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Determining the mechanism of orexin A induced neuroprotection in an *ex vivo* arcuate nucleus model

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Introduction

High fat diets (HFD) rich in saturated fatty acids such as palmitic acid (PA) increase oxidative stress, apoptosis, and pro-inflammatory cytokines in both peripheral tissue and brain [1]. In *in vitro* models, PA increases lipid peroxidation and cell death. High-fat diets have been shown to induce neuronal degeneration of hypothalamic sites important in regulating energy balance *in vivo*, but the underlying cause of this dysregulation remains unclear.

Recent evidence suggests that orexin A (OXA; hypocretin 1), a hypothalamic neuropeptide, protects against oxidative stress and neuroinflammation [2, 3]. In an *in vitro* hypothalamic cell culture model, we showed that OXA reduces lipid peroxidation, decreases PA-induced apoptosis, and stabilizes the pro-apoptotic protein B-cell lymphoma-2 (Bcl-2) [2, 4]. Data suggest these effects rely in part on orexin-induced elevations in hypoxia-inducible factor 1 alpha (HIF1-α), a transcription factor modulating oxidative phosphorylation and ATP production [2, 5, 6].

To validate and extend these findings, we performed several studies on orexin and PA in an *"ex vivo"* organotypic explant model using heterogeneous brain tissue. We focused on two hypothalamic regions, the arcuate nucleus (Arc) and caudal lateral hypothalamus (cLH), due to their roles in energy metabolism and feeding behaviors. We also tested PA effects in the BV-2 immortalized microglial cell line, as pilot studies showed microglial activation affected neuronal response to PA-induced toxicity. Together, these studies were designed to answer three main questions:

1) **Does OXA increase HIF1-α in arcuate nucleus and caudal lateral hypothalamic explants?**

2) **Does PA decrease pro-survival gene expression and increase cytotoxicity in Arc and cLH explants?**

3) **Does PA increase mitochondrial activity and microglial activation in immortalized microglial cells?**

Methods

Tissue collection: All animal use was approved by the Minneapolis VA Health Care System Animal Care and Use Committee. Three month old Male Sprague Dawley rats (Charles River) were decapitated and brain tissue was rapidly dissected, then placed in ice cold Hibernate A (HIB-A, Invitrogen) supplemented with 2% B27, 0.25% Glutamax (Glu), and 1% penicillin streptomycin neomycin (PSN). Selected hypothalamic nuclei were isolated by micropunch, keeping tissue in HIB-A at all times.

Ex vivo explant studies: On day 1, explant punches were transferred to 24 well plates with 2 ml Neurobasal-A (NBA; with 2% B27, 0.25% Glu, and 1% PSN) and incubated for 24 h at 37°C with 5% CO_2. On day 2, half of the media was removed and replaced with fresh NBA with palmitic acid (0.075 or 0.1 mM, suspended in 0.01% DMSO; Sigma). Control tissue was incubated with NBA + 0.01% DMSO vehicle.

Lactate dehydrogenase (LDH) cell toxicity assay: Cell viability was evaluated using an LDH assay (Abcam) on culture media from ex vivo samples. LDH activity was measured with a spectrophotometer (λmax = 450 nm).

Western blots: Protein samples isolated from tissue lysate (15 µg) were separated on 8-16% polyacrylamide-SDS gels, transferred onto a nitrocellulose membrane, and blocked (SuperBlock; Pierce Thermo-Scientific) using the Snap ID system (Millipore). Antibodies used: 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 (West Pico-Super Signal; Pierce), membranes were placed on x-ray film and developed. Band density was determined using the Eagle Eye II (Stratagene) and normalized to GAPDH.

Real-time RT-PCR: Total mRNA from cultured cells was isolated using a commercial kit (Qiagen). Primers for the following genes were designed using MacVector 12: Akt3 (NM_031575.1), Bcl-2 (NM_016993.1), BDNF (NM_012513), GAPDH (NM_017008), and Iba-1 (NM_017196). PCR reactions were performed in a Roche LightCycler. Relative mRNA levels were normalized to GAPDH using the Δ-ΔCT method.

PA in microglial cells: 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% CO_2. Cells (1.0×10^5/well) were grown in 6 well plates overnight and then incubated in PA (DMEM + 0.1 mM PA in 0.01% DMSO) or control media (DMEM + 0.01% DMSO).

Tissue metabolic activity: Metabolic activity was determined using the Presto Blue resazurin-based assay (Invitrogen). Tissue or microglial cells were incubated for 2 or 4 h. Media was collected and read using a spectrophotometer (λmax = 570 nm).

Statistical Methods: Explant studies were analyzed using one way ANOVA. *In vitro* studies and PCR data were analyzed using unpaired two-tailed *t* tests. Analysis for LDH and Presto Blue assays was performed on raw relative fluorescent or luminescent values. Data were normalized against control values and graphed as percent change relative to control. GraphPad Prism 5 was used for all analyses.

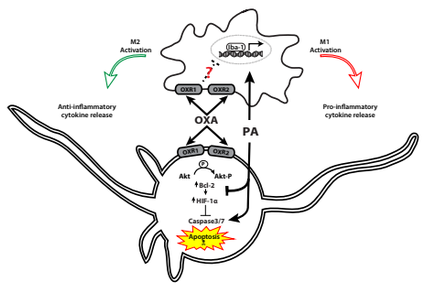

Figure 1: Schematic illustrating hypothesized pathways of orexin-mediated neuroprotection and palmitic acid-induced neuronal insult.

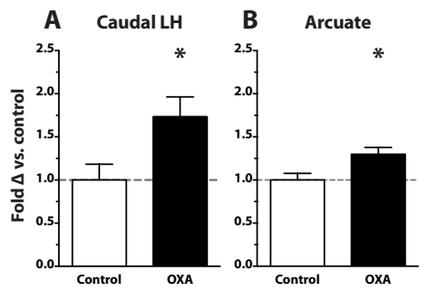

Figure 2: Orexin A (150 µM) significantly increases HIF1-α expression in caudal lateral hypothalamus (**A**) and arcuate nucleus (**B**) hypothalamic tissue explants. *Tissue treated for 24 h; * $p < 0.05$.*

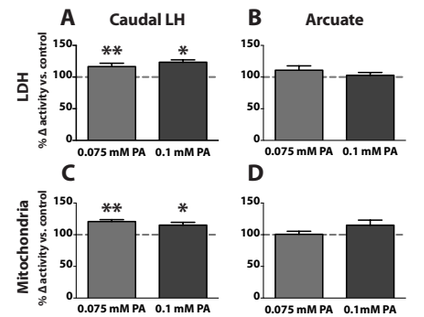

Figure 3: Palmitic acid treatment (0.075 or 0.1 mM for 24 h) significantly increased lactate dehydrogenase (LDH) activity (a marker of cytotoxicity) and mitochondrial activity in cLH (**A, C**) but not arcuate explants (**B, D**). * $p < 0.05$; ** $p < 0.01$.*

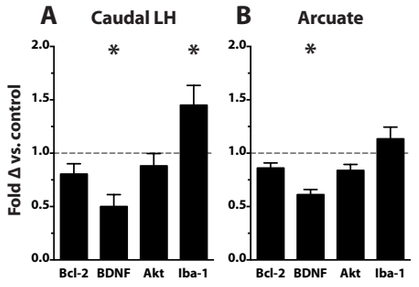

Figure 4: Palmitic acid significantly reduces gene expression of BDNF and Bcl-2 (pro-survival genes) in cLH (**A**), and BDNF in arcuate tissue (**B**). Palmitic acid significantly increases Iba-1 mRNA (marker of microglial activation) in cLH (**A**). * $p < 0.05$.*

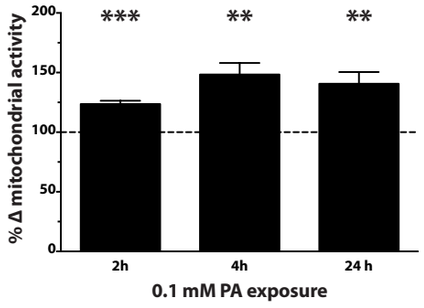

Figure 5: Palmitic acid (0.1 mM; 2, 4, and 24 h treatment) significantly increases mitochondrial activity of BV-2 immortalized microglial cells compared to respective controls. ** $p < 0.01$; *** $p < 0.001$.*

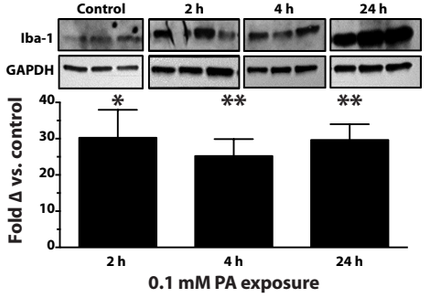

Figure 6: Palmitic acid treatment (0.1 mM; 2, 4, and 24 h) significantly increases Iba-1 protein in BV-2 immortalized microglial cells. * $p < 0.05$; ** $p < 0.01$.*

Acknowledgements

We thank Drs. Philippe Marambaud and Weihua Zhao for providing the BV-2 cell line. Funding was provided by the Department of Veterans Affairs RR&D, VA BLR&D BX001686, and NIH grant DK078985.

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Results

We show here that OXA increases HIF1-α in hypothalamic explants (**Fig. 2**), while PA increases cell death (indicated by LDH activity; **Fig. 3A–B**), and decreases mRNA expression of the pro-survival genes BDNF (brain-derived neurotrophic factor) and Bcl-2 (**Fig. 4**). We also show that PA increases mitochondrial activity (**Fig. 3C–D**, **Fig. 5**) and Iba-1 (a marker of microglial activity; **Fig. 4**, **Fig. 6**) in *ex vivo* and *in vitro* models.

Discussion

Diets high in saturated fatty acids (such as PA) have been linked to increased neuroinflammation, pro-inflammatory cytokine release, apoptosis and gliosis [1], characteristics of neurodegenerative diseases [7]. The increased expression of Iba-1 observed in explants, along with increased mitochondrial activity and Iba-1 in BV-2 microglial cells, suggest that PA contributes to neuroinflammation by inducing microglial activation in hypothalamic tissue. Following ischemia or saturated fatty acid exposure, resting microglia are activated to an M1 toxic phenotype [8, 9], mediated by the TLR-4/NFκB pathway, resulting in release of the pro-inflammatory cytokine TNF-α [9]. In cortical tissue, orexin reduces inflammation and infarct size following cerebral ischemia through attenuation of microglial TNF-α [3]. The neuroprotective effects of OXA *in vivo* may thus rely on both protecting neurons via HIF1-α and through suppression of M1 microglial activation. Further elucidating the control of microglial phenotypes in response to orexin will provide insight on how the hypothalamus adapts to HFD-induced changes.

In summary, these data indicate that neuroprotection in intact brain tissue cannot be fully evaluated without considering the role of non-neuronal components. Understanding the contribution from all components within the brain will bridge the gap between environmental changes and immunomodulary responses within the central nervous system. We are currently studying OXA signaling mechanisms in microglia and neurons to further investigate the dual role of OXA in microglial immunomodulation and endogenous neuroprotection.

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