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Orexin A protects against fatty acid induced apoptosis in hypothalamic cells

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Introduction

High fat diets (HFD) and sedentary lifestyles have been linked to obesity and related comorbidities[1-3]. HFDs, specifically those high in saturated fatty acids (SFA) such as palmitic acid (PA), increase oxidative stress, insulin resistance, pro-inflammatory cytokines, and apoptosis in both peripheral tissue and in the central nervous system[4-9]. Recently, such HFD-induced neuronal changes in the hypothalamus have been linked to obesity[10-12].

Orexin A (OxA; hypocretin 1) is a hypothalamic neuropeptide important in regulating eating behavior, the sleep/wake cycle and physical activity, and promotes obesity resistance[13]. Recently, OxA was shown to decrease apoptosis in cerebral cortex tissue[14,15]. Work from our laboratory has also shown OxA to be neuroprotective in hypothalamic cells by decreasing caspase 3/7 activity, an important marker of apoptosis[9]. Proteins involved in anti- and pro-apoptotic pathways, such as B cell lymphoma 2 (Bcl-2) and Bcl-2 associated X protein (Bax), could be potential mediators involved in the neuroprotective effect of OxA. Bcl-2 activity is influenced by the serine/threonine protein kinase Akt. Additionally, neuronal survival has been correlated to the phosphatidylinositol 3-kinase (PI3K)/Akt pathway[12]. Furthermore, Bcl-2 and Akt activity is decreased by PA[6,8,16]. Bcl-2 inhibits Bax by preventing a conformational change that would otherwise cause the release of cytochrome C and further induce apoptosis[17].

Previous data show that OxA decreases caspase 3/7 activity when challenged with the oxidative stressors hydrogen peroxide (H_2O_2) or PA[9, 18]. However, the mechanism by which OxA exerts neuroprotective effects remains unknown. Here we tested the hypothesis that **OxA promotes neuroprotection against PA-induced apoptosis by increasing Bcl-2 anti-apoptotic proteins.**

Methods

Cell line Maintenance and Reagents: Differentiated immortalized adult mouse hypothalamic (A1/2) cells were purchased from CELLutions-Cedarlane and were maintained in DMEM medium supplemented with 10% FBS at 37°C with 5% CO_2. OxA peptide (American Peptides) was dissolved in PBS, stored at -20°C, and diluted to final concentration in DMEM before use. PA (Sigma) was prepared fresh each time in DMSO and diluted to a final concentration in DMEM.

Caspase Activity: Cells were challenged with increasing doses of PA for 2 hours, then caspase-3/7 activity was determined by the addition of a luminogenic caspase substrate DEVD based assay (Caspase-Glo 3/7, Promega). Changes in relative luminance units (RLU) were analyzed using a microplate spectrometer reader.

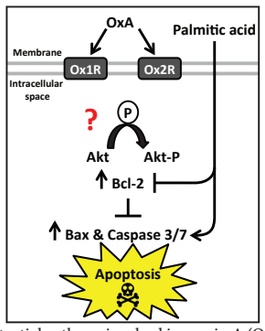

Figure 1: Potential pathway involved in orexin A (OxA) induced neuroprotection. Ox1R, Ox2R: Orexin receptors 1 and 2; Bcl-2: B cell lymphoma 2; Bax: Bcl-2 associated X protein; Akt: serine/threonine protein kinase.

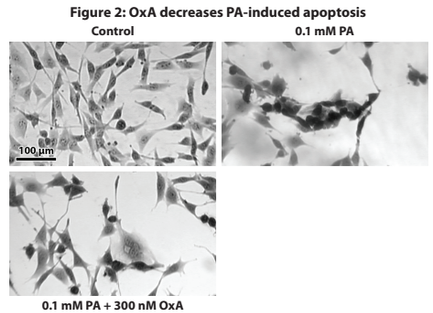

Figure 2: Photomicrograph of A1/2 cells in culture in the presence/absence of Orexin A (OxA) and palmitic acid (PA). Cells pretreated with OxA and then challenged with PA remain viable and resist the onset of apoptosis[9, 20].

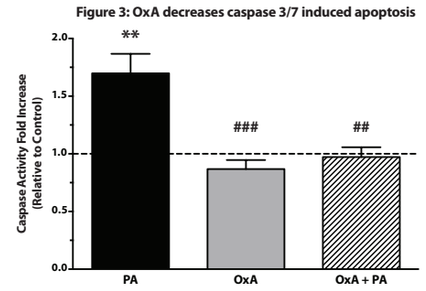

Figure 3: OxA decreases caspase 3/7 induced apoptosis. A12 cells were pretreated with OxA for 24 h and incubated with PA with or without OxA for 2 h. Caspase 3/7 activity was determined and normalized to cell number.

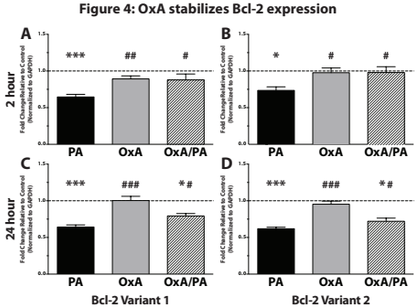

Figure 4: A12 cells were pretreated with OxA for 24 h and challenged with PA with or without OxA for 2 (A, B) or 24 h (C, D). Gene expression was determined via qRT-PCR. Fold change relative to control was determined and normalized to GAPDH.

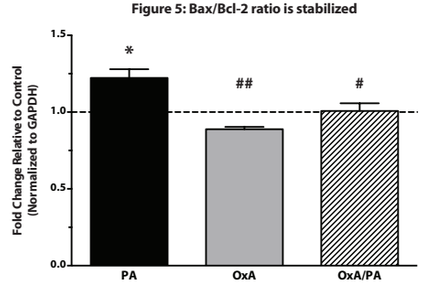

Figure 5: OxA stabilizes Bax/Bcl-2 ratio. Data represent the Bax/Bcl-2 ratio after 24 h OxA pretreatment, followed by PA in the presence or absence of OxA for 2 h.

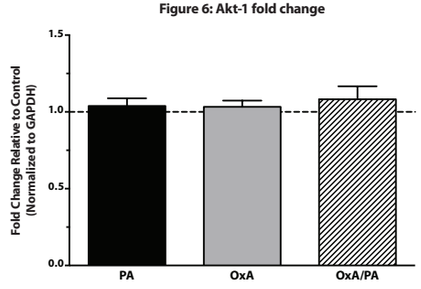

Figure 6: Akt isoform-1 expression is unchanged in the presence of OxA.

Real-time RT-PCR: Total mRNA from cultured cells was isolated using a commercial extraction kit (Qiagen). RNA was analyzed spectrophotometrically at 100 nm. Primers were designed using MacVector 12. Bcl-2: (NM_177410.2; Forward: 5'-CACCGCGAGGGGACGCTTTG-3'; Reverse: 5'-AGGTCGCATGCTGGGGCCATA-3') Bax: (NM_007527.3; Forward: 5'-GCTGAGCGAGTGTCTCCGGC-3'; Reverse: 5'- ACGCGGCCCCAGTTGAAGTT-3') Akt: (NM_009652.3; Forward: 5'- GGAGTGTGTGGACAGTGAGCGG -3'; Reverse: 5'- TCGGCAATGCAGAGGAGCGT -3'). Isolated mRNA was measured by real-time RT-PCR using a Roche LightCycler. Relative mRNA levels were normalized to GAPDH using the Δ-ΔCT method[19].

Statistical Methods: Significant differences were determined by unpaired, two-tailed t-test using Graph Pad Prism 5. (*$p < 0.05$ and ***$p < 0.001$ vs. control; #$p < 0.05$, ##$p < 0.005$, and ###$p < 0.0005$ vs. PA-treated cells.)

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Results

- OxA exerts neuroprotection by decreasing caspase 3/7 activity and apoptosis (**Figures 2–3**)
- OxA appears to stabilize both Bcl-2 transcript variants 1 and 2 expression when cells are pretreated with OxA and challenged with PA (**Figure 4**)
- OxA stabilizes Bax/Bcl-2 ratio (**Figure 5**)
- Akt isoform-1 expression does not appear to be altered by OxA (**Figure 6**)

Discussion

Maintaining hypothalamic integrity is important because the hypothalamus controls multiple important body functions such as eating behavior, sleeping and metabolic processes[21]. Diets rich in SFAs such as PA not only induce apoptosis in hypothalamic cells, but also have been linked to obesity and other comorbidities such as decreased insulin sensitivity, diabetes, and stroke[2, 4].

The neuroprotective effects of OxA are intriguing, but little is known about the mechanistic pathways involved. Bcl-2 is an important modulator in the anti-apoptotic process of cells and is tightly regulated by post-translational modifications resulting in two transcription variants[22]. We analyzed both variants and show here that pretreatment with OxA appears to stabilize both Bcl-2 transcription variants 1 and 2 expression when cells are challenged with PA. Although data are inconclusive, it appears that the Bax/Bcl-2 ratio is increased in cells treated with PA and stabilized in those treated with OxA.

Ongoing work will involve measures of pro-apoptotic expression for caspase 3 and caspase 7, which increase with high fat diets[6]. We will continue to analyze Akt activity and its involvement in OxA–induced neuroprotection. Planned studies include Western blot analysis of Akt activity and microarray analysis to analyze change in gene expression of pro- and anti-apoptotic proteins involved in OxA's neuroprotective mechanisms.

Acknowledgements

Funding for these experiments provided by the US Department of Veterans Affairs Rehabilitation Research & Development, a University of Minnesota Undergraduate Research Opportunity Program grant, and NIDDK grant DK078985.