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Palmitate induces neuroinflammation, ER stress, and *Pomc* mRNA expression in hypothalamic mHypoA-POMC/GFP neurons through novel mechanisms that are prevented by oleate

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ABSTRACT

Dietary fats can modulate brain function. How free fatty acids (FFAs) alter hypothalamic pro-opiomelanocortin (POMC) neurons remain undefined. The saturated FFA, palmitate, increased neuroinflammatory and ER stress markers, as well as *Pomc* mRNA levels, but did not affect insulin signaling, in mHypoA-POMC/GFP-2 neurons. This effect was mediated through the MAP kinases JNK and ERK. Further, the increase in *Pomc* was dependent on palmitoyl-coA synthesis, but not *de novo* ceramide synthesis, as inhibition of SPT enhanced palmitate-induced *Pomc* expression, while methylpalmitate had no effect. While palmitate concomitantly induces neuroinflammation and ER stress, these effects were independent of changes in *Pomc* expression. Palmitate thus has direct acute effects on *Pomc*, which appears to be important for negative feedback, but not directly related to neuroinflammation. The monounsaturated FFA oleate completely blocked the palmitate-mediated increase in neuroinflammation, ER stress, and *Pomc* mRNAs. This study provides insight into the complex central metabolic regulation by FFAs.

1. Introduction

Two thirds of North American adults are either overweight or obese, and the increased consumption of a high-fat diet is one contributing factor in this epidemic (Jequier, 2002). Consumption of high levels of dietary fat increases levels of the saturated free fatty acid (FFA) palmitate in the serum (Gambino et al., 2016) and brain, specifically the hypothalamus (Posey et al., 2009). Consequently, accumulation of this 16-carbon saturated FFA and its metabolites in the hypothalamus results in inflammation and endoplasmic reticulum (ER) stress, leading to a decrease in insulin signaling, weight gain, and metabolic dysfunction (Benoit et al., 2009; Posey et al., 2009). The deleterious effects of palmitate in the hypothalamus are similarly demonstrated *in vitro*. Specifically, cultured hypothalamic neuropeptide Y (NPY) neurons exposed to palmitate have decreased insulin signaling illustrated by reduced insulin-stimulated phospho-Akt (Benoit et al., 2009; Mayer and Belsham, 2010) and increased phospho-JNK and cleaved caspase-3 protein levels (Mayer and Belsham, 2010). Palmitate also increases the mRNA expression of pro-inflammatory cytokines *Il6*, *Tnfα, and Il1β*, the ER stress markers *Chop*, *Bax/Bcl2* ratio, and *Grp78*, and other proteins involved in the unfolded protein response (Dalvi et al., 2017; McFadden et al., 2014; Ye et al., 2016) in hypothalamic neurons *in vitro*. These effects of palmitate in the hypothalamus are shown, at least in part, to be mediated through toll-like receptor 4 (TLR4) and the downstream IKKβ/NF-κB signaling pathway (Milanski et al., 2009; Shi et al., 2006), as well as the mitogen-activated protein (MAP) kinase signaling pathways (Holzer et al., 2011). Additionally, it is suggested that palmitate may act in the hypothalamus by increasing ceramide levels; a palmitate metabolite associated with insulin resistance and apoptosis (Chavez et al., 2003; Contreras et al., 2014; Gao et al., 2011).

The arcuate nucleus of the hypothalamus contains opposing orexigenic NPY/agouti-related peptide (AgRP) neurons and anorexigenic pro-opiomelanocortin (POMC) neurons that regulate food intake and energy expenditure by integrating signals of energy status from hormones and nutrients, including FFAs (Dziedzic et al., 2007; Morgan et al., 2004). Consumption of a high-fat diet or exposure to palmitate treatment directly alters hypothalamic

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neuropeptide expression. Exposure of cultured NPY neurons to palmitate increases *Npy* mRNA expression and this effect is blocked with pre-treatment with anti-inflammatory sodium salicylate (Ye et al., 2016) or the IKKβ inhibitor PS-1145 (Dalvi et al., 2017), implicating FFA-induced inflammation as a mediator for altered *Npy* mRNA expression. In the whole hypothalamus, early stages of high-fat diet-induced obesity are accompanied by an increase in *Pomc* mRNA expression (Ziotopoulou et al., 2000), whereas *Pomc* levels are decreased with prolonged diet (Lee et al., 2010). As described above, the effects of palmitate are well documented in the hypothalamus and in hypothalamic NPY-expressing neurons however, the direct effects of palmitate on POMC-expressing neurons has not been investigated. In the hypothalamus the pro-hormone POMC is enzymatically cleaved to produce alpha-melanocyte stimulating hormone (α-MSH), which acts as a satiety signal by activating the melanocortin 4 receptor (MC4R) (Gantz and Fong, 2003). Dysregulation of POMC by sustained nutrient excess decreases α-MSH-inhibited feeding; resulting in increased food intake and body weight (Cakir et al., 2013; Ziotopoulou et al., 2000). Further, the response to high-fat feeding differs among populations of hypothalamic neurons. For instance, it is reported that POMC neurons are selectively targeted for apoptosis compared to NPY neurons (Moraes et al., 2009) suggesting that distinct neuronal populations differ in their sensitivity and response to FFAs.

Therefore, to investigate the effects of palmitate directly on POMC neurons, an immortalized, non-clonal POMC-expressing cell line, derived from the hypothalamus of an 8-week-old male transgenic mouse with green fluorescence protein (GFP) under the control of the promoter for the *Pomc* gene was used in this study. POMC neurons were selected for fluorescence to produce a non-clonal, mixed population of POMC-expressing neurons. This mHypoA-POMC/GFP-2 neuronal cell line was previously generated and characterized by our laboratory, and was shown to express *Pomc*, as well as a host of inflammatory receptors, mediators, and cytokines (Nazarians-Armavil et al., 2014). Additionally, the mHypoA-POMC/GFP neurons represent functional POMC models as both *Pomc* mRNA and α-MSH mature protein levels are increased with insulin and sodium nitroprusside treatments (Nazarians-Armavil et al., 2014; Wellhauser et al., 2016)

In this study, we report that acute palmitate treatment increases *Pomc* mRNA expression and concomitantly increases the mRNA expression of pro-inflammatory and ER stress markers in POMC neurons. The induction of *Pomc* mRNA expression by palmitate is dependent on JNK and ERK signaling, and unlike palmitate-mediated *Npy* mRNA expression, is independent of IKKβ/NF-κB signaling. The metabolism of palmitate to palmitoyl-coA, but not ceramide, is required to increase *Pomc* mRNA expression. Lastly, co-treatment of the saturated FFA palmitate with mono-unsaturated oleate blocks the effects of palmitate on *Pomc*, *Chop*, and *Il6* mRNA expression. Together, these results provide evidence that POMC neurons respond directly to an acute exposure to palmitate resulting in increased *Pomc* mRNA expression, and the mechanisms described provide insight into central metabolic regulation by FFAs.

2. Material and methods

2.1. Cell culture and reagents

mHypoA-POMC/GFP-1, -2, -3, and -4 neurons were cultured in DMEM (Sigma-Aldrich; St. Louis, MO, USA) containing 5.5 mM glucose and supplemented with 2% fetal bovine serum (Gibco; Grand Island, NY, USA) and 1% penicillin-streptomycin (Gibco). The mHypoA-POMC/GFP neuronal cell lines were previously generated and characterized by our laboratory (Nazarians-Armavil et al., 2014). In brief, hypothalamic tissue was extracted from an 8-week-old male transgenic mouse with green fluorescence protein (GFP) under the control of the promoter for the *Pomc* gene. The hypothalamic tissue was dispersed into culture and treated with ciliary neurotrophic factor to induce neuroproliferation prior to immortalizion by the retroviral transfer of SV40 T-antigen. Neurons were then selected for geneticin resistance and fluorescence-activated cell (FAC)-sorted to produce a non-clonal, mixed population of POMC-expressing neurons. Sodium palmitate, sodium oleate, methylpalmitate, C16-ceramide, myriocin, and L-cycloserine were purchased from Sigma. TAK-242 was purchased from Calbiochem (San Diego, CA, USA). SP 600125, SB 202190, and PD 0325901 were purchased from Tocris Bioscience (Ellisville, MO, USA). Insulin was a gift from Novo Nordisk (Mississauga, ON, CAN). Sodium palmitate, methylpalmitate, and sodium oleate were dissolved in molecular grade water (Thermo Scientific; Nepean, ON, CAN) and heated to 70 C to dissolve, as described previously (Ye et al., 2016). L-cycloserine was dissolved in sterile water (Thermo Scientific). C16-ceramide was dissolved in ethanol with 2% dodecane (Sigma). TAK-242, SP 600125, SB 202190, PD 0325901, and myriocin were dissolved in dimethyl sulfoxide (DMSO); final treatment containing 0.1% DMSO.

2.2. MTT assay

Cell viability after palmitate treatment was assessed by MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (Vybrant® MTT Cell Proliferation Assay Kit V-13154; Life Technologies; Eugene, OR, USA). Briefly, neurons were grown on 96-well plates and treated with 10 μM, 50 μM, or 100 μM palmitate. After 24 h, treatment was removed and neurons were incubated in 1.1 mM MTT for 2 h. Formazan was then solubilized by dimethyl sulfoxide and concentration determined by optical density at 570 nm.

2.3. Quantitative RT-PCR

Total RNA was isolated using the PureLink RNA Mini Kit with on-column PureLink DNase (Ambion, Streetsville, ON, CAN). cDNA was synthesized with 1000 ng RNA using the SuperScript VILO cDNA synthesis kit (Invitrogen, Carlsbad, CA, USA). 12.5 ng cDNA was amplified using qRT-PCR master mix (Platinum SYBR Green qPCR SuperMix-UDG with ROX; Invitrogen) with gene specific primers (Table 1) on an Applied Biosystem Prism 7000 machine. qRT-PCR quantities were determined by a standard curve and normalized to the reference gene, histone 3a.

2.4. Western blotting

Total protein was harvested using 1X lysis buffer (Cell Signaling; Danvers, MA, USA) with 1 mM PMSF, 1% phosphatase inhibitor, and 1% protease inhibitor and supernatant isolated after centrifugation at 14000 rpm for 10 min at 4 C. Protein concentration was determined using the BCA protein assay kit (Thermo Scientific). 20–25 μg protein was run on a 10% SDS-PAGE gel and transferred to a PVDF membrane (Biorad). Membranes were blocked for 1 h in 5% milk in tris-buffered saline with tween (TBS-T), then incubated in primary antibody (1:1000) at 4 C overnight. Primary antibodies were purchased from Cell Signaling, and include phospho-Akt (Ser473), Akt, phospho-SAPK/JNK (Thr183/Tyr185), and SAPK/JNK. Membranes were washed in TBS-T and incubated in secondary anti-rabbit antibody (1:7500, Cell Signaling) for 1 h, then imaged using the Signal Fire ECL Reagent (Cell Signaling) and the Kodak Image Station 2000R. Densitometry was performed using ImageJ software (National Institute of Mental Health; Bethesda, MD, USA).

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