000 IU of vitamin A (8 mg retinol as retinyl palmitate) per Kg of diet [control (Co) group]. Feeding the animals with a vitamin A-free diet during 3 months, guarantees subclinical plasma retinol concentration and depleted retinol stores in liver (Anzulovich et al., 2000; Aguilar et al., 2009; Vega et al., 2009; Oliveros et al., 2000). Rats were kept in a controlled environment and were given free access to food and water throughout the entire 3 months of the experimental period. Body weight and food intake were registered daily. At the end of the 3 months, four rats from each group were sacrificed every 5 hours during a 24-h period, at the zeitgeber times (ZT): ZT2, ZT7, ZT12, ZT17 and ZT22 (with ZT0 when light is on). Liver was removed on an ice-chilled plate, weighed, and samples of 250 and 100 mg were immediately placed in liquid nitrogen. All experiments were conducted in accordance with the National Research Council's Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Research, Commission on Life Sciences, National Research Council, 1996) and the National University of San Luis Committee's Guidelines for the Care and Use of Experimental Animals. Diets were prepared according to the AIN-93 for laboratory rodents (Reeves et al., 1993) as described previously (Anzulovich et al., 2000; Aguilar et al., 2009; Vega et al., 2009; Oliveros et al., 2000; Navigatore Fonzo et al., 2009). Both, vitamin A-deficient and control diets had the following composition (g/kg): 397.5 cornstarch, 100 sucrose, 132 dextrinized cornstarch, 200 lactalbumin, 70 soybean oil, 50 cellulose fiber, 35 AIN-93 mineral mix, 10 AIN-93 vitamin mix (devoided of vitamin A for the vitamin A-deficient diet), 3 L-cystine, 2.5 choline bitartrate, and 0.014 tert-butylhydroquinone.

Daily locomotor activity analysis

Locomotor activity of individually housed Co and VAd rats was recorded using Archon®, an Acquisition System for Rodent Activity, during last week of treatment period. Activity counts were sampled and stored on a computer hard disk using time frames of 5 min. Data from an ASCII files were graphed in double-plotted actograms at modulo 24 h. For rats locomotor activity data, a rhythm was considered as synchronized with lights-off when activity regularly started within a maximum range of two hours.

RNA isolation and Reverse Transcriptase (RT) reaction

Total RNA was extracted from 100 mg liver samples using the Trizol reagent (Invitrogen Co). All RNA isolations were performed as directed by the manufacturers. The yield and purity of total RNA were determined spectrophotometrically at 260 and 280 nm. Gel electrophoresis and ethidium bromide staining confirmed the integrity of RNA. Three micrograms of total RNA were reverse-transcribed with 200 units of MMLV RT (Promega Inc.) using random primer hexamers in a 25 µl reaction mixture, following the manufacturer's instructions.

Real-Time PCR

Relative quantification of basal RARα, RXRα and RXRβ mRNA levels was performed by Real-Time PCR using the ABI Prism® 7500 thermocycler (Applied Biosystems, USA) as described in Fonzo et al. (2009). Gene-specific primers are shown in Table 1. Relative expression of the real-time PCR products was determined by the $\Delta\Delta C_t$ method. Each sample was run in triplicate, and the mean C_t was used in the $\Delta\Delta C_t$ equation. Data for the normalized transcript levels of RARα, RXRα and RXRβ are shown as means $\pm$ S.E.M.

PCR amplification

Fragments coding for β -actin, RARα, RXRα, RXRβ, GR and GPx, were amplified by PCR as previously described in Fonzo et al. (2009). The sequences of the specific primers are shown on Table 1. The amplified fragments were visualized and photographed under ultraviolet (UV) transillumination. The mean of gray value for each band was measured using NIH ImageJ software (Image Processing and Analysis in Java from <http://rsb.info.nih.gov/ij/>) and the relative abundance of each band was normalized according to the housekeeping β -actin gene, calculated as the ratio of the mean of gray value of each product to that of β -actin.

Total, reduced and oxidized glutathione levels

Total (Total GSH), reduced (GSH) and oxidized (GSSG) glutathione levels were determined in liver samples isolated from control and vitamin A-deficient rats at ZT2, ZT7, ZT12, ZT17 and ZT22, following Akerboom & Sies (1981). Briefly, total GSH was measured in neutralized acid extracts by a kinetic assay using NADPH, 5,5'-dithiobisnitrobenzoic acid, and GR. GSSG was determined in the same extracts by following the oxidation of NADPH at 340 nm after addition of GR. GSH values were obtained by difference. GSH and GSSG values were expressed as μ moles /g of tissue.

Tissue homogenates and enzyme activity assays

Liver samples (250 mg) isolated from control and vitamin A-deficient rats at ZT2, ZT7, ZT12, ZT17 and ZT22, were homogenized in 1/5 (w/v) dilution of 120 mM KCl and 30 mM phosphate buffer, pH 7.2 at 4°C. Suspensions were centrifuged at 800 x g for 10 min at 4°C. The pellets were discarded and supernatants were used to determine antioxidant enzyme activities. GPx, and GR activities were determined by the methods of Flohe & Gunzler (1984) and Schaedle (1977), respectively. All reagents were from Sigma-Aldrich Co.

Scanning of antioxidant genes upstream regions for putative E-box, RARE and RXRE sites

To identify putative retinoic acid-responsive (RARE: AGGTCANNNNNAGGTCA and RXRE: AGGTCANAGGTCA) as well as clock-responsive (perfect E-box: CACGTG, or E-box like: CANNTG) DNA consensus regulatory sites, 1200 bp upstream of the translation start codon of GPx (NCBI GenBank Acc. #: AB004231) and GR (NCBI GenBank Acc. #: NC_005115) genes, were scanned for significant matches using the MatInspector® software from Genomatix (<http://www.genomatix.de>; Quandt et al., 1995).

Immunoblotting assays

Protein extracts were prepared by homogenizing 250 mg liver samples in buffer C (20 mM HEPES, pH 7.9, 0.42 M NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 1 µg/ml leupeptin, 1 µg/ml pepstatin, 1 mM sodium fluoride, 5 µM sodium orthovanadate, and 25% glycerol). Aliquots containing 40 µg of total protein were subjected to electrophoresis in 4–12% NuPage™ Bis-Tris gels (Invitrogen Life Technologies, Carlsbad, CA), and then transferred to Immobilon-PTM transfer membranes (Millipore, Bedford, MA). Immunoblot analyses were performed following the manufacturers' protocols for the detecting antibodies and as described in Fonzo et al. (2009). Briefly, membranes were blocked in Blotto (5% nonfat dry milk, 10 mM Tris-HCl, pH 8.0, and 150 mM NaCl) followed by 3h incubation at RT with either goat anti-CAT, goat anti-GPx1, goat anti-BMAL1 or rabbit anti-PER1 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) in Blotto containing 0.05% thimerosal. After incubation with primary antibody, the membranes were washed in TBS (10 mM Tris-HCl, pH 8.0, and 150 mM NaCl) containing 0.05% Tween-20, before incubation with horseradish-peroxidase-conjugated donkey anti-goat, or goat anti-rabbit, IgG (Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:10,000 in Blotto for 1 hour at room temperature. After washing, antibody/protein complexes on membranes were detected using Vectastain® DAB Peroxidase Substrate kit from Vector Laboratories (Burlingame, CA) and following the manufacturers' indications. The mean of intensity of each band was measured using the NIH ImageJ® software (Image Processing and Analysis in Java from <http://rsb.info.nih.gov/ij/>). BMAL1 and PER1 protein levels were normalized against ACTIN (endogenous control).

Statistical Analyses

Time point data were expressed as means ± standard errors of the mean (SE) and pertinent curves were drawn. Time series were computed by one-way ANOVA followed by Tukey's post-hoc test for specific comparisons. A $P < 0.05$ was considered to be significant. When amplitude or phase was required, a fitting technique was applied. Data were fitted by the following function: $c + a \cos[2\pi(t - \phi)/24]$, where c is the mesor, a is the amplitude of the cosine wave, t is time in hours, and ϕ is the phase in hours from ZT 0 in the imposed light cycle. The fitting was performed using Nonlinear Regression from GraphPad Prism 3.0 software (CA, USA). The routine also estimates the standard error of the fit parameters. The standard error arises from scatter in the data and from deviations of the data from cosine form. Note that the frequency was taken as the 1 cycle per 24 h of the light regime.

Results

RAR α , RXR α and RXR β expression levels

In order to assess whether the vitamin A-free diet modifies the retinoid receptors expression in the liver, we first measured basal RAR α , RXR α and RXR β mRNA levels at the beginning of the day. We observed RXR β transcript level decreased in the liver of vitamin A-deficient rats ($P < 0.05$; Figure 1A). After that, we studied the temporal patterns of retinoid receptors expression and observed RAR α , RXR α and RXR β mRNA levels vary

significantly throughout a 24-h period ($P<0.01$ and $P<0.05$). We found retinoid receptors expression peaks around the end-of-the-day-beginning-of-the-night in the rat liver (acrophases: $11:48\pm0:15$, $14:33\pm0:43$ and $13:21\pm0:25$, respectively, Table 2). Interestingly, VAD had a differential effect on the oscillating retinoid receptors expression. On one hand, it abolished daily rhythmicity of $RAR\alpha$ and $RXR\beta$ expression (Figure 1B). However, on the other hand, VAD phase-shifted cycling $RXR\alpha$ expression (acrophases: $14:33 \pm 0:43$ vs $10:55 \pm 0:26$, $P<0.05$; Figure 1B and Table 2).

Daily variation of GSH and GSSG levels

GSH and GSSG levels were analyzed in the liver of control rats, throughout a 24-h period. We observed a significant daily rhythm of GSH, GSSG and GSH/GSSG ratio ($P<0.0001$, $P<0.01$ and $P<0.001$, respectively; Figure 2A–C) with GSH levels and GSH/GSSG ratio peaking during the first half of the light period (acrophases: $4:57\pm0:20$ and $3:10\pm0:59$ h, respectively; Figures 2A and C and Table 3). As expected, daily GSH levels were in antiphase with GSSG, being the last maximal at the beginning of the dark phase (Figure 2B and Table 3).

VAD decreased GSH levels and abolished their rhythmic variation in the rat liver (Figure 2A). On the other hand, daily rhythmicity of GSSG levels was phase shifted in the liver of vitamin A-deficient rats (acrophase: $12:59\pm1:36$ vs $2:23\pm1:17$, $P<0.01$; Figure 2B and Table 3). As a consequence, mesor and amplitude of rhythmic GSH/GSSG ratio were significantly reduced (1.13 ± 0.06 vs 0.62 ± 0.003 , $P<0.001$, and 0.52 ± 0.09 vs 0.22 ± 0.03 , $P<0.01$) and its acrophase shifted from $3.10 \pm 0:59$ to $13:02 \pm 0:28$ ($P<0.05$) in the vitamin A-deficient rats (Figure 2C and Table 3).

Temporal expression and daily activity of GR and GPx

GR and GPx mRNA expression oscillates significantly in a 24 h cycle ($P<0.05$; Figures 3A and 4A, respectively) with the highest mRNA levels occurring at ZT $11:46\pm0:59$ and ZT $10:30\pm1:12$, respectively (Table 4). Consistently, we found GR and GPx enzymatic activities also follow diurnal rhythms in the rat liver ($P<0.001$ and $P<0.01$) with their acrophases occurring at ZT $0:52\pm0:11$ and ZT $11:30\pm0:30$, respectively (Figures 3B and 4B and Table 4).

Day-night oscillating expression of GR and GPx was differentially affected by the nutritional VAD. On one hand, VAD dampened rhythmic GR expression (Figure 3A) and enzymatic activity (Figure 3B). On the other hand, the nutritional deficiency phase shifted the daily pattern of GPx mRNA (acrophase: $10:30 \pm 01:12$ vs $6:40 \pm 01:20$, Figure 4A and Table 4) as well as daily GPx activity (acrophase: $11:30 \pm 0:30$ vs $05:00 \pm 0:15$, $P<0.001$, Figure 4B and Table 4) in comparison to controls.

RARE, RXRE and E-box sites on GPx and GR genes upstream region

Scanning of 1200 bp upstream of the translation start codon of GPx and GR genes in the Genomatix database is shown in Figure 5. This analysis revealed five RXREs and one E-box site on the GPx gene upstream region while three RAREs and three E-boxes were found on the GR regulatory region. Additionally, one and three retinoic acid-related orphan receptor α (ROR α) responsive elements (ROREs) were found on the GPx and GR promoters, respectively.

Daily rhythms of BMAL1 and PER1 expression in the liver of vitamin A-deficient rats

Having found clock-responsive E-box sites on the GR and GPx gene promoters, led us to test whether, and to which extent, vitamin A deficiency could modify the circadian expression of core clock factors. We analyzed the variation of BMAL1 and PER1 protein

levels during a 24-h period, in the liver of control and vitamin A-deficient rats. As expected, BMAL1 and PER1 protein expression varies throughout a day in the rat liver with their acrophases occurring at ZT $12:42 \pm 0:30$ and $20:13 \pm 0:34$ h, respectively (Figures 6A and B and Table 5). Interestingly, vitamin A deficiency abolished BMAL1 rhythmicity (Figure 6A) and shifted PER1 acrophase from ZT $20:13 \pm 0:34$ h to ZT $02:13 \pm 01:14$ h (Figure 6B and Table 5).

Daily locomotor activity

As expected, locomotor activity was synchronized to the nocturnal phase in the control rats (Figure 7A). Analyzing the corresponding actograms, we observed the activity onset was at ZT $11:35 \pm 0:52$ while the offset occurred at ZT $23:26 \pm 0:22$. Feeding animals with a vitamin A-free diet during three months dampened their daily motor activity ($1,735 \pm 262$ vs 496.5 ± 150.4 total activity counts, $P < 0.02$; Figure 7B).

Discussion

Vitamin A, an essential micronutrient with a wide range of vital functions, can modulate antioxidant enzyme expression by activating specific retinoid nuclear receptors, RARs and RXRs (Xia et al., 1996). Previous results from our lab indicate that three months of feeding the vitamin A-free diet described in this work, causes a well established VAD, with depleted vitamin A stores in the rat liver, a significant reduction of the serum vitamin levels, and a decreased RXR α and β expression in the rat heart and brain, associated to alterations in non enzymatic and enzymatic antioxidant defense systems (Anzulovich et al., 2000; Aguilar et al., 2009; Vega et al., 2009; Oliveros et al., 2000; Fonzo et al., 2009). Additionally, here, we observed VAD reduced significantly basal RXR β levels in the rat liver (Figure 1A).

On the other hand, and for the first time at our knowledge, we observed RAR α , RXR α and RXR β expression displays a daily oscillation profile in the rat liver (Figure 1B), suggesting a rhythmic transcriptional activation of their target genes. Interestingly, VAD abolished daily oscillation of RAR α and RXR β mRNA and phase-shifted daily rhythm of RXR α in the liver (Figures 1B and Table 2). Thus, VAD might alter daily rhythmicity of retinoic acid target genes by modifying the daily patterns of retinoid receptors expression.

Aerobic organisms developed a complex and efficient network of antioxidant defenses to protect themselves against deleterious effects of reactive oxygen species and maintenance of tissue and cellular homeostasis. Glutathione plays a key role in the antioxidant network and its dynamic metabolism is determinant of an optimal cellular redox state. We and others have observed circadian rhythmicity of antioxidant enzymes activity and GSH levels in different tissues and species (Baydas et al., 2002; Hardeland et al., 2003; Pablos et al., 1998; Subramanian et al., 2008; Fonzo et al., 2009; Filipski et al., 2004). In this study, we found glutathione redox cycle displays a robust daily rhythmicity in the rat liver, with GSH levels peaking at the beginning of the light (mainly anabolic) period in rats (Figure 2A and Table 3), similar to the observed by Filipski et al. (2004) in the mouse liver. Consistently, GSH peak is preceded by the maximal GR activity and followed by the highest GPx activity, in the liver of control rats (Figures 3B and 4B and Table 4). GPx activity peak at the end of the light period is, as expected, followed by an increase in GSSG levels, which are maximal at ZT $12:59 \pm 1:36$ (Figure 2B and Table 3) and decrease GSH/GSSG ratio at this time of the day. These facts generate an oxidant cellular environment which, as shown by Lee et al. (2004), could activate GR transcription and explain the GR mRNA peak at the end-of-the-day-beginning-of-the-night seen in this study (Figure 3A). All above observations lead us to propose the existence of a very well temporally orchestrated GSH cycle in the rat liver, with a reduced cellular environment occurring during the rest (anabolic) period whereas an

oxidant status concurs with the activity (catabolic) phase. Such temporal organization is, however, susceptible to oxidative stress.

Interestingly, VAD exerted differential effects on the daily rhythmicity of glutathione cycle in the liver. On one hand, daily oscillation of GSH became arrhythmic in the liver of vitamin A-deficient rats in comparison to the control group (Figure 2A). This could be a consequence of an attenuated or even abolished temporal fluctuation of GR expression and activity (Figure 3) and/or to the significant increase in GPx activity, and thus a higher GSH consumption, observed at ZT2 and ZT7 in the liver of the vitamin A-deficient animals (Figure 4B). The last observation is consistent with our previous work (Anzulovich et al., 2000). However, it differs from what we observed in the rat hippocampus under the same vitamin deficiency (Fonzo et al., 2009), suggesting tissue-specific effects of the nutritional VAD and thus, probably, a tissue-specific role for the vitamin A.

It has been demonstrated that vitamin A modulates the upregulation of some major scavenger enzyme genes, such as glutathione-S-transferase (GST) (Xia et al., 1996) while VAD decreases GST expression and activity in the rat liver (Sohlenius-Sternbeck et al., 2000; McClintick et al., 2006). Here, temporal changes in the enzymatic GR and GPx activity followed changes in mRNA levels, suggesting VAD affects the circadian expression of GR and GPx at the transcriptional level.

Interestingly, we found three RARE and three E-box sites within 1200 bp upstream of the translation site in the GR gene while five RXRE and only one E-box were found in the GPx gene (Figure 5). The presence of different putative retinoid- and clock- responsive elements on the GR and GPx genes upstream region (Figure 5), would explain, at least in part, a differential transcriptional regulation of these genes as well as their distinct response to the VAD seen in this study. Thus, while GR promoter seems more responsive to a RAR- and clock-mediated regulation, GPx promoter might be more sensitive to changes in RXR levels.

Consistently, maximal GR mRNA expression at the end-of-the-day/beginning-of-the-night (ZT12) concurs with the BMAL1 protein peak in the control rats (Figures 3A, and 6A) while the lowest level of GR expression occurs following the clock negative regulator, PER1, protein peak (Figures 3A and 6B).

Even though there are many reports that associate vitamin deficiencies with altered daily expression patterns, just a very few report the effect of nutritional deficiencies on circadian clock gene expression, and only one of them determines the effect of vitamin A deficiency on the oscillating Bmal1 and Per2 mRNA expression in mice (Shirai et al., 2006). It is known the central clock in the SCN controls the daily activity-rest cycles via a direct route (Schibler and Sassone-Corsi, 2002). Noteworthy, we observed vitamin A deficiency dampened rats' daily locomotor activity rhythm (Figure 7). Additionally, and similarly to what we previously observed in a different peripheral clock (Fonzo et al., 2009) daily oscillation of BMAL1 protein was abolished (Figure 6A) while PER1 rhythm was phase shifted (Figure 6B and Table 5) in the liver of vitamin A-deficient rats. All above observations might indicate a putative role for vitamin A in the regulation of the endogenous clock activity. Thus, and since VAD also abolished daily variation of RAR α and RXR β expression in the rat liver (Figure 1B), the loss of GR daily rhythmicity could be a consequence of the sum of clock and retinoid receptors rhythms alterations observed in the liver of vitamin A-deficient animals. Moreover, taking into account clock-related repressor Rev-Erba, on one hand, represses Bmal1 transcription and, on the other hand, would bind to RORE sites on the GR promoter, the shallower GR rhythmicity might be also explained by an increase in Rev-Erba expression as observed by McClintick under similar VAD conditions (2006).

GPx mRNA and activity rhythms were phase shifted in the liver of VAd animals (Figures 4A and B and Table 4) probably as a consequence of changes in the RXR α expression and PER1 levels acrophases (Figure 6B and Table 5). Additionally, and similar to what we and others previously observed in other rat tissues (Vega et al., 2009; Husson et al., 2004) VAD significantly reduced RXR β transcript levels in the rat liver (Figures 1A and B). Taking into account that in the absence of RA, RAR:RXR heterodimers have been shown to be associated with corepressor molecules (Klein et al., 2000), the significant increase observed in GPx mRNA levels at ZT2 could be due to the reduction in RXR β levels seen in the rat vitamin A depleted liver (Figure 1A) and thus to a desrepression of GPx transcription.

Although others have tested and demonstrated the effects of nutritional factors, such as aspartate, glutamate, or changes in feeding schedule (Selmaoui and Thibault, 2002; Manivasagam and Subramanian, 2004; Sivaperumal et al., 2007) on the circadian expression of antioxidant enzymes, this would be, at least at our knowledge, the first published report on the effects of the nutritional vitamin A deficiency on the daily rhythmicity of GR and GPx expression and activity and its putative impact on the circadian functionality of the liver.

Oxidative stress is usually defined as an imbalance between antioxidants and prooxidants. From a mechanistic standpoint, it has also been described as a disruption of redox signaling and control (Jones, 2006). Circadian regulation of GSH cycle might reveal an interesting strategy to respond to the challenge of daily oxidative stress. Loss of daily GSH, GR, RAR α and RXR β receptors and clock BMAL1 rhythmicity induced by the nutritional VAD, suggests retinoids would participate in the circadian regulation of the cellular redox state in the liver, a peripheral clock with a relevant function in the control of circadian metabolism as well as in the temporal synchronization of other peripheral oscillators.

Nutritional VAD is a serious concern and has a clinical and a socio-economical significance worldwide. Learning how VAD affects the rhythmic expression of genes involved in the glutathione reduction-oxidation cycle may have an impact on the nutritional and chronobiology fields, emphasizing for the first time the importance of nutritional factors, such as dietary micronutrients, in the daily regulation of GSH cycle and the cellular redox state in the liver.

Given the relevant role of GSH in the cellular physiology, modifications in its regulation and homeostasis can be associated to the etiology and progression of several pathologies. We would expect emerging data from these and future studies will also highlight retinoid and redox signaling pathways as potential novel therapeutic targets for circadian rhythms disorders.

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Figure 1.

Transcript levels of RAR α , RXR α and RXR β in the liver of control and vitamin A-deficient rats. A) Basal mRNA levels were determined by Real-Time PCR and normalized to β -actin. Each bar represents the mean $\pm$ SE of 4 samples in triplicates with * $P < 0.05$ in comparison to controls. B) Average cosinor fit for rhythmic RAR α , RXR α and RXR β expression. Temporal profiles of RAR α , RXR α and RXR β mRNA levels were determined by RT-PCR on liver samples isolated from control and vitamin A-deficient rats at zeitgeber times ZT2, ZT7, ZT12, ZT17 and ZT22. Horizontal bars represent the distribution of light (open) and dark (closed) phases of a 24 h (ZT0-ZT24) photoperiod. Curves represent normalized mRNA levels versus ZT. Each point represents the mean $\pm$ SE of four liver samples. Statistical analysis was performed using one-way ANOVA followed by Tukey test with * $P < 0.05$ and ** $P < 0.01$ when indicated means were compared to the corresponding maximal value in each group.

Figure 2.

Day-night cycles of GSH (A), GSSG (B) and GSH/GSSG ratio (C) in control and vitamin A-deficient rats. Average cosinor fit for oscillating GSH, GSSG and GSH/GSSG ratio. GSH and GSSG levels were determined in liver samples isolated from control and vitamin A-deficient rats at ZT2, ZT7, ZT12, ZT17 and ZT22, following a kinetic assay. Horizontal bars represent the distribution of light (open) and dark (closed) phases of a 24 h (ZT0-ZT24) photoperiod. Curves represent hepatic GSH and GSSG levels versus zeitgeber times. Each point represents the mean $\pm$ SE of six liver samples. Statistical analysis was performed using one-way ANOVA followed by Tukey test with * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ when indicated means were compared to the corresponding maximal value in each group.

Figure 3.

Daily rhythms of GR mRNA expression and enzymatic activity in the liver of control and vitamin A-deficient rats. Cosine fitting curves for normalized GR mRNA levels (A) and GR enzymatic activity (B) throughout a day. Horizontal bars represent the distribution of light (open) and dark (closed) phases of a 24-h (ZT0-ZT24) photoperiod. Each point represents the mean $\pm$ SE of four liver samples at a given ZT (with ZT=0 when light is on). Statistical analysis was performed using one-way ANOVA followed by Tukey test with * P <0.05 and *** P <0.001 when indicated means were compared to the corresponding maximal value in each group.

Figure 4.

Daily rhythms of GPx mRNA expression and enzymatic activity in the liver of control and vitamin A-deficient rats. Cosine fitting curves for normalized GPx mRNA levels (A) and GPx enzymatic activity (B) throughout a day. Horizontal bars represent the distribution of light (open) and dark (closed) phases of a 24-h (ZT0-ZT24) photoperiod. Each point represents the mean $\pm$ SE of four liver samples at a given ZT (with ZT=0 when light is on). Statistical analysis was performed using one-way ANOVA followed by Tukey test with * $P < 0.05$ and ** $P < 0.01$ when indicated means were compared to the corresponding maximal value in each group.

Figure 5.

Schematic representation of RARE, RXRE and E-box sites on the 5' regulatory region of GR and GPx genes. The accession # for the sequences taken from the NCBI Nucleotide database are: GPx (Acc. #: AB004231) and GR (Acc. #: NC_005115). Arrows indicate the first translation codon, gray boxes represent exons, dashed circles are RARE sites, white circles are RXREs, black ovals are perfect E-boxes (CACGTG) and grey ovals are RORE sites. Negative (-) numbers indicate regulatory sites positions relative to the start of translation (+1).

Figure 6.

Daily rhythms of BMAL1 and PER1 protein levels in the liver of control and vitamin A-deficient rats. (A) Cosine fitting curves for rhythmic normalized BMAL1 and PER1 protein levels obtained from the densitometric quantitation of the Immunoblots representative data. Each point represents the mean $\pm$ SE of three liver samples at a given ZT (with ZT=0 when light is on). Horizontal bars represent the distribution of light (open) and dark (closed) phases of the 24-h photoperiod. (B) Immunoblot analysis of protein extracted from control and vitamin A-deficient rat livers isolated at ZT2, ZT6, ZT10, ZT14, ZT18, and ZT22.

Figure 7.
Representative actograms of control and vitamin A-deficient rats. Double-plot representation of daily locomotor activity of (A) Co and (B) VAd male Holzman rats, maintained in 12h:12h light:dark (LD) conditions during 7 days.

Table 1Primer pairs used for RT-PCR^{*} and Real-Time PCR[§]

Gene name	GenBank Accession No	Forward primer 5'- 3'	Reverse primer 5'- 3'	Fragment size
GPx1	NM_030826	CGGTTTCCCGTGCAATCAGTT	ACACCGGGGACCAAATGATG	225 bp
GR	NM_053906	AGCCACAGCGGAAGTCAAC	CAATGTAACCGGCACCCACA	186 bp
RARα	NM_031528	CGCCTGTGAGGGCTGTAAG	ATGCCCACTTCGAAGCATTT	150 bp
RXRα	NM_012805	GCCCACCCCTCAGGAAATAT	CACCGGTCCGCTGTCTCT	200 bp
RXRβ	NM_206849	CGAAGCTCAGGCAAGCACTA	TCCTGTACCGCCTCCCTTTT	200 bp

^{*} antioxidant enzymes genes[§] retinoid receptors genes

Rhythm parameters of daily retinoic acid receptors mRNA oscillating levels in the liver of Co and VAd rats

Table 2

Rhythm Parameters	Mesor (mean ± SE)			Amplitude (mean ± SE)			Acrophase (mean ± SE)		
	Co	VAd	P	Co	VAd	P	Co	VAd	P
RARα	0.82± 0.02	N/A	-	0.11 ± 0.03	N/A	-	11:48± 0:15	N/A	-
RXRα	0.81 ± 0.03	0.76 ± 0.02	0.21	0.06 ±0.03	0.06± 0.03	0.89	14:33 ± 0:43	10.55± 0:26	0.023
RXRβ	0.82 ± 0.02	N/A	-	0.06 ± 0.004	N/A	-	13:21 ± 0:25	N/A	-

N/A: it does not apply, since daily mRNA levels became arrhythmic.

Table 3

Rhythm parameters of daily glutathione oscillating levels in the liver of Co and VAd rats

Rhythm Parameters	Mesor (mean $\pm$ SE)			Amplitude (mean $\pm$ SE)			Acrophase (mean $\pm$ SE)		
	Co	VAd	P	Co	VAd	P	Co	VAd	P
GSH	52.90 $\pm$ 2.45	N/A	-	19.61 $\pm$ 3.55	N/A	-	04:57 $\pm$ 0:20	N/A	-
GSSG	48.44 $\pm$ 2.83	41.86 $\pm$ 1.83	0.06	15.23 $\pm$ 4.10	15.63 $\pm$ 3.33	0.94	12:59 $\pm$ 1:36	02:23 $\pm$ 1:17	0.01
GSH/GSSG	1.13 $\pm$ 0.06	0.62 $\pm$ 0.03	0.00	0.52 $\pm$ 0.09	0.22 $\pm$ 0.03	0.01	03:10 $\pm$ 0:59	13:02 $\pm$ 0:28	0.02

N/A: it does not apply, since daily GSH levels became arrhythmic.

Table 4

Rhythm parameters of daily GR and GPx oscillating expression and activity in the liver of Co and VAd rats

Rhythm Parameters	Mesor (mean $\pm$ SE)		Amplitude (mean $\pm$ SE)		Acrophase (mean $\pm$ SE)	
	Co	VAd	P	Co	VAd	P
GR mRNA	0.92 $\pm$ 0.02	N/A	-	0.10 $\pm$ 0.03	N/A	-
GR activity	43.90 $\pm$ 0.61	N/A	-	5.75 $\pm$ 0.88	N/A	-
GPx mRNA	0.66 $\pm$ 0.12	0.93 $\pm$ 0.16	0.26	0.21 $\pm$ 0.05	0.43 $\pm$ 0.12	0.18
GPx activity	0.20 $\pm$ 0.01	0.19 $\pm$ 0.02	0.74	0.04 $\pm$ 0.01	0.06 $\pm$ 0.03	0.58
				11:30 $\pm$ 0:30	06:40 $\pm$ 1:20	0.05
				11:30 $\pm$ 0:30	5:00 $\pm$ 0:14	0.001

N/A: it does not apply, since daily mRNA levels and activity became arrhythmic.

Table 5

Rhythm parameters of daily BMAL1 and PER1 protein levels in the liver of Co and VAd rats

Rhythm Parameters	Mesor (mean $\pm$ SE)			Amplitude (mean $\pm$ SE)			Acrophase (hh:mm)		
	Co	VAd	P	Co	VAd	P	Co	VAd	P
BMAL1	1.07 $\pm$ 0.03	N/A	-	0.14 $\pm$ 0.00	N/A	-	12:42 $\pm$ 0:30	N/A	-
PER1	0.69 $\pm$ 0.02	0.79 $\pm$ 0.03	0.048	0.08 $\pm$ 0.01	0.11 $\pm$ 0.03	0.427	20:13 $\pm$ 0:34	02:35 $\pm$ 1:14	0.001

N/A: it does not apply, since daily protein levels became arrhythmic.