International Journal of Immunopathology & Pharmacology

Original Research Article

Orexin-A protects SH-SY5Y cells against H_2O_2-induced oxidative damage via the PI3K/$MEK_{1/2}$/$ERK_{1/2}$ signaling pathway

International Journal of Immunopathology and Pharmacology
Volume 32: 1–10

DOI: 10.1177/2058738418785739
journals.sagepub.com/home/iji
SAGE

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Abstract

Orexin-A elicits multiple potent effects on a variety of tumor cells via different signaling pathways. However, it is unknown whether it has a neuroprotective effect on SH-SY5Y human neuroblastoma cells. This study investigated the neuroprotective effect of Orexin-A against hydrogen peroxide (H_2O_2)-induced oxidative damage in SH-SY5Y cells and the underlying mechanism. H_2O_2 treatment decreased the viability of SH-SY5Y cells, induced apoptosis, and decreased superoxide dismutase activity. Orexin-A attenuated these effects, indicating that it protects SH-SY5Y cells against H_2O_2-induced oxidative damage. Pre-treatment with Orexin-A also attenuated H_2O_2-induced increases in phosphorylation of $MEK_{1/2}$ and $ERK_{1/2}$. Moreover, these effects of Orexin-A were reduced in the presence of the PI3K inhibitor LY294002. Finally, pre-treatment with LY294002 abrogated attenuation of the H_2O_2-induced decrease in cell viability and increase in caspase-3/7 activity by Orexin-A. These results show that the PI3K/$MEK_{1/2}$/$ERK_{1/2}$ signaling pathway is involved in the neuroprotective effects of Orexin-A against H_2O_2-induced oxidative damage in SH-SY5Y cells. Our findings provide insight into the neuroprotective effects of Orexin-A and the underlying mechanism, which will be useful for the treatment of nervous system diseases.

Keywords

Orexin-A, neuroprotective effect, oxidative damage, PI3K/MEK/ERK pathway

Date received: 6 March 2018; accepted: 6 June 2018

Introduction

Orexins, officially named hypocretins, are peptides that were identified simultaneously by two groups in 1998.[1,2] There are two structural forms of orexins, Orexin-A and Orexin-B, which are derived from prepro-orexin by hydrolysis and contain 33 and 28 amino acids, respectively.[3] The amino acid homology of Orexin-A and -B is 46%.[2]

Orexins were recently reported to inhibit growth and induce apoptosis of a variety of tumor cells.[4–7] The effects of Orexin-A are particularly pronounced.[8–10] This peptide significantly reduces the viability of HCT-116 human colon cancer cells.[10] Orexin-A strongly delays tumor growth and promotes apoptosis of tumor cells in nude mice xenografted with colon cancer cells.[6] Moreover, Orexin-A markedly inhibits growth of rat C6 glioma cells by activating the caspase pathway.[8]

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However, the effects of Orexin-A on SH-SY5Y human neuroblastoma cells are relatively few. This study demonstrates that Orexin-A protects SH-SY5Y cells against hydrogen peroxide (H_2O_2)-induced oxidative damage and discusses the possible underlying molecular mechanism. These results will facilitate the clinical application of orexins to treat nervous system diseases.

Materials and methods

Materials

Human Orexin-A was obtained from Phoenix Pharmaceuticals (Belmont, CA, USA). Dulbecco's Modified Eagle's Medium and fetal bovine serum were purchased from Gibco Life Technologies (Grand Island, NY, USA). An anti-β-actin antibody was obtained from BZSGB Technology (Beijing, China). Primary antibodies against p-$MEK_{1/2}$, p-$ERK_{1/2}$, total $MEK_{1/2}$ (t-$MEK_{1/2}$), and total $ERK_{1/2}$ (t-$ERK_{1/2}$) were purchased from Cell Signaling Technology (Danvers, MA, USA). The PI3K inhibitor LY294002 was purchased from Sigma (St. Louis, MO, USA).

Cell culture

SH-SY5Y cells were purchased from the Cell Resource Center Chinese Academy of Sciences (Shanghai, China). Cells were grown in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37°C in a humidified atmosphere containing 5% CO_2.

Cell viability assay

Cells were seeded into 96-well plates at a density of 1×10^4 cells/well, cultured for 24 h, and then treated with 100, 200, 300, and 500 μM H_2O_2 for 12 and 24 h to induce neurotoxicity. Cell viability was determined using the Cell Counting Kit-8 (CCK-8) assay (KeyGEN BioTECH Corp., Nanjing, China). Briefly, each well was incubated with 10 μL of CCK-8 for 2 h at 37°C and then absorption at 420 nm was measured using a microplate reader (Bio-Rad, Hercules, CA, USA). All assays were repeated at least three times. Cell viability was expressed as a percentage of that in the non-treated control.

The protective effect of Orexin-A against H_2O_2-induced neurotoxicity was evaluated by pre-treating cells with 10, 100, and 1000 nM Orexin-A for 6 h and then treating them with 200 μM H_2O_2 for 24 h. Cell viability was determined using the CCK-8 assay as described above. In experiments incorporating LY294002, cells were treated with this inhibitor for 30 min prior to Orexin-A.

Real-time cell analysis

The effect of Orexin-A on SH-SY5Y cells was assessed by determining the cell index using an xCELLigence Real-Time Cell Analyzer (RTCA) DP system (ACEA Biosciences, San Diego, CA, USA) at 37°C in 5% CO_2. To determine the baseline, 100 μL of culture media was added to each well of an E-Plate 16 (ACEA Biosciences), and the plate was monitored using the RTCA for 30 min at 37°C. Next, SH-SY5Y cells were seeded at a density of 2×10^4 cells/well into an E-plate 16 containing 100 μL of medium per well. When cells entered log phase, Orexin-A was added to a final concentration of 100 nM, and then, cells were cultured for 3 h, treated with H_2O_2 and continuously monitored for 48 h.

Analysis of intracellular superoxide dismutase

The intracellular level of superoxide dismutase (SOD) was measured using a SOD Assay Kit (Jiancheng Bioengineering Institute, Nanjing, China). Cells were seeded into six-well plates at a density of 1×10^5 cells/well, pre-treated with 100 nM Orexin-A for 6 h, and then treated with 200 μM H_2O_2 for 24 h. Thereafter, cells were washed three times with phosphate-buffered saline (PBS), cell lysis buffer was added, and samples were incubated for 30 min on ice. The detailed testing steps of the samples were carried out according to the manufacturer's instructions. Finally, absorption at 450 nm was measured using a microplate reader.

Apoptosis assay

Cells were seeded into six-well plates at a density of 1×10^5 cells/well, cultured for 24 h, pre-treated with 100 nM Orexin-A for 6 h, and then treated with 200 μM H_2O_2 for 24 h. Flow cytometry was performed using an Annexin V-fluorescein-5-isothiocyanate (FITC) Apoptosis Detection Kit (KeyGEN BioTECH Corp.). Cells were trypsinized, washed twice with cold PBS, and resuspended in

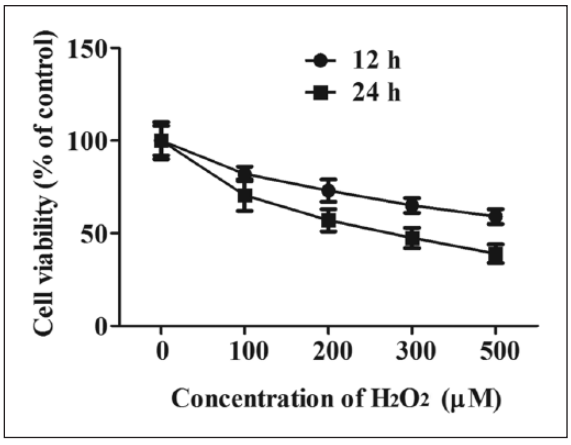

Figure 1. Effects of treatment with various concentrations of H_2O_2 for various durations on the viability of SH-SY5Y cells. SH-SY5Y cells were treated with 100, 200, 300, and 500 μM H_2O_2 for 12 and 24 h. Cell viability was determined using the CCK-8 assay and is expressed as a percentage of that in the non-treated control.

binding buffer at a density of 1×10^5 cells/mL. Thereafter, 5 μL of annexin V-FITC and 5 μL of propidium iodide (PI) were added. Samples were incubated for 30 min in the dark and then analyzed by flow cytometry (FACSCalibur; BD Biosciences, Franklin Lakes, NJ, USA). The percentage of apoptotic cells was calculated. Each sample was run in triplicate.

In situ caspase activation

Cells were seeded into six-well plates at a density of 10^6 cells/well and cultured for 24 h. Thereafter, the culture medium was replaced by fresh medium containing or lacking 100 nM Orexin-A, cells were cultured for 6 h, and then, 200 μM H_2O_2 was added. After 24 h, caspase activation was detected using a Caspase-3/7 Assay Kit (Promega, Madison, WI, USA) according to the manufacturer's instructions.

Western blot analysis

Cells were seeded into six-well plates at a density of 1×10^6 cells/well, cultured for 24 h, pre-treated with 100 nM Orexin-A for 6 h, and then treated with 200 μM H_2O_2 for 12 h. Thereafter, cells were lysed on ice and centrifuged, and the supernatants were collected. Protein concentrations were measured using a Bicinchoninic Acid Assay (BCA) Kit (Beyotime Biotechnology Corp., Shanghai, China). Equal amounts of protein (~30 mg) were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride (PVDF) membrane. The membrane was blocked with 5% non-fat milk prepared in tris-buffered saline with Tween 20 (TBST) for 1 h at room temperature and then incubated with a primary antibody (1:1000) overnight at 4°C. After being washed three times in TBST, the membrane was incubated with a secondary horseradish peroxidase–conjugated goat anti-rabbit IgG antibody (1:2000) for 1 h at room temperature. Finally, the membrane was washed three times and signals were visualized using an enhanced chemiluminescence kit. The band densities were quantified from three different observations using ImageJ software. For normalization, the same membrane was submerged in stripping buffer for 30 min, washed three times with TBST, blocked with 5% non-fat milk prepared in TBST for 1 h, and incubated with an anti-β-actin antibody.

Statistical analysis

All statistical data are expressed as mean ± standard error of the mean from at least three repeats. Data were analyzed using GraphPad Prism 5. Differences between groups were tested using a one-way analysis of variance (ANOVA); $p < 0.05$ was considered significant.

Results

Determination of the optimal concentration and duration of H_2O_2 treatment to elicit toxic effects on SH-SY5Y cells

To determine the optimal concentration and duration of H_2O_2 treatment to elicit toxic effects on SH-SY5Y cells, cells were treated with 100, 200, 300, and 500 μM H_2O_2 for 12 and 24 h. Cell viability was measured using the CCK-8 assay and was expressed as a percentage of that in the non-treated control. H_2O_2 treatment for 12 or 24 h decreased cell viability in a concentration-dependent manner (Figure 1). Cell viability was 82.32% ± 5.66%, 74.51% ± 6.32%, 65.46% ± 4.59%, and 59.52% ± 5.05% following treatment with 100, 200, 300, and 500 μM H_2O_2 for 12 h, respectively. In addition, cell viability was 70.56% ± 8.06%, 57.75% ± 4.35%, 47.50% ± 6.59%, and 39.24% ± 4.05% following treatment with 100, 200, 300, and 500 μM H_2O_2 for 24 h, respectively. Based on these results,

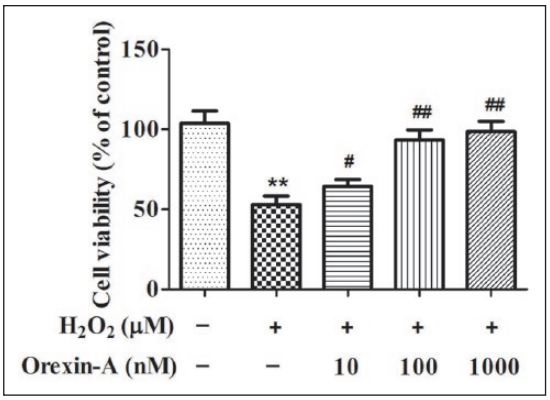

Figure 2. Effects of treatment with various concentrations of Orexin-A on the H_2O_2-induced decrease in the viability of SH-SY5Y cells. SH-SY5Y cells were pre-treated with 10, 100, and 1000 nM Orexin-A for 6 h and then treated with 200 μM H_2O_2 for 24 h. Cell viability was determined using the CCK-8 assay and is expressed as a percentage of that in the non-treated control. (**$p < 0.01$ vs non-treated group; #$p < 0.05$, and ##$p < 0.01$ vs H_2O_2-treated group.)

treatment with 200 μM H_2O_2 for 24 h was considered optimal and was performed in subsequent experiments.

Determination of the optimal concentration of Orexin-A to protect SH-SY5Y cells against H_2O_2-induced neurotoxicity

To determine the optimal concentration of Orexin-A to protect SH-SY5Y cells against H_2O_2-induced neurotoxicity, cells were pre-treated with 10, 100, and 1000 nM Orexin-A for 6 h and then treated with 200 μM H_2O_2 for 24 h. Cell viability was significantly higher in the groups pre-treated with 10, 100, and 1000 nM Orexin-A than in the group treated with H_2O_2 alone ($p < 0.05$ or $p < 0.01$; Figure 2). Moreover, cell viability in the group pre-treated with 100 nM Orexin-A was almost as high as that in the non-treated group. Based on these results, treatment with 100 nM Orexin-A for 6 h was considered optimal and was performed in subsequent experiments.

Orexin-A attenuates the H_2O_2-induced decrease in cell viability

The effect of Orexin-A on SH-SY5Y cells was determined by measuring the cell index using an RTCA, which monitors cell growth in real time. The cell index was much lower in the H_2O_2-treated group than in the non-treated group, but was higher in the group pre-treated with Orexin-A than in the H_2O_2-treated group (Figure 3(a)). These data suggest that Orexin-A attenuates the H_2O_2-induced decrease in the viability of SH-SY5Y cells.

Orexin-A attenuates the H_2O_2-induced decrease in SOD activity

A SOD Assay Kit was used to assess the effects of H_2O_2 and Orexin-A on SOD activity in SH-SY5Y cells. SOD activity was significantly lower in H_2O_2-treated cells (125.31 ± 11.75 U/mgprot) than in non-treated cells (428.12 ± 22.03 U/mgprot; Figure 4, $p < 0.001$). By contrast, SOD activity was significantly higher in cells pre-treated with Orexin-A (256.78 ± 9.72 U/mgprot) than in those treated with H_2O_2 alone ($p < 0.01$). These results suggest that Orexin-A attenuates the H_2O_2-induced decrease in SOD activity.

Orexin-A attenuates the H_2O_2-induced increase in apoptosis

Flow cytometry was performed to investigate whether Orexin-A protects SH-SY5Y cells against H_2O_2-induced apoptosis (Figure 5). The percentage of apoptotic cells was significantly higher in the H_2O_2-treated group (13.85% ± 0.95%) than in the non-treated group (7.2% ± 0.4%). By contrast, the percentage of apoptotic cells was significantly lower in the group pre-treated with Orexin-A (9.97% ± 0.52%) than in the group treated with H_2O_2 alone ($p < 0.05$). These results indicate that Orexin-A significantly inhibits H_2O_2-induced apoptosis.

Based on this result, we speculated that Orexin-A alters expression of pro-apoptotic proteins, such as caspase-3/7. To investigate this, caspase-3/7 activity was detected via in situ caspase activation. Caspase-3/7 activity was 103% higher in H_2O_2-treated cells than in non-treated cells ($p < 0.001$), but was 84.33% lower in cells pre-treated with Orexin-A than in H_2O_2-treated cells ($p < 0.01$; Figure 6). These results suggest that Orexin-A prevents H_2O_2-induced apoptosis by decreasing caspase-3/7 activity.

Orexin-A protects against H_2O_2-induced neurotoxicity via $MEK_{1/2}$/$ERK_{1/2}$ signaling

p-$MEK_{1/2}$ and p-$ERK_{1/2}$ were detected by western blotting to determine whether the $MEK_{1/2}$/$ERK_{1/2}$

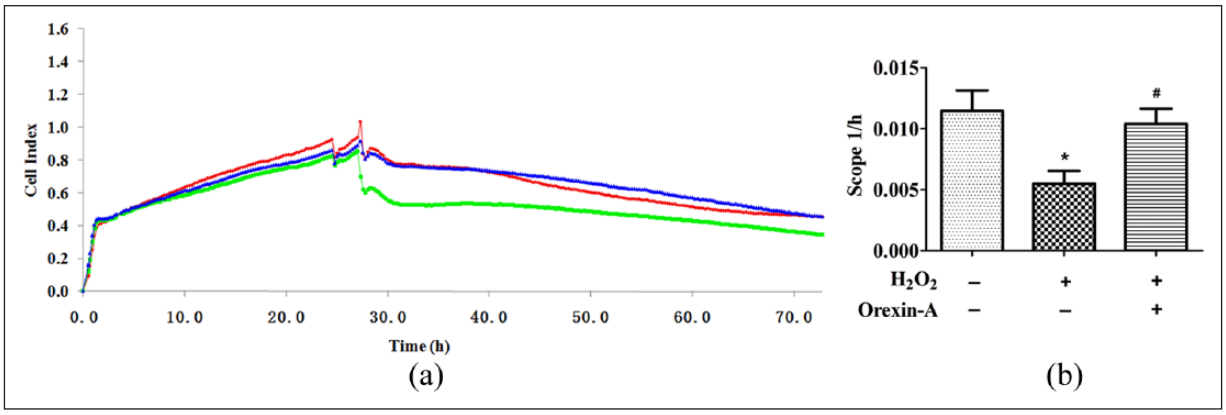

Figure 3. Evaluation of the effects of H_2O_2 and Orexin-A on cell viability using a RTCA. (a) SH-SY5Y cells were treated with H_2O_2 alone or pre-treated with Orexin-A, and the cell index was determined for up to 72 h using RTCA Software 2.0. The mean of triplicates is plotted. Red, green, and blue denote control, H_2O_2, and Orexin-A, respectively. (b) The statistical analysis according to Scope of three groups. (*$p < 0.05$ vs non-treated group; #$p < 0.05$ vs H_2O_2-treated group.)

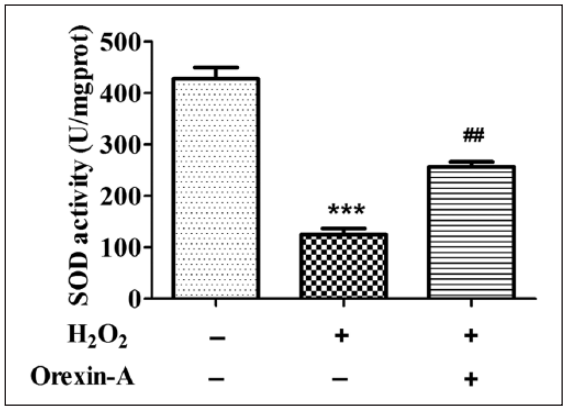

Figure 4. Orexin-A attenuates the H_2O_2-induced decrease in SOD activity. SH-SY5Y cells were pre-treated with 100 nM Orexin-A for 6 h and then treated with 200 μM H_2O_2 for 24 h. SOD activity was assessed and is presented as units of SOD activity per milligram of protein. Data are expressed as mean ± standard error of the mean of three experiments. (***$p < 0.001$ vs non-treated group; ##$p < 0.01$ vs H_2O_2-treated group.)

signaling pathway is involved in the neuroprotective effects of Orexin-A. Levels of p-$MEK_{1/2}$ and p-$ERK_{1/2}$ were significantly higher in H_2O_2-treated cells than in non-treated cells ($p<0.001$), but were significantly lower in cells pre-treated with Orexin-A than in H_2O_2-treated cells ($p<0.05$ or $p<0.01$; Figure 7). Meanwhile, levels of p-$MEK_{1/2}$ and p-$ERK_{1/2}$ were significantly higher in cells treated with 25 mM LY294002 for 30 min prior to Orexin-A and H_2O_2 than in cells treated with LY294002 and H_2O_2 only ($p<0.01$ or $p<0.05$). These data indicate that Orexin-A elicits neuroprotective effects via the PI3K/$MEK_{1/2}$/$ERK_{1/2}$ signaling pathway.

Orexin-A attenuates the H_2O_2-induced decrease in cell viability and increase in apoptosis via PI3K/$MEK_{1/2}$/$ERK_{1/2}$ signaling

The role of PI3K/$MEK_{1/2}$/$ERK_{1/2}$ signaling in the neuroprotective effects of Orexin-A were further evaluated using the PI3 K inhibitor LY294002. Cell viability was significantly higher in the group pre-treated with Orexin-A than in the H_2O_2-treated group ($p<0.01$), and this protective effect of Orexin-A was abolished by LY294002 ($p<0.05$; Figure 8(a)). Consistently, Orexin-A did not attenuate the H_2O_2-induced increase in caspase-3/7 activity in the presence of LY294002 (Figure 8(b), $p<0.05$). These data suggest that Orexin-A increases the viability of SH-SY5Y cells and inhibits their apoptosis via PI3K/$MEK_{1/2}$/$ERK_{1/2}$ signaling.

Discussion

H_2O_2 treatment is commonly used to induce oxidative stress, which can cause oxidative injury.[11] In this study, we established an in vitro model of H_2O_2-induced oxidative injury using SH-SY5Y cells. H_2O_2 treatment decreased cell viability and SOD activity, but increased apoptosis and caspase-3/7 activity, consistent with previous reports.[12–14]

The neuroprotective peptide Orexin-A ameliorates ischemia–reperfusion injury by decreasing the number of apoptotic cells.[15] In a cellular model of Parkinson's disease, Orexin-A inhibits neurotoxicity induced by 6-hydroxydopamine and elicits anti-apoptotic effects.[16] Orexin-A increases proliferation

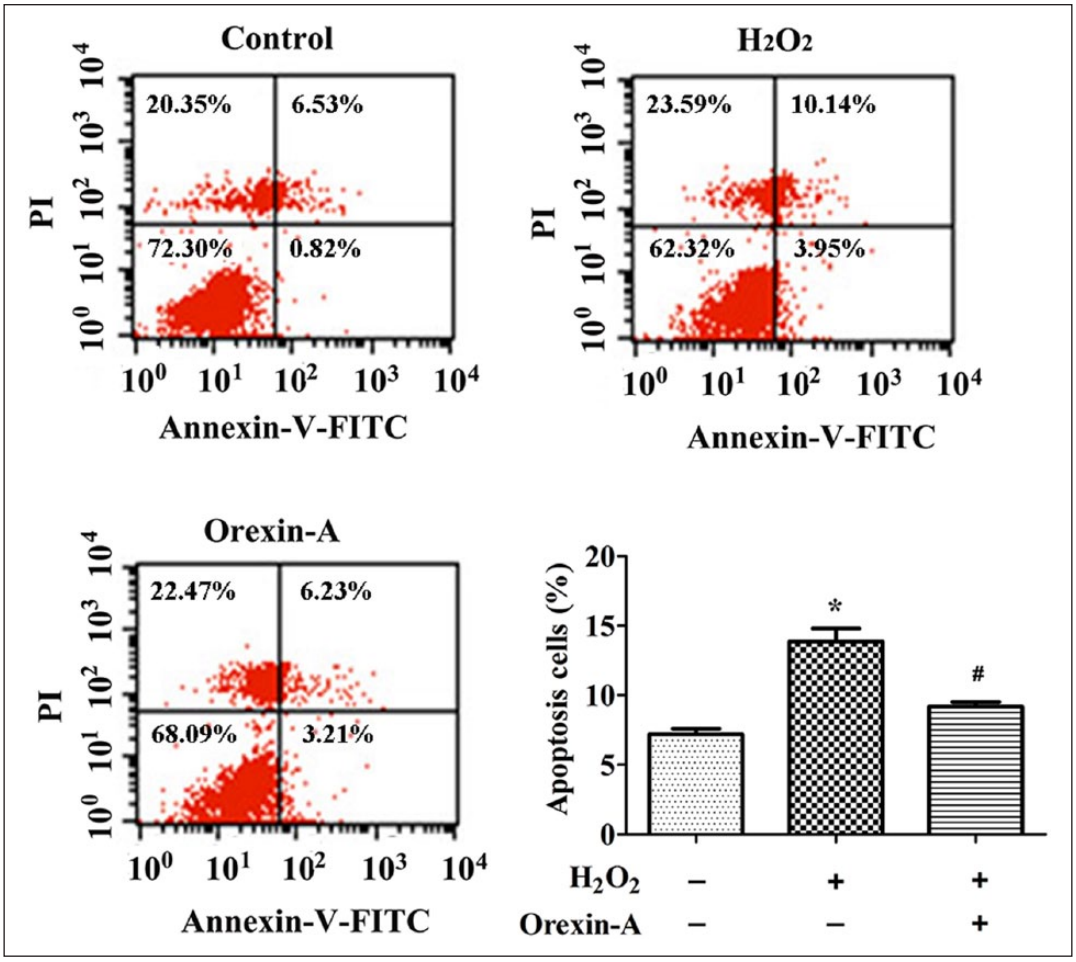

Figure 5. Orexin-A attenuates the H_2O_2-induced increase in apoptosis. SH-SY5Y cells were pre-treated with 100 nM Orexin-A for 6 h and then treated with 200 μM H_2O_2 for 24 h. Caspase-3/7 activity was assessed using an annexin V-FITC Apoptosis Detection Kit. The percentage of apoptotic cells is shown. (*$p < 0.05$ vs non-treated group; #$p < 0.05$ vs H_2O_2-treated group.)

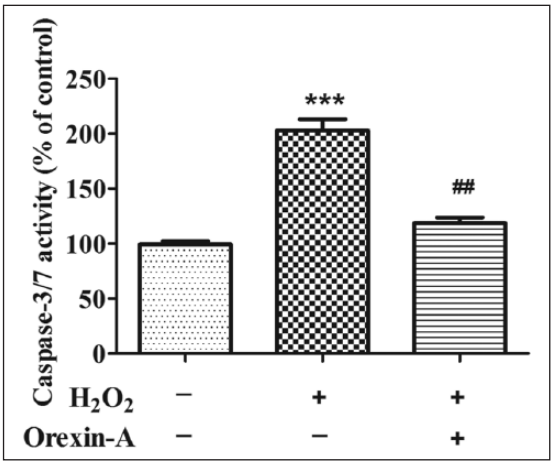

Figure 6. Orexin-A attenuates the H_2O_2-induced increase in caspase-3/7 activity. SH-SY5Y cells were pre-treated with 100 nM Orexin-A for 6 h and then treated with 200 μM H_2O_2 for 24 h. Caspase-3/7 activity was measured using a Caspase-3/7 Assay Kit. (***$p < 0.001$ vs non-treated group; ##$p < 0.01$ vs H_2O_2-treated group.)

and decreases caspase-3 activity in H295R adrenocortical cells.[17] To the best of our knowledge, this study is the first to evaluate the neuroprotective effects of Orexin-A in H_2O_2-treated SH-SY5Y cells. The effects of increasing concentrations of Orexin-A on the H_2O_2-induced decrease in SH-SY5Y cell viability were evaluated. Orexin-A dose-dependently attenuated the H_2O_2-induced decrease in cell viability. Moreover, Orexin-A markedly attenuated the H_2O_2-induced increase in apoptosis. Consistently, caspase-3/7 activity, which was measured using a Caspase-3/7 Assay Kit, was lower in cells pre-treated with Orexin-A than in H_2O_2-treated cells. These results indicate that Orexin-A protects SH-SY5Y cells against H_2O_2-induced neurotoxicity. H_2O_2 treatment significantly decreased SOD activity in SH-SY5Y cells and this was attenuated by pre-treatment with Orexin-A, consistent with a study by Bihamta et al.[18]

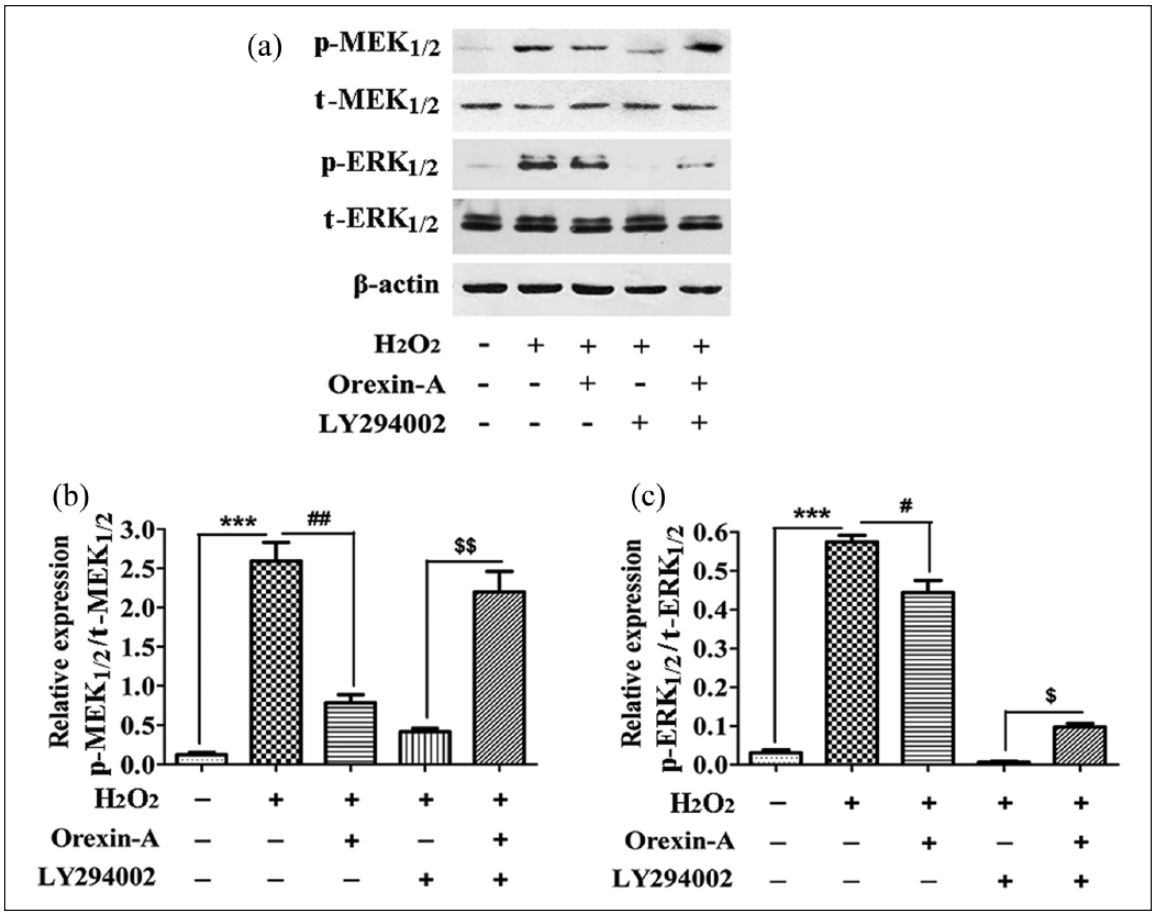

Figure 7. Effects of H_2O_2, Orexin-A, and LY294002 on phosphorylation of $MEK_{1/2}$ and $ERK_{1/2}$. (a) Representative western blots of p-$MEK_{1/2}$ and p-$ERK_{1/2}$ in SH-SY5Y cells treated with H_2O_2, Orexin-A, and/or LY294002. (b) Quantification of the band intensities of p-$MEK_{1/2}$ and p-$ERK_{1/2}$ in SH-SY5Y cells treated with H_2O_2, Orexin-A, and/or LY294002. (***$p < 0.001$ vs non-treated group; #$p < 0.05$ and ##$p < 0.01$ vs H_2O_2-treated group; $$p < 0.05$ and $$$p < 0.01$ vs H_2O_2 plus LY294002-treated group.)

ERK signaling is involved in oxidative stress in various cell lines.[19,20] H_2O_2 treatment increases phosphorylation of $ERK_{1/2}$ in human umbilical vein endothelial cells and this is associated with apoptosis of these cells.[21,22] GYY4137 protects against H_2O_2-induced death and apoptosis of MC3T3-E1 osteoblastic cells by suppressing activation of $ERK_{1/2}$.[20,23] Buchang Naoxintong Capsule protects H9c2 rat cardiomyoblasts against H_2O_2-induced oxidative injury by activating $ERK_{1/2}$ and blocking mitochondria-mediated apoptosis.[24] Many groups have reported that H_2O_2 induces phosphorylation of $ERK_{1/2}$ in SH-SY5Y cells.[23,25,26] In this study, phosphorylation of $ERK_{1/2}$ in SH-SY5Y cells was increased by H_2O_2, and this effect was attenuated by Orexin-A. Furthermore, phosphorylation of $ERK_{1/2}$ was accompanied by phosphorylation of $MEK_{1/2}$. These results are consistent with those of other studies.[27–29] Our data indicate that the neuroprotective effects of Orexin-A involve suppression of $MEK_{1/2}$/$ERK_{1/2}$ activity.

Orexin-A exerts physiological and pharmacological effects by regulating the PI3K/Akt signaling pathway in various cell types. For example, Orexin-A protects rat hepatocytes against apoptosis by regulating FoxO1 and mTORC1 via the PI3K/Akt signaling pathway.[30] Orexin-A promotes proliferation and reduces the pro-apoptotic activity of caspase-3 in H295R adrenocortical cells via the Akt pathway.[31] It was reported that Orexin-A protects SH-SY5Y cells against 6-hydroxydopamine-induced neurotoxicity, an in vitro model of Parkinson's disease, via PI3 K signaling pathways.[32] However, we did not detect phosphorylation of Akt at serine 308/serine 473 in SH-SY5Y cells treated with H_2O_2 and Orexin-A. To investigate the relationship between PI3K and $ERK_{1/2}$ signaling, cells were pre-treated with LY294002, an inhibitor of

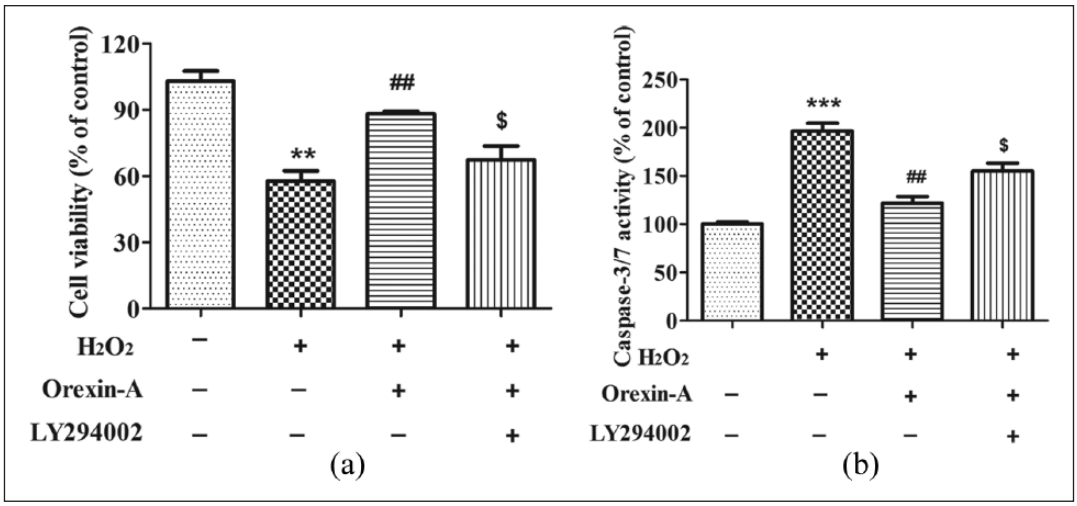

Figure 8. Treatment with the PI3K inhibitor LY294002 attenuates the protective effects of Orexin-A against H_2O_2-induced neurotoxicity. SH-SY5Y cells were pre-treated with 25 mM LY294002 for 30 min, treated with 100 mM Orexin-A for 6 h, and then exposed to H_2O_2 for 24 h. Cell viability and caspase-3/7 activity were measured using (a) the CCK-8 assay and (b) a Caspase-3/7 Assay Kit, respectively. (**$p < 0.01$ and ***$p < 0.001$ vs non-treated group; ##$p < 0.01$ vs H_2O_2-treated group; $$p < 0.05$ vs H_2O_2 plus Orexin-A-treated group.)

PI3K, for 30 min. H_2O_2-induced phosphorylation of $MEK_{1/2}$ and $ERK_{1/2}$ was abolished in SH-SY5Y cells pre-treated with LY294002, while it was higher in cells pre-treated with Orexin-A and LY294002 than in those pre-treated with only LY294002. We speculate that Orexin-A protects SH-SY5Y cells against H_2O_2-induced oxidative stress via the PI3K/$MEK_{1/2}$/$ERK_{1/2}$ signaling pathway. Furthermore, attenuation of the H_2O_2-induced decrease in cell viability by Orexin-A was abolished upon pre-treatment with LY294002. Orexin-A also failed to attenuate the H_2O_2-induced increase in caspase-3/7 activity in the presence of LY294002. These results support the notion that Orexin-A protects against H_2O_2-induced oxidative stress via the PI3K/$MEK_{1/2}$/$ERK_{1/2}$ signaling pathway.

Taken together, this study demonstrates that Orexin-A protects against H_2O_2-induced oxidative damage via the PI3K/$MEK_{1/2}$/$ERK_{1/2}$ signaling pathway and attenuates the H_2O_2-induced increase in apoptosis and decrease in cell viability. Consequently, Orexin-A may be useful to treat neurodegenerative diseases associated with oxidative damage. However, further in vivo studies are required to evaluate the clinical significance of Orexin-A prior to its clinical use.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

This work was supported by grants from the National Nature Science Foundation of China (No. 81501018), the Shandong Province Natural Science Foundation (No. ZR2013CQ031), and the Science and Technology Project of Colleges and Universities in Shandong Province, China (No. J15LE19).

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