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Research Paper

Role of Inflammatory Signaling in the Differential Effects of Saturated and Poly-unsaturated Fatty Acids on Peripheral Circadian Clocks

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ABSTRACT

Inflammatory signaling may play a role in high-fat diet (HFD)-related circadian clock disturbances that contribute to systemic metabolic dysregulation. Therefore, palmitate, the prevalent proinflammatory saturated fatty acid (SFA) in HFD and the anti-inflammatory, poly-unsaturated fatty acid (PUFA), docosahexaenoic acid (DHA), were analyzed for effects on circadian timekeeping and inflammatory responses in peripheral clocks. Prolonged palmitate, but not DHA, exposure increased the period of fibroblast *Bmal1-dLuc* rhythms. Acute palmitate treatment produced phase shifts of the *Bmal1-dLuc* rhythm that were larger in amplitude as compared to DHA. These phase-shifting effects were time-dependent and contemporaneous with rhythmic changes in palmitate-induced inflammatory responses. Fibroblast and differentiated adipocyte clocks exhibited cell-specific differences in the time-dependent nature of palmitate-induced shifts and inflammation. DHA and other inhibitors of inflammatory signaling (AICAR, cardamonin) repressed palmitate-induced proinflammatory responses and phase shifts of the fibroblast clock, suggesting that SFA-mediated inflammatory signaling may feed back to modulate circadian timekeeping in peripheral clocks.

1. Introduction

Over-nutrition, especially through the consumption of a HFD, is a critical factor in the rapidly increasing incidence of obesity. HFD-induced obesity is associated with systemic insulin resistance and the corresponding development of metabolic disorders such as type 2 diabetes, cardiovascular disease and non-alcoholic fatty liver disease (Angulo, 2002; Hossain et al., 2007; Jensen et al., 2008). Increasing evidence indicates that chronic low-grade inflammation in peripheral tissues contributes to HFD-mediated obesity and insulin resistance. HFD increases plasma levels of saturated fatty acids, which can trigger proinflammatory responses through the induction of NF-κB and JNK signaling. Thus, the precise modulation of free fatty acid levels and inflammatory signaling are vital for the maintenance of metabolic homeostasis.

Cell-autonomous circadian clocks in peripheral tissues are involved in the regulation of inflammatory responses and metabolic homeostasis. Endogenous clocks in immune cells provide for the circadian control of their abundance in the circulation, production of inflammatory factors, and functional responses to inflammatory challenge (Keller et al., 2009; Lange et al., 2010). Tissue- and cell-specific clocks also provide for the local coordination of circadian rhythms in the metabolism of fatty acids (Stenvers et al., 2012). For example, circulating levels of free fatty acids and adipose tissue expression of genes mediating lipolysis and fatty acid biosynthesis or transport are characterized by clock-controlled rhythmicity (Shostak et al., 2013). Moreover, genetic or environmental disruption of circadian clock function has been shown to potentiate inflammatory responses and to produce obesity and other metabolic disorders (Turek et al., 2005; Marcheva et al., 2010; Gibbs et al., 2012; Paschos et al., 2012). Our recent findings demonstrate

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that circadian clock disruption in bone marrow cells exacerbates HFD-induced tissue inflammation, adiposity, and systemic insulin dysregulation (Xu et al., 2014).

While the hierarchy of circadian clocks throughout the body is clearly involved in regulating inflammatory and metabolic processes, this interaction is not strictly unidirectional as inflammatory signals and fatty acid metabolism have been conversely implicated in the feedback modulation of the circadian clock mechanism. In this regard, the cytokines, TNF-α and IL-1β induce phase delays and HFD increases the free-running period of the activity rhythm in mice (Kohsaka et al., 2007; Leone et al., 2012; Xu et al., 2014). Thus, mutual interactions between circadian clocks and key mediators of inflammation may be important in maintaining metabolic homeostasis and in linking clock dysregulation and metabolic phenotypes in diet-associated obesity. Because inflammatory responses and corresponding metabolic disturbances in diet-induced obesity are differentially engaged by specific types of fatty acids found in HFDs, we compared the differential effects of palmitate, the prevalent proinflammatory SFA in HFD versus the anti-inflammatory, PUFA DHA on circadian clock function. The extent of the coupling between diet-mediated inflammatory responses and alterations in fundamental clock properties was examined by analyzing the phase-shifting effects of acute palmitate and DHA treatment at different times throughout the circadian cycle and then determining their coincidence with fatty acid-mediated induction of inflammatory signaling. To examine the role of inflammatory signaling in the mechanism by which SFAs modulate clock properties, experiments were also conducted to determine whether inhibition of proinflammatory responses blocks palmitate-induced phase shifts of peripheral circadian clocks.

2. Materials and Methods

2.1. Cell Culture

Bmal1-dLuc fibroblasts (Dr. Andrew Liu, University of Memphis, Memphis, TN; Ramanathan et al., 2012) were propagated on 60 mm dishes in Dulbecco's Modified Eagle Medium (DMEM; HyClone) containing 292 μg/ml L-glutamine, 10% Fetal Bovine Serum (FBS), 100 units/ml penicillin, and 100 μg/ml streptomycin and maintained at 37 °C and 5% CO_2. Medium was replaced every 48 h and cultures were split 1:4 every 3 days. As established previously (Huo et al., 2010, 2012), adipocytes were differentiated from *Bmal1-dLuc* fibroblasts maintained in DMEM containing 10 μg/ml insulin, 1 μM dexamethasone, and 0.5 mM 3-isobutyl-1-methylxanthine for 48 h, and then incubated for 4 additional days in medium supplemented with 10 μg/ml insulin. Following differentiation, adipocytes were maintained for 2 days in normal growth medium prior to experimentation. Cell differentiation into adipocytes was verified by positive staining with Oil Red O (Fig. S1A) and by upregulated expression of PPARγ and adiponectin (Fig. S1B). While our analysis of these phenotypic markers indicates that *Bmal1-dLuc* fibroblasts exhibit many adipocyte-like properties following differentiation, it is unclear whether the cells are fully differentiated into mature adipocytes, thus warranting their subsequent designation as 'differentiated adipocytes'.

2.2. Fatty Acid/Drug Preparation and Treatment

Palmitate (Sigma) and DHA (Nu-Chek-Prep, Inc.) were dissolved in ethanol and then diluted (1:5.4 ratio) with 10% BSA (fatty acid-free and low endotoxin) diluted in 0.1 M phosphate-buffered saline (PBS). Palmitate and DHA treatment in these studies was based on physiological concentrations that have been previously observed in vivo or used for in vitro studies (Ajuwon and Spurlock, 2005; Han et al., 2010; Puri et al., 2009; Weldon et al., 2007). Controls for fatty acid treatment contained BSA diluted in PBS with an equivalent ratio of ethanol.

AICAR (Tocris) or cardamonin (Tocris) were dissolved in DMSO and then diluted 1:400 and 1:10000 in culture medium to achieve final concentrations of 500 μM or 5 μM, respectively. Vehicle controls for AICAR and cardamonin treatment consisted of cultures in which an equivalent amount of DMSO was added to the medium.

2.3. Effect of Prolonged Fatty Acid Treatment on Circadian Period

Bmal1-dLuc fibroblasts were plated onto 35 mm dishes and ≈24 h later treated with either BSA (10% in PBS with EtOH), palmitate (150 μM), or DHA (150 μM) for 48 h. Following fatty acid treatment, cultures were rinsed and then maintained in recording media for 6–7 days during real-time analysis of *Bmal1-dLuc* bioluminescence.

2.4. Time-dependent Variation in the Phase Shifting and Proinflammatory Effects of Acute Fatty Acid Treatment

Bmal1-dLuc fibroblast cultures on 35 mm dishes were exposed for 2 h to medium containing 15 μM forskolin to facilitate circadian oscillation synchronization across cultures (Menger et al., 2007) and then treated for 4 h with BSA (10% in PBS with EtOH), palmitate (250 μM) or DHA (250 μM) at 6 h intervals throughout the circadian cycle. Cultures were subjected to control or fatty acid treatments at 6, 12, 18 or 24 h after forskolin administration and then placed in recording media for bioluminescence analysis of treatment-induced phase shifts of *Bmal1-dLuc* oscillations.

For parallel analyses of inflammatory responses to acute fatty acid treatment, confluent cultures of *Bmal1-dLuc* fibroblasts on 6-well plates were exposed for 2 h to 15 μM forskolin and then 6, 12, 18 or 24 h later

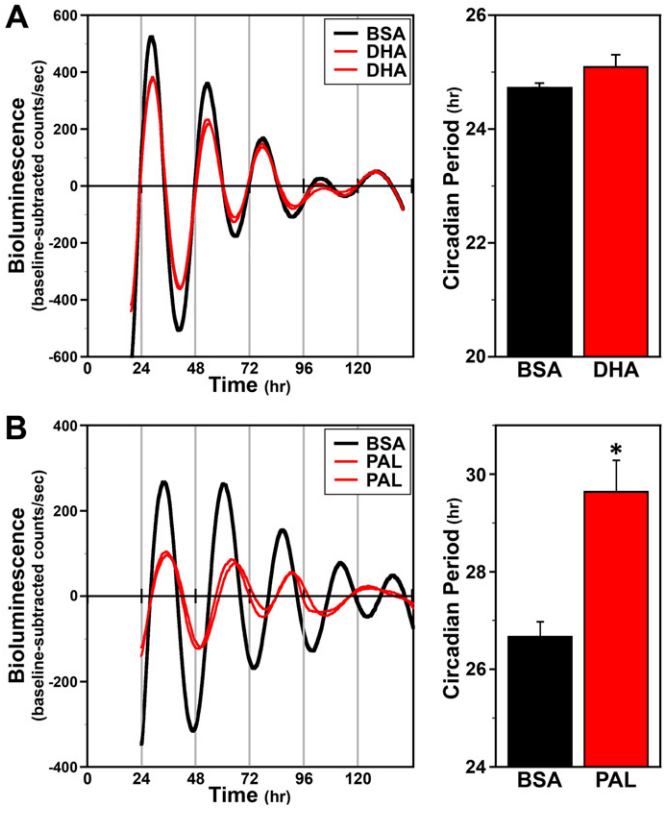

Fig. 1. Effects of prolonged PUFA and SFA treatment on ensemble *Bmal1-dLuc* rhythms in cultured fibroblasts. Individual recordings of ensemble bioluminescence (expressed as detrended baseline-subtracted counts per second) from representative cultures of *Bmal1-dLuc* fibroblasts treated with: (A) BSA (n = 6) or 150 μM DHA (n = 8), and (B) BSA (n = 6) or 150 μM palmitate (n = 8). Bar graphs depict comparisons of the circadian period (mean ± SEM) of the *Bmal1-dLuc* rhythms in BSA control and DHA- or palmitate-treated fibroblasts. Asterisk indicates that the period of the *Bmal1-dLuc* rhythms in palmitate-treated cultures was significantly greater ($p < 0.05$) than that in BSA controls.