Int J Clin Exp Med 2019;12(6):6757-6767
www.ijcem.com /ISSN:1940-5901/IJCEM0078850

Original Article

Orexin A stimulates Foxo1 phosphorylation via OX1R-induced PI3K/AKT and MAPK/ERK signaling pathways in hepatocytes

Xuhao Yang[1*], Shujing Ju[2*], Dongxiao Fan[1], Yinan Li[1], Lei Guo[1]

Departments of [1]Orthopedic Surgery, [2]Endocrinology, First Affiliated Hospital, China Medical University, Shenyang 110001, Liaoning, P. R. China. [*]Equal contributors.

Received November 12, 2017; Accepted February 12, 2019; Epub June 15, 2019; Published June 30, 2019

Abstract: Orexin A and B are multifunctional neuropeptides implicated in the regulation of food intake, energy metabolism, glucose regulation, and wakefulness. They signal through two G-protein-coupled receptors (GPCR), orexin receptor 1 and orexin receptor 2 (OX1R and OX2R). Many previous studies have shown that orexins interact with PI3K/AKT and ERK1/2 MAPK signaling pathways through OX1R-coupling in other cell types, but are seldom involved in hepatocytes. Present study analysis showed that OX1R mRNA and protein was expressed in rat hepatocytes and upregulated by exogenous orexin A, in a dose-dependent manner. RT-PCR analysis showed an orexin A-induced reduction in PEPCK and G6Pase mRNA levels, two key enzymes in hepatic gluconeogenesis. Western blot analysis was used to detect the effects of exogenous orexin A acting on Forkhead box O1 (Foxo1) phosphorylation in hepatocytes. OX1R antagonist, inhibitors of PI3K/AKT, adenylyl cyclase (AC), protein kinase C (PKC), protein kinase A (PKA), and extracellular regulated protein kinases 1/2 (ERK1/2) were used in present experiments to investigate the intracellular mechanisms of two important signaling pathways, PI3K/AKT and MAPK/ERK1/2. Orexin A increased AKT phosphorylation and blockage of OX1R or PI3K reduced AKT production. Additionally, this study observed that orexin A concentration-dependently upregulated ERK1/2 phosphorylation, coupled with OX1R-induced AC/PKC, but not PKA signaling transduction in hepatocytes. Foxo1 phosphorylation was upregulated with orexin A stimulation through OX1R-induced PI3K/Akt signaling and became inactivated. Using an ERK1/2 inhibitor, it was found that ERK1/2 is also responsible for Foxo1 phosphorylation. Results revealed possible intracellular mechanisms of orexin A in regulating PI3K/AKT and ERK1/2 MAPK signaling cascades in hepatocytes, as well as its role in hepatic gluconeogenesis through deactivating two key enzymes, PEPCK and G6Pase.

Keywords: Orexin A, orexin receptor 1, PI3K/Akt, ERK1/2, Foxo1, PEPCK, G6Pase

Introduction

Orexin A and orexin B are neuropeptides discovered by separate groups in 1998 [1, 2]. Both orexins are derived from one precursor, prepro-orexin [1, 2]. They regulate cellular function by binding to the receptors, orexin receptor 1 and orexin receptor 2 (OX1R and OX2R) [3]. Orexin A and orexin B synthesizing neurons are found in the lateral hypothalamus and project to many other brain regions [4, 5]. Orexins and their receptors are involved in many physiological processes, including food intake, sleep, wakefulness, reproductive behavior, energy homeostasis, cell proliferation, and apoptosis [6-10]. OX-1R has a wide tissue distribution in the central nervous system and peripheral tissues [11-14], including adipose tissue, intestines, pancreas, adrenal glands, and testes. Orexin-mediated intracellular signaling pathways have been under intensive investigation [15-20]. Recent studies have shown that orexins are tightly interacted with PI3K/AKT and MAPKs signaling transductions [17, 20-22]. Despite the important biological actions of orexins, the effects of orexins on PI3K/AKT and MAPKs cascades are seldom involved in hepatocytes.

Forkhead box O1 (Foxo1) is a prominent member of the Forkhead box family and subfamily O of transcription factors [23]. Foxo1 is produced from the FKHR gene and involved in regulating

metabolism, oxidative stress, immune homeostasis, cell proliferation, differentiation, cell cycle progression, apoptosis, and cell death [24, 25]. Foxo1 is a downstream target of PI3K/AKT signaling pathways [23]. AKT changes Foxo1 transcriptional activities by phosphorylating threonine and serine of Foxo1 [26-30]. Previous studies have shown that hepatic gluconeogenic gene expression is mainly controlled by Foxo1, as it positively regulates transcription of G6-Pase and PEPCK, two key enzymes in hepatic gluconeogenesis [31, 32]. Phosphorylated Foxo1 is translocated from the nucleus to cytosol and loses its transcriptional activity [31-35]. Studies have proven that ERK1/2 could phosphorylate Foxo1 because Foxo1 contains 15 consensus phosphorylation sites for MAPKs in many other cell types [36]. To date, however, no further studies have been published characterizing the interaction of orexin A with hepatocytes. The current study, therefore, investigated the roles of orexin A on hepatocytes in normal rats, characterizing underlying signal transduction mechanisms. To further characterize molecular pathways conferring the inhibition of OX1R, PI3K/AKT, adenylyl cyclase, protein kinases, and ERK1/2, this study treated hepatocytes with orexin A in the presence of the above signaling inhibitors. The versatility of orexin A-mediated signaling pathways creates the need to study interactions related to cellular responses observed upon receptor stimulation.

Materials and methods

Animals

Thirty male Sprague-Dawley rats (3~4 weeks old and 200-250 g) were obtained from China Medical University and bred in the laboratory. Temperature was maintained at 22 ± 2°C in housing, with a constant 12-hour light-dark cycle (6:00 AM-6:00 PM). All rats received a normal diet (from Commercial Diet) of standard laboratory chow (20% protein, 15% fat, 65% carbohydrate diet) (China Medical University Laboratory Animal Center, Shenyang, China). All animal experiments were approved by the Ethics Committee of the First Affiliated Hospital of China Medical University.

Reagents

Orexin A was obtained from Sigma-Aldrich (St. Louis, MO). Rabbit anti-phosphor-OX1R, ERK 1/2, AKT and Foxo1 polyclonal antibodies were purchased from Abcam (Cambridge, UK). Anti-total-OX1R, ERK1/2, AKT, and Foxo1 polyclonal antibodies were purchased from Abcam (Cambridge, UK). Horseradish peroxidase-conjugated anti-species secondary antibodies were obtained from Zhongshan Jinqiao (Beijing, China). Beta-actin antibody was purchased from Abcam (Cambridge, UK). The OX1R antagonist SB334867 was obtained from TOCRIS Bioscience (Missouri, USA). Adenylyl cyclase (AC) inhibitor SQ-22536 was obtained from Biomol Research Laboratories (Milan, Italy). Protein kinase C (PKC) inhibitor GF-109203X was purchased from Calbiochem (La Jolla, CA). Protein kinase A (PKA) inhibitor KT5720 was obtained from TORIS Bioscience (Missouri, USA). PI3K inhibitor LY 294002 and ERK inhibitor U0126 were obtained from Cell Signaling Technology (Beverly, MA).

Isolation and culturing of rat hepatocytes

Rat livers were decapsulated and incubated with 0.5 mg/mL collagenase type IV (Invitrogen, Grand Island, NY, USA) for 30 minutes at 37°C in a shaker, at 160 cycles/minute. Cell suspension was collected by centrifugation at 800 rpm for 10 minutes. To obtain purified hepatocytes, the crude cell suspension was centrifuged on a Percoll gradient (20%, 40%, 60%, and 90% Percoll in PBS solution; Sigma, St. Louis, MO) and subsequently centrifuged at 800 rpm for 20 minutes at 4°C. Fractions containing hepatocytes were collected and centrifuged in a continuous and self-generating density gradient, starting with 60% Percoll at 2,000 rpm for 30 minutes at 4°C. Purified hepatocytes were cultured with RPMI Medium 1640 (Invitrogen) supplemented with 10% bovine serum albumin (HyClone, Beijing), 100 IU/mL penicillin, and 100 μg/mL streptomycin (Xianfeng, Shanghai). They were cultured (10^6 cells/mL per dish) at 37°C in a humidified incubator with 5% CO_2 for 24 hours.

Total RNA isolation and real-time PCR

Total RNA was extracted from hepatocytes using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). Expression of OX1R mRNA in hepatocytes was detected by real-time PCR using TaqMan reagents (Takara, Otsu, Japan). The following specific primers were used: OX1R-forward (5'-TGC GGC CAA CCC TAT CAT CTA-3') and

OX1R-reverse (5'-ACC GGC TCT GCA AGG ACA A-3'); PEPCK-forward (5'-AGC TGA CAG ACT CGC CCT AT-3') and PEPCK-reverse (5'-GCA GTG AGT TCC CAC CGT AT-3'); G6Pase-forward (5'-TAC CTT GCG GCT CAC TTT-3') and G6Pase-reverse (5-CTC CTT TGC AGC TCT TGC-3'). As an internal control for reverse transcription (RT) and reaction efficiency, 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 GAPDH-reverse (5'-ATG GTG GTG AAG ACG CCA GTA-3'). PCR reactions were carried out using the following conditions: 95°C for 30 seconds, then 40 cycles of 95°C for 5 seconds, 60°C for 30 seconds, and 95°C for 15 seconds. All primers and TaqMan probes, specific to OX1R and GAPDH, were designed using Primer Premier 5.0 software (Premier Biosoft International, Palo Alto, CA, USA).

Protein preparations and Western blot analysis

Cell lysates were incubated on ice for 30 minutes and centrifuged at 12,000 × g for 10 minutes at 4°C. The supernatant was collected and mixed with 5 × loading buffer, then denatured by boiling for 10 minutes. Lysate protein samples (~5 μg) were separated by SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membranes, at 70 V for 1.5 hours, in a transfer buffer containing 20 mM Tris, 150 mM glycine, and 20% methanol. The membranes were incubated in non-fat dry milk for 120 minutes at room temperature, then washed three times with TBST for 30 minutes. PVDF membranes were incubated in TBST with phosphor/total-OX1R at a 1:250 dilution, phosphor/total-ERK1/2 at a 1:1000 dilution, phosphor/total-AKT at a 1:1000 dilution, and phosphor/total-Foxo1 at a 1:1000 dilution overnight at 4°C, respectively. The membranes were washed and incubated with horseradish peroxidase-conjugated anti-species secondary antibody for 1.5 hours at room temperature. They were then washed three times with TBST for 30 minutes. Proteins were visualized by ECL and densities were measured using Quantity-One software.

Statistical analysis

Data are presented as mean ± SEM (M ± SEM). One-way analysis of variance (ANOVA) was used for multiple group comparisons. Unpaired t-tests were used for two-group comparisons. Correlation analysis was carried out using Pearson's correlation analysis. Statistical significance is set at a level of $P < 0.05$. N = 6. Statistical analysis was performed using SPSS 15.0 software.

Results

Expression of OX1R in mRNA and protein levels in hepatocytes

Primary hepatocytes were extracted from rats on a normal-diet and cultured for 24 hours at 37°C. Cells were treated with orexin A at concentrations of 0, 10^{-10}, 10^{-8}, and 10^{-6} M for 30 minutes, respectively. Quantitative RT-PCR analysis, with the use of specific couples of primers, was used to demonstrate OX1R mRNA expression in hepatocytes. Orexin A (10^{-10} M, 10^{-8} M, and 10^{-6} M) induced a significant increase in OX1R mRNA levels, in a dose-dependent manner. Moreover, 10^{-6} M orexin A-induced OX1R mRNA expression in the presence of OX1R antagonist SB334867 (10^{-5} M, 30 minutes), showing no significant differences, compared with 10^{-6} M orexin A treatment alone (**Figure 1A**). Levels of OX1R phosphorylation were determined by Western blot analysis. It was observed that exogenous orexin A upregulated phosphorylated OX1R, in a dose-dependent manner. Maximum stimulation was seen with 10^{-6} M orexin A treatment, while minimum stimulation was seen with 10^{-10} M treatment (**Figure 1B**). OX1R expression was reduced in the presence of 10^{-5} M SB334867, an OX1R-specific antagonist (**Figure 1B**).

Effects of orexin A on PEPCK and G6Pase mRNA expression in hepatocytes

Primary hepatocytes were extracted from rats on a normal-diet and cultured for 24 hours at 37°C. Cells were treated with orexin A at concentrations of 0, 10^{-10}, 10^{-8}, and 10^{-6} M for 30 minutes, respectively. Quantitative RT-PCR analysis was used to demonstrate orexin A-induced PEPCK and G6Pase (two key enzymes in hepatic gluconeogenesis) mRNA expression in hepatocytes. Orexin A induced a significant decrease of PEPCK and G6Pase mRNA expression. Moreover, 10^{-6} M orexin A induced the maximum reduction and 10^{-10} M induced the minimum reduction of PEPCK and G6Pase mRNA levels (**Figure 2A** and **2B**).

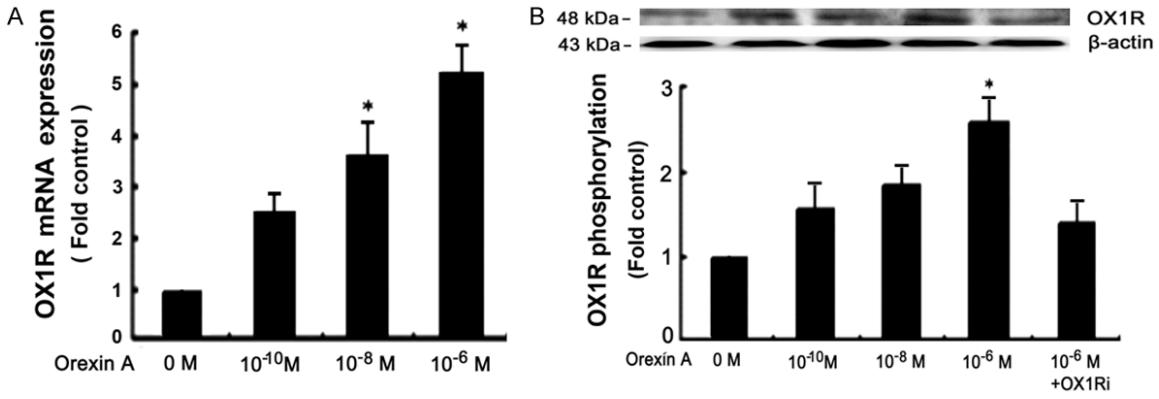

Figure 1. Expression of OX1R in mRNA and protein levels in hepatocytes. Primary hepatocytes were extracted from rats on a normal-diet and cultured for 24 hours at 37°C. Cells were treated with orexin-A at concentrations of 0, 10^{-10}, 10^{-8}, and 10^{-6} M for 30 minutes, respectively. OX1R expression in mRNA and protein levels was detected by RT-PCR (A) and Western blot analysis (B), respectively. OX1R phosphorylation was then assessed following treatment with 10^{-8} M ORA and 10^{-5} M SB334867 for 30 minutes. Results are expressed by mean ± SEM. $P < 0.05$ indicates statistical significance. *$P < 0.05$; **$P < 0.01$ vs. control. n = 6.

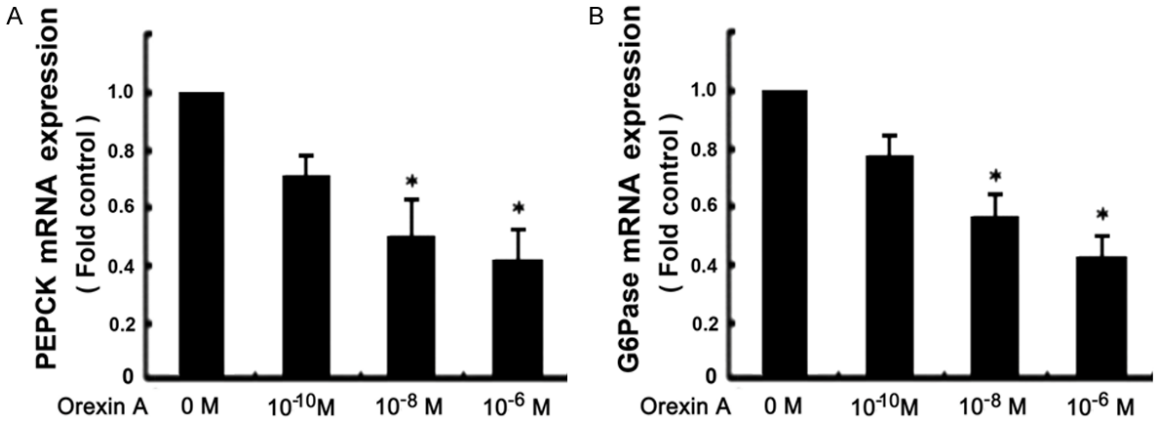

Figure 2. Primary hepatocytes were extracted from rats on a normal-diet and cultured for 24 hours at 37°C. Cells were treated with orexin A at concentrations of 0, 10^{-10}, 10^{-8}, and 10^{-6} M for 30 minutes, respectively. RT-PCR analysis was used to demonstrate an orexin A-induced PEPCK (A) and G6Pase (B) (two key enzymes in hepatic gluconeogenesis) mRNA expression in hepatocytes. Results are expressed by mean ± SEM. $P < 0.05$ indicates statistical significance. *$P < 0.05$; **$P < 0.01$ vs. control. n = 6.

Effects of orexin A on Foxo1 phosphorylation in hepatocytes

One of the downstream targets of PI3K/AKT pathways is Foxo1, which becomes phosphorylated (inactivated) by AKT. Hepatocytes were extracted and cultured for 24 hours at 37°C. To determine the effects of orexin A on Foxo1 phosphorylation in hepatocytes, cells were treated with orexin A at different concentrations (0, 10^{-10} M, 10^{-8} M, 10^{-6} M) for 30 minutes. Total-Foxo1 and phosphor-Foxo1 levels were determined by Western blotting. Compared to basal levels, Western blot analysis showed significant increases in Foxo1 phosphorylation at any concentration, in a dose-dependent manner. Orexin A induced maximum Foxo1 phosphorylation at a concentration of 10^{-6} M and minimum phosphorylation at 10^{-10} M. Total Foxo1 remained unchanged (**Figure 3A**). OX1R antagonist (SB334867, 10^{-5} M) was used to pre-incubate hepatocytes for 30 minutes in the presence of orexin A (10^{-6} M, 30 min). Foxo1 phosphorylation was abolished in cells treated with SB334867. Thus, orexin A induced Foxo1 phosphorylation through OX1R-mediated signaling cascades. This study then treated hepatocytes with orexin A (10^{-6} M) in the presence of

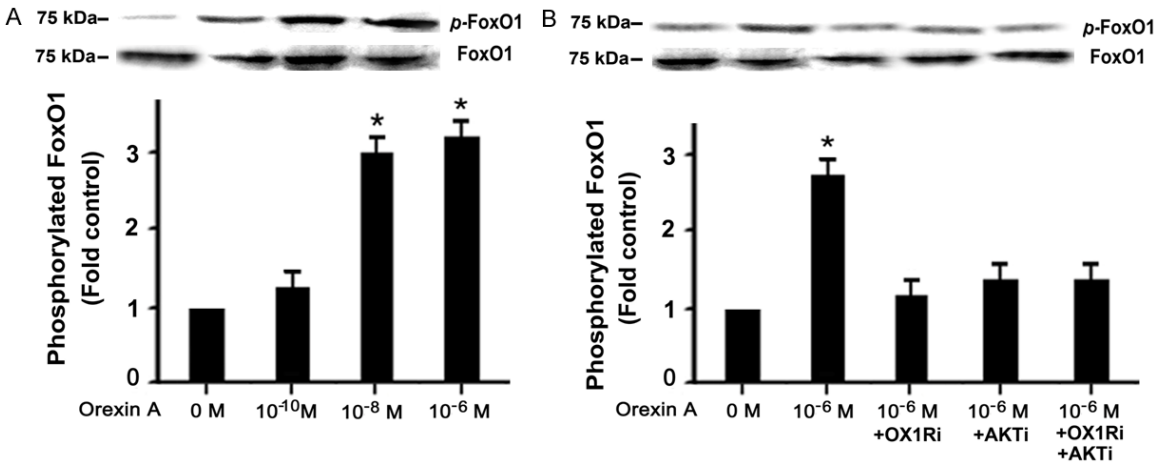

Figure 3. Effects of orexin A on Foxo1 phosphorylation in hepatocytes of normal rats. Cells were extracted from normal rats and treated with orexin A at different concentrations of 0, 10^{-10} M, 10^{-8} M, and 10^{-6} M for 30 minutes. OX1R antagonist SB334867 (10^{-5} M, 30 minutes) and PI3K inhibitor LY294002 (25 μmol/L, 24 hours before) were used to detect the intracellular mechanisms of OX1R-induced Foxo1 phosphorylation. Western blot analysis was used to determine total-Foxo1 and phosphor-Foxo1. Results are expressed by mean ± SEM. $P < 0.05$ indicates statistical significance. *P < 0.05; **P < 0.01 vs. control. n = 6.

PI3K/AKT inhibitor LY294002, pretreatment (25 μmol/L, 24 hours before), and measured Foxo1 phosphorylation via Western blotting. In the presence of LY294002, the phosphorylated activity of PI3K/AKT was blocked. Thus, Foxo1 phosphorylation was reduced significantly, compared with cells incubated with orexin A (10^{-6} M) alone. Additionally, hepatocytes were pretreated with the combination of SB334867 and LY294002, simultaneously, then orexin A (10^{-6} M) was treated for 30 minutes. Western blot analysis showed that either SB334867 or LY294002 significantly suppressed orexin A-induced Foxo1 phosphorylation. There were no differences between the two inhibitory effects. Blockage of either OX1R or PI3K/AKT could suppress Foxo1 phosphorylation. With the combination of SB334867 and LY294002, simultaneously, Foxo1 phosphorylation was further suppressed to a lower level, compared with treatment with either SB334867 or LY294002 alone. Results suggest that the excitatory effects of orexin A on Foxo1 phosphorylation are through OX1R-mediated PI3K/AKT signaling pathways (**Figure 3B**).

Effects of orexin A on AKT phosphorylation in hepatocytes

In this experiment, hepatocytes of normal-diet rats were extracted and cultured. To determine the correlation of OX1R-induced AKT phosphorylation in hepatocytes, cells were treated with orexin A of different concentrations (0, 10^{-10} M, 10^{-8} M, 10^{-6} M) for 30 minutes. Total-AKT and phosphor-AKT levels were determined by Western blotting. Compared to basal levels, Western blot analysis showed significant increases in AKT phosphorylation at any concentration, in a dose-dependent manner. Orexin A induced maximum AKT phosphorylation at a concentration of 10^{-6} M and minimum phosphorylation at 10^{-10} M. Total AKT remained unchanged (**Figure 4A**). Next, OX1R antagonist (SB334867, 10^{-5} M, 30 minutes) and AKT inhibitor (LY294002, 25 μmol/L, 24 hours) were used to detect the intracellular mechanisms of orexin-induced signaling pathways. AKT phosphorylation was abolished in cells treated with the two blockers. Thus, orexin A induced AKT production through an OX1R-dependent signaling pathway in hepatocytes (**Figure 4B**).

Effects of orexin A on ERK1/2 phosphorylation through OX1R-mediated AC/PKC signaling transduction in hepatocytes

ERK1/2 has been proven to be an important OX1R-induced signaling element in many other cell types. The current study treated hepatocytes of a normal diet with different concentrations of orexin A (0, 10^{-10} M, 10^{-8} M, and 10^{-6} M) for 30 minutes. ERK1/2 phosphorylation was measured by Western blot analysis. It was

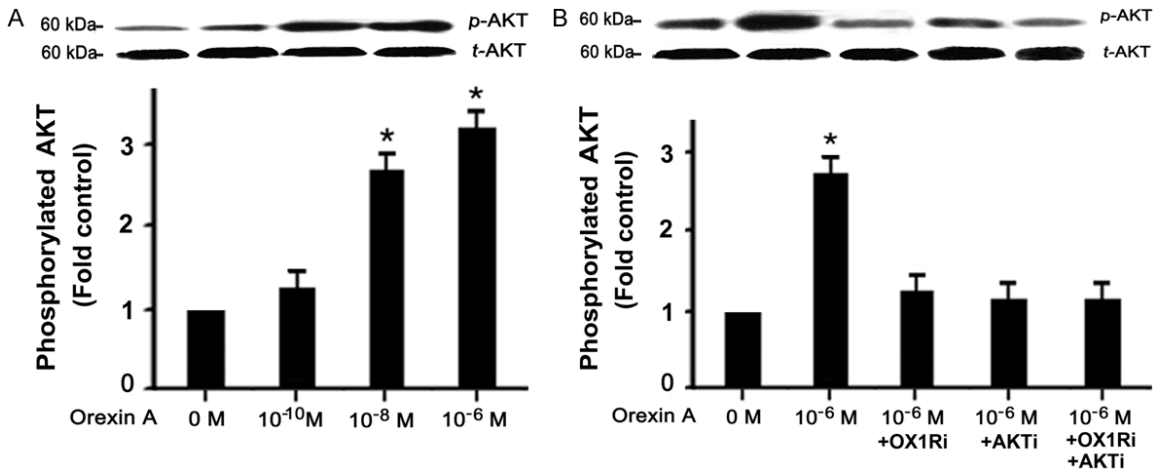

Figure 4. Effects of orexin A on AKT phosphorylation in hepatocytes. Cells were extracted from normal rats and treated with orexin A at different concentrations of 0, 10^{-10} M, 10^{-8} M, and 10^{-6} M for 30 minutes (A). OX1R antagonist SB334867 (10^{-5} M, 30 minutes) and PI3K inhibitor LY294002 (25 μmol/L, 24 hours) were used to detect the intracellular mechanisms of the OX1R-induced AKT phosphorylation (B). Western blot analysis was used to determine total AKT and phosphor-AKT. Results are expressed by mean ± SEM. $P < 0.05$ indicates statistical significance. n = 6.

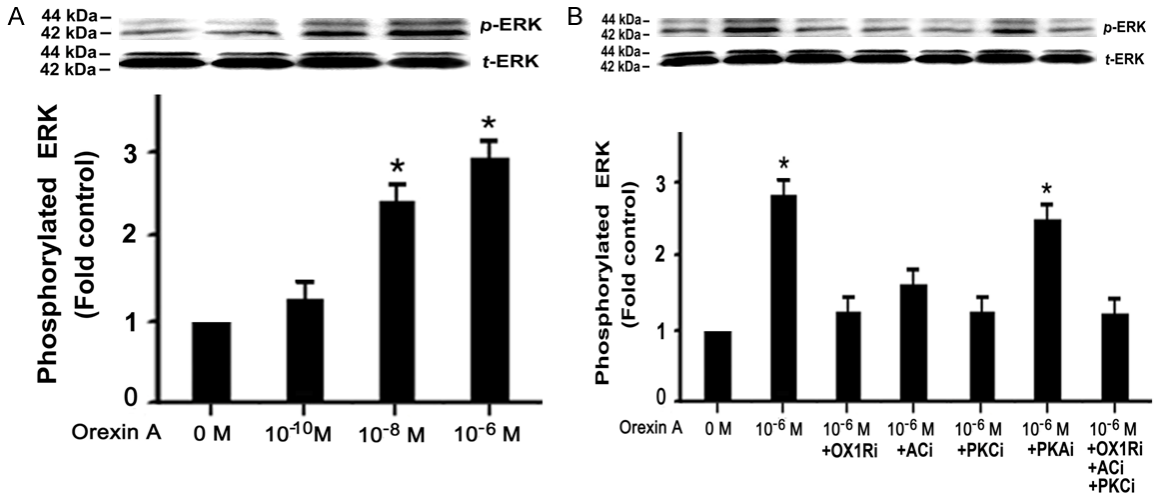

Figure 5. Effects of orexin A on ERK1/2 phosphorylation through OX1R-mediated AC/PKC signaling transduction in hepatocytes. Cells were extracted from rats and treated with orexin A at different concentrations of 0, 10^{-10} M, 10^{-8} M, and 10^{-6} M for 30 minutes. OX1R antagonist SB334867 (10^{-5} M, 30 minutes), AC inhibitor SQ-22536 (10^{-4} M, 40 minutes), PKA inhibitor KT5720 (10^{-7} M, 60 minutes), and PKC inhibitor GF-109203X (10^{-6} M, 60 minutes) were used to detect the intracellular mechanisms of the OX1R-induced ERK1/2 phosphorylation. Western blot analysis was used to determine total and phosphor-ERK1/2. Results are expressed by mean ± SEM. $P < 0.05$ indicates statistical significance. n = 6.

observed that orexin A stimulated ERK1/2 phosphorylation in a dose-dependent manner (**Figure 5A**). Additionally, blockage of OX1R, adenylate cyclase (AC), PKC, and PKA was used to investigate the intracellular mechanisms of OX1R-induced ERK1/2 signaling. This study pre-incubated cells with OX1R antagonist SB-334867 (10^{-5} M, 30 minutes), AC inhibitor SQ-22536 (10^{-4} M, 40 minutes), PKC inhibitor GF-109203X (10^{-6} M, 60 minutes), and PKA inhibitor KT5720 (10^{-7} M, 60 minutes), respectively. Hepatocytes were then treated with 10^{-6} M orexin A for 30 minutes. ERK1/2 phosphorylation was determined by Western blotting with antibody against p-ERK. Results showed that PKC inhibitors suppressed ERK1/2 phosphory-

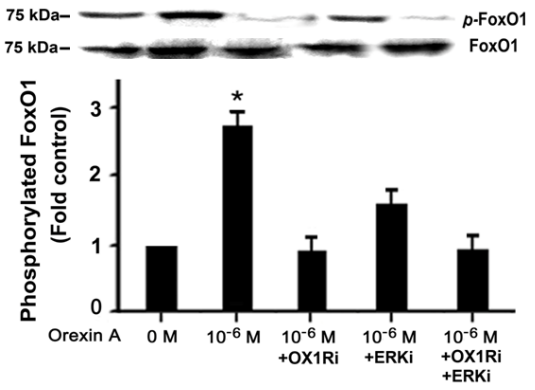

Figure 6. ERK1/2 phosphorylated Foxo1 via OX1R-induced signaling pathway in hepatocytes. Cells were extracted from normal rats and treated with orexin A at concentrations of 10^{-6} M for 30 minutes. OX1R antagonist SB334867 (10^{-5} M, 30 minutes) and ERK1/2 inhibitor U0126 (10^{-5} M, 30 minutes) were used to detect the intracellular mechanisms of the OX1R-induced Foxo1 phosphorylation. Western blot analysis was used to determine total and phosphor-Foxo1. Results are expressed by mean ± SEM. $P < 0.05$ indicates statistical significance. n = 6.

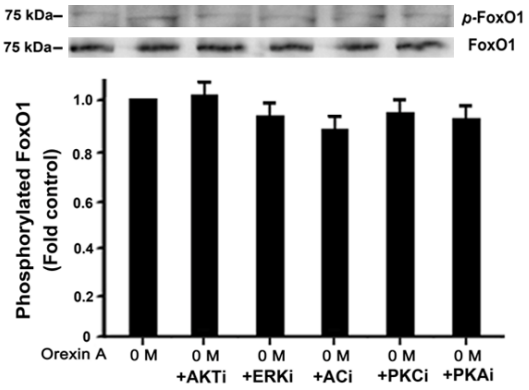

Figure 7. Effects of AKT inhibitor and ERK1/2 inhibitor on Foxo1 phosphorylation in hepatocytes Cells were treated with AC inhibitor SQ-22536 (10^{-4} M, 40 minutes), PKC inhibitor GF-109203X (10^{-6} M, 60 minutes), PKA inhibitor KT5720 (10^{-7} M, 60 minutes), AKT inhibitor, and ERK1/2 inhibitor U0126, respectively. Total-Foxo1 and phosphor-Foxo1 levels were determined by Western blot. Results are expressed by mean ± SEM. $P < 0.05$ indicates statistical significance. n = 6.

lation, significantly. PKA inhibitors did not affect ERK1/2 phosphorylation. Therefore, it is suggested that PKC is an upstream kinase of ERK1/2 in OX1R-mediated signaling transduction and PKA appears not to be the upstream kinase in this pathway. In the presence of SQ-22536, ERK1/2 phosphorylation decreased significantly, compared with orexin A treatment alone. Results indicate that AC was involved in ERK1/2 phosphorylation and regulated ERK-1/2 positively. When treated with the three inhibitors (SB334867, GF-109203X, and SQ-22536), simultaneously, ERK1/2 phosphorylation furthermore decreased significantly. Present results indicate that orexin A was able to activate OX1R-mediated ERK1/2 phosphorylation via AC/PKC coupling (**Figure 5B**).

Orexin A phosphorylated Foxo1 via OX1R-induced signaling pathway in hepatocytes

The next experiment focused on the interaction of ERK1/2 and Foxo1 in hepatocytes of normal-diet rats. Cells were preincubated with orexin A (10^{-6} M) for 30 minutes. It was observed that Foxo1 phosphorylation was reduced by either SB334867 or U0126. The co-inhibitory effects of the two inhibitors led an enhanced reduction of Foxo1 phosphorylation. Results demonstrated that orexin A affected Foxo1, not only via PI3K/AKT, but also via ERK1/2, indicating that it is a key upstream element as well (**Figure 6**).

Effects of AKT inhibitor, AC inhibitor, and ERK1/2 inhibitor on Foxo1 phosphorylation in hepatocytes

Cells were treated with AC inhibitor SQ-22536 (10^{-4} M, 40 minutes), PKC inhibitor GF-109203X (10^{-6} M, 60 minutes), PKA inhibitor KT5720 (10^{-7} M, 60 minutes), AKT inhibitor, and ERK1/2 inhibitor U0126, respectively. Total-Foxo1 and phosphor-Foxo1 levels were then determined via Western blotting. Compared to basal levels (cells without any treatment), Western blot analysis showed no significant decreases in Foxo1 phosphorylation with the five inhibitors above. There were no significant differences between the two inhibitor treatments (**Figure 7**).

Discussion

Orexin A has been implicated in the activation of PI3K/AKT and MAPK signaling pathways in many peripheral organs and cells [3, 16]. Orexin receptors are expressed widely in the hypothalamus, adrenal glands, adipose tissue, and pancreas [11, 12]. Orexins elicit their biological effects via GPCRs, OX1R, and OX2R, which can signal through multiple G proteins [37, 38]. RT-

PCR and Western blotting showed expression of OX1R in mRNA and protein levels in hepatocytes. It seems to be hypersensitive to exogenous orexin A stimulation, in a dose-dependent manner. Results of this study suggest that orexin A exerted its biological effects by ligand-induced upregulation of OX1R. Higher concentrations of orexin A increased more OX1R expression. Several studies have demonstrated expression of OX1R in many cell types in human and rodents, suggesting that the effects of orexin A are mediated through direct interaction with OX1R [22]. The effects of orexin A may be mediated through a specific interaction with the corresponding GPCR. However, the physiological relevance of OX1R expression in hepatocytes requires further investigation.

PI3K/AKT and MAPK/ERK1/2 are two important signaling transductions in metabolisms. Activation of PI3K/AKT signal transduction pathways have been proven to decrease proglucagon gene transcription in InR1-G9 [39]. The current study presents orexin A-induced and OX1R-mediated PI3K/AKT and MAPK/ERK1/2 signaling in hepatocytes. Orexin A upregulated ERK1/2 and AKT phosphorylation, in a dose-dependent manner. This effect was blocked by the OX1R-specific antagonist SB334867. Thus, it was concluded that orexin A stimulates ERK1/2 and AKT via OX1R. It has been reported that orexin receptors couple with multiple G protein signaling pathways, including Gq, Gs, and Gi, and activate downstream targets in other cell types [37, 38]. In hepatocytes, upregulation of both ERK1/2 and AKT by orexin A may be involved in multiple G proteins and its downstream targets signaling pathways. However, further investigation is necessary.

Foxo1 is a key transcription factor in the liver. The involvement of Foxo1 in glucose metabolism has already been demonstrated by showing Foxo1-dependent effects of insulin on glucose metabolism, in response to alterations of the feeding state [40, 41]. Foxo1 activity is reduced by insulin-induced phosphorylation of Foxo1 via AKT signal transduction pathways [42]. Data indicates that Foxo1 is an important subcellular regulator of glucose metabolism. The current study demonstrated orexin A-induced phosphorylation of Foxo1 in hepatocytes, which suppressed Foxo1 transcriptional activity via phosphorylation-dependent nuclear exclusion [31-34]. Foxo1 is regulated by orexin A, in a PI3K/AKT-dependent manner. Inhibition of either OX1R or PI3K reduced AKT and Foxo1 phosphorylation, indicating OX1R-mediated PI3K/AKT-dependent signaling cascades.

Previous studies have shown that Foxo1 promotes hepatic glucose production [32-35]. PEPCK and G6Pase are two key enzymes in hepatic gluconeogenesis. It has been proven that Foxo1 positively regulates transcription of PEPCK and G6Pase genes [33-35]. In the current experiment, orexin A decreased PEPCK and G6Pase activity through deactivated Foxo1 transcriptional activity. This novel observation suggests that orexin A-induced inhibition of hepatic gluconeogenesis by altering key enzyme (PEPCK and G6Pase) activity in hepatocytes is mediated through an interaction with transcription factor Foxo1. Protein expression levels of PEPCK and G6Pase should be further studied, examining the roles of orexin A in hepatic gluconeogenesis.

MAPK/ERK1/2 is a key signaling pathway in metabolism regulation [36, 43]. ERK1/2 phosphorylation, induced by OX1R activation, has been investigated in several studies [20, 44-46]. ERK1/2 has been shown in response to OX1R stimulation. In the adrenals, where the signaling mechanisms have been studied in more detail, OXRs appear to activate G proteins associated with AC regulation [47, 48]. Activation of AC pathways in response to orexins has been reported in rat and human adrenal cortices and in CHO cells heterologously expressing OX1R [16, 49]. Regulation of AC activity is also considered as an important component of orexin receptors signaling [16, 18, 39, 50, 51]. Orexin A induced a dose-dependent increase in ERK1/2 phosphorylation. The current investigation provides insight into the signaling cascades involved in the AC-mediated ERK1/2 action with stimulation of orexin A. ERK1/2 phosphorylation was blocked by OX1R antagonist, AC inhibitors, or PKC inhibitors, respectively. Furthermore, treatment with protein kinase A (PKA) failed to suppress ERK1/2 phosphorylation. This suggests that orexin A triggered ERK1/2 phosphorylation through PKC, but not through PKA. PKA appears to not play a role in this OX1R-mediated ERK1/2 signaling pathway. Thus, PKC was identified as an upstream kinase of ERK1/2 phosphorylation in OX1R-

mediated signal transduction in hepatocytes. OX1R-induced ERK1/2 phosphorylation in this cell type did not couple to PKA. This is not in agreement with previous demonstrations. In adrenal cells, ERK1/2 was partially activated through adenlyate cyclase (cAMP) following stimulation by orexin A. It was PKA-dependent for orexin A [17]. This may be a good indicator of cell types as an important consideration when assessing the intracellular mechanisms of orexin cell signaling.

AC is also correlated with ERK1/2 activity and regulates ERK1/2 phosphorylation positively. Data suggests that PKC coupling of OX1R occurs via activation of AC. Taken together, these findings strongly suggest that orexin A stimulated ERK1/2 production from cultured hepatocytes through OX1R coupled to AC/PKC-dependent cascades. In agreement with this is the finding that the stimulatory effects of OX1R on ERK1/2 phosphorylation require an interaction of AC and PKC in hepatocytes of normal rats.

ERK1/2 has been proven to be an upstream element in regulating Foxo1 phosphorylation in other cell types [36]. As Foxo1 contains 15 consensus phosphorylation sites for MAPKs [36], it was expected that ERK1/2 could phosphorylate Foxo1 in hepatocytes. The current study tested whether induction of Foxo1 phosphorylation by orexin A stimulation is due to ERK1/2. The next experiment provided evidence approving this possibility. Using ERK1/2 inhibitor (U0126), orexin A-induced Foxo1 phosphorylation was suppressed. Present data indicates that ERK1/2 regulated Foxo1 phosphorylation by acting as a co-regulator. In hepatocytes, orexin A induced Foxo1 phosphorylation is mediated through both PI3K/AKT and MAPK/ERK signaling pathways.

In summary, the current study identified orexin A, for the first time, as a potent physiological regulator of hepatic gluconeogenesis by deactivating two important enzymes, PEPCK and G6Pase, in hepatocytes. It was demonstrated that hepatocytes express OX1R and identify AKT and ERK1/2 as intracellular effector molecules of orexin A action. Furthermore, this study described Foxo1 as a transcription factor mediating orexin A effects on hepatic gluconeogenesis inhibition through deactivating two key enzymes, PEPCK and G6Pase. Two important signaling transductions, PI3K/AKT and MAPK/ERK1/2, play key roles in this process in hepatocytes. Understanding the cellular mechanisms of action of orexins in hepatocytes is an important step in gaining insight into the therapeutic roles of orexin A in the regulation of a variety of homeostatic events. The activity of orexin A deserves further evaluation as a potential principle toward improving glucose control in type 2 diabetes.

Acknowledgements

We are thankful to China Medical University Affiliated Hospital Laboratory Center for kindly providing equipment needed.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Lei Guo, Department of Orthopedic Surgery, First Affiliated Hospital, China Medical University, Shenyang 110001, Liaoning, P. R. China. Tel: +86 15241818899; E-mail: guolei@cmu.edu.cn

References

[1] de Lecea L, Kilduff TS, Peyron C, Gao X, Foye PE, Danielson PE, Fukuhara C, Battenberg EL, Gautvik VT, Bartlett FS 2nd, Frankel WN, van den Pol AN, Bloom FE, Gautvik KM and Sutcliffe JG. The hypocretins: hypothalamus-specific peptides with neuroexcitatory activity. Proc Natl Acad Sci U S A 1998; 95: 322-327.

[2] Sakurai T, Amemiya A, Ishii M, Matsuzaki I, Chemelli RM, Tanaka H, Williams SC, Richardson JA, Kozlowski GP, Wilson S, Arch JR, Buckingham RE, Haynes AC, Carr SA, Annan RS, McNulty DE, Liu WS, Terrett JA, Elshourbagy NA, Bergsma DJ and Yanagisawa M. Orexins and orexin receptors: a family of hypothalamic neuropeptides and G protein-coupled receptors that regulate feeding behavior. Cell 1998; 92: 573-585.

[3] Karteris E and Randeva HS. Orexin receptors and G-protein coupling: evidence for another "promiscuous" seven transmembrane domain receptor. J Pharmacol Sci 2003; 93: 126-128.

[4] Date Y, Ueta Y, Yamashita H, Yamaguchi H, Matsukura S, Kangawa K, Sakurai T, Yanagisawa M and Nakazato M. Orexins, orexigenic hypothalamic peptides, interact with autonomic, neuroendocrine and neuroregulatory systems. Proc Natl Acad Sci U S A 1999; 96: 748-753.

[5] Nambu T, Sakurai T, Mizukami K, Hosoya Y, Yanagisawa M and Goto K. Distribution of orex-

in neurons in the adult rat brain. Brain Res 1999; 827: 243-260.
[6] Yokobori E, Kojima K, Azuma M, Kang KS, Maejima S, Uchiyama M and Matsuda K. Stimulatory effect of intracerebroventricular administration of orexin A on food intake in the zebrafish, danio rerio. Peptides 2011; 32: 1357-1362.
[7] Ramanjaneya M, Conner AC, Chen J, Stanfield PR and Randeva HS. Orexins stimulate steroidogenic acute regulatory protein expression through multiple signaling pathways in human adrenal H295R cells. Endocrinology 2008; 149: 4106-4115.
[8] Takahashi N, Okumura T, Yamada H and Kohgo Y. Stimulation of gastric acid secretion by centrally administered orexin-A in conscious rats. Biochem Biophys Res Commun 1999; 254: 623-627.
[9] Ida T, Nakahara K, Murakami T, Hanada R, Nakazato M and Murakami N. Possible involvement of orexin in the stress reaction in rats. Biochem Biophys Res Commun 2000; 270: 318-323.
[10] Spinazzi R, Ziolkowska A, Neri G, Nowak M, Rebuffat P, Nussdorfer GG, Andreis PG and Malendowicz LK. Orexins modulate the growth of cultured rat adrenocortical cells, acting through type 1 and type 2 receptors coupled to the MAPK p42/p44- and p38-dependent cascades. Int J Mol Med 2005; 15: 847-852.
[11] Beiras-Fernandez A, Gallego R, Blanco M, Garcia-Caballero T, Dieguez C and Beiras A. Merkel cells, a new localization of prepro-orexin and orexin receptors. J Anat 2004; 204: 117-122.
[12] Johren O, Neidert SJ, Kummer M, Dendorfer A and Dominiak P. Prepro-orexin and orexin receptor mRNAs are differentially expressed in peripheral tissues of male and female rats. Endocrinology 2001; 142: 3324-3331.
[13] Kirchgessner AL. Orexins in the brain-gut axis. Endocr Rev 2002; 23: 1-15.
[14] Nakabayashi M, Suzuki T, Takahashi K, Totsune K, Muramatsu Y, Kaneko C, Date F, Takeyama J, Darnel AD, Moriya T and Sasano H. Orexin-A expression in human peripheral tissues. Mol Cell Endocrinol 2003; 205: 43-50.
[15] Hoang Q, Bajic D, Yanagisawa M, Nakajima S and Nakajima Y. Effects of orexin (hypocretin) on GIRK channels. J Neurophysiol 2003; 90: 693-702.
[16] Holmqvist T, Johansson L, Ostman M, Ammoun S, Akerman KE and Kukkonen JP. OX1 orexin receptors couple to adenylyl cyclase regulation via multiple mechanisms. J Biol Chem 2005; 280: 6570-6579.
[17] Ramanjaneya M, Conner AC, Chen J, Kumar P, Brown JE, Johren O, Lehnert H, Stanfield PR and Randeva HS. Orexin-stimulated MAP kinase cascades are activated through multiple G-protein signalling pathways in human H295R adrenocortical cells: diverse roles for orexins A and B. J Endocrinol 2009; 202: 249-261.
[18] Zhu Y, Miwa Y, Yamanaka A, Yada T, Shibahara M, Abe Y, Sakurai T and Goto K. Orexin receptor type-1 couples exclusively to pertussis toxin-insensitive G-proteins, while orexin receptor type-2 couples to both pertussis toxin-sensitive and -insensitive G-proteins. J Pharmacol Sci 2003; 92: 259-266.
[19] Karteris E, Chen J and Randeva HS. Expression of human prepro-orexin and signaling characteristics of orexin receptors in the male reproductive system. J Clin Endocrinol Metab 2004; 89: 1957-1962.
[20] Ammoun S, Johansson L, Ekholm ME, Holmqvist T, Danis AS, Korhonen L, Sergeeva OA, Haas HL, Akerman KE and Kukkonen JP. OX1 orexin receptors activate extracellular signal-regulated kinase in Chinese hamster ovary cells via multiple mechanisms: the role of Ca2+ influx in OX1 receptor signaling. Mol Endocrinol 2006; 20: 80-99.
[21] Tsuneki H, Murata S, Anzawa Y, Soeda Y, Tokai E, Wada T, Kimura I, Yanagisawa M, Sakurai T and Sasaoka T. Age-related insulin resistance in hypothalamus and peripheral tissues of orexin knockout mice. Diabetologia 2008; 51: 657-667.
[22] Goncz E, Strowski MZ, Grotzinger C, Nowak KW, Kaczmarek P, Sassek M, Mergler S, El-Zayat BF, Theodoropoulou M, Stalla GK, Wiedenmann B and Plockinger U. Orexin-A inhibits glucagon secretion and gene expression through a Foxo1-dependent pathway. Endocrinology 2008; 149: 1618-1626.
[23] Kaestner KH, Knochel W and Martinez DE. Unified nomenclature for the winged helix/forkhead transcription factors. Genes Dev 2000; 14: 142-146.
[24] Schmidt M, Fernandez de Mattos S, van der Horst A, Klompmaker R, Kops GJ, Lam EW, Burgering BM and Medema RH. Cell cycle inhibition by FoxO forkhead transcription factors involves downregulation of cyclin D. Mol Cell Biol 2002; 22: 7842-7852.
[25] Dijkers PF, Birkenkamp KU, Lam EW, Thomas NS, Lammers JW, Koenderman L and Coffer PJ. FKHR-L1 can act as a critical effector of cell death induced by cytokine withdrawal: protein kinase B-enhanced cell survival through maintenance of mitochondrial integrity. J Cell Biol 2002; 156: 531-542.
[26] Tran H, Brunet A, Griffith EC and Greenberg ME. The many forks in FOXO's road. Sci STKE 2003; 2003: Re5.
[27] Daitoku H, Hatta M, Matsuzaki H, Aratani S, Ohshima T, Miyagishi M, Nakajima T and Fukamizu A. Silent information regulator 2 potenti-

ates Foxo1-mediated transcription through its deacetylase activity. Proc Natl Acad Sci U S A 2004; 101: 10042-10047.
[28] Matsuzaki H, Daitoku H, Hatta M, Tanaka K and Fukamizu A. Insulin-induced phosphorylation of FKHR (Foxo1) targets to proteasomal degradation. Proc Natl Acad Sci U S A 2003; 100: 11285-11290.
[29] Gross DN, Wan M and Birnbaum MJ. The role of FOXO in the regulation of metabolism. Curr Diab Rep 2009; 9: 208-214.
[30] Kim DH, Park CH, Park D, Choi YJ, Park MH, Chung KW, Kim SR, Lee JS and Chung HY. Ginsenoside Rc modulates Akt/Fox01 pathways and suppresses oxidative stress. Arch Pharm Res 2014; 37: 813-820.
[31] Zheng X, Yang Z, Yue Z, Alvarez JD and Sehgal A. FOXO and insulin signaling regulate sensitivity of the circadian clock to oxidative stress. Proc Natl Acad Sci U S A 2007; 104: 15899-15904.
[32] Thorn SR, Brown LD, Rozance PJ, Hay WW Jr, Friedman JE. Increased hepatic glucose production in fetal sheep with intrauterine growth restriction is not suppressed by insulin. Diabetes 2013; 62: 65-73.
[33] Miao H, Zhang Y, Lu Z, Liu Q and Gan L. FOXO1 involvement in insulin resistance-related proinflammatory cytokine production in hepatocytes. Inflamm Res 2012; 61: 349-358.
[34] Matsumoto M, Pocai A, Rossetti L, Depinho RA and Accili D. Impaired regulation of hepatic glucose production in mice lacking the forkhead transcription factor Foxo1 in liver. Cell Metab 2007; 6: 208-216.
[35] Matsumoto M, Han S, Kitamura T and Accili D. Dual role of transcription factor FoxO1 in controlling hepatic insulin sensitivity and lipid metabolism. J Clin Invest 2006; 116: 2464-2472.
[36] Asada S, Daitoku H, Matsuzaki H, Saito T, Sudo T, Mukai H, Iwashita S, Kako K, Kishi T, Kasuya Y and Fukamizu A. Mitogen-activated protein kinases, Erk and p38, phosphorylate and regulate Foxo1. Cell Signal 2007; 19: 519-527.
[37] Malendowicz LK, Tortorella C and Nussdorfer GG. Orexins stimulate corticosterone secretion of rat adrenocortical cells, through the activation of the adenylate cyclase-dependent signaling cascade. J Steroid Biochem Mol Biol 1999; 70: 185-188.
[38] Lopez M, Senaris R, Gallego R, Garcia-Caballero T, Lago F, Seoane L, Casanueva F and Dieguez C. Orexin receptors are expressed in the adrenal medulla of the rat. Endocrinology 1999; 140: 5991-5994.
[39] Schinner S, Barthel A, Dellas C, Grzeskowiak R, Sharma SK, Oetjen E, Blume R and Knepel W. Protein kinase B activity is sufficient to mimic the effect of insulin on glucagon gene transcription. J Biol Chem 2005; 280: 7369-7376.
[40] Imae M, Fu Z, Yoshida A, Noguchi T and Kato H. Nutritional and hormonal factors control the gene expression of FoxOs, the mammalian homologues of DAF-16. J Mol Endocrinol 2003; 30: 253-262.
[41] Barthel A, Schmoll D and Unterman TG. FoxO proteins in insulin action and metabolism. Trends Endocrinol Metab 2005; 16: 183-189.
[42] Nakae J, Park BC and Accili D. Insulin stimulates phosphorylation of the forkhead transcription factor FKHR on serine 253 through a Wortmannin-sensitive pathway. J Biol Chem 1999; 274: 15982-15985.
[43] Ouedraogo R, Naslund E and Kirchgessner AL. Glucose regulates the release of orexin-a from the endocrine pancreas. Diabetes 2003; 52: 111-117.
[44] Ziolkowska A, Spinazzi R, Albertin G, Nowak M, Malendowicz LK, Tortorella C and Nussdorfer GG. Orexins stimulate glucocorticoid secretion from cultured rat and human adrenocortical cells, exclusively acting via the OX1 receptor. J Steroid Biochem Mol Biol 2005; 96: 423-429.
[45] Tang J, Chen J, Ramanjaneya M, Punn A, Conner AC and Randeva HS. The signalling profile of recombinant human orexin-2 receptor. Cell Signal 2008; 20: 1651-1661.
[46] Wenzel J, Grabinski N, Knopp CA, Dendorfer A, Ramanjaneya M, Randeva HS, Ehrhart-Bornstein M, Dominiak P and Johren O. Hypocretin/orexin increases the expression of steroidogenic enzymes in human adrenocortical NCI H295R cells. Am J Physiol Regul Integr Comp Physiol 2009; 297: R1601-1609.
[47] Shin HS, Cho HS, Sung KW and Yoon BJ. Orexin-A increases cell surface expression of AMPA receptors in the striatum. Biochem Biophys Res Commun 2009; 378: 409-413.
[48] Ammoun S, Lindholm D, Wootz H, Akerman KE and Kukkonen JP. G-protein-coupled OX1 orexin/hcrtr-1 hypocretin receptors induce caspase-dependent and -independent cell death through p38 mitogen-/stress-activated protein kinase. J Biol Chem 2006; 281: 834-842.
[49] Mazzocchi G, Malendowicz LK, Gottardo L, Aragona F and Nussdorfer GG. Orexin A stimulates cortisol secretion from human adrenocortical cells through activation of the adenylate cyclase-dependent signaling cascade. J Clin Endocrinol Metab 2001; 86: 778-782.
[50] Karteris E, Randeva HS, Grammatopoulos DK, Jaffe RB and Hillhouse EW. Expression and coupling characteristics of the CRH and orexin type 2 receptors in human fetal adrenals. J Clin Endocrinol Metab 2001; 86: 4512-4519.
[51] Randeva HS, Karteris E, Grammatopoulos D and Hillhouse EW. Expression of orexin-A and functional orexin type 2 receptors in the human adult adrenals: implications for adrenal function and energy homeostasis. J Clin Endocrinol Metab 2001; 86: 4808-4813.