Article

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Abstract

The circadian timing system regulates key aspects of mammalian physiology. Here, we analyzed the effect of the endogenous antioxidant paraoxonase 1 (PON1), a high-density lipoprotein-associated lipolactonase that hydrolyses lipid peroxides and attenuates atherogenesis, on circadian gene expression in C57BL/6J and PON1KO mice fed a normal chow diet or a high-fat diet (HFD). Expression levels of core-clock transcripts Nr1d1, Per2, Cry2 and Bmal1 were altered in skeletal muscle in PON1-deficient mice in response to HFD. These findings were supported by circadian bioluminescence reporter assessments in mouse C2C12 and human primary myotubes, synchronized in vitro, where administration of PON1 or pomegranate juice modulated circadian period length.

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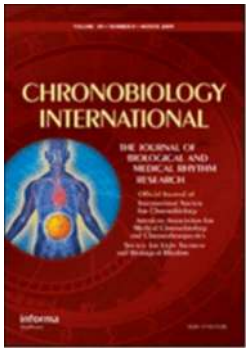

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Paraoxonase 1 (PON1) and pomegranate influence circadian gene expression and period length

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ABSTRACT

The circadian timing system regulates key aspects of mammalian physiology. Here, we analysed the effect of the endogenous antioxidant paraoxonase 1 (PON1), an HDL-associated lipo-lactonase that hydrolyses lipid peroxides and attenuates atherogenesis, on circadian gene expression in C57BL/6J and PON1KO mice fed a normal chow diet (ND) or a high-fat diet (HFD). Expression levels of core-clock transcripts *Nr1d1*, *Per2*, *Cry2* and *Bmal1* were altered in skeletal muscle in PON1-deficient mice in response to HFD. These findings were supported by circadian bioluminescence reporter assessments in mouse C2C12 and human primary myotubes, synchronized *in vitro,* where administration of PON1 or pomegranate juice modulated circadian period length.

Keywords: Circadian clock, PON1, pomegranate, skeletal muscle, circadian bioluminescence recording

INTRODUCTION

Paraoxonases belong to a family of ester hydrolyses, involved in the detoxification of organophosphates and lactones. The three members of the paraoxonase family (PON1-3) have multifunctional roles in various biochemical pathways, such as protection against oxidative damage and lipid peroxidation, innate immunity, detoxification of reactive molecules, modulation of endoplasmic reticulum stress and regulation of cell proliferation and apoptosis (Aviram et al, 1998; Martinelli et al, 2013; Rosenblat & Aviram, 2009). Paraoxonase-1 (PON1) is the most studied enzyme of the family, based on its antioxidative properties and its protective role in oxidative stress, inflammation and liver diseases. PON1 is found mainly in serum where its association with high-density lipoprotein (HDL) is responsible for many of the anti-atherogenic and cardioprotective characteristics of HDL (Rosenblat & Aviram, 2009). PON2 on the other hand is not present in serum and is ubiquitously expressed in all body cells, including skeletal muscle and heart (Mackness et al, 2010).

Accumulating evidence has shown that PON1 has a protective effect on the development of cardiovascular diseases (CVD) and diabetes (Aviram et al, 2000b; Koren-Gluzer et al, 2011). Studies in *Pon1*-knockout and *Pon1*-transgenic mice suggest that PON1 protects against the development of diabetes and its cardiovascular complications mostly through its antioxidant properties (Aviram et al, 1999; Rozenberg et al, 2008). Furthermore, it was demonstrated that in skeletal muscle PON1 attenuates insulin resistance and promotes glucose uptake by enhancing GLUT4 expression (Koren-Gluzer et al, 2013). These data are further supported by the protective role of HDL–associated PON1 and pomegranate juice (which activates PON1) against CVD and diabetes in humans (Aviram et al, 2000a; Aviram et al, 2000b; Mastorikou et al, 2006).

In peripheral organs a large number of key metabolic functions are subject to daily oscillations, such as carbohydrate and lipid metabolism (Adamovich et al, 2015) but also xenobiotic detoxification by the liver, kidney or small intestine (Bass, 2012; Dibner & Schibler, 2015). These rhythmic oscillations are organized by the circadian clock and have evolved to anticipate diurnal variations and to provide the organism with an adaptive advantage. The circadian clock is driven by a master pacemaker, located in the suprachiasmatic nucleus (SCN) of the hypothalamus, which is orchestrating subsidiary oscillators in peripheral organs (Albrecht, 2012). At the molecular level, circadian rhythms rely on a signaling network of transcriptional and translational feedback loops. In mammals, the core clock machinery is driven by a functional interplay of the BMAL1 and CLOCK activators and the PER and CRY repressors proteins. In addition, the CLOCK/BMAL1 complex also activates a second auxiliary feedback loop, involving the nuclear orphan receptors REV-ERB and ROR, which contributes to the cyclic transcription of *Bmal1* and *Clock* (Dibner & Schibler, 2015).

Recent data have indicated a critical role for the circadian clock in both muscle health and whole body homeostasis. More than 2300 genes in mouse skeletal muscle are expressed in a circadian pattern and participate in a wide range of functions, such as myogenesis, and metabolism (Harfmann et al, 2015). Furthermore, disruption of *Clock* or *Bmal1* leads to structural and functional alterations at the cellular level in skeletal muscle. In $Clock^{\Delta 19}$ and $Bmal1^{-/-}$ mutant mice, the observed effects include alteration in myofilament organization and reduction in mitochondrial volume and respiration (Andrews et al, 2010). Moreover, muscle specific loss of *Bmal1* results in impaired insulin-stimulated glucose uptake due to reduced protein levels of GLUT4 (Dyar et al, 2014). In addition, we have recently demonstrated that the circadian clock, operative in human primary skeletal myotubes, is regulating basal myokine secretion (Perrin et al, 2015).

In view of the emerging role of PON1 in insulin resistance, we aimed in this study to explore the connection between PON1, its activator, the polyphenolic, punicalagin-rich pomegranate juice, and the circadian clock in skeletal muscle. For this, we analyzed circadian gene expression *in vivo* in the PON1KO mouse model and further investigated whether PON1, or pomegranate, can modulate period length in mouse skeletal muscle cells (C2C12) and human primary myotubes synchronized *in vitro*.

MATERIALS AND METHODS

Animals and Diets

Eight-week-old male C57BL/6 or PON1KO mice were fed a normal or a high-fat diet for 8 weeks. C57BL/6 mice were purchased from Jackson Laboratories (Bar Harbor, ME). Generation of PON1KO mice with the C57BL/6 background has been previously described (Shih et al, 1998) and mice were generously given by Dr. Diana M. Shih, Department of Medicine, University of California, Los Angeles, CA. All animal studies were conducted according the National Institutes of Health guideline and were approved by the Technion Ethics Committee for Experimentation in Animals.

The diets, TD06416 (10% kcal, low fat) and TD06414 (60% kcal, high fat), were purchased from Harlan (Madison, WI, USA). A detailed description of the diets can be found in Supplementary Table 1. The mice were individually housed under controlled temperature with 12 h light-dark cycles and had free access to water and a standard rodent diet for 7 days. For the study, the mice were divided into four groups. Group 1 57BL/6c control mice (n=10) were fed a normal diet (control ND); group 2 57BL/6c control mice (n=6) were fed a high-fat diet (control HFD); group 3 *Pon1* knockout mice (n=6) were fed a normal diet (PON1KO ND); and group 4 *Pon1* knockout mice (n=6) were fed a high-fat diet (PON1KO HFD). The

food was changed at 3-day intervals to avoid oxidation of the fat or other dietary components. Weekly body weight and fasting glucose levels for each mouse were determined throughout the study.

Cell culture

C2C12 mouse myoblast cells (Yaffe & Saxel, 1977) were maintained at a subconfluent condition in growth medium containing DMEM GlutaMAX (Thermo Fisher) with 4.5 g/L glucose, 100 µg/ml streptomycin, and 10% fetal calf serum (Sigma). Differentiation into myotubes was induced in near-confluent cells (~80% confluence) by lowering the serum concentration to 2%. Cells were maintained in the differentiation medium for 5-7 days until myoblasts had fused into polynucleated myotubes. For bioluminescence recording, C2C12 cells were transduced with lentiviral particles harboring the *Bmal1-luc* reporter construct (Pulimeno et al, 2013). Cells were treated with Blasticidin (5 µg/ml) to select for resistant colonies.

Primary skeletal myoblasts were derived from donor biopsies with informed consent obtained from all participants (see Supplementary Table 2 for donor characteristics). Cells were purified and differentiated into myotubes as previously described (Perrin et al, 2015). Briefly, myoblast cells were cultured in growth medium (HAM F-10 supplemented with 20% fetal bovine serum (FBS; Thermo Fisher)), 1% penicillin/streptomycin (Invitrogen), 0.5% Gentamycin (AppliChem) and 0.5% Fongizone (Thermo Fisher) at 37°C. After reaching confluence, myoblasts were differentiated into myotubes during 7 - 10 days in DMEM with 1g/L glucose, supplemented with 2% FBS. Muscle cell differentiation was characterized by the fusion of myoblasts into polynucleated myotubes.

Real Time PCR

mRNA levels of *Nr1d1*, *PER2*, *Cry2* and *Bmal1* in mice skeletal muscle were determined in all groups by quantitative PCR (qPCR). For this, mice were fasted for 4 hours before sacrifice. Mouse *triceps brachii* muscles were collected and immediately frozen in liquid nitrogen. Total RNA was extracted using the MasterPure^{TM} RNA purification kit (Epicentre Biotechnologies). cDNA was generated from 1 µg of total RNA using the Thermo Fisher Verso^{TM} cDNA kit. Products of the reverse transcription were subjected to qPCR using TaqMan gene expression analysis. Quantitative PCR was performed on the Rotor-Gene 6000 Corbett Life science instrument (Qiagen). Results were normalized to GAPDH expression. The primers for all analyzed genes were designed by PrimerDesign (South Hampton, UK).

Recombinant PON1 (rePON1), pomegranate juice

RePON1was generated by directed evolution as described previously (Aharoni et al, 2004) and stored at 4°C. PON1 storage buffer (50mM Tris, pH 8.0, 50mM NaCl, 1mM $CaCl_2$, and 0.1% v/v Tergitol) was supplemented with 0.02% (w/v) sodium azide. Before adding rePON1 to cells tergitol was removed using Bio-Beads SM-2 (Bio-RAD). 30 mg beads were added to 100 µl of PON1 in an Eppendorf tube. The mixture was incubated for 2 hours at 4°C in a rotating instrument, followed by centrifugation at 10,000 rpm and collection of the supernatant. This was repeated twice and the final protein concentration was determined by the Lowry protein assay. For the bioluminescence assay, myotubes were incubated for 24 hours with 8 µg/ml of PON1 followed by synchronization with forskolin for 1h and medium change.

Pomegranate juice (PJ) was supplied by POM Wonderful. The concentrated juice was diluted 1/5 in H_2O before use.

Bioluminescence recording

C2C12 or human primary myoblasts were transduced with lentiviral particles expressing the *Bmal1-luc* reporter, as described in (Perrin et al, 2015). Cells were differentiated into myotubes and synchronized with forskolin (Sigma, Saint-Louis, MO, USA) at a final concentration of 10 µM. Following 60 min forskolin incubation at 37°C, the medium was changed to the phenol red - free recording medium containing 100 µM luciferin (Prolume LTD, USA) and cells were transferred to a 37°C light-tight Lumicycle incubator (Actimetrics, USA) as previously described by us (Perrin et al, 2015). Bioluminescence from each dish was continuously monitored using a Hamamatsu photomultiplier tube (PMT) detector assembly.

Data analysis

Actimetrics LumiCycle analysis software (Actimetrics LTD) was used for bioluminescence data analysis (Perrin et al, 2015; Pulimeno et al, 2013). Statistical analyses were performed using a paired Student's *t* test. Differences were considered significant for $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***).

RESULTS

Chronic circadian misalignment, as it occurs in shift work, is associated with a higher prevalence of insulin resistance and obesity (Dibner & Schibler, 2015; Marcheva et al, 2013). Given the tight connection between PON1 and metabolic diseases, we aimed in this study to explore the potential connection between PON1 and the circadian clock in skeletal muscle. As previously demonstrated, PON1 deficiency caused enhanced insulin resistance in both ND

and HFD mice compared to their controls (Koren-Gluzer et al, 2013). To unravel whether this correlation might be due to changes in the molecular makeup of circadian oscillator, we examined the expression levels of the core-clock transcripts *Nr1d1*, *PER2*, *Cry2* and *Bmal1* in skeletal muscle, using the same groups of mice. While the expression levels of *Per2*, *Cry2* and *Bmal1* transcripts were similar between wild type and PON1KO mice fed a normal diet (Figure 1A), *Nr1d1* expression was upregulated in PON1KO mice compared to their control counterparts. Moreover, HFD significantly reduced the expression levels of *Nr1d1* and *Per2* in comparison to the control diet. In addition, the expression levels of *Per2* became significantly different between wild type and PON1KO mice under HFD. Interestingly, while in control mice, *Nr1d1* and *Per2* levels were reduced upon HFD diet, the same two genes were elevated in PON1KO mice under HFD. *Cry2* expression was upregulated in control mice fed a HFD but remained unchanged in PON1KO mice fed a HFD. Finally, *Bmal1* levels were not significantly altered in response to the different diets.

Next, we asked whether exogenous supply of PON1 could directly influence the circadian clock machinery. To this end, *in vitro* experiments were performed using differentiated C2C12 myotubes, cultured for 24 hours in the presence of increasing concentrations of recombinant PON1 (0-10 arylesterase U/ml), with subsequent assessment of circadian transcript expression levels. Incubation with PON1 led to a dose-dependent decrease in *Per2* expression whereas it caused a dose-dependent increase in *Cry2* expression in C2C12 myotubes (see Figure 1B).

Circadian bioluminescence recording in living cells allows for the study of molecular clocks in mammalian peripheral tissues as previously demonstrated by us (Mannic et al, 2013; Pulimeno et al, 2013). We applied this powerful methodology to assess clock properties in mouse C2C12 cells and human primary skeletal myotubes established from human donor biopsies and differentiated *in vitro* (see Supplementary Table 2 for donor characteristics).

C2C12 cells, stably expressing the circadian *Bmal1-luc* reporter were differentiated into myotubes, incubated for 24 hours with 8 µg/ml of recombinant PON1 (rePON1), and subsequently synchronized with a forskolin pulse. High-amplitude self-sustained oscillations were recorded for the *Bmal1-luc* reporter. Preincubation with rePON1 for 24h extended the period length by 0.47h ± 0.11h (Figure 2A). Next, we assessed the influence of pomegranate juice, a major effector of paraoxonase gene expression, on the period length in human primary muscle cells. To this end, human primary muscle cells from 4 donors with an average BMI of 25.75 ± 1.69 and an average donor age of 62.5 ± 4 (Supplementary Table 2) were analyzed. Differentiated human skeletal myotubes were pretreated with pomegranate juice for 24h with subsequent forskolin synchronization and bioluminescence recording performed as described in (Perrin et al, 2015). Pomegranate incubation led to a significant increase in period length from 25.9h ± 0.25h in untreated cells to 27.3h ± 0.29h ($p < 0.05$) in pomegranate treated cells (Figure 2B and C).

DISCUSSION

In recent years, there has been a tremendous amount of interest in the circadian regulation of metabolic processes. Misalignment of circadian rhythms as it occurs due to social jet-lag, shift work or frequent time-zone changes, is associated with an increased risk of metabolic, endocrine, and cardiovascular abnormalities (Scheer et al, 2009). Furthermore, a number of metabolites display a rhythmic profile, as has been recently shown for lipids (Adamovich et al, 2015). Mice with circadian clock ablation develop hyperphagia, obesity, hyperglycemia and hypoinsulinemia (Turek et al, 2005). Moreover, mice fed a high-fat diet show an increase in period length as early as one week following the start of the calorie dense chow (Kohsaka

et al, 2007). Here, we show that high fat diet feeding affects circadian gene expression in skeletal muscle in comparison to regular chow (Figure 1). These data confirm previous results from hypothalamus, fat and liver of mice fed a high calorie diet (Kohsaka et al, 2007). Interestingly, the response to HFD differed between wild type and PON1 deficient animals (Figure 1). In PON1KO mice *Pon2*, *Pon3* and potentially other genes may have been modulated as a compensatory mechanism in response to loss of PON1. These compensatory effects might play a role in the metabolic response to high fat diet and might influence circadian gene expression in skeletal muscle in PON1KO mice in response to HFD.

In an agreement with these findings, we further demonstrate that incubation with increasing doses of PON1 modulates core-clock gene expression in mouse C2C12 skeletal myotubes (Figure 1). However questions remain concerning the mechanism underlying this effect. Incubation of recombinant PON1 with macrophages results in cellular binding and internalization of PON1, leading to PON1 localization in the cytoplasmic compartment (Efrat & Aviram, 2008). Whether skeletal muscle cells are able to internalize rePON1 protein is not known, but it has been demonstrated that exogenous PON1 upregulates GLUT4 expression and enhances glucose uptake in C2C12 myotubes at a concentration of 4.5 U/ml (Koren-Gluzer et al, 2013). Therefore, PON1 has beneficial effects on mouse skeletal muscle cells in the context of insulin resistance.

Interestingly, we observed differential changes in *Per2* and *Cry2* expression levels in response to HFD in mouse skeletal muscle *in vivo*, but also in the response to PON1 incubation of C2C12 myotubes *in vitro* (Figure 1). PER2 and CRY proteins are usually considered co-repressors of the circadian clock. However, it has been recently shown that PER2 is rather a modulator than a co-repressor of CRY2 and might play different roles at different circadian phases. It was proposed that PER suppresses CRY activity during an early phase and acts as a transcriptional repressor with CRY at a later phase thereby buffering the

effect of CRY (Akashi et al, 2014). Opposite effects on expression levels as we have observed here with regard to gene expression could be due to this interaction.

It has been reported that loss of *Cry1* results in short circadian periods, whereas a loss of *Cry2* results in longer periods, indicating that these proteins have an important regulatory role in the control of circadian period length (van der Horst et al, 1999). We therefore questioned whether the observed effects on *Cry2* and *Per2* gene expression would translate into changes in period length. To investigate this connection, we performed circadian bioluminescence recordings in skeletal muscle cells pre-incubated with either PON1 or pomegranate juice (Figure 2). Of note, both PON1 and pomegranate increased circadian period length in skeletal muscle. However, the effect of pomegranate might not be restricted to PON1 alone, as pomegranate not only increases *Pon1* gene expression and activity but also leads to an increase in *Pon2* gene expression and *Pon3* activity (Rosenblat et al, 2003; Shiner et al, 2007).

Taken together, these data show in a convincing manner that PON1 is able to modulate circadian clock properties. However, whether *Pon1* expression itself follows a circadian pattern is not known. To address this question, we queried two computational resources, which were recently developed for the analysis of large-scale circadian data sets whether the expression of *Pon1* in skeletal muscle follows a circadian pattern. Both databases, the CircaDB database (circadb.hogeneschlab.org), comprising of mammalian circadian gene expression profiles (Pizarro et al, 2013) and the CircadiOmics database (http://circadiomics.igb.uci.edu/), which integrates transcriptomics data with proteomic and metabolomics datasets (Patel et al, 2012), identified *Pon1* gene expression in skeletal muscle as being circadian by JTK_CYCLE. *Pon1* expression levels in skeletal muscle were 100 times lower compared to liver (Zhang et al, 2014), however, these results indicate that PON1 might have an additional tissue specific function. Further analysis using CircaDB and the

JTK_CYCLE algorithm identified circadian expression of *Pon1* also in aorta, liver and adrenal gland. *Pon2* was circadian in distal colon, adrenal gland, aorta, white adipose tissue and liver and *Pon3* expression was circadian in lung, aorta, adrenal gland, liver and macrophages. Taken together these results suggest that the paraoxonase gene family is expressed in a circadian manner, which might play an important role in the cellular response to oxidative stress. This is further supported by a recent pilot study suggesting that the PON1 effector pomegranate extract can affect daily rhythms of serum lipids and oxidative stress markers (Hayek et al, 2014). In their function as biomarkers of oxidative stress paraoxonases resemble peroxiredoxins, a highly conserved class of peroxidases that are rhythmic across all domains of life including bacteria, archae and eukaryota (Edgar et al, 2012). There is emerging evidence that the circadian oscillator is driving daily redox cycles that involve the antioxidant peroxiredoxin proteins (Reddy & Rey, 2014). Remarkably, these circadian cycles of peroxiredoxin oxidation/reduction are operative in human and mouse red blood cells, which are incapable of transcription and translation (Cho et al, 2014; O'Neill & Reddy, 2011). The potential role of such peroxiredoxin cycles in coupling the circadian oscillators to the metabolic clock stays to be unravelled. In view of the functional resemblance between peroxiredoxins and paraoxanases, this exciting discovery sheds an interesting light on our newly described link between circadian clock and PON1.

This work is the first to characterize the effect of PON1 and pomegranate on the skeletal muscle circadian oscillator and its critical impact on period length. It might therefore pave the way for future studies that may link defects in these pathways with insulin resistance, obesity, and T2D.

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DECLARATION OF INTEREST

The authors have no conflict of interest to declare.

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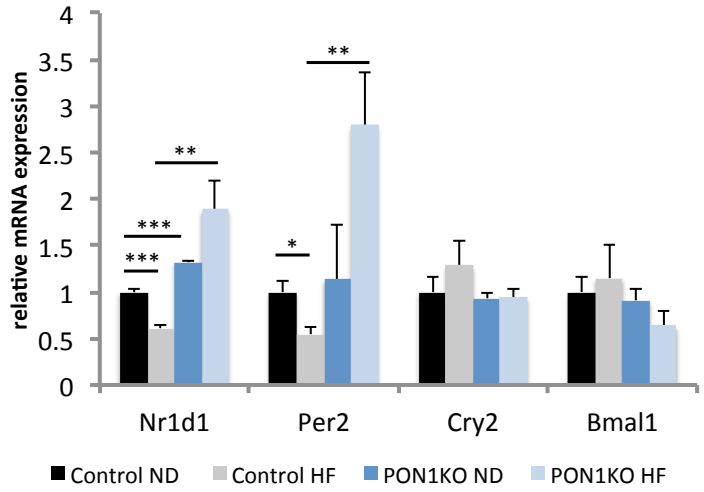
Figures

A



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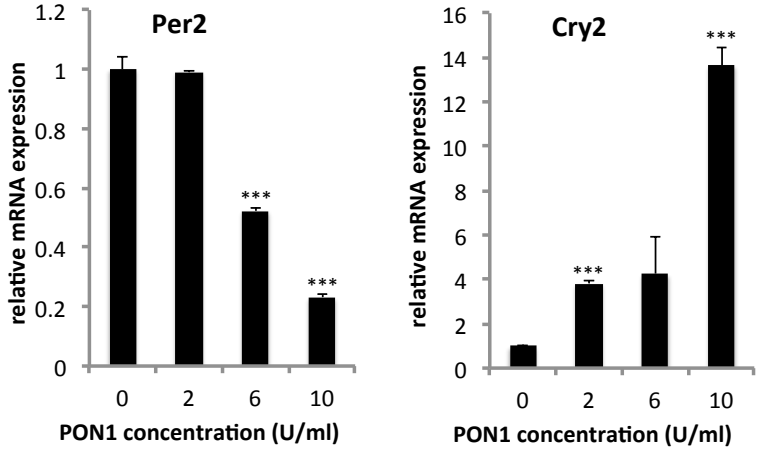

Figure 1. PON1 and HFD modulate circadian gene expression (A) Eight-week-old male C57BL/6 or PON1KO mice were fed normal or high fat diet for 8 weeks. The mice were fasted for 4 hours before sacrifice. Mouse *triceps brachii* muscles were collected and frozen in liquid nitrogen. mRNA levels of *Nr1d1*, *PER2*, *Cry2* and *Bmal1* in mice skeletal muscle were determined by quantitative PCR. Values were normalized to GAPDH expression. Results are represented as mean ± SEM, ($n \geq 5$), *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. **(B)** Differentiated C2C12 myoblasts were cultured for 24 h in the presence of increasing PON1 concentrations (0–10 arylesterase U/ml) in the medium. *Per2* and *Cry2* mRNA expression levels were assessed by qPCR. Results represent mean ± SEM, ($n = 3$), ***$p < 0.001$.

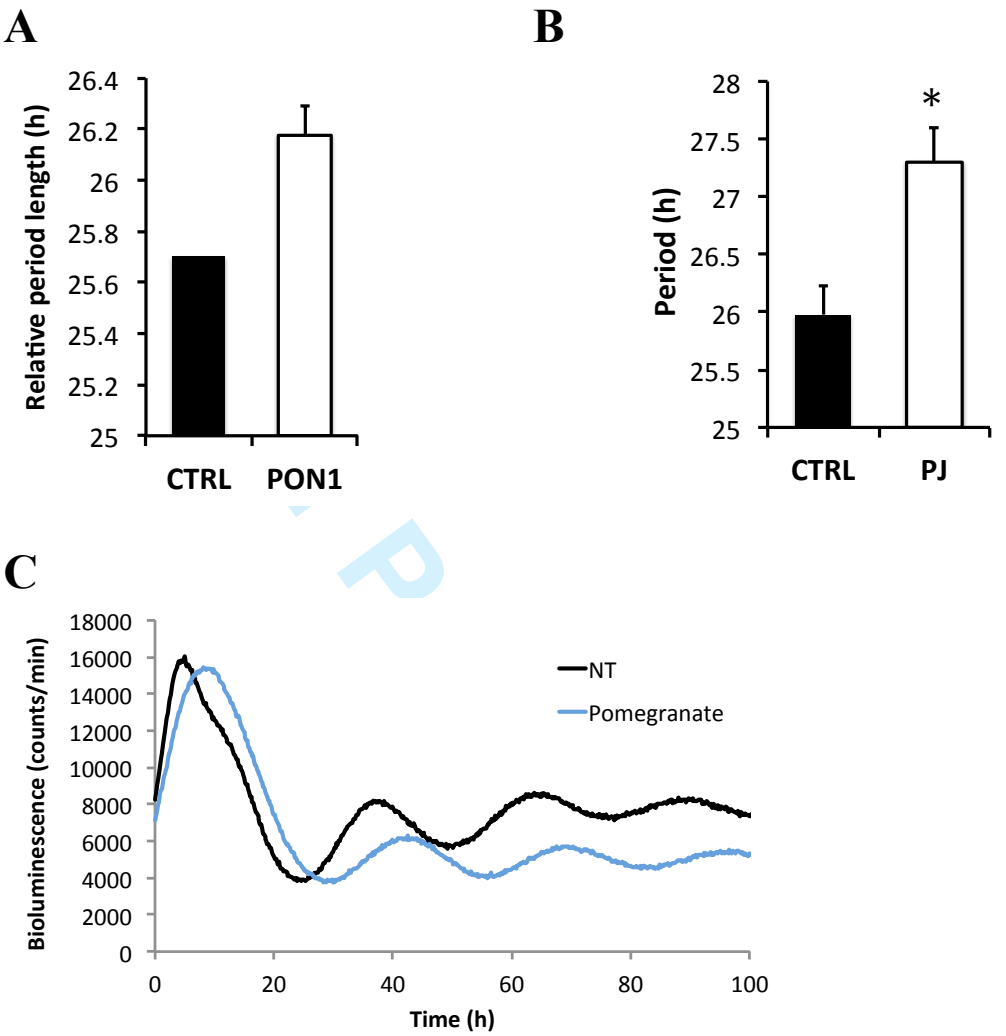

Figure 2. PON1 and pomegranate increase circadian period length. (A) C2C12 cells, stably expressing the *Bmal-luc* reporter, were differentiated into myotubes. Differentiated cells were incubated for 24 hours with 8 µg/ml of PON1 followed by synchronization with forskolin (10 µM, 60 min) and transfer to the Actimetrics LumiCycle for bioluminescence recording. Data show the fold increase in period length in the PON1 treated cells compared to the average period length of the control cells (n=4). Data represent the mean ± SD. **(B)** Human primary myoblasts were transduced with lentiviral particles expressing the *Bmal1-luc* reporter. Cells were differentiated into myotubes, preincubated with pomegranate juice (PJ) for 24h, followed by synchronization with forskolin and transferred to the Actimetrics LumiCycle for bioluminescence recording (n=4). Data represent the mean ± SEM. *$p < 0.05$. **(C)** Oscillation profile of human myotubes representative of 4 independent experiments (one donor per experiment).

Supplementary data to:

Paraoxonase 1 (PON1) and pomegranate influence circadian gene expression and period length

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Supplementary Table 1. Composition of the diets

Ingredients	Low fat diet (g%)	High fat diet (g%)
Casein	210.0	265.0
L-Cystine	3.0	4.0
Maltodextrin	50.0	160.0
Sucrose	325.0	90.0
Lard	20.0	310.0
Soybean Oil	20.0	30.0
Cellulose	37.15	65.5
Mineral Mix, AIN-93G-MX (94046)	35.0	48.0
Calcium Phosphate, dibasic	2.0	3.4
Vitamin Mix, AIN-93-VX (94047)	15.0	21.0
Corn Starch	280.0	0

Supplementary Table 2. Characteristics of human donors

Characteristics of donors for skeletal muscle biopsies				
Donor	**Sex**	**Age (years)**	**BMI (kg/m^2)**	**Biopsy source**
#1	M	57	28.10	*Gluteus maximus*
#2	M	62	24.30	*Gluteus maximus*
#3	F	65	25.85	*Gluteus maximus*
#4	F	66	24.77	*Gluteus maximus*
N=4	**M=2, F=2**	**62.5 ± 4**	**25.75 ± 1.69**	