Role of Orexin A Signaling in Dietary Saturated Fatty Acid–Activated Microglial Cells

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Significance

Excess saturated fatty acids (SFAs) such as palmitic acid (PA) contribute to hypothalamic inflammation and subsequent metabolic dysregulation.[1, 2] In rodent models, high fat diets activate microglial nuclear factor-κB (NFκB) nuclear translocation, initiating release of the pro-inflammatory cytokines tumor necrosis factor alpha (TNF-α) and interleukin-6 (IL-6).[1,3] Further, SFA–activated microglia induce cell death in nearby neurons.[3] Conversely, the neuroprotective peptide orexin A (OXA, hypocretin) prevents neurodegeneration and inflammation through a microglial mediated pathway,[4] reducing apoptosis through activation of orexin receptors 1 and 2 (OX1R, OX2R).[4,5] We present here results of a study investigating modulation of microglial activation by OXA after exposure to SFAs.

Overall Hypothesis

Orexin A attenuates palmitic acid–induced microglial activation and reduces release of pro-inflammatory cytokines.

Objectives

Aim 1: Characterize PA induced inflammatory changes in microglial cells.

Aim 2: Determine if OXA reduces pro-inflammatory cytokine release in microglial cell

Methods

Cell culture: The murine microglial cell line BV-2 was grown in Dulbecco's modified Eagle's medium plus 10% FBS and 1% PSN (DMEM; Invitrogen) and maintained at 37°C with 5% CO2. Cells (7.8 X 10^4) were grown in T-25 flasks overnight. Cells were serum starved for 24h prior to 1 h OXA (300 nM) pretreatment and 4 h PA (0.1 mM) or lipopolysaccharide (LPS; 0.4 ug).

Western blots: Protein samples (15 µg) separated on polyacrylamide-SDS gels, transferred to nitrocellulose membrane, and blocked using Snap ID system (Millipore). Antibodies: Iba-1 (ab5076; Abcam) and GAPDH (NB100-56875; Novus) primary; HRP conjugated anti-goat IgG (ab97100; Abcam) or anti-rabbit IgG (NB710H, Novus) secondary. After addition of HRP substrate (Pierce), membranes were developed and band density was determined (Eagle Eye II, Stratagene); data normalized to GAPDH.

Real-time RT-PCR: PCR reactions of total mRNA from cultured cells were performed in a Roche LightCycler for the following genes were used: OX1R (NM_198959), OX2R (NM_198962), TNF-α (NM_0213693), IL-4 (NM_021283), IL-6 (NM_031168) GAPDH (NM_017008). Relative mRNA levels were normalized to GAPDH using the Δ-ΔCT method.

ELISA: TNF-α in culture media was determined using a commercial ELISA kit (BioLegend Inc.). Absorbance at 450 nm was read using a spectrophotometer (SpectraMax-M5, Molecular Probes). Concentrations determined using standard curve; data presented as pg TNFα/ml.

Cell Viability Assay for Hypothalamic Cells: Cell survival was determined using Presto Blue (Invitrogen), a resazurin-based assay producing a fluorescent signal proportional to number of living cells [5]. Activity was determined using SpectraMax-M5; data reported as relative fluorescence units (RFU) % change vs. control.

Statistical Methods: Significance differences were determined by unpaired, two-tailed t-test using Graph Pad Prism 5 * p<0.05 vs. control, ***p<0.0001 vs. control, + p<0.05 vs. OXA only, ++p<0.001 vs. OXA only, +++ p<0.0001 vs. OXA, ## p<0.001 vs. PA only, ###p<0.0001 vs. PA only.

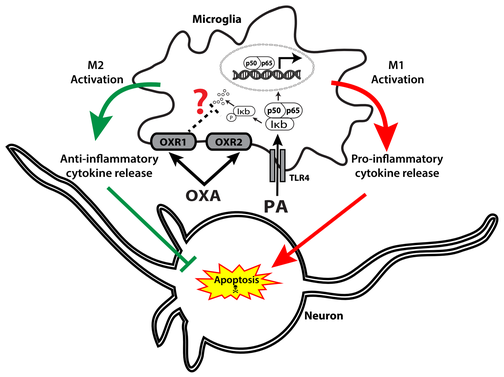

Figure1: Hypothesized orexin A (OXA) microglial immunomodulation pathway.

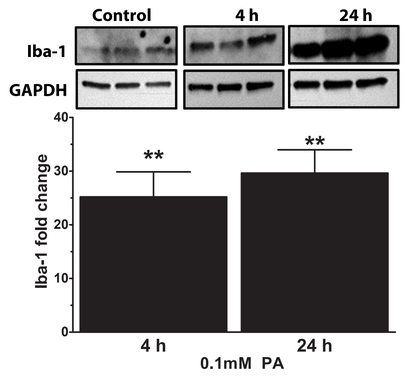

Figure 2: Palmitic acid–induced microglial activation. Palmitic acid treatment (0.1 mM) significantly increases Iba-1 protein in BV-2 microglial cells.

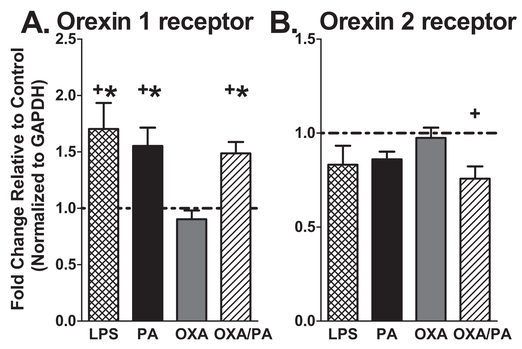

Figure 3: PA increases OX1R expression in microglia. Orexin receptor 1 (A) expression but not OX2R (B) is increased following PA and LPS stimulation.

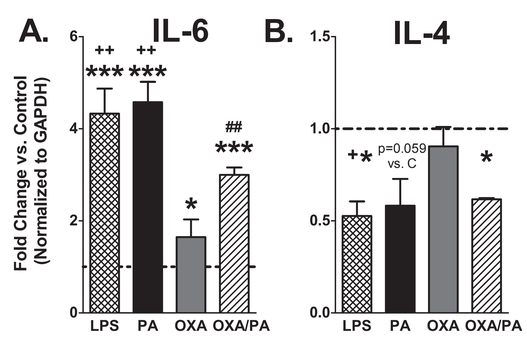

Figure 4: Orexin suppresses pro-inflammatory cytokine expression in microglial cells. Pro-inflammatory cytokine IL-6 (A) expression is increased following PA and LPS exposure, OXA plus PA reduces IL-6 expression compared to PA only but not vehicle control. Orexin plus PA and LPS exposure reduce anti-inflammatory IL-4 (B) expression.

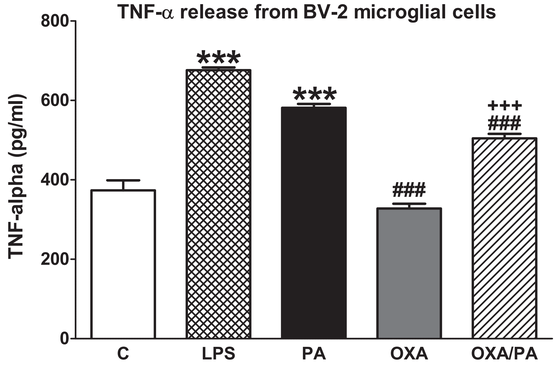

Figure 5: Orexin reduces TNF-α secretion in microglia. Palmitic acid and LPS treatment increases TNF-α secretion compared to control. Orexin reduces TNF-α secretion compared to PA only treatment.

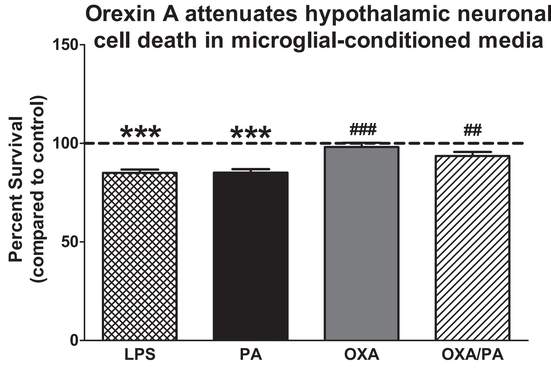

Figure 6: Orexin A attenuates hypothalamic neuronal cell death in microglia–conditioned media. Adult mouse hypothalamic cells (A12) have increased viability when conditioned media (CM) from OXA stimulated microglial was added. Cell viability is reduced following the addition of PA and LPS stimulated microglial CM.

Summary

- PA increases microglial activation through increased Iba-1 **(Fig 2)**.
- Orexin receptor 1, but not OXR2 is increased with PA and LPS stimulation **(Fig 3A-B)**, suggesting OX1R primarily participates in inflammatory responses.
- Pro-inflammatory cytokines IL-6 **(Fig 4A)** and TNF-α **(Fig 5)** are increased by PA and LPS.
- OXA pretreatment increases IL-6 and reduces TNF-α expression **(Figs. 4A, 5)**.
- Anti-inflammatory IL-4 expression is reduced by PA and LPS **(Fig 4B)**.
- OXA attenuates PA–activated microglia–induced cell death in neurons exposed to conditioned media **(Fig 6)**.

Neuron–microglia crosstalk is essential to maintain brain homeostasis. To our knowledge, this is the first report demonstrating PA increases OX1R expression in microglia. Increased microglial OX1R expression in response to pro-inflammatory stimuli could represent a compensatory response to reduce release of inflammatory cytokines. As OXA is neuroprotective,[4, 5] acting upon microglia may be one mechanism through which OXA protects against apoptosis and lipid peroxidation. Future studies will further explore this mechanism and focus on defining the role of OXA as an immunomodulator.

Acknowledgements

We thank Drs. Philippe Marambaud (Feinstein Institute for Medical Research, Manhasset, NY) and Weihua Zhao (Methodist Hospital, Houston, TX) for providing the BV-2 cell line, and Angelo (Ce) Yuan and Lauren Wisdorf for assistance in ongoing studies. Funding provided by the Department of Veterans Affairs RR&D BX#### (to CMK), BLR&D BX001686 (to TAB), and a grant from the University of Minnesota Healthy Foods, Healthy Lives Institute (to CMD)

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