Orexin-A stimulates 3β-hydroxysteroid dehydrogenase expression and cortisol production in H295R human adrenocortical cells through the AKT pathway

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Abstract. Orexin-A is a regulatory peptide involved in the regulation of food intake, sleep-wakefulness, and it has various endocrine and metabolic functions. It orchestrates diverse central and peripheral processes through the stimulation of two G-protein coupled receptors, orexin receptor type 1 (OX_1 receptor) and orexin receptor type 2 (OX_2 receptor). In this study, human adrenocortical cells (NCI-H295R cells) were incubated with various concentrations of orexin-A (10^{-10} to 10^{-6} M) *in vitro*, and the mRNA and protein expression of OX_1 receptor was determined in the cells. In addition, NCI-H295R cells treated with 10^{-6} M orexin-A were then treated with or without OX_1 receptor specific antagonist (SB334867), AKT antagonist (PF-04691502), or a combination of both. Subsequently, cell proliferation, the cortisol content in the medium and the mRNA and protein expression expression of 3β-hydroxysteroid dehydrogenase (3β-HSD) were analyzed. The activity of the AKT signaling pathway was also determined in the NCI-H295R cells. We observed that the increase in the mRNA and protein expression of OX_1 receptor was orexin-A concentration-dependent, with 10^{-6} M orexin-A exerting the most potent effect. Orexin-A enhanced cell proliferation and cortisol production, and increased the mRNA and protein expression of 3β-HSD in the NCI-H295R cells; however, these effects were partly blocked by the OX_1 receptor antagonist, the AKT antagonist and the combination of both. Furthermore, orexin-A significantly increased the phosphorylation of AKT, with the levels of total AKT protein remaining unaltered. This effect was blocked in the presence of PF-04691502 (10^{-6} M), SB334867 (10^{-6} M) and the combination of both. On the whole, our data demonstrate that the effects of orexin-A on the survival and function of human adrenocortical cells are mediated through the AKT signaling pathway.

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Abbreviations: OX_1 receptor, orexin receptor type 1; OX_2 receptor, orexin receptor type 2; AKT/PKB, protein kinase B; MAPK, mitogen-activated protein kinase; PI3K, phosphatidylinositol 3-kinase; PKA, protein kinase A; PKC, protein kinase C; 3β-HSD, 3β-hydroxysteroid dehydrogenase

Key words: orexin-A, orexin receptor type 1, cortisol, AKT signaling pathway, 3β-hydroxysteroid dehydrogenase, adrenocortical cells

Introduction

Orexin-A and orexin-B, also known as hypocretin-1 and hypocretin-2, have been implicated in a wide range of central, as well as peripheral functions (1-4). Orexins were initially characterized as neuropeptides restricted to hypothalamic neurons in the brain that projected to nuclei involved in the control of food intake, sleep-wakefulness, neuroendocrine homeostasis and autonomic regulation (5). The functions of orexins are mediated by two membrane bound G-protein coupled receptors, the orexin receptor type 1 (OX_1 receptor) and the orexin receptor type 2 (OX_2 receptor). While both peptides bind with almost equal affinity to the OX_2 receptor, the OX_1 receptor seems to be selective for orexin-A (4). Further observations have indicated that orexins and their receptors are not restricted to the hypothalamus, but are also expressed in a few peripheral tissues (5), including the adrenal glands, gastrointestinal tract and the pancreas (4). To date, compelling evidence indicates an interaction of the orexin system with the hypothalamus-pituitary-adrenal (HPA) axis on a central, as well as peripheral level (6). Orexins (10^{-10} to 10^{-6} M) exert a stimulatory effect on glucocorticoid release, adrenocortical cell growth and, in some cases, mineralocorticoid release from the adrenal cortex of various species (7-13). This fact seems to be related to orexin-A (10^{-6} M) enhancing the expression of 3β-hydroxysteroid dehydrogenase (3β-HSD) (14). The enzymes of the 3β-HSD family that catalyze the conversion of Δ^5-3β-hydroxysteroids into Δ^4-3-ketosteroids are located in the endoplasmic reticulum and the mitochondrial membrane (14-17). Thus, 3β-HSD plays a key role in glucocorticoid synthesis and is expressed in a number of tissues, including the adrenal glands, gonads and the brain (17). Wenzel *et al* (14) found that orexins stimulated glucocorticoid secretion from human adrenocortical NCI-H295R cells, and that this increase was accompanied by a simultaneous increase in the mRNA levels of 3β-HSD. However, little is known

regarding the mechanisms involved in the effects of orexins leading to an increased level of 3β-HSD in adrenal cells.

AKT, also known as protein kinase B (PKB), is essential for cell survival and growth during development and carcinogenesis. AKT is a serine-threonine kinase that is regulated mainly following the activation of the second messenger, phosphatidylinositol 3-kinase (PI3K). Abundant evidence indicates that AKT is a key regulator of multiple cell survival mechanisms (18-24). 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 number of cellular proteins. For example, AKT phosphorylates and activates mammalian target of rapamycin (mTOR) in response to growth factors and oncogenes (19-21). As such, AKT plays key roles in cell survival (22), cell proliferation (23), cell growth (24) and apoptosis (22). To date, certain studies have indicated the involvement of orexins in the regulation of cell viability, cell proliferation and cortisol production (25,26). These effects can be mediated through multiple signaling pathways, including protein kinase A (PKA), protein kinase C (PKC) and mitogen-activated protein kinase (MAPK) cascade-dependent mechanisms (14,25,26). However, little is known of 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 (27). The cells were exposed to various concentrations of orexin-A (10^{-10} to 10^{-6} M), in the presence of OX_1 receptor antagonist, AKT antagonist or a combination of both, and cell proliferation assays were then performed to assess the effects of orexin-A on adrenocortical cell growth. Our results revealed that orexin-A significantly enhanced the expression of 3β-HSD and the production of cortisol, and increased the phosphorylation of AKT in the NCI-H295R cells. Our data present evidence for a functional role of orexin-A in human adrenocortical cells through the OX_1 receptor-stimulated AKT signaling pathway.

Materials and methods

Reagents. Orexin-A was obtained from Sigma (St. Louis, MO, USA). RPMI-1640 medium and fetal bovine serum were purchased from Gibco (Grand Island, NY, USA). The AKT inhibitor, PF-04691502, was purchased from Selleck Chemicals LLC (Houston, TX, USA). The OX_1 receptor-specific antagonist, SB334867, was obtained from Tocris Bioscience (Minneapolis, MN, USA). The cell proliferation enzyme-linked immunosorbent assay (ELISA) BrdU colorimetric kit was purchased from Roche Diagnostics (Penzberg, Germany). Total-AKT polyclonal antibody (ab8805), phospho-AKT (s473) polyclonal antibody (ab8932) and OX_1 receptor antibody (ab68718) were obtained from Abcam (Cambridge, UK). The Cortisol Express EIA kit was purchased from Alpco (Paris, France). β-actin antibody (C4; sc-47778) and 3β-HSD antibody (37-2; sc-100466) were obtained from Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA.

Cell culture. Human NCI-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, L-glutamine, penicillin (50 μg/ml) and streptomycin (100 μg/ml). The cells were grown in a humidified atmosphere containing 5% CO_2 at 37˚C. Prior to the experiments, the cells were grown in petri dishes in serum-free medium for 24 h .The following day, the cells ($4x10^3$ cells/well in 96-well plates, $5x10^5$ cells/well in 6-well plates) were treated with various concentrations of orexin-A (0, 10^{-10}, 10^{-8} and 10^{-6} M), or 10^{-6} M orexin-A with either SB334867 or PF-04691502, or a combination of both.

Cell proliferation assays. The adrenocortical NCI-H295R cells were seeded ($2x10^3$ cells/well) in 96-well plates and cultured for 24 h. To synchronize the cell cycle, the cells were serum-deprived for 24 h and then treated with the test agents for a further 24 h. BrdU incorporation into DNA was measured using the Cell Proliferation ELISA BrdU colorimetric kit (Roche Diagnostics). The cells were incubated with BrdU fresh medium 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. Peroxidase-conjugated BrdUrd antibody was then added to each well followed by incubation for 1 h. After washing thoroughly, the bound peroxidase-conjugated BrdUrd antibody was quantified with peroxidase substrate tetramethylbenzidine. Finally the BrdUrd 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.

Cortisol measurements. For cortisol release experiments, the NCI-H295R cells were cultured in 6-well plates until the cells were at approximately 80-85% confluence. The cells were serum starved overnight, then washed and incubated in fresh serum-free medium containing various concentrations of orexin-A and the different inhibitors for 24 h. At the end of the incubation period, the supernatant was removed and snap-frozen immediately in liquid nitrogen until the cortisol measurements were taken. Cortisol levels were assessed using the ELISA kit according to the manufacturer's instructions.

Total RNA preparations and reverse transcription quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from the NCI-H295R cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The mRNA expression of OX_1 receptor and OX_2 receptor was detected by real-time PCR using TaqMan reagents (Takara, Otsu, Japan). The following specific primers were used: OX_1 receptor forward, 5'-TGC GGC CAA CCC TAT CAT CTA-3' and reverse, 5'-ACC GGC TCT GCA AGG ACAA-3'; OX_2 receptor forward, 5'-ATC GCA GGG TAT ATC ATC GTG TTC-3' and reverse, 5'-TGA CTG TCC TCA TGT GGT GGT TC-3'; 3β-HSD forward, 5'-AGC AAA AAG ATG GCC GAG AA-3' and reverse, 5'-GGC ACA AGT ATG CAA TGT GCC-3'. As an internal control for reverse transcription (RT) and reaction efficiency, the amplification of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was carried out in parallel for each sample. The following specific primers were used: GAPDH forward, 5'-GGC ACA GTC AAG GCT GAG AAT G-3' and reverse, 5'-ATG GTG GTG AAG ACG CCA GTA-3'. The PCR reactions were carried out 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 OX_1 receptor, OX_2 receptor, 3β-HSD and GAPDH were

designed using Primer Premier 5.0 software (Premier Biosoft International, Palo Alto, CA, USA).

Protein preparations and western blot analysis. The NCI-H295R cells were washed with cold phosphate-buffered saline (PBS) and harvested in RIPA buffer containing protease inhibitors. The cell lysates were incubated on ice for 30 min and were collected and centrifuged at 12,000 x g for 10 min at 4˚C. The supernatants were collected and mixed with 5X loading buffer, then denatured by boiling for 10 min. The samples were separated by sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) and transferred onto PVDF membranes at 60 V for 2.5 h in transfer buffer containing 20 mM Tris, 150 mM glycine and 20% methanol. The membranes were incubated in non-fat dry milk for 120 min at room temperature, and then washed 3 times with TBST for 30 min, then incubated with primary antibody against OX_1 receptor at a 1:250 dilution, phospho/total-AKT at a 1:1,000 dilution or antibody against 3β-HSD at a 1:1,000 dilution in TBST overnight at 4˚C. The membranes were washed and incubated with a secondary antibody (source: rabbit) for 1.5 h at room temperature, and then washed 3 times with TBST for 30 min. Protein was visualized using the ECL method. Band densities were measured using Quantity One software.

Statistical analysis. The results are expressed as the means ± standard error of the mean (SEM) and differences between the means were analyzed by one-way analysis of variance (ANOVA). A value of $P \leq 0.05$ was considered to indicate a statistically significant difference.

Results

Orexin-A receptor expression in NCI-H295R cells. RT-qPCR assays demonstrated that OX_1 receptor mRNA was expressed in the NCI-H295R cells (Fig. 1A). However, OX_2 receptor mRNA was not detectable under the same conditions (data not shown). Orexin-A (10^{-10}, 10^{-8} and 10^{-6} M) induced a significant increase in the mRNA and protein levels of OX_1 receptor in a dose-dependent manner (Fig. 1). Treatment with orexin-A increased OX_1 receptor protein expression in the NCI-H295R cells, an increase that was dependent upon the concentration of orexin-A, with 10^{-6} M of orexin-A exerting the most potent effect (Fig. 1B). This increase in expression was attenuated in the presence of 10^{-6} M SB334867, a high-affinity, OX_1 receptor-specific, non-peptide antagonist (Fig. 1B).

Orexin-A enhances the proliferation of NCI-H295R cells. To determine the effects of orexin-A on cell survival, as well as the involvement of the AKT signaling pathway in the effects of orexin-A in the NCI-H295R cells, we employed BrdU incorporation assay to examine cell proliferation. The NCI-H295R cells were treated with various concentrations (0, 10^{-10}, 10^{-8} and 10^{-6} M) of orexin-A; the promoting effects on cell proliferation were dose-dependent. Concentrations of 10^{-6} and 10^{-8} M of orexin-A led to a 0.8- and 0.6-fold increase in cell proliferation, respectively. However, these effects were partly blocked when the cells were treated with 10^{-6} M orexin-A and the OX_1 receptor antagonist, SB334867 (10^{-6} M), or the AKT antagonist, PF-04691502 (10^{-6} M), as well as with a combination of both antagonists. Taken together, these data suggest that AKT participates in the orexin-A-induced stimulation of the proliferation of NCI-H295R cells (Fig. 2).

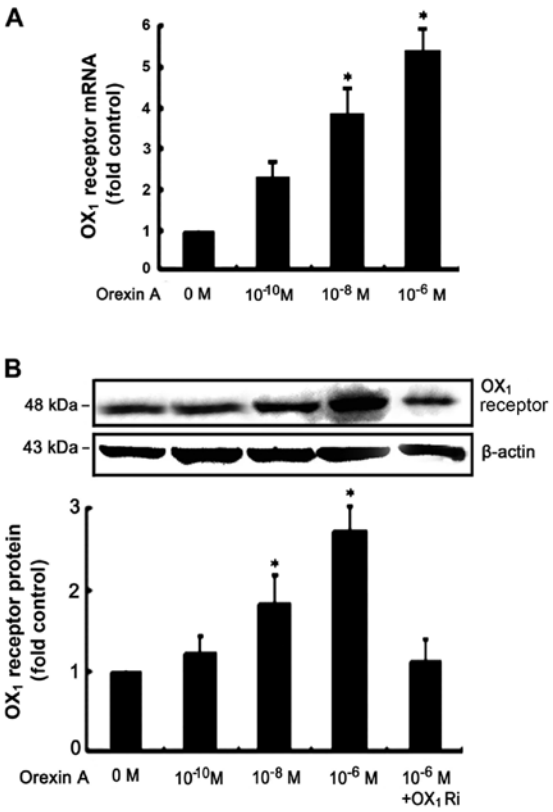

Figure 1. Orexin-A receptor expression in NCI-H295R cells. The cells were exposed to orexin-A at concentrations of 0, 10^{-10}, 10^{-8} and 10^{-6} M for 24 h. Another treatment group consisted of 10^{-6} M orexin-A in the presence of the orexin receptor type 1 (OX_1) receptor antagonist, SB334867 (OX_1Ri) (10^{-6} M). The expression of OX_1 receptor mRNA (A) and protein (B) was measured by RT-qPCR and western blot analysis. Data are presented as the means ± standard error of the mean (SEM) based on triplicate determinations from a representative experiment. Asterisks indicate significant differences compared to the controls (*$P<0.05$).

Orexin-A induces cortisol secretion from NCI-H295R cells. To determine whether the production of the cortisol is affected in orexin-A-stimulated NCI-H295R cells, cortisol levels in the culture medium were assessed using an ELISA kit. The dose-dependent effects of orexin-A on the cortisol content in the medium were determined from the cell culture supernatants. The effects of 10^{-6} and 10^{-8} M orexin-A reached statistical significance, increasing cortisol secretion by 1.0- and 0.5-fold, respectively compared to the controls (untreated cells). This affect was blocked in the presence of PF-04691502 (10^{-6} M), SB334867 (10^{-6} M) and the combination of both antagonists (Fig. 3).

Effects of orexin-A on 3β-HSD mRNA and protein expression. Follwoing starving overnight in serum-free medium, the NCI-H295R cells were incubated with various concentrations (0, 10^{-10}, 10^{-8} and 10^{-6} M) of orexin-A, and the cells were then treated with 10^{-6} M orexin-A and the OX_1 receptor antagonist, SB334867 (10^{-6} M), or the AKT antagonist, PF-04691502 (10^{-6} M), or a combination of both antagonists. Treatment with orexin-A increased the mRNA and protein expression

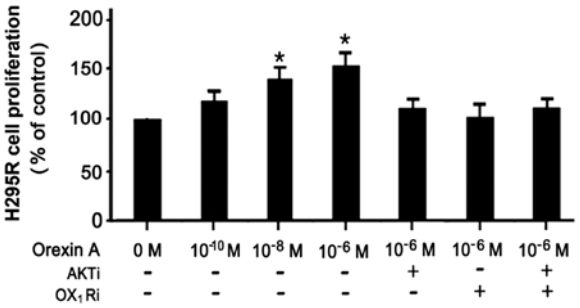

Figure 2. Orexin-A enhanced the proliferation of NCI-H295R (H295R) cells via stimulation of the AKT signaling pathway. Cells were exposed to orexin-A at concentrations of 10^{-10}, 10^{-8} and 10^{-6} M for 24 or 48 h in the presence or absence of PF-04691502 (AKT antagonist; AKTi), SB334867 (OX_1 receptor antagonist; OX_1Ri), or a combination of both. Proliferation was determined by BrdU assay. Data are presented as the means ± standard error of the mean (SEM) based on quadruplicate determinations from a representative experiment. Asterisks indicate significant differences when compared to the controls (*P<0.05).

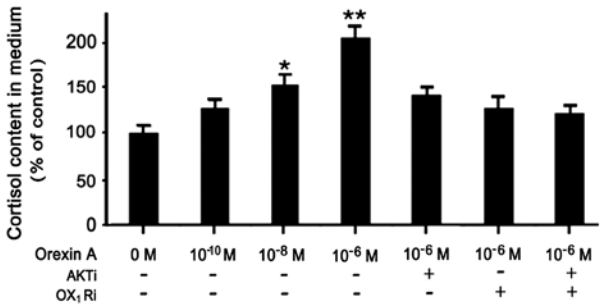

Figure 3. Orexin-A induces cortisol secretion from NCI-H295R cells. Cells were exposed to orexin-A at concentrations of 0, 10^{-10}, 10^{-8} and 10^{-6} M for 24 h. Cells were cultured with 10^{-6} M orexin-A for 24 h in the presence or absence of PF-04691502 (AKT antagonist; AKTi), SB334867 (OX_1 receptor antagonist; OX_1Ri) or a combination of both antagonists. Cortisol content was assessed using an enzyme-linked immunosorbent assay (ELISA) kit. Data are presented as the means ± standard error of the mean (SEM) based on triplicate determinations from a presentative experiment. Asterisks indicate significant differences compared to the controls (*P<0.05 and **P<0.01).

levels of 3β-HSD in the NCI-H295R cells, an increase that was dependent on the concentration of orexin-A, with 10^{-6} M of orexin-A exerting the most potent effect. Concentrations of 10^{-6} M and 10^{-8} M orexin-A led to a 2.1- and 2.4-fold increase in the mRNA levels, respectively (Fig. 4A). As regards protein expression, 10^{-6} M orexin-A and 10^{-8} M orexin-A induced a 1.8- and 1.2-fold increase in expression, respectively compared to the controls (Fig. 4B). This increase was attenuated in the presence of 10^{-6} M SB334867 or 10^{-6} M PF-04691502, or the combination of both antagonists.

Orexin-A signals through the AKT pathway. To determine wether the orexin-A-stimulation of NCI-H295R cells induces the activation of AKT, the NCI-H295R cells were stimulated with various concentrations (0, 10^{-6}, 10^{-8} and 10^{-10} M) of orexin-A. The data demonstrated a specific increase in the levels of phospo-AKT in the NCI-H295R cells treated with 10^{-6} and 10^{-8} M orexin-A, increasing by 0.7- and 0.45-fold, respectively, compared to the untreated controls (Fig. 5). The levels of total AKT, however, remained unaltered by treatment. In addition, the relative increase in AKT activation in response to orexin-A was abolished by treatment with AKT antagonist

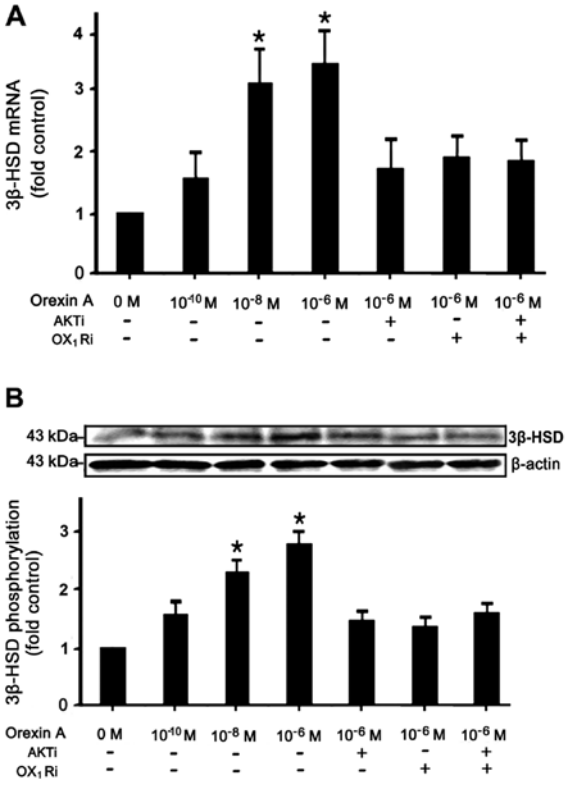

Figure 4. Effects of orexin-A on 3β-hydroxysteroid dehydrogenase (3β-HSD) mRNA and protein expression. Cells were exposed to orexin-A at concentrations of 0, 10^{-6}, 10^{-8} and 10^{-10} M for 24 h. In addition, a separate group of cells was treated with 10^{-6} M orexin-A in the presence of the 10^{-6} M SB334867 (OX_1 receptor antagonist; OX_1Ri) or 10^{-6} M PF-04691502 (AKT antagonist; AKTi), or a combination of both antagonists. The expression of 3β-HSD mRNA (A) and protein (B) was measured by RT-qPCR and western blot analysis. Data are presented as the means ± standard error of the mean (SEM) based on quadruplicate determinations from a representative experiment. Asterisk indicate significant differences as compared to the controls (*P<0.05).

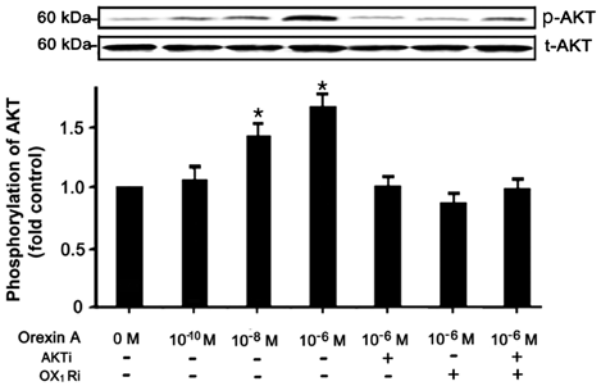

Figure 5. Orexin-A signals through the AKT pathway. Cells were stimulated with orexin-A at concentrations of 0, 10^{-6}, 10^{-8} and 10^{-10} M for 20 min, in the presence of PF-04691502 (AKT antagonist; AKTi; 10^{-6} M), SB334867 (OX_1 receptor antagonist; OX_1Ri; 10^{-6} M) or the combination of both. Protein activation was measured by western blot analysis. Autophosphorylation of AKT was evaluated along with the total protein activation. Total AKT protein expression was used as an internal control for equal protein loading. Data are presented as the means ± standard error of the mean (SEM) based on quintuplicate determinations from a representative experiment. Asterisks indicate significant differences compared to the controls (*P<0.05).

(PF-04691502, 10^{-6} M), OX_1 receptor antagonist (SB334867, 10^{-6} M), as well as the combination of both antagonsits (Fig. 5).

Above all, these data suggest that the regulation of the AKT signaling pathway is intimately associated with the orexin-A-stimulated NCI-H295R cell survival through the OX_1 receptor.

Discussion

Studies have indicated an association between the hypocretin/orexin system and the HPA axis on a central, as well as peripheral level (7). In this study, we dertermined the effects of orexin-A on the expression of OX_1 receptor and the proliferation of human adrenocortical cells. Consistent with the study by Blanco *et al* (28), we did not observe the expression of OX_2 receptor in the NCI-H295R cells. Furthermore, we found a marked increase in cortisol production and 3β-HSD expression following the stimulation of the NCI-H295R cells with orexin-A, that was associated with an increased activity of AKT. This finding indicates that another signaling pathway, the AKT pathway, partly regulates the survival and functions of adrenocortical cells stimulated with orexin-A through the OX_1 receptor.

Previous studies addressing the effects of orexins on adrenal function focused on the effects of the HPA axis. One of the first *in vivo* studies described an increase in both adrenocorticotropic hormone (ACTH) and corticosterone plasma levels 1-2 h following a single systemic administration of orexin-A in rats, while orexin-B had no stimulating effect (9). Other studies clearly demonstrated an additional direct action of orexins on the adrenal cortex without the involvement of the HPA axis (7,12). Orexin-A enhances the production of mineralocorticoids, glucocorticoids and androgens in the adrenal glands (29). Furthermore, studies have demonstrated the important role of orexin-A in adrenocortical cell proliferation (8,12). However, data on whether orexins regulate the growth of carcinoma cells are inconlcusive. Certain studies have found that orexins promote the growth of cancer cells (8,10), athough others have shown inhibitory effects (30,31). The different cell types used may be the reason for these conflicting results. Consistent with previous studies, we found that orexin-A stimulated NCI-H295R cell proliferation (8,10) and promoted the release of cortisol in a dose-dependent manner, while the effects were blunted by co-treatment with the OX_1 receptor antagonist, SB334867. The synthesis of glucocorticoids in human adrenal glands is achieved by the selective expression of some steroid-synthesizing enzymes, such as 3β-HSD. As previously reported, the selective upregulation of 3β-HSD mRNA expression following prolonged stimulation with orexins indicated the existence of a specific pathway for the transcriptional regulation of orexins (14). In this study, we found the highest cortisol synthesis rate after 24 h of treatment with orexin-A, possibly due to the stimulation of the expression of 3β-HSD and the proliferation of adrenocortical cells. This was consistent with the results of a previous study by Wenzel *et al*, who demonstrated that orexins increased the expression of steroidogenic enzymes at the transcriptional level and that orexins played a role in the long-term regulation of adrenal steroid production (14).

As is well known, among mechanisms controlling the cell growth and survival, the PI3K signaling pathway is often activated. The serine/threonine kinase AKT, acting downstream of PI3K signaling, is a key regulator of multiple survival routes (32-37). Previous studies focused on the effects of orexin-A on the MAPK pathway (6,25). Göncz *et al* (38) found that orexin-A modulated glucagon secretion and gene expression through the PI3K/AKT-dependent pathway in clonal pancreatic A-cells (InR1-G9 cells). They found an increase in the phosphorylation of AKT and phosphoinositide-dependent kinase-1 (PDK-1) proteins in response to treatment with orexin-A (10^{-5} M) in InR1-G9 cells. Our study demonstrated that orexin-A (10^{-10} to 10^{-6} M) stimulated NCI-H295R cell proliferation and promoted the release of cortisol and increased the expression of 3β-HSD. In addition we observed a specific increase in the levels of phospho-AKT in the NCI-H295R cells treated with 10^{-6} and 10^{-8} M orexin-A. The levels of total AKT, however, remained unaltered by treatment. The increase in AKT phosphorylation may be involved in the regulation of the expression of 3β-HSD. However, these effects were partly blocked by co-treatment with the OX_1 receptor antagonist, SB334867, or the AKT antagonist, PF-04691502, as well as with the combination of both antagonists. With respect to the concentration of orexin-A, it is possible that the cell type may be an important factor contributing to the physiological effects of orexin-A. Further studies are required in order to further determine to what extent the cell type plays a role in the effects of orexin-A. Taken together, these data suggest that orexin-A stimulates 3β-HSD expression and cortisol production in human adrenocortical cells through the OX_1 receptor mediated through the AKT pathway.

In conclusion, we demonstrate that the AKT signaling pathway is involved in the orexin-A stimulated increase in the synthesis of cortisol in human adrenocortical cells. Although this interaction of orexins with adrenocortical cell functions, particularly with glucocorticoid production, has now bee established, more comprehensive and specific mechanisms remain to be elucidated to clarify the nature of the signaling pathway. On the whole, orexins, together with leptin, may comprise a counter-regulatory system that controls body weight and energy homeostasis through the regulation of adrenocortical steroid production.

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