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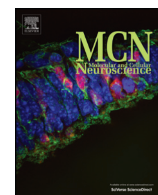

Orexin A attenuates palmitic acid-induced hypothalamic cell death

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ABSTRACT

Palmitic acid (PA), an abundant dietary saturated fatty acid, contributes to obesity and hypothalamic dysregulation in part through increase in oxidative stress, insulin resistance, and neuroinflammation. Increased production of reactive oxygen species (ROS) as a result of PA exposure contributes to the onset of neuronal apoptosis. Additionally, high fat diets lead to changes in hypothalamic gene expression profiles including suppression of the anti-apoptotic protein B cell lymphoma 2 (Bcl-2) and upregulation of the pro-apoptotic protein B cell lymphoma 2 associated X protein (Bax). Orexin A (OXA), a hypothalamic peptide important in obesity resistance, also contributes to neuroprotection. Prior studies have demonstrated that OXA attenuates oxidative stress induced cell death. We hypothesized that OXA would be neuroprotective against PA induced cell death. To test this, we treated an immortalized hypothalamic cell line (designated mHypoA-1/2) with OXA and PA. We demonstrate that OXA attenuates PA-induced hypothalamic cell death via reduced caspase-3/7 apoptosis, stabilization of Bcl-2 gene expression, and reduced Bax/Bcl-2 gene expression ratio. We also found that OXA inhibits ROS production after PA exposure. Finally, we show that PA exposure in mHypoA-1/2 cells significantly reduces basal respiration, maximum respiration, ATP production, and reserve capacity. However, OXA treatment reverses PA-induced changes in intracellular metabolism, increasing basal respiration, maximum respiration, ATP production, and reserve capacity. Collectively, these results support that OXA protects against PA-induced hypothalamic dysregulation, and may represent one mechanism through which OXA can ameliorate effects of obesogenic diet on brain health.

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1. Introduction

Diets rich in the saturated fatty acid palmitic acid (PA) promote hypothalamic dysregulation and contribute to obesity through disruption of appetite regulating signals (Benoit et al., 2009; Kleinridders et al., 2009). Mechanisms triggered by PA exposure include increased oxidative stress, insulin resistance, release of pro-inflammatory cytokines, and apoptosis (Benoit et al., 2009; Moraes et al., 2009; Thaler et al., 2012; Zhang et al., 2008). In the hypothalamus, diet-induced obesity also alters genes regulating the apoptotic pathway. For example, hypothalamic expression of anti-apoptotic protein B cell lymphoma 2 (Bcl-2) is decreased, while expression of the pro-apoptotic protein B cell lymphoma 2 associated X protein (Bax) is upregulated (Moraes et al., 2009). These gene expression changes are linked to obesity pathogenesis. High fat diets (HFD) also contribute to the overproduction of reactive oxygen species (ROS) in the brain, resulting in increased oxidative stress and cell damage (Pipatpiboon et al., 2013, 2012; Zhang et al., 2005). Because production of ROS is a crucial signal resulting in cell death, oxidative stress and apoptosis are closely related pathways. Further, ROS suppresses neuronal anti-apoptotic proteins including Bcl-2 (Pugazhenthi et al., 2003).

Because dietary components such as PA can directly and negatively affect brain health, it is likely that protection against obesity involves neural mechanisms capable of ameliorating diet effects on central nervous system signaling. One potential candidate are the orexins (hypocretins), hypothalamic peptides important in maintaining energy metabolism, sleep/wake cycles, and promoting obesity resistance (Butterick et al., 2013; Kotz et al., 2012). Orexin A (OXA) and orexin B (OXB) act through two G-protein coupled receptors (orexin receptors 1 and 2; OX1R and OX2R, respectively) to alter intracellular metabolic functions (Harada et al., 2011; Sellayah et al., 2011; Sikder and Kodadek, 2007) and promote cell survival against oxidative stress and ischemic events (Butterick et al., 2012; Yuan et al., 2011) in part through activation of Akt (protein kinase B) (Sokolowska et al., 2014). Here we focus on elucidating neuroprotective pathways through which orexin alters brain response to PA, a major component of obesogenic diets.

Work from our lab has demonstrated that OXA attenuates caspase-3/7 mediated apoptosis in response to H_2O_2, an initiator of oxidative

Abbreviations: orexin A, (OXA); palmitic acid, (PA); reactive oxygen species, (ROS); high fat diet, (HFD); B cell lymphoma 2, (Bcl-2); B cell lymphoma 2 associated X protein, (Bax); orexin receptor 1, (OX1R); orexin receptor 2, (OX2R); dual orexin receptor antagonist, (DORA).

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stress (Butterick et al., 2012). Because HFDs are known to induce oxidative stress and neuronal cell death, (Moraes et al., 2009; Wu et al., 2004) we sought to determine whether OXA protects against PA-induced cell death. To test this, we evaluated the response of an immortalized murine hypothalamic cell line (designated mHypoA-1/2) (Belsham et al., 2009) to OXA. We demonstrate here that OXA attenuates PA-induced apoptosis via reducing caspase-3/7 activity, increasing Akt activation, stabilizing expression of the pro-survival gene Bcl-2, and inhibiting ROS production. Finally, we show that OXA alters intracellular metabolic function in mHypoA-1/2 cells in real-time via increased basal respiration, maximum respiration, ATP production, and reserve capacity.

2. Methods

2.1. Cell culture and reagents

Differentiated immortalized adult mouse hypothalamic (mHypoA-1/2, cited elsewhere as CLU172; CELLutions-Cedarlane, Burlington ON CAN) (Belsham et al., 2009; Guo and Feng, 2012) were maintained in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad CA USA) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic cocktail (penicillin, streptomycin and neomycin) at 37 °C with 5% CO_2. The mHypoA-1/2 cells do not express endogenous orexin (Belsham et al., 2004), independently confirmed by our laboratory (data not shown). Orexin A peptide (ThermoFisher Scientific, Waltham MA USA) was suspended in phosphate buffered saline (PBS; Invitrogen) and diluted to a final concentration of 300 nM in DMEM. Palmitic acid (Sigma-Aldrich, St Louis, MO USA) was suspended in dimethyl sulfoxide (DMSO) and diluted to a final concentration 0.1 mM in DMEM. Dual orexin receptor antagonist (DORA; TCS 1102; Tocris Bioscience, Avonmouth GBR) was reconstituted in DMSO and diluted to a final concentration of 1.16 nM in DMEM (Callander et al., 2013). The dual orexin receptor antagonist used in this study binds similarly to both OX1R and OX2R rapidly with high affinity (Callander et al., 2013).

2.2. Treatments

In general for treatment assays, cells underwent two steps: First, a 24 h pretreatment with a test substance (e.g. orexin or vehicle); and second, a 2 to 24 h challenge with palmitic acid or vehicle, with or without the test substance used for 24 h pretreatment. Specifically, for cell viability assays, neurons were treated with DORA or DMSO vehicle for 20 min to block orexin receptors, subjected to a 24 h treatment with OXA or PBS vehicle, and finally challenged with vehicle (PBS + DMSO), PA, DORA, or OXA for 24 h. For the caspase-3/7, ROS, and gene expression experiments, cells underwent 24 h pretreatment with OXA or PBS vehicle, followed by 2 h challenge with PA or DMSO vehicle in the presence of OXA or PBS vehicle. For the mitochondrial respiration assays without PA challenge, cells were exposed to vehicle (PBS + DMSO), OXA (50, 150, or 300 nM) or DORA for 2 h. For the mitochondrial assay with PA challenge, cells were first pretreated with OXA (300 nM) or vehicle control (PBS) for 24 h followed by a 6 h challenge with vehicle (PBS + DMSO), PA, or OXA. All concentrations and time points were based on previous studies (Butterick et al., 2014, 2012; Callander et al., 2013; Xu et al., 2015).

2.3. Cell viability assay

Cell survival was determined using a resazurin-based assay (Presto Blue, Invitrogen) as previously described (Butterick et al., 2012). Viable cells produce a fluorescent signal ($560_{EX}/590_{EM}$) as determined using a spectrophotometer (SpectraMax-M5; Molecular Devices, Sunnyvale CA USA). Data are reported as percent relative fluorescence units (RFU) change vs. control.

2.4. Caspase activity

Caspase 3/7 activity was determined as previously described (Butterick et al., 2014, 2012). Briefly, caspase-3/7 activity was determined by the addition of a luminogenic caspase substrate DEVD based assay (Caspase-Glo 3/7, Promega, Madison WI USA). Changes in relative luminance units (RLU; 650 nm) were analyzed using a microplate spectrometer reader (SpectraMax-M5). Data are presented as fold increase in caspase activity normalized to cell number.

2.5. Reactive oxygen species assay

Intracellular ROS (superoxide and hydroxyl radical) production was determined using a commercially available deep red fluorescence kit following manufacturer's protocol (Abcam, Cambridge GBR). Cells were pretreated with OXA (or vehicle) for 24 h, then challenged with PA (or vehicle) plus OXA (or vehicle) for 2 h. During the last hour of challenge, the cell-permeable deep red dye was added to cells and allowed to incubate at 37 °C and 5% CO_2. Intracellular superoxide and hydroxyl radicals oxidize the deep red dye producing a fluorescent signal (Gliyazova et al., 2013). Fluorescence was measured at $650_{EX}/675_{EM}$ using a spectrophotometer (SpectraMax-M5). Data are presented as fold change vs. control.

2.6. Real time RT-PCR

Total RNA was extracted from cultured mHypoA-1/2 cells with the aid of Trizol reagent (Invitrogen). A final concentration of mRNA (100 ng/μl) was determined using 260 and 280 nm readings on a spectrophotometer (Nanodrop ND-8000; ThermoFisher). Primers were designed using MacVector 12 (MacVector Inc, Cary NC USA) and sequences are listed in Table 1. Relative expression of target genes was determined with SYBR Green using the $2^{-\Delta\Delta CT}$ method, normalized to GAPDH (Livak and Schmittgen, 2001).

2.7. Cell-based enzyme-linked immunosorbent assay (ELISA)

Changes in Akt activation were determined using a commercially available kit (R&D Systems, Minneapolis, MN). Briefly, cells were pretreated with OXA (or vehicle) for 24 h and challenged with PA (or vehicle) and an additional dose of OXA (or vehicle) for 1 h. Time point was based on Sokolowska et al., 2014. Cells were fixed with 4% formaldehyde, blocked, and incubated with primary antibodies (anti-phospho-Akt (S473) and anti-total Akt) overnight. Unbound primary antibodies were washed away and secondary antibodies (HRP-conjugated or AP-conjugated) were added. Unbound secondary antibodies were washed away and fluorogenic substrates were added.

Table 1
Real-time qPCR primer sequences.

Target and accession number	Forward primer (5′ to 3′)	Reverse primer (5′ to 3′)
Bcl-2 (NM_177410.2)	CACCGCGAGGGGACGCTTTG	AGGTCGCATGCTGGGGCCATA
Bax (NM_007527.3)	GCTGAGCGAGTGTCTCCGGC	ACGCGGCCCCAGTTGAAGTT
GAPDH (NM_017008)	GACATCAAGAAGGTGGTGAAGCAG	AAGGTGGAAGAGTGGGAGTTGC

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