Abstract. Numerous studies have demonstrated the ability of orexin-A to regulate adrenocortical cells through the mitogen-activated protein kinase signaling pathway. In the present study, human H295R adrenocortical cells were exposed to orexin-A (10^{-10} - 10^{-6} M), with orexin receptor type 1 (OX_{1} receptor) antagonist SB334867 or AKT antagonist PF-04691502. It was found that orexin-A stimulated H295R cell proliferation, reduced the pro-apoptotic activity of caspase-3 to protect against apoptotic cell death and increased cortisol secretion. Furthermore, phospho-AKT protein was increased by orexin-A. SB334867 (10^{-6} M) and PF-04691502 (10^{-6} M) abolished the effects of orexin-A (10^{-6} M). These results suggested that the orexin-A/OX_{1} receptor axis has a significant pro-survival function in adrenal cells, which is mediated by AKT activation. Further studies investigating the effects of orexin-A upregulation may further elucidate the diverse biological effects of orexin-A in adrenal cells.

Introduction

Orexin-A and -B, also known as hypocretin-1 and -2, are encoded by a single gene and are derived from a common pre-propeptide (1,2). The orexins are hypothalamic peptides implicated in sleep regulation, wakefulness, neuroendocrine homeostasis and feeding (3). More recent studies have demonstrated that the expression of the orexins is not restricted to the hypothalamus only, but that they are also expressed in peripheral tissues, including adrenal glands, the gastrointestinal tract and the pancreas (2).

Orexin-A regulates cell apoptosis in human H295R adrenocortical cells via orexin receptor type 1 through the AKT signaling pathway

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The activities of orexins are mediated by two membrane-bound G-protein-coupled receptors, orexin receptor type 1 (OX_{1} receptor) and -2 (OX_{2} receptor), which are found in the central nervous system as well as in peripheral organs, including the hypothalamus, adrenal glands, the gastrointestinal tract and the pancreas (3-7). It has been recently shown that the activated OX_{1} receptor may exist in a homodimeric form (8). Orexin-A is a high-affinity agonist of the OX_{1} receptor, whereas orexin-B has significantly lower affinity for the OX_{1} receptor. Previous studies have shown that orexin-A stimulates the proliferation, contributes to the viability and protects against apoptotic cell death in 3T3-L1 fibroblasts and adrenocortical cells (9,10). Of note, a recent study has demonstrated the ability of orexins to induce apoptosis in cancer cells in culture (11). OX_{1} receptor is expressed in cancer cells and was shown to be responsible for regulating the apoptotic effects of orexins (12,13). Thus, it has been proposed that pro-apoptotic activity is an intrinsic property of orexin receptors (14).

AKT, also known as protein kinase B (PKB), is a 56-kDa member of the AGC serine/threonine protein kinase family. AKT was first characterized for its function in regulating cell proliferation and survival, which may be due to the direct or indirect effects of AKT on a large number of cellular proteins. AKT is mainly regulated through the activation of the second messenger, phospholipid kinase phosphatidylinositol 3-kinase (PI3K). Abundant evidence indicated that AKT is a key regulator of multiple cell survival mechanisms (15). For example, AKT can contribute to the inactivation of the tumor suppressor p53 (promoter of apoptosis), in response to cellular stress, possibly by the phosphorylation of Mdm2, which is a direct regulator of p53 (16). The pro-apoptotic B-cell lymphoma-2 (Bcl-2) family member Bcl-2-associated death domain (17), as well as Forkhead box and cyclic adenosine monophosphate-response element binding protein transcription factors can be phosphorylated and inactivated by AKT (18,19). Furthermore, AKT phosphorylates and activates mammalian target of rapamycin (mTOR) in response to growth factors and oncogenes (20-22). AKT leads to cell cycle dysregulation and inhibition of pro-apoptotic pathways that are typical hallmarks of human tumors (23). As such, AKT has key roles in tumor cell survival (24), proliferation (25), growth (26), apoptosis (27),
migration (27) and polarity (28). Recent studies have shown that AKT might be a potential therapeutic target for innovative treatments of cancer, and some AKT inhibitors are now being tested in clinical trials in cancer patients (29,30).

To date, compelling evidence has indicated an interaction of the orexin system with the hypothalamus-pituitary-adrenal axis on a central as well as peripheral level (14). More recent studies have shown that orexins (10^{-6}-10^{-8} M), acting through orexin receptors, can regulate the viability and proliferation of adrenal cells (30,31). These effects can be mediated through multiple signaling pathways, including protein kinase A, protein kinase C, and mitogen-activated protein kinase (MAPK) cascade-dependent mechanisms (31,32). However, little is known regarding the ability of orexins to activate the PKB/AKT pathway in adrenal cells.

In the present study, human NCI-H295R cells were used as an adrenocortical cell model (33). A cell proliferation assay was performed to assess the effect of orexin-A on adrenocortical cell growth. Furthermore, the apoptotic rate and caspase-3 activation were examined to assess the effect of orexin-A on protecting against apoptosis. In addition, to ascertain the involvement of the PKB/AKT pathway, the present study examined the expression of total AKT and phosphorylated AKT after cells were treated with serial concentrations of orexin-A and inhibitors. The results provided evidence for a functional role of orexin-A in human adrenocortical cells via an OX_{1} receptor-stimulated AKT signaling pathway.

Materials and methods

Reagents. The orexin-A and caspase-3 colorimetric assay kits were obtained from Sigma-Aldrich (St Louis, MO, USA). RPMI 1640 medium and fetal bovine serum were purchased from Gibco Life Technologies (Carlsbad, CA, USA). The AKT inhibitor PF-04691502 was purchased from Selleck (Houston, TX, USA). The OX_{1} receptor-specific antagonist SB334867 was obtained from Tocris (Minneapolis, MA, USA). The cell Proliferation ELISA brodmodeoxyuridine (BrdU) colorimetric kit was purchased from Roche Diagnostics (Basel, Switzerland). Total-AKT rabbit polyclonal antibody (ab8805), phospho- (p)-AKT (T308+S473) rabbit polyclonal antibody (ab66134) and OX, receptor rabbit polyclonal antibody (ab68718) were obtained from Abcam (Cambridge, UK). β-Actin mouse monoclonal antibody (C4) (sc-47778) was obtained from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). The Cortisol Express ELISA kit was purchased from Alpco (Paris, France). The Annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit was obtained from BD Biosciences (Franklin Lakes, NJ, USA).

Cell culture. Human H295R adrenocortical cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and maintained in RPMI 1640 medium supplemented with 10% (wt/vol) fetal bovine serum (Gibco Life Technologies), L-glutamine, penicillin (50 µg/ml) and streptomycin (100 µg/ml; Xianfeng, Shanghai, China). The cells were grown in a humidified atmosphere containing 5% CO_{2} at 37°C. Prior to each experiment, cells were grown in petridishes in serum-free medium for 24 h. The next day, cells (4x10^{3} cells/well in 96-well plates or 5x10^{5} cells/well in six-well plates) were treated with various concentrations of orexin-A (0, 10^{-10}, 10^{-8} or 10^{-6} M; Sigma-Aldrich) or 10^{-6} M orexin-A with SB334867 and/or PF-04691502.

Cell proliferation assays. H295R adrenocortical cells were seeded (2x10^{3} cells/well) in 96-well plates and cultured for 24 h. To synchronize the cells, they were serum-deprived for 24 h and then treated with the respective test agents for a further 24 h. BrdU incorporation into DNA was measured by the Cell Proliferation ELISA BrdU colorimetric kit (Roche Diagnostics). The cells were incubated with BrdU fresh media at 37°C and 5% CO_{2} for 12 h and fixed with 200 µl of fixative/denaturing solution for 30 min at room temperature. The peroxidase-conjugated BrdU antibody was then added to each well followed by incubation for 1 h. After washing thoroughly with cold phosphate-buffered saline (PBS) three times, the bound peroxidase-conjugated BrdU antibody was quantified with peroxidase substrate tetramethylbenzidine. Finally, the BrdU absorbance was measured at 440 nm using an ELISA plate reader (BioTek Instruments, Winooski, VT, USA). A control without cells was used to measure the background absorbance of the medium and was subtracted from the results.

Annexin V/propidium iodide (PI) assays for apoptosis. For Annexin V/PI assays, H295R cells were stained with Annexin V-FITC and PI, and evaluated for apoptosis by flow cytometry according to the manufacturer’s instructions (BD Biosciences). Cells were treated with various concentrations of orexin-A in the absence of serum for 48 h. Briefly, 1x10^{5} cells were washed twice with PBS and stained with 5 µl Annexin V-FITC and 10 µl PI in 500 µl binding buffer for 15 min at room temperature in the dark. Quantification of apoptosis was determined by counting the number of cells stained by FITC-labeled Annexin V. The apoptosis of cells was detected using the Annexin V/PI Apoptosis Detection kit by fluorescence-assisted cell sorting. The data was quantified and analyzed using FACSScan flow cytometry and Cellquest software, version 3.3 (Becton Dickinson, San Jose, CA, USA).

Early apoptotic cells were identified by negative PI staining and positive FITC-Annexin V staining, while cells in late apoptosis or necrotic cells were FITC-Annexin V- and PI-positive.

Activity of caspase-3 in H295R cells. H295R cells were cultured in serum-free medium in six-well plates (1.5x10^{3} 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.

Assessment of cortisol. For cortisol release experiments, H295R cells were cultured in six-well plates until the cells were ~80-85% confluent. Cells were serum-starved overnight and then washed and incubated in fresh serum-free media containing various concentrations of orexin-A and the respective inhibitors for 24 h. At the end of the incubation period, the supernatant was preserved by immediate snap-freezing in liquid nitrogen until cortisol measurements were performed. Cortisol levels were assessed using the ELISA kit according to the manufacturer’s instructions.
Reverse transcription quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from H295R cells using TRIzol reagent (Invitrogen Life Technologies). Following spectrophotometric quantification, 1 μg total RNA was reverse-transcribed into cDNA using the PrimeScript™ RT reagent kit with gDNA Eraser (TaKaRa Bio Inc., Otsu, Japan) according to the manufacturer’s instructions. cDNA aliquots corresponding to equal amounts of RNA were used for the quantification of mRNA by qPCR using the LightCycler 96 real time quantitative PCR detection system (Roche, Indianapolis, IN, USA). The following specific primers were used: OX1 receptor forward, 5'-TGCCGCGCAAACCTATCATCTA-3' and reverse, 5'-ACCGGCTCTGCAAGGACAA-3'. As an internal control for reverse transcription and reaction efficiency, amplification of GAPDH mRNA was performed in parallel for each sample. The following specific primers were used: GAPDH forward, 5'-GGCACTGTCAGGCTGAGATG-3' and reverse, 5'-ATGGTGGAAGACGCCCAGTA-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 OX1 receptor and GAPDH were designed using Primer Premier 5.0 software (Premier Biosoft International, Palo Alto, CA, USA). Relative changes in gene expression were calculated using the equation: ΔΔCt = Ct target - Ct GAPDH and ΔΔCt = ΔCt Unmethylated - ΔCt control.

Protein preparation and western blot analysis. H295R cells were washed with cold PBS and harvested in radioimmunoprecipitation assay buffer (Beyotime Institute of Biotechnology, Haimen, China) containing protease inhibitors. Cell lysates were incubated on ice for 30 min and were collected and centrifuged at 12,000 xg for 10 min at 4°C. The supernatants were collected and mixed with 5X loading buffer (Beyotime Institute of Biotechnology), then denatured by boiling for 10 min. Samples were separated by 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes (Beyotime Institute of Biotechnology) at 60 V for 2.5 h in a transfer buffer containing 20 mM Tris, 150 mM glycine and 20% methanol (Beyotime Institute of Biotechnology). Membranes were incubated in non-fat dry milk for 120 min at room temperature and then washed three times with Tris-buffered saline containing Tween 20 (TBST) for 30 min. The membranes were incubated with primary antibody against OX1 receptor at a 1:250 dilution of phospho/total-AKT and β-actin at a 1:1,000 dilution in TBST overnight at 4°C. The membranes were washed and incubated with a secondary goat anti-rabbit immunoglobulin G antibody at a 1:2,000 dilution in TBST for 1.5 h at room temperature, and then washed three times with TBST for 30 min. Protein was visualized using the enhanced chemiluminescence method with an ECL detection kit (Beyotime Institute of Biotechnology). Band densities were measured using Quantity-One (v 4.6.2) software (Bio-Rad Laboratories, Hercules, CA, USA).

Statistical analysis. Values are expressed as the mean ± standard error of the mean and differences between values 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

Effects of orexin-A on OX1 receptor protein expression in H295R cells. PCR analysis demonstrated that OX1 receptor mRNA was expressed in H295R cells (Fig. 1A). Orexin-A...
(10^{-10}, 10^{-8} and 10^{-6} M) induced a significant increase of OX1 receptor mRNA levels in a dose-dependent manner (Fig. 1A). Similarly, orexin-A treatment increased OX1 receptor protein expression in H295R cells in a dose-dependent manner, with 10^{-6} M orexin-A being the most potent (Fig. 1B). This increase in expression was attenuated in the presence of 10^{-8} M SB334867, a high-affinity, non-peptidic OX1 receptor-specific antagonist (Fig. 1B). However, the increase of OX1 receptor mRNA levels was not significantly abolished in the presence of SB334867, compared with that in the group treated with 10^{-6} M orexin-A only (Fig. 1A; *P<0.05, vs. control).

Effects of orexin-A on proliferation of H295R cells. To determine the effects of orexin-A on cell proliferation, H295R cells were stimulated with various concentrations of orexin-A (0, 10^{-6}, 10^{-8} and 10^{-10} M) or 10^{-8} M orexin-A along with 10^{-6} M OX1 receptor antagonist SB334867 and subjected to a BrdU incorporation assay. The cell proliferation-promoting effect of orexin-A was dose-dependent. Concentrations of 10^{-6}, 10^{-8} and 10^{-10} M of orexin-A led to 1.6-, 1.5- and 1.3-fold increases, respectively, in cell proliferation, which was attenuated in the presence of SB334867 (Fig. 2; *P<0.05, **P<0.01, vs. control).

Effects of orexin-A on cortisol secretion by H295R cells. After starvation over night in serum-free media, H295R cells were incubated with various concentrations (0, 10^{-10}, 10^{-8} and 10^{-6} M) orexin-A, and the cells were treated with 10^{-6} M orexin-A and OX1 receptor antagonist SB334867 (10^{-6} M). Dose-dependent effects of orexin-A on the cortisol content in the medium were identified from the cell culture supernatants. The effect of 10^{-6} and 10^{-8} M orexin-A reached statistical significance, increasing cortisol secretion by 2- and 1.5-fold, respectively, compared to that of the control (P<0.01). This effect was ameliorated in the presence of SB334867 (10^{-6} M) (Fig. 4A). Furthermore, the AKT antagonist PF-04691502 (10^{-6} M), the OX1 receptor antagonist SB334867 (10^{-6} M), as well as their combination abolished the relative increases in the cortisol secretion in response to orexin-A (Fig. 4B; *P<0.05, **P<0.01, vs. control).
Orexin-A improves proliferation of H295R cells via an OX<sub>1</sub> receptor-stimulated AKT signaling pathway. As the P13K/AKT signaling pathway is involved in cell survival signaling, the present study examined whether orexin-A-stimulation of H295R cells induced activation of AKT. The results confirmed a specific increase in p-AKT protein in H295R cells treated with 10<sup>-8</sup> M orexin-A, which was 1.6-fold increased compared with that in the untreated control (Fig. 5). Total AKT levels, however, remained unaffected by orexin-A treatment. Furthermore, the AKT antagonist PF-04691502 (10<sup>-8</sup> M), the OX<sub>1</sub> receptor antagonist SB334867 (10<sup>-6</sup> M), as well as their combination, abolished the relative increases in AKT activation in response to orexin-A (Fig. 5). These results suggested that the regulation of the AKT pathway was closely associated with orexin-A-induced proliferation via the OX<sub>1</sub> receptor ("P<0.05, vs. control).

Effects of orexin-A on proliferation of H295R cells via activation of the AKT signaling pathway. To confirm the involvement of the AKT signaling pathway in orexin-A-mediated proliferation in H295R cells, the BrdU incorporation assay was employed to test cell proliferation. The proliferation of H295R cells was significantly increased following incubation with 10<sup>-6</sup> M orexin-A. However, these effects were blocked by AKT antagonist PF-04691502, OX<sub>1</sub> receptor antagonist SB334867, or their combination. In comparison, cell proliferation was not affected by the AKT antagonist or the OX<sub>1</sub> receptor antagonist in the absence of orexin-A (Fig. 6). These results suggested that AKT participates in orexin-A-induced stimulation of proliferation in H295R cells ("P<0.05, "P<0.01, vs. control).

Effects of orexin-A on caspase-3 activation in H295R cells. To test whether the activation of the caspase pathway was involved in the orexin-A-mediated anti-apoptotic effect in H295R cells, caspase-3 activity (part of the cell death cascade) was measured. Orexin-A treatment (10<sup>-6</sup> M) caused a significant decrease in caspase-3 activity. This effect was blunted in the presence of PF-04691502 (10<sup>-6</sup> M), SB334867 (10<sup>-6</sup> M), and the with two inhibitors administered simultaneously. These results indicated that the anti-apoptotic effect of orexin-A was mediated, at least in part, through caspase-3 (Fig. 7).

Discussion

The present study demonstrated that orexin-A had a crucial effect on the proliferation and apoptosis of human H295R adrenocortical cells through the OX<sub>1</sub> receptor and the AKT signaling pathway. In agreement with studies performed on adrenocortical cells (31,32), the present study reported that orexin-A regulates the biological activity of H295R cells via the OX<sub>1</sub> receptor. In particular, it was identified that orexin-A activates the MAPK signaling cascades in adrenocortical cells. It was also demonstrated that the effects of orexin-A on survival and apoptosis via the OX<sub>1</sub> receptor are mediated through an additional signaling pathway, namely the AKT pathway.

Orexin-A is considered to be a high-affinity agonist of the OX<sub>1</sub> receptor, whereas orexin-B has a significantly lower affinity for the OX<sub>1</sub> receptor. However, the two orexins show similar affinities for the OX<sub>2</sub> receptor (5,34). The OX<sub>2</sub> receptor is expressed in cancer cells and was shown to be responsible for the pro-apoptotic effect of orexins (14). In the present study, the effects of orexin-A on OX<sub>2</sub> receptor expression were investigated. The increase in OX<sub>2</sub> receptor expression was orexin-A concentration-dependent, and the increase induced by 10<sup>-6</sup> and 10<sup>-8</sup> M orexin-A was significant, with 10<sup>-6</sup> M orexin-A having the most potent effect.

In agreement with studies performed on 3T3-L1 fibroblasts and adrenocortical cells (9,10), the present study found that orexin-A stimulated H295R cell proliferation, protected against apoptotic cell death and promoted the release of cortisol. These effects were blunted by co-treatment with the OX<sub>2</sub> receptor antagonist SB334867. Consistent with previous studies, the effects of promoting proliferation and cortisol release were dose-dependent. High concentrations of orexin-A (10<sup>-6</sup> and 10<sup>-8</sup> M) led to a statistically significant decrease in the rate of apoptosis of H295R cells, with the concentration of 10<sup>-8</sup> M of orexin-A being the most potent. All these findings demonstrate that orexin-A and its receptor are closely associated with the survival and function of adrenal cortical cells. Following further research, orexin-A and its receptor may become a therapeutic target for regulating adrenal cortical...
dysfunction. It is important to note, however, that certain studies discovered that orexin-A was a potent pro-apoptotic peptide in colon cancer cell lines and neuroblastoma cells (12). Therefore, it is possible that the cell type is an important factor contributing to the physiological effects of orexin-A-induced proliferation and apoptosis. Future studies may further determine these cell type-specific effects.

Numerous previous studies have focused on the effects of the MAPK pathway, which is one pathway via which the biological effects of orexin-A are mediated (9,10,35). The AKT signaling pathway is often activated via mechanisms controlling cell growth and survival. Increased AKT activation or dysregulation due to elevated AKT activation, as well as indirect changes in AKT regulation, result in enhanced cell survival signaling, which is a common feature in various forms of human cancers (36,37). These changes directly or indirectly regulate apoptosis (38). The present study investigated the association between the regulation of apoptosis by orexin-A and the AKT pathway. 10^8 and 10^8 M orexin-A led to statistically significant decreases in the rate of apoptosis of H295R cells, and this effect was confirmed by a reduction in caspase-3 activation. Caspase-3 is a key molecule involved in the execution of apoptosis and acts downstream in the apoptotic cascade (39). Other caspase pathways may be assessed in future studies in order to investigate whether caspases involved in the extrinsic (receptor-mediated) pathway of apoptosis are also implicated. Orexin-A failed to protect H295R cells against apoptosis in the presence of PF-04691502, a TP-competitive PI3K/mTOR dual inhibitor. The PF-04691502 inhibitor reduced phosphorylation of AKT T308 and AKT S473 and inhibited cell proliferation.

The present study has shed new light on the mechanisms whereby orexin-A mediates the biological activity of adrenocortical cells. The findings demonstrated that orexin-A regulates H295R cell proliferation and survival, promotes the release of cortisol, reduces the pro-apoptotic activity of caspase-3 and also protects against apoptotic death via the AKT pathway. While more comprehensive and specific mechanisms remain to be elucidated, the present study has provided the first evidence of orexin-A regulating biological functions of human H295R adrenocortical cells via the AKT signaling pathway. Together with further studies utilizing orexin receptor inhibitors, the results of the present study may provide a novel and promising target for the treatment of diseases caused by orexin in the adrenal gland.

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