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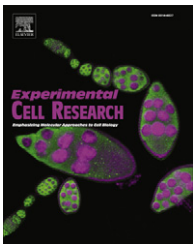

OX2R activation induces PKC-mediated ERK and CREB phosphorylation

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ABSTRACT

Deficiencies in brain orexins and components of mitogen activated protein kinase (MAPK) signaling pathway have been reported in either human depression or animal model of depression. Brain administration of orexins affects behaviors toward improvement of depressive symptoms. However, the documentation of endogenous linkage between orexin receptor activation and MAPK signaling pathway remains to be insufficient. In this study, we report the effects of orexin 2 receptor (OX2R) activation on cell signaling in CHO cells over-expressing OX2R and in mouse hypothalamus cell line CLU172. Short-term extracellular signal-regulated kinase (ERK) phosphorylation and long-term cyclic adenosine monophosphate (cAMP) response element binding protein (CREB) phosphorylation were subsequently observed in CHO cells that over-express OX2R while 20 min of ERK phosphorylation was significantly detected in mouse adult hypothalamus neuron cell line CLU172. Orexin A, which can also activate OX2R, mediated ERK phosphorylation was as the same as orexin B in CHO cells. A MAPK inhibitor eliminated ERK phosphorylation but not CREB phosphorylation in CHO cells. Also, ERK and CREB phosphorylation was not mediated by protein kinase A (PKA) or calmodulin kinase (CaMK). However, inhibition of protein kinase C (PKC) by GF 109203X eliminated the phosphorylation of ERK and CREB in CHO cells. A significant decrease in ERK and CREB phosphorylation was observed with 1 μM GF 109203X pre-treatment indicating that the conventional and novel isoforms of PKC are responsible for CREB phosphorylation after OX2R activation. In contrast, ERK phosphorylation induced by orexin B in CLU172 cells cannot be inhibited by 1 μM of protein kinase C inhibitor.

From above observation we conclude that OX2R activation by orexin B induces ERK and CREB phosphorylation and orexin A played the same role as orexin B. Several isoforms of PKC may be involved in prolonged CREB phosphorylation. Orexin B induced ERK phosphorylation in mouse hypothalamus neuron cells differs from CHO cell line and cannot be inhibited by PKC inhibitor GF 109203X. And hypothalamus neuron cells may use different downsteam pathway for orexin B induced ERK phosphorylation. This result supports findings that orexins might have anti-depressive roles.

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Introduction

Orexins, including orexin A and orexin B (also called hypocretin 1 and hypocretin 2), are peptides found in the hypothalamus that contain 33 and 28 amino acids [1]. While the orexinergic neuronal system is important in wake/sleep regulation [2–4], eating behavior [5], energy metabolism [6,7], and the pathology of narcolepsy [8–11], evidence also implicates the orexinergic system in the pathology of depression. Our previous studies showed a significant reduction of orexins in a rat model of depression at the younger stage and an increase of orexins in mature rats [12]. Allard et al. reported that the number and size of orexin A-immunostained neurons were decreased in a genetic rat model of depression [13]. Orexin 2 receptor (OX2R) knockout mice displayed an increase in behavioral despair [14]. Furthermore, decreased levels of orexin were found in the cerebrospinal fluid of human patients diagnosed with depression [15,16]. Additionally, intracerebroventricular administration of orexin A induced an antidepressive effect in a rodent model [17]. Evidence that orexins promote wakefulness and that orexin levels are lower in human depression and animal models of depression provide biological support to the findings that sleep deprivation improves the symptoms of depression [18–23].

Orexins activate MAPK signaling pathway [24], which has been demonstrated as deficiency in suicide victim of human depression [25] and animal model of depression [26]. Both total extracellular signal-regulated kinases (ERK) and phosphorylated ERK (p-ERK) are significantly decreased in the frontal cortex and hippocampus in a rat model of depression compared to the controls [26]. In human studies, samples from victims of depression-related suicide had impaired signaling pathways, including significantly decreased phosphorylation of cyclic adenosine monophosphate (cAMP) response element binding protein (CREB) [27,28]. Also, antidepressive treatment increased the expression of CREB [29–31]. This evidence prompted interest in how orexins affect cell signaling.

Like most of GPCR signaling, orexin binds on orexin receptors to activate G proteins [32,33]. Orexin A and orexin B induces a phospholipase C (PLC)-mediated release of calcium from intracellular storages in different cell lines over expressing orexin 1 receptor (OX1R) and OX2R [32,34]. The OX1R linked to the influx of Ca^{2+} may go through receptor-operated calcium channels and conventional phospholipase C (PLC)-Ca^{2+} release-store-operated Ca^{2+} channel (SOC) pathways [35] and involves diacylglycerol-activated transient receptor potential canonical (TRPC) channels in neuronal cells [36]. Orexins had different effects on cAMP in different type of sources. Orexins may not stimulate cAMP accumulation [34] or inhibit the PACAP-induced increase in the cAMP level in PC12 cells [37]. Conversely, orexin significantly increased the cAMP level in human adrencortical cells [38]. Orexins induce a rapid, dose and time dependent increase in activation of ERK1/2 and p38 MAPK in variable type of cell lines and tissues [24,39]. This activation is through multiple G-proteins and different intracellular signaling pathways. ERK1/2 activation involves Gq/PLC/protein kinase C (PKC), but not PKA [40]. Inhibition of PKC and, in part, PKA prevents orexin induced ERK1/2 phosphorylation [41].

Although evidence has demonstrated that orexin induces ERK phosphorylation, how orexin induced ERK phosphorylation link to CREB phosphorylation is not known. Furthermore, systemic information regarding orexin induced cell signaling remains to be demonstrated. In the following, we report the result regarding orexins induced cell signaling in a CHO cell line over expressing OX2R and in mouse hypothalamus neuron cell line.

Material and methods

Materials

[Ala11, D-Leu15]-Orexin B (OB_{DL}), orexin B and orexin A (Cat# 2142, 1457 and 1455) was purchased from TOCRIS Bioscience (Missouri, USA). The HCRTR2-G_{α}15-NFAT-*bla* CHO-K1 (CHO-OX2R) cell line was purchased from Invitrogen (Cat# K1246, California, USA). Mouse hypothalamus neuron cell line CLU172 was purchased from Cellutions Biosystems Inc. Antibodies were purchased from sources as shown in the figure legends. The thawing media used for cell restoration was 10% dialyzed FBS-DMEM supplemented with penicillin (100 U/mL), streptomycin (100 μg/mL), non-essential amino acids (0.1 mM), and HEPES buffer (25 mM). The cell growth medium was 10% dialyzed FBS-DMEM supplemented with penicillin (100 U/mL), streptomycin (100 μg/mL), zeocin (100 μg/mL), blasticidin (5 μg/mL), hygromycin (600 μg/mL), non-essential amino acids (0.1 mM), and HEPES buffer (25 mM). The assay medium was 1% dialyzed FBS-DMEM supplemented with penicillin (100 U/mL), streptomycin (100 μg/mL), non-essential amino acids (0.1 mM), and HEPES buffer (25 mM). The FACE assay kit was purchased from Active Motif (California, USA). SuperSignal West Femto reagent was purchased from Pierce Biotechnology Inc (Cat# 34096, Illinois, USA).

Cell culture

The CHO-OX2R cell line was restored with thawing medium for 2–3 days. Once the cells began growing, they were passaged with growth medium twice a week. For the Fast-Activation Cell-based ELISA (FACE) assay, 30,000 CHO-OX2R cells were seeded into 96-well plates. For Western blotting, 500,000 cells were seeded into 6-well plates for treatment with protein kinase inhibitors and orexin B as indicated. Mouse hypothalamus neuron cell line CLU172 was restored and cultured in DMEM medium with 10% FBS. 500,000 CLU172 cells were seeded into 6-well plates for orexin B treatment and protein kinase C inhibitor treatment.

Immunofluorescence staining of monolayer cells

Phosphorylation of ERK and expression of OX2R were observed using immunofluorescence staining [42]. About 300,000 to 500,000 CHO-OX2R cells were seeded into 35-mm plates containing a cover slip and allowed to adhere overnight. After treatment, cells were fixed with 100% ice-cold methanol at room temperature for 15 min. The cells were rinsed twice with PBS for 5 min. For OX2R staining, the cells were blocked with 1% BSA-1% horse serum-PBS at room temperature for 1 h. For p-ERK staining, the cells were blocked with 5% non-fat milk-PBS at room temperature for 1 h. After blocking, the cells were rinsed twice for 5 min and incubated with primary antibody diluted in 1% BSA-PBS at 4 °C overnight. Secondary antibody incubation was