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

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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 hypothalamic (LH) deceases spontaneous food intake and obesity resistance.1,2,3


The mechanism by which this occurs is unknown and little is known about OxA signaling in the hypothalamus.

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 to best to determine distinct neuronal phenotypes and their contributions to such SPA in response to OxA. Ultimately determining OxA induced changes to short- and long-term intracellular metabolic capacity of activated neurons would result in therapies that could maintain elevated SPA and increase obesity resistance. OxA has pleiotropic effects and can increase ATP and the transcription factor hypoxia inducible factor-1α (HIF-1α) in hypothalamic tissue under normoxic conditions.4

In various models OxA has been shown to decrease lipid peroxidation (LP) and apoptosis 5. Whether these effects are important to OxA-mediated SPA is not known. If put into context physiologically, OxA could stimulate neuroneuroprotective mechanisms and increase intracellular metabolism in response to HIF-1A mRNA. A higher level of OxA responses 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 cloned immortalized embryonic rat hypothalamic cell line.

Methods

Cell line and Maintenance: A immortalized hypothalamic embryonic rat hypothalamic (C1) cell line was purchased from Cell Culture Calabasas (North Hollywood, CA) and maintained in EBM2 medium supplemented with 8% heat inactivated FBS (HyClone, Logan, UT). The OX-A peptide (American Peptides, Sunnyvale, CA) was dissolved in artificial cerebral spinal fluid (ACSF) that was stored at -20°C until the day of use. C1 cells were cultured in Neurobasal/A (Invitrogen, Carlsbad, CA) supplemented with 2 mM L-glutamine. Cells (20000 cells/cm²) were seeded into flared 24-well plates in 0.5 ml of Neurobasal media for 24 h. Then media was replaced with 0.5 ml of fresh Neurobasal media supplemented with 8% heat inactivated FBS. The OX-A peptide (100 µM) was injected into the media well. Cells were pretreated with OxA for 24 h prior to a 24 h H2O2 challenge. Cells were used as a control group of cells not treated with OxA. Cells were incubated with 100 µM OxA for 24 h and then challenged with 50 μM H2O2 and 0.05% Triton X-100 for 24 h. This was repeated two times. Intracellular Ca2+ levels were measured by a Fura2-based fluorimetry in response to incubation with 500 μM OxA and 500 μM H2O2. ATP levels were measured using the bioluminescence-based luciferase assay. Metabolic rate was determined using an oxygen consumption-based assay. HIF-1δ (nuclear) was determined using a fluorimetric dual-color lysosomal assay. Apoptosis was determined by a colorimetric caspase-3 kit. Apoptotic cells were determined using a flow cytometry assay. Cells were cultured in a 2 cm² area of a surface. Cells were fixed with 4% FA and stained for OX-A immunohistochemistry (IHC) using a dilution of 1:100 of the OX-A antibody or 1:50 (Invitrogen, Carlsbad, CA) antibody followed by a standard protocol.

Viability Assay: Cells were plated at 5000 cells per well in a 24 well plate overnight prior to assay. Changes in intracellular Ca2+ concentration in response to incubation with 500 μM OxA were measured using the fluorescent dye fura-2. Changes in intracellular ATP were measured using a fluorimetric protocol that evaluates intracellular ATP levels by the luciferase-based assay. Changes in intracellular ATP were measured using a fluorimetric protocol. Cells were preincubated for 20 min with 50 mg/liter p-aminohippurate and 50 mg/liter NaC1 and then challenged with 0.05% Triton X-100. Intracellular ATP levels were measured following incubation with 50 μM OxA in cells grown to 90% confluence by a colorimetric-based assay. In this case, the signal is proportional to the number of viable cells. Changes in intracellular ATP (nM) 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 caspase-3/7 substrate, DEVD-AFC (Caspase 3/7: Promega). The activation of intracellular caspase 3/7 is a biomarker product is produced. A change in luminescence is proportional to caspase activity and the induction of apoptosis.

Oxidative stress was induced by a reactive oxygen stressor H2O2 which is known to induce apoptosis. H2O2 was added to the media at a concentration of 50 μM. Cells were pretreated with OxA for 24 h prior to a 24 h H2O2 (50 μM) challenge. OxA-induced neuronal apoptosis was determined by determining caspase activity as compared to H2O2 challenged cells.

Conclusions

• OxA decreases apoptosis, lipid peroxidation and is neuroprotective against the oxidative stressor H2O2.

• 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. Changes in intracellular total (ATP) were utilized and plotted as percent changes relative to controls.

• Intracellular Reactive Substrates (1:1000). ATP levels were measured using the bioluminescence-based luciferase assay. Determining the generation of reactive metabolite (H2O2) using a commercially available luminol substrate was measured by a microtiter plate reader. The enzyme samples were reacted with luminol/acid (TBAB) under high temperature and low pH conditions to form a luminol chemiluminescent signal that was quantified spectrophotometrically at 450 nm.

• One-step real time PCR: 100 ng of RNA using the iScript cDNA Amplification Kit (Bio-Rad Green Healthcare, Micrococal, CA). All changes in expression were normalized to GAPDH.

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