Effect of orexin A on apoptosis in BGC-823 gastric cancer cells via OX1R through the AKT signaling pathway

JING WEN1, YUYAN ZHAO1, YANG SHEN1 and LEI GUO2

Departments of 1Endocrinology and 2Orthopedic Surgery, First Affiliated Hospital, China Medical University, Shenyang, Liaoning 110001, P.R. China

Received January 11, 2014; Accepted July 22, 2014

DOI: 10.3892/mmr.2015.3190

Abstract. Orexins are a class of peptides involved in the regulation of food intake, energy homeostasis, the sleep-wake cycle and gastrointestinal function. Recent studies have demonstrated that orexin A may influence apoptosis and proliferation in numerous types of cancer cells. However, the effect of orexin A on gastric cancer cells and its mechanisms of action remain elusive. In the present study, BGC-823 gastric cancer cells were treated with orexin A (10⁻¹⁰-10⁻⁶ M) in vitro and the expression levels of orexin receptor 1 (OX1R) protein in cells was then determined. The proliferation, viability and apoptosis of BGC-823 cells were detected. In addition, BGC-823 cells were treated with AKT inhibitor PF-04691502 or OX1R-specific antagonist SB334867 in combination with orexin A, in order to examine the activation of AKT and caspase-3. The results showed that orexin A (10⁻¹⁰-10⁻⁶ M) stimulated the OX1R protein expression in BGC-823 cells, which improved the proliferation and viability of the cells as well as protected them from apoptosis. Phosphorylated AKT protein was significantly increased in BGC-823 cells following treatment with orexin A. Moreover, 10⁻⁶ M orexin A reduced the proapoptotic activity of caspase-3 (by ≤30%). The OX1R antagonist SB334867 (10⁻⁶ M) and AKT antagonist PF-04691502 (10⁻⁶ M), when used individually or in combination, abolished the effect of orexin A (10⁻⁶ M) on BGC-823 cells. In conclusion, the results of the present study demonstrated that orexin A inhibited gastric cancer cell apoptosis via OX1R through the AKT signaling pathway.

Introduction

Orexin A and B are neuropeptides derived from the proteolytic cleavage of a common 130-amino acid precursor peptide prepro-orexin. In humans their amino acids are 46% identical (1). These peptides act via two closely associated G-protein coupled receptors, the orexin receptors 1 (OX1R) and 2 (OX2R) for orexins (1,2). OX1R appears to be highly selective for orexin A, whereas OX2R binds orexin A and B with similar affinity (3). Orexins are involved in the regulation of numerous body functions, including food intake (4), the sleep-wake cycle (4), breathing (5), the reward system (4,6) and drug addiction (6,7). Orexin-producing neurons are present in the dorsal and lateral hypothalamus, where they project to and excite numerous structures of the brain (8). It has been reported that orexins are not restricted to the hypothalamus, but may also be expressed in peripheral tissues, including adrenals, gastrointestinal tract and endocrine pancreas (9).

It has been reported that orexins may also have a role in the proliferation of certain types of cancer cells (10). For example, orexins induced apoptosis in human colon cancer cell lines, which greatly reduced cell growth. This effect was observed in human neuroblastoma cells (10) and rat pancreatic tumor cells (11). However, orexin A stimulated cell proliferation in adrenal gland tumor cells, the effects of which were more remarkable in cultured adenomatous cells than in normal adrenocortical cells (12). In addition, orexin A had no effect on proliferation of rat C6 glioma cells (13). These studies added a novel dimension to the biological functions of orexins. However, to the best of our knowledge, the effects of orexin A on proliferation and apoptosis have not been demonstrated in gastric cancer cells.

Numerous studies have demonstrated that disorders of the phosphatidylinositol 3-kinase (PI3K)/Akt/mechanistic target of rapamycin (mTOR) signaling pathway are associated with the process of proliferation and apoptosis in various tumor cells (14-18). Phosphorylation of Akt occurs primarily in gastric cancer cells and activation of these cells can prolong their survival and increase proliferation, thereby promoting tumor development and angiogenesis (19). Moreover, phosphorylated Akt exerts anti-apoptotic effects through the phosphorylation of B-cell lymphoma-associated death promoter (Bad) (20) and caspase-9 (21). Therefore, Akt may be involved in the regulation of gastric cancer cell survival and apoptosis.

In the present study, BGC-823 gastric cancer cell apoptosis and proliferation assays were performed to evaluate the effect of orexin A on gastric cancer cell growth. Furthermore,
cell death levels and caspase-3 activation were examined in order to determine the protective effect of orexin A against apoptosis. In addition, in order to verify the involvement of the PKB/AKT pathway, the activation of phosphorylated AKT and total AKT were examined following the treatment of cells with a series of concentrations of exogenous orexin A and associated inhibitors. The results of the present study provided evidence for the functional role of orexin A in gastric cancer cells via OX1R-stimulated AKT signaling pathway.

Materials and methods

Reagents. The orexin A and caspase-3 assay kits were obtained from Sigma (St. Louis, MO, USA). RPMI 1640 medium and fetal bovine serum (FBS) were purchased from Gibco-BRL (Grand Island, NY, USA). The AKT inhibitor PF-04691502 was purchased from Selleck (Houston, TX, USA). OX1R-specific antagonist SB334867 was obtained from Tocris (Minneapolis, MA, USA). The Cell Death Detection ELISA kit and Cell Proliferation ELISA bromodeoxyuridine (BrdU) colorimetric kit were purchased from Roche Diagnostics (Penzberg, Germany). Total/phospho-AKT (s473) polyclonal antibodies, β-actin (c4): sc-47778 mouse monoclonal antibody was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Total/phospho-AKT (s473) rabbit polyclonal antibodies and the OX1R rabbit polyclonal antibody were obtained from Abcam (Cambridge, UK).

Cell culture. Human BGC-823 gastric cancer cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and maintained in RPMI 1640 medium supplemented with 10% (wt/vol) FBS, L-glutamine (2 mM), penicillin (50 µg/ml) and streptomycin (100 µg/ml) (Xianfeng, Shanghai, China). The cells were grown in a humidified atmosphere containing 5% CO₂ at 37°C. Prior to an experiment, cells were grown in petri dishes in serum-free medium for 24 h. The next day, cells were treated with different concentrations of orexin A (0, 10⁻⁸, 10⁻⁷ and 10⁻⁶ M), or 10⁻⁸ M orexin A with either SB334867, PF-04691502 or their combination for 20 min.

Cell proliferation assays. BGC-823 gastric cancer cells were seeded (2x10⁵ cells/well) in 96-well plates and cultured for 24 h. To synchronize cell cycles, cells were serum-deprived for 24 h and then treated with various concentrations (0, 10⁻⁸, 10⁻⁷ and 10⁻⁶M) of orexin A or 10⁻⁸ M orexin A along with 10⁻⁸ M OX1R antagonist SB334867 for a further 24 h. BrdU solution (10⁻⁶ M) was then added and cells were incubated for 2.5 h. BrdU incorporation into DNA was measured using the Cell Proliferation ELISA BrdU colorimetric kit (Roche Diagnostics).

Cell viability. BGC-823 gastric cancer cells were seeded (2x10⁵ cells/well) in 96-well plates and cultured for 24 h. Following incubation in a serum free RPMI 1640 medium supplemented with test agents for 48 h, MTT (Sigma) solution (0.5 mg/ml) was added. After 3 h, the culture medium was removed and the formazan crystals that formed were dissolved in 100 µl dimethyl sulfoxide (Merck KGaA, Darmstadt, Germany). Optical density was measured using a plate reader (SpectraMax Plus384 microplate reader; Molecular Devices, Ismaning, Germany) at 570 and 650 nm (reference wave/length).

Annexin V/propidium iodide (PI) assays for apoptosis. Apoptotic cells were quantified using the Annexin V/PI Apoptosis Detection kit and evaluated for apoptosis by BD Accuri™ C6 Flow Cytometer according to the manufacturer's instructions (BD Pharmingen, San Diego, CA, USA). Cells were treated with different concentrations of orexin A in the absence of serum for 48 h. 1x10⁵ cells were briefly washed twice with phosphate-buffered saline, then stained with 5 µl Annexin V-fluorescein isothiocyanate (FITC) and 10 µl PI in 500 µl binding buffer for 15 min at room temperature in the dark. Apoptosis was determined by counting the number of cells stained by FITC-labeled Annexin V by fluorescence-activated cell sorting analysis. Early apoptotic cells were identified as PI-negative and FITC-Annexin V positive; cells that were in late apoptosis or already dead were positive for PI as well as FITC-Annexin V.

Activity of caspase-3 in BGC-823 cells. BGC-823 cells were cultured in serum-free medium using six-well plates (1.5x10⁵ cells/well). Culture medium was then replaced with fresh culture medium with or without orexins. After 24 h, caspase-3 activity was assessed using a Caspase-3 Colorimetric Assay kit following the manufacturer's instructions (Sigma).

Polymerase chain reaction (PCR). Total RNA was extracted from BGC-823 cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The expression of OX1R and OX2R messenger RNA (mRNA) was detected using PCR and TaqMan reagents (Takara, Otsu, Japan). The following specific primers were used: OX1R forward, 5'-TGC GGC CAA CCC TAT CAT CTA-3' and reverse, 5'-ACC GGC TCT GCA AGG ACA A-3'; OX2R forward, 5'-ATG GTA GTA GTG GTA-3' and reverse, 5'-ATG GCA TTC ACA GAG GTA GTA-3'. The PCR reactions were performed using the following conditions: 95°C for 30 sec, then 40 cycles of 95°C for 5 sec, 60°C for 30 sec and 95°C for 15 sec. All primers and TaqMan probes specific to OX1R, OX2R and GAPDH were designed using Primer Premier 5.0 software (Premier Biosoft International, Palo Alto, CA, USA).

Protein preparations and western blot analysis. BGC-823 cells were washed with cold PBS and harvested in radioimmunoprecipitation assay buffer (Beyotime Biotechnology, Jiangsu, China) containing protease inhibitors like phenylmethylsulfonyl fluoride (Beijing, Jiangsu, China) and phosphatase inhibitors (KeyGEN Biotech Co., Ltd., Nanjing, China). Cell lysates were incubated on ice for 30 min, then collected and centrifuged at 12,000 x g for 10 min at 4°C. The supernatants were collected, mixed with 5X loading buffer and then denatured by boiling for 10 min. Samples were separated by SDS-PAGE and transferred to polyvinylidene fluoride membranes at 60 V for 2.5 h in a transfer buffer containing 20 mM Tris (bioWORLD,
Dublin, OH, USA), 150 mM glycine (Solarbio, Beijing, China) and 20% methanol (Xinxing, Liaoning, China). Membranes were then incubated with a primary antibody against OX1R at a 1:250 dilution or phospho/total-AKT at a 1:1,000 dilution in TBST overnight at 4˚C. The membranes were washed and incubated with horseradish peroxidase-conjugated anti-species secondary antibody for 1.5 h at room temperature, and then washed three times with TBST for 30 min. Protein were visualized by BeyoECL (Beyotime Biotechnology, Jiangsu, China). Band densities were measured using Quantity-One V4.6.2 software (Bio-Rad, Hercules, CA, USA).

Statistical analysis. Values are expressed as the mean ± standard error of the mean and differences between the means were analyzed by one-way analysis of variance. P<0.05 was considered to indicate a statistically significant difference between values. Statistical analysis was performed using the SPSS 15.0 software package (SPSS Inc., Chicago, IL, USA).

Results

Orexin A stimulates OX1R protein expression in BGC-823 cells. BGC-823 cells were cultured for 24 h at 37˚C and treated with orexin A at concentrations of 0, 10⁻⁸, 10⁻⁶ and 10⁻⁴ M, for 24 h. PCR assays demonstrated that OX1R mRNA was expressed in BGC-823 cells (Fig. 1A). However, OX2R mRNA was not detected under identical conditions (data not shown). Treatment with Orexin A caused a dose-dependent increase of OX1R protein expression in BGC-823 cells with 10⁻⁶ M being the most potent concentration of orexin A (~2.1-fold increase) (Fig. 1B). Orexin A (10⁻⁸ M) in the presence of OX1Ri (10⁻⁶ M) significantly inhibited the expression of OX1R protein (Fig. 1B).

Effects of orexin A on proliferation and viability of BGC-823 cells. To determine the effects of orexin A on cell survival, BGC-823 cells were stimulated with orexin A at concentrations (0, 10⁻⁸, 10⁻⁶ and 10⁻¹⁰ M) in combination with OX1R antagonist SB334867 (10⁻⁶ M). The cells were serum-starved for 24 h prior to exposure to the tested compounds in order to avoid interactions with growth factors and other mediators present in serum. An MTT assay showed that orexin A, at all the tested concentrations, significantly promoted the proliferation and viability of BGC-823 cells (P<0.05; Fig. 2). The present study determined that treatment with 10⁻⁸ M orexin A increased cell proliferation and viability by 1.5-fold compared with that of the control. The proliferation and viability of the group of cells treated with orexin A (10⁻⁸ M) and OX1R-specific antagonist SB334867 were not significantly higher than those of the control. This therefore indicated that...
SB334867 significantly inhibited the proliferation and viability of BGC-823 cells in comparison with the cells exposed to orexin A (10⁻⁸ M) at an identical concentration (Fig. 2).

Orexin A protects BGC-823 cells from apoptosis. Orexin A treatment (10⁻⁸, 10⁻⁴ and 10⁻⁶ M) resulted in a decreased apoptotic index, measured using the Cell Death Detection ELISA kit. Concentrations of 10⁻⁸ and 10⁻⁶ M orexin A resulted in a significant decrease in the apoptotic rate of H295R cells compared to that of the control (P<0.05) (Fig. 3); however, orexin A (10⁻⁸M) failed to protect cells against apoptosis in the presence of 10⁻⁶ M SB334867 (Fig. 3).

Orexin A enhances proliferation of BGC-823 cells via OX1R-evoked AKT signaling pathway. It is known that the PI3K/AKT signaling pathway is involved in cell survival and apoptotic signaling; therefore, the present studies investigated whether orexin A-stimulation of BGC-823 cells induced activation of AKT (22,23). The data confirmed a 1.5-fold increase of p-AKT protein in BGC-823 cells treated with 10⁻⁴ M orexin A, compared to that of the untreated control cells (P<0.05) (Fig. 4). However, the total AKT levels remained unaffected by the aforementioned treatment. Moreover, 10⁻⁶ M AKT antagonist PF-04691502 and 10⁻⁶ M OX1R antagonist SB334867 abolished the relative increase in AKT activation in response to orexin A, independently and in combination (Fig. 4).

Effects of orexin A on proliferation and viability of BGC-823 cells through the AKT signaling pathway. In order to confirm the involvement of the AKT signaling pathway in orexin A-mediated proliferation and viability in BGC-823 cells, cell survival rates were determined using BrdU and MTT analysis. 10⁻⁶ M orexin A significantly promoted the proliferation and viability of BGC-823 cells (P<0.05) (Fig. 5). However, these effects were blocked with AKT antagonist (PF-04691502), OX1R antagonist (SB334867) or their combination (Fig. 5). Moreover, the proliferation and viability were not changed significantly when treated with AKT antagonist or OX1R antagonist in the absence of orexin A co-treatment (Fig. 5). The data suggested that AKT participated in orexin A-induced stimulation of proliferation and viability of BGC-823 cells.

Effects of orexin A on caspase-3 activity in BGC-823 cells. To determine whether the activation of the caspase pathway was affected by orexin A, leading to the protection of BGC-823 cells from apoptosis, caspase-3 activity was measured. Treatment of BGC-823 cells with 10⁻⁴ M orexin A significantly decreased caspase-3 activity (30% below that of the control) (Fig. 6). This effect was reversed in the presence of PF-04691502 (10⁻⁶ M), SB334867 (10⁻⁶ M) and a combination of the inhibitors (Fig. 6). These results indicated that apoptosis induced by orexin A was mediated, at least in part, through caspase-3.

Discussion

The present study demonstrated, for the first time, to the best of our knowledge, that OX1R was expressed at mRNA and protein levels in BGC-823 gastric cancer cells. In order to explore the potential role of orexins in BGC-823 gastric...
cancer cells, the effects of orexin A on BGC-823 cell proliferation and apoptosis were examined. Orexin A stimulated the proliferation and viability of BGC-823 gastric cancer cells and protected them from apoptosis via the AKT signaling pathway. Evidence suggested that the effects of orexin A on proliferation and apoptosis may vary dependent on the type of cancer cells (10-13). For example, orexin A suppressed cell growth by inducing apoptosis in human colon cancer, neuroblastoma cells and rat pancreatic tumor cells (10,11). However, orexin A has also been reported to stimulate cell proliferation in adrenal gland tumor cells; the effects were more marked in cultured adenomatus than those in normal adrenocortical cells (12). In addition, orexin A had no effect on proliferation of rat C6 glioma cells, as assessed using a (3H) thymidine incorporation assay (13). PI3K/PKB activators stimulated cell proliferation and viability in 3T3-L1 cells (24,25). However, a more recent study showed that PI3K/PKB was not essential for orexin A-induced stimulation of proliferation and viability in 3T3-L1 preadipocytes (26). The mechanisms by which orexin A has opposing effects on apoptosis in different cancer cell types remains to be elucidated. One possible explanation may be that cells have different intrinsic sensitivities to cytochrome c. The differential sensitivity to cytochrome c may be due to high levels of apoptotic protease activating factor 1 (Apaf-1) in tumor tissues, such as neuroblastoma, in comparison with low levels of Apaf-1 in the adjacent brain tissue (27). Cytochrome c, once released from the mitochondria, binds to Apaf-1, leading to the formation of the apoptosome and the recruitment of procaspase-9. Activated caspase-9 activates caspase-3 and caspase-7, thereby promoting cell death (28). Another possible explanation of what determines the influence of orexins on cell apoptosis may be the activation of mitogen-activated protein kinase (MAPK) signaling pathways. Studies have reported the expression of stable OX1R in human embryonic kidney-293 and Chinese hamster ovary cells. These studies have shown that orexins can exert convergent effects on cell apoptosis through activation of the classical MAPK signaling pathways (29,30). The extracellular signal-regulated kinase 1/2 pathway was shown to protect against apoptosis, whereas p38 was a key promoter of cell death (29,30). In addition, it has also been reported that activation of OX1R resulted in mobilization of intracellular calcium through a Gq-dependent mechanism (31). Although increases of cytosolic calcium are well known to occur during cell apoptosis, this does not provide sufficient evidence to explain the proapoptotic effect of orexins (32). Numerous GPCRs in human colon cancer cells are known to promote intracellular Ca\(^{2+}\) mobilization (33-35). These receptors include NT1 receptors for neurotensin (33), protease-activated receptors 1 for thrombin (34), protease-activated receptors 2 for trypsin (35) or muscarinic M3 receptors for acetylcholine (36). These receptors do not trigger apoptosis but conversely stimulate cell proliferation.

In the present study, orexin A, at all tested concentrations, significantly promoted the proliferation and viability of BGC-823 gastric cancer cells. In addition, the effects of orexin A on proliferation and apoptosis in BGC-823 gastric cancer cells were blocked by AKT-specific inhibitors. This suggested the involvement of the activated PI3K/AKT signaling pathway in gastric cell proliferation and apoptosis induced by orexin A. The PI3K pathway has an important role in cell growth, proliferation, survival and apoptosis. Abnormal cell signaling via this pathway occurs in diverse types of cancer (37,38). PI3K is activated by both receptor tyrosine kinases and Ras, and in turn activates multiple downstream signaling pathways. The AKT family, a multifunctional serine-threonine protein kinase, is a major downstream signaling molecule of PI3K and a critical target of PI3K in human cancer. PI3K/AKT signaling has been shown to be activated in various types of cancer. Activated AKT phosphorylates Bad and caspase-9 and the activated nuclear factor kappa-light-chain-enhancer of activated B cells pathway may promote resistance to apoptosis in cancer cells (39-43). In the present study, it was demonstrated that BGC-823 gastric cancer cells treated with orexin A had lower rates of apoptosis than the control treated cells. Orexin A treatment caused a significant decrease in caspase-3 activity in BGC-823 cells. Caspase-3 is a key molecule involved in the execution of apoptosis and acts downstream in the apoptotic cascade (44). Although not documented in the present study, other caspase pathways can be studied in the future. It is necessary to investigate whether caspases are involved in the extrinsic (receptor-mediated) pathway of apoptosis. Furthermore, by employing the AKT-specific inhibitor PF-04691502, the present study demonstrated that orexin A inhibited apoptosis and regulated apoptosis-associated proteins in BGC-823 gastric cancer cells via AKT signaling pathways. Overall, the results of the present study suggested that orexin A promoted the activation of the PI3K/AKT pathway to inhibit the apoptosis of gastric cancer cells. However, further studies are required to clarify the mechanism by which orexin A modulates activation of PI3K/AKT and other crucial signaling pathways for cancer cell survival and chemoresistance.

In conclusion, the present study provided the first evidence for the presence of orexin receptors in BGC-823 gastric cancer cells. Furthermore, the study showed that orexin A regulates BGC-823 gastric cancer cell proliferation and survival, reduced pro-apoptotic caspase-3 activity, and protected against apoptotic death via OX1R through the AKT signaling pathway.

Acknowledgements

The authors would like to thank The China Medical University Affiliated Hospital Laboratory Center for kindly providing the equipment. This study was supported by the National Natural Science Foundation of China (grant nos. 30872724, 81071460 and 81271996) and the Natural Science Foundation of Liaoning Province (grant no. 201202292).

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