Orexin A Influences Lipid Peroxidation and Neuronal Metabolic Status in a Novel Immortalized Hypothalamic Cell Line

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Introduction

The hypothalamic neuropeptide orexin A (OxA) regulates diverse physiological processes by activation of two related G-protein coupled receptors (OxR1 and OxR2). OxA injected into rostral lateral hypothalamus (rLH) elevates spontaneous physical activity (SPA) and confers obesity resistance [1, 2]. The mechanism by which this occurs is unknown and little is known about OxA signaling in the hypothalamus.

Diets high in saturated fats have been thought to contribute to adverse changes in brain mechanisms that contribute to several disease processes including obesity, albeit controversial, through the induction of neurodegenerative pathways [3-5]. Mechanisms that have been reported to increase included the increase in lipid peroxiadative metabolites and increases oxidative stress induced apoptosis [6, 7]. It is unclear if these disturbances alter SPA and OxA signaling in the hypothalamus.

OxA has pleiotropic effects and can increase ATP and the transcription factor hypoxia inducible factor-1α (HIF-1α) in hypothalamic tissue under normoxic conditions [8]. In various models OxA has been shown to decrease lipid peroxidation (LP) and apoptosis [6, 7]. Whether these effects are important to OxA-mediated SPA is not known. If put into context physiologically, OxA could stimulate neuroprotective mechanisms and increase intracellular metabolism in responsive rLH OxA neurons. A higher level of OxA responsiveness could increase resistance to high fat diet induced neurodegeneration and obesity. To address this issue we tested if OxA is neuroprotective against oxidative induced apoptosis in the hypothalamus utilizing a novel clonal immortalized embryonic rat hypothalamic cell line.

Methods

Cell line and Maintenance: Differentiated immortalized embryonic rat hypothalamic (R7) cells were purchased from CELLutions-Cedarlane (North Carolina USA) and were maintained in DMEM medium supplemented with 10% FBS at 37°C 5% CO_2. The OxA peptide (American Peptides, Sunnyvale, CA) was dissolved in artificial cerebrospinal fluid was stored at -20°C until the day it was used. Final concentrations were diluted in DMEM. Hydrogen peroxide (30%, Sigma) was prepared fresh each time it was used and diluted to a final concentration in DMEM.

Intracellular Ca^{2+} and ATP Assays: Cells were plated at ~ 25,000 per well in a 96 well plate overnight prior to use. Changes in intracellular Ca^{2+} concentration cells in response to incubation with 300 nM OxA was measured using the fluorescent based Fluo-4 Direct Calcium Assay Kit (F10471, Invitrogen, Inc.) according to manufacturer's instructions. Intracellular ATP levels were assayed following incubation with 50 nM OxA in cells grown to ~80% confluency as previously described [8]. ATP values were normalized to cell counts done by using a hemocytometer.

OXR Immunofluorescence: Embryonic rat hypothalamic (R7) and human embryonic kidney 293 (HEK293) were maintained in DMEM medium at 37°C 5% CO_2. HEK 293 cells were used as a negative control. Cells were fixed with PFA 4% and used for for OXR immunofluorescence (IF) using a dilution 1:500 of the OX1R (Novus, Inc) or OX2R (Chemicon, Inc) antibodies following standard IF protocols.

Viability Assay: Cell were plated at 7000 per well and cell viability was determined by using a resazurin based assay (Presto Blue, Invitrogen). Briefly, resazurin is a cell permeable compound that undergoes enzymatic reduction by mitochondria in metabolically active cells. Viable/living cells continuously convert resazurin to resorufin and this produces a fluorescence signal that is proportional to the number of viable cells. Changes in relative fluorescent units (RFU) were recorded and data was reported as percent changes relative to controls.

Caspase Activity: Caspase 3/7 activity was determined by the addition of the luminogenic caspase substrate, DEVD (Caspase-Glo 3/7, Promega). Upon the activation of intracellular caspase-3/7 a luminescent product is produced. A change in luminescence is proportional to caspase activity and the induction of apoptosis. Changes in relative luminance units (RLU) were collected and reported as percent changes relative to controls.

Tiobarbituric Acid Reactive Substances (TBARS): Lipid peroxidation was measured by determining the generation of malondialdehyde (MDA) using a commercially available kit according to manufacturer's instructions (Cayman Chemical). Briefly samples were incubated with thiobarbituric acid (TBA) under high temperature and acidic conditions to form a MDA-TBA complex that were quantified spectrometrically at 530 nm/ 550 nm.

One-step real-time RT-PCR: 100 ng of total RNA using the Roche RNA Amplification Kit SYBR Green I and the Roche LightCycler (Roche Applied Science, Indianapolis, IN). Fold changes in expression were normalized to GAPDH.

Statistical Methods: Significant differences were determined by unpaired, two-tailed t-test using Graph Pad Prism software. *P < 0.05, ** P < .001 or *** P < .0001.

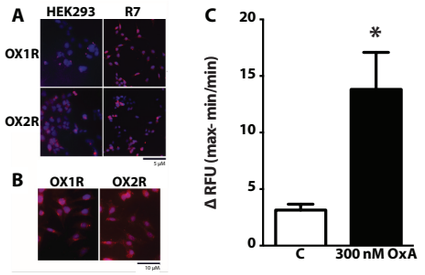

Figure 1: Characterization of R7 cells. (A,B) OXR expression in HEK293 and R7 cells. (C) Changes in intracellular Ca^{2+} after bath application of OxA in R7. This data supports that that R7 cells are a suitable in vitro model for studying OxA changes in neuronal intracellular metabolism.

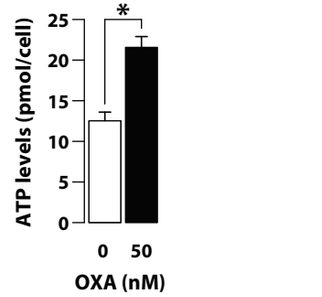

Figure 2: Intracellular ATP was assayed following 2 h incubation with 50 nM OxA to determine if OxA is able to increase intracellular neuronal metabolic responses. These data match published data demonstrating that 100 nM OxA increases ATP over 2 h in mouse hypothalamic tissue slices [8].

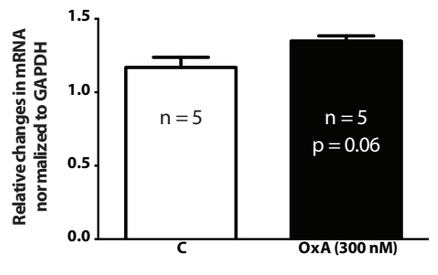

Figure 3: HIF-1α expression in normoxic R7 cells following 2h OxA incubation. Although not significant, trend for increase suggests that significance may be reached once optimum time and dose is determined. These data agree with current literature showing that OxA increases neuronal HIF-1α [7, 8].

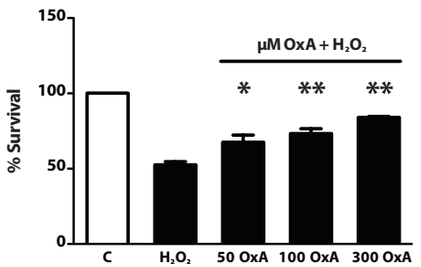

Figure 4: OxA is neuroprotective against oxidative stress in R7 neurons. Cells were pretreated with OxA for 24 h prior to a 24 h H_2O_2 (50 μM) challenge. H_2O_2 concentration and incubation time match other in vitro models demonstrating that the concentration used in this experiment predominately induces apoptosis and not necrosis [9].

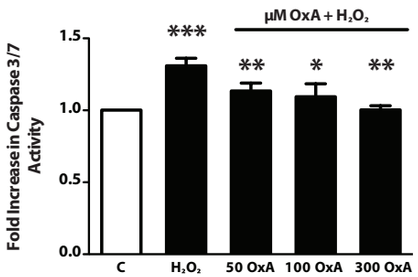

Figure 5: OxA decreases oxidative induced apoptosis. Caspase 3/7 activity was assayed to verify that H_2O_2-induced cell death was initiated by an apoptotic pathway, rather than via necrosis. OxA-induced neuroprotection was determined by decreased caspase activity as compared to H_2O_2-challenged cells.

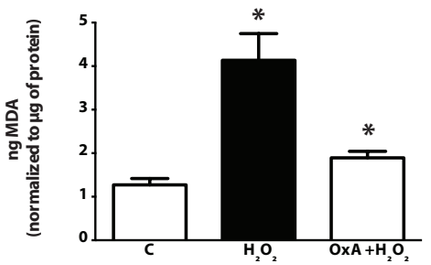

Figure 6: Pretreatment with OxA for 24 h attenuates lipid peroxidation. Cells were incubated with 300 nM OxA for 24 h and were given a second treatment just prior to H_2O_2 (50 μM) challenge. Lipid peroxidation was determined by the generation of MDA using the TBARS assay.

Conclusions

- OxA decreases apoptosis, lipid peroxidation and is neuroprotective against the oxidative stressor H_2O_2.
- OxA increases ATP and HIF-1α, indicating that OxA can positively alter intracellular metabolic responses. This would suggest that OxA can alter proteins or genes critical to maintaining neuronal intracellular metabolic responsiveness.

Discussion

The novel rat hypothalamic cell line designated as R7 represents an in vitro model that can be used to evaluate OxA induced changes in cell survival and intracellular metabolism. The advantage of this model it is derived from differentiated primary hypothalamic neurons immortalized using a genetic technique rather than from hypothalamic tumor-derived cell lines. The advantage of this approach is that the resulting cells are less likely to exhibit characteristics atypical of a normal hypothalamic cell.

Little is known about the short and long term effects of its signaling on intracellular neuronal metabolic status and its physiological relevance to SPA. Collectively, emerging evidence indicates that OxA also alters proteins involved in intracellular metabolic function. However, the complexity of neuronal networks within the hypothalamus can make it difficult at best to determine distinct neuronal phenotypes and their contributions to behaviors such SPA in response to OxA. Ultimately determining OxA induced changes in short- and long-term intracellular metabolic capacity of activated neurons would result in therapies that could maintain elevated SPA and increase obesity resistance.

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