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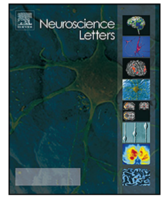

Research paper

Role of orexin A signaling in dietary palmitic acid-activated microglial cells

Cayla M. Duffy [a,b], Ce Yuan [b], Lauren E. Wisdorf [b], Charles J. Billington [a,c,d], Catherine M. Kotz [a,b,d], Joshua P. Nixon [a,b], Tammy A. Butterick [a,b,*]

[a] Veterans Administration Medical Center, Minneapolis, MN, USA
[b] Department of Food Science and Nutrition, University of Minnesota, St. Paul, MN, USA
[c] Department of Medicine, University of Minnesota, Minneapolis, MN, USA
[d] Minnesota Obesity Center, St Paul, MN USA

HIGHLIGHTS

- Palmitic acid (PA) increases microglial orexin receptor 1 expression.
- PA increases microglial pro-inflammatory cytokine release.
- Orexin A reduces microglial pro-inflammatory cytokine release.
- Orexin A attenuates hypothalamic cell death induced by PA-activated microglia.
- Orexin A may function as an immunomodulatory regulator of microglia.

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ABSTRACT

Excess dietary saturated fatty acids such as palmitic acid (PA) induce peripheral and hypothalamic inflammation. Hypothalamic inflammation, mediated in part by microglial activation, contributes to metabolic dysregulation. In rodents, high fat diet-induced microglial activation results in nuclear translocation of nuclear factor-kappa B (NFκB), and increased central pro-inflammatory cytokines tumor necrosis factor alpha (TNF-α) and interleukin-6 (IL-6). The hypothalamic neuropeptide orexin A (OXA, hypocretin 1) is neuroprotective in brain. In cortex, OXA can also reduce inflammation and neurodegeneration through a microglial-mediated pathway. Whether hypothalamic orexin neuroprotection mechanisms depend upon microglia is unknown. To address this issue, we evaluated effects of OXA and PA on inflammatory response in immortalized murine microglial and hypothalamic neuronal cell lines. We demonstrate for the first time in microglial cells that exposure to PA increases gene expression of orexin-1 receptor but not orexin-2 receptor. Pro-inflammatory markers IL-6, TNF-α, and inducible nitric oxide synthase in microglial cells are increased following PA exposure, but are reduced by pretreatment with OXA. The anti-inflammatory marker arginase-1 is increased by OXA. Finally, we show hypothalamic neurons exposed to conditioned media from PA-challenged microglia have increased cell survival only when microglia were pretreated with OXA. These data support the concept that OXA may act as an immunomodulatory regulator of microglia, reducing pro-inflammatory cytokines and increasing anti-inflammatory factors to promote a favorable neuronal microenvironment.

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1. Introduction

The orexins (orexins A and B; hypocretin 1 and 2) are hypothalamic peptides produced in lateral hypothalamic neurons and released widely throughout the CNS [1,2]. Orexin A (OXA) and B (OXB) regulate homeostatic mechanisms of energy balance and metabolism [3] through activation of two G-protein coupled receptors, orexin receptors 1 and 2 (OX1R and OX2R, respectively) [2]. Recent studies have shown that orexin plays a role in neuropro-

Abbreviations: PA, palmitic acid; TNF-α, tumor necrosis factor alpha; NFκB, nuclear factor-kappa B; OXA, orexin A/hypocretin 1; OX1R, orexin receptor 1; OX2R, orexin receptor 2; IL-6, interleukin 6; LPS, lipopolysaccharide; TLR-4, toll like receptor 4; RT-PCR, real time polymerase chain reaction; RFU, relative fluorescence units; ELISA, enzyme-linked immunosorbent assay; BSA, bovine serum albumin.

* Corresponding author.
E-mail address: butte017@umn.edu (T.A. Butterick).

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tection [3,4], in part by reducing lipid peroxidation, apoptosis, and inflammation [5–8]. Data suggest that the neuroprotective effects of orexin could rely on modulation of microglia, the resident immune cells of the brain.

Microglia are initiators of the neuroinflammatory response and are highly reactive to endogenous signaling. Microglia are highly dynamic, transitioning between neurotoxic pro-inflammatory (M1) and neuroprotective (M2) phenotypes. For example, following cerebral ischemic events, microglia are first activated to a neuroprotective M2 phenotype as oxygen levels decrease, and then switch to a pro-inflammatory M1 phenotype, inducing cell death [9]. While inflammation is a component of a normal immune response, chronic activation to M1 pro-inflammatory phenotypes can cause microglia to become refractory and contribute to subsequent neuronal dysfunction [10].

Several lines of evidence suggest a role for orexin in modulation of microglia. In cerebral ischemia models, pretreatment with OXA reduces infarct size through a microglial-mediated pathway [8]. Microglia may also become more sensitive to orexin signaling after activation. The potent pro-inflammatory agonist lipopolysaccharide (LPS) increases tumor necrosis factor alpha (TNF-α) in microglia, but also increases OX1R expression, and OXA treatment prior to LPS exposure reduces TNF-α in microglia [8]. These data indicate that increased microglial OX1R could enhance responsiveness to orexin, thus enhancing capability to counter an inflammatory insult. We are especially interested in how these orexin–microglia dynamics might impact brain health in the context of diet-induced obesity.

Dietary intake influences neuronal function, overall brain health, and cognition [11]. High fat diet increases circulating pro-inflammatory cytokines released from microglial cells, resulting in hypothalamic neuroinflammation and neurodegeneration [12,13]. Chronic intake of saturated fatty acids (SFA) such as palmitic acid (PA, C16:0), activate microglia to an M1 phenotype, eliciting the release of pro-inflammatory cytokines [14,15]. High fat diets cause this response by activating nuclear translocation of microglial nuclear factor-kappa B (NFκB), initiating release of pro-inflammatory cytokines such as TNF-α and interleukin-6 (IL-6) [13–15]. Palmitic acid activates microglia through a toll like receptor 4 (TLR-4)-dependent pathway, inducing the release of TNF-α and IL-6 [14]. Further, microglia activated by SFA via TLR-4 induce neuronal cell death [14]. Given the above findings on orexin action in microglia, orexin signaling might promote microglia switching to a protective M2 phenotype, protecting against palmitic acid induced inflammation.

The objective of these studies was to determine if orexin reduces PA-induced neuroinflammation by altering microglial M1/M2 phenotype dynamics. To test whether orexin treatment influences microglial phenotype, we evaluated the effect of OXA on PA-induced release of pro-inflammatory cytokines in an immortalized murine microglial cell line (designated BV2). We first validated that PA activates microglia to an M1 state via TLR-4. In our next set of experiments, we tested whether OXA pretreatment influenced levels of the M1 pro-inflammatory markers IL-6, TNF-α; inducible nitric oxide synthase (iNOS); and the M2 anti-inflammatory marker arginase-1 in microglia. Finally, we performed a series of studies to determine how conditioned media from these prior tests altered hypothalamic neuronal survival.

2. Materials and methods

2.1. Cell culture and reagents

Immortalized murine microglial cells (BV2) and adult murine hypothalamic cells (mHypoA-1/2, cited elsewhere as CLU172; CELLutions Biosystems) [16–18] were grown in Dulbecco's modified Eagle's medium (DMEM) plus 10% fetal bovine serum and 1% penicillin, streptomycin, and neomycin (Invitrogen) and maintained at 37 °C with 5% CO_2. Orexin A peptide (American Peptides) was suspended in phosphate buffered saline (PBS, Invitrogen) and diluted to 300 nM in DMEM. Palmitic acid (Sigma–Aldrich) was conjugated to fatty acid free bovine serum albumin (BSA) [19] and diluted to 0.1 mM in DMEM. Lipopolysaccharide (Sigma–Aldrich) was reconstituted in PBS and diluted to 0.4 μg (100 ng/ml) in DMEM. The TLR-4 inhibitor TAK-242 (EMD Millipore) was reconstituted in DMSO and diluted to 100 nM in DMEM.

For microglial experiments with OXA, BV2 cells were seeded in T-25 flasks at 7×10^5 cells and grown to ~80% confluency. Concentrations of OXA and PA are based on Xiong et al. and Wang et al. [8,14]. For all assays, cells were serum starved for 24 h. The experiment was completed in two stages: a 1 h pre-incubation followed by a 4 h challenge. Pre-incubation used either vehicle (PBS) or OXA (300 nM). For challenge, cells were exposed to vehicle (fatty acid free BSA), PA (0.1 mM), or LPS (0.4 μg; 100 ng/ml). There were a total of 5 treatment groups: vehicle–vehicle (control), vehicle-LPS, vehicle-PA, OXA-vehicle, and OXA-PA. After treatment, supernatant and cells were rapidly collected and stored at −20 °C. Supernatant from microglial cultures treated as described here was used as conditioned media for hypothalamic neuronal cultures (described below).

For microglial experiments with TAK-242, cells were seeded in 6 well plates at 3.5×10^5 cells per well and grown to ~80% confluency. Concentrations and time points were based on Matsunaga et al. and Takashima et al. [20,21]. Cells were serum starved for 24 h. TAK-242 (100 nM) or vehicle was added 20 min prior to incubation with PA (0.1 mM) or vehicle for 4 h.

2.2. Real-time RT-PCR

Total RNA was extracted from BV2 cells with Trizol (Invitrogen) as previously described [5,22]. Concentrations were determined using spectrophotometric readings at 260 and 280 nm (Nanodrop 8000, Thermo Fisher Scientific) and 2.5 μg RNA was used for each reaction. Primer sequences were generated using MacVector 15 for OX1R (NM_198959), OX2R (NM_198962), IL-6 (NM_031168), iNOS (NM_010927), arginase-1 (NM_007482) and GAPDH (NM_017008). Relative mRNA expression of target genes was determined using SYBR Green detection normalized to GAPDH using the $^{\Delta\text{-}\Delta}$CT method [23].

2.3. Enzyme-linked immunosorbent assay (ELISA)

TNF-α level in culture media was determined using an ELISA kit (BioLegend Inc.). Concentrations were determined using a spectrophotometer (SpectraMax-M5; Molecular Probes). Data are presented as picograms of TNF-α/ml.

2.4. Cell viability assay for hypothalamic cells

mHypoA-1/2 cells were seeded in a 96 well plate at 5×10^3 cells per well overnight. Amicon Filters (Millipore) were used to remove PA and OXA and concentrate conditioned media (supernatant from microglial cultures described above) [24]. Concentrated conditioned media was used at a six-fold concentration and added to mHypoA-1/2 cells for 24 h. Time points were based on previously described studies [14,25,26]. Cell survival was determined using a resazurin-based assay (Presto Blue, Invitrogen) producing a fluorescent signal [5]. Activity was determined using a spectrophotometer (SpectraMax-M5) and presented as percent relative fluorescence units (RFU) change vs. control.

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