Activation of α7 Nicotinic Acetylcholine Receptor Protects Against Oxidant Stress Damage Through Reducing Vascular Peroxidase-1 in a JNK Signaling-Dependent Manner in Endothelial Cells

Dong-Jie Li a,b,c Ting Zhao b,c Ru-Juan Xin b Yuan-Yuan Wang a Yi-Bo Fei a Fu-Ming Shen a

aDepartment of Clinical Pharmacy, Shanghai Tenth People’s Hospital, Tongji University; bDepartment of Pharmacology, Second Military Medical University, Shanghai, China; cThese two authors contribute equally to this work

Key Words
Alpha7 nicotinic acetylcholine receptor • Vascular peroxidase-1 • Oxidation • Apoptosis • Endothelial cell • JNK signaling

Abstract
Aim: Alpha7 nicotinic acetylcholine receptor (α7nAChR), a subtype of nAChR regulating neurotransmission in central nervous system, is an essential regulator of cholinergic anti-inflammatory pathway in periphery. The present study was to determine the effects of activation of α7nAChR on oxidant stress-induced injury in endothelial cells. Methods: Cultured human umbilical vein endothelial cells were treated with H2O2 (400 µM) or H2O2 plus PNU-282987 (10 µM). Cell viability and membrane integrity were measured. Annexin V + PI assay, immunoblotting of bcl-2, bax and cleaved capase-3, and immunofluorescence of apoptosis inducing factor (AIF) were performed to evaluate apoptosis. Protein expression of vascular peroxidase-1 (VPO-1) and phosphor-JNK were measured by immunoblotting. Results: Activation of α7nAChR by a selective agonist PNU-282987 prevented H2O2-induced decrease of cell viability and increase of lactate dehydrogenase release. Activation of α7nAChR markedly reduced cell apoptosis and intracellular oxidative stress level. Moreover, activation of α7nAChR reduced H2O2-induced VPO-1 protein upregulation and JNK1/2 phosphorylation. The inhibitory effect of α7nAChR activation on VPO-1 was blocked by JNK inhibitor SP600125. In addition, pretreatment of α7nAChR antagonist methyllycaconitine blocked the cytoprotective effect of PNU-282987. Conclusion: These results provide the first evidence that activation of α7nAChR protects against oxidant stress-induced damage by suppressing VPO-1 in a JNK signaling pathway-dependent manner in endothelial cells.

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Introduction

Reactive oxygen species (ROS) are a family of molecules including molecular oxygen and its derivatives produced in cellular metabolism. Excessive production of ROS, outstripping endogenous antioxidant defense mechanisms, is commonly referred to as oxidant stress. A large number of studies showed that one of major cause of endothelial dysfunction is the excessive oxidant stress [1, 2]. And an increasing body of evidence suggests that oxidant stress-caused endothelial dysfunction is involved in the pathogenesis of many cardiovascular diseases, including hypertension, hypercholesterolemia, atherosclerosis, diabetes, stroke and heart failure [1-4].

The nicotinic acetylcholine receptors (nAChRs) are a group of ligand-gated ion channels found in both the periphery and the central nervous system [5]. The main function of these receptors is to transmit signals for the neurotransmitter acetylcholine at neuromuscular junctions in the central and peripheral nervous systems [5]. The α7 subtype of nAChR (α7nAChR), which was isolated and sequenced in 1990, was once believed to be a neuronal specific nAChR subunit that is distinguished from other nAChRs by its rapid desensitization and low probability of channel opening [6]. Recently, α7nAChR has attracted great attention due to its essential role in the cholinergic anti-inflammatory pathway [7, 8]. In cardiovascular system, the α7nAChR was reported as an important protector in myocardial ischemia/reperfusion injury [9], stroke [10], atherosclerosis [11] and hypertension [12, 13]. Our group previously showed that activation of α7nAChR protects against lipopolysaccharide-induced septic shock via inhibiting release of inflammatory cytokines [14]. We also demonstrated that downregulation of α7nAChR might contribute to the development of end organ damage through inducing cardiovascular inflammation in both genetic and experimental hypertension [12, 13]. Besides this intrinsic anti-inflammatory characteristic, we hypothesized that there might be more molecular mechanisms underlying the protective effects of α7nAChR in cardiovascular system. In this study, we investigated the effect of activation of α7nAChR on oxidant stress-induced damage in endothelial cells. Our results provide the first evidence that activation of α7nAChR protects against oxidant stress damage through reducing vascular peroxidase-1 (VPO-1) in a JNK signaling-dependent manner in endothelial cells.

Materials and Methods

Reagents

Antibodies against cleaved caspase-3, phospho-JNK1/2 and total-JNK1/2 were purchased from Cell Signaling Biotechnology (Danvers, MA). Antibody against α7nAChR and VPO-1 were purchased from Abcam (Cambridge, MA). Antibody against apoptosis-inducing factor (AIF) was purchased from Santa-Cruz Biotechnology (Santa-Cruz, CA). Antibody against β-actin was from Sigma. Cell viability assay (Cell Counting Kit-8) was from Dojindo Molecular Technologies, Inc. (Kumamoto, Japan). LDH cytotoxic kit was purchased from Promega (Madison, WI). SP600125, Malondialdehyde (MDA), MnSOD activity assay and Annexin V + PI kits were purchased from Beyotime (Haimen, Jiangsu, China). Dichloro-dihydro-fluorescein diacetate (DCFH-DA) was purchased from Invitrogen (Carlbad, CA). Enhanced chemiluminescence and protease/phosphatase inhibitors were purchased from Pierce (Rockford, IL).

Cell Culture and treatment

Human umbilical vein endothelial cell line (HUVECs) was provided and licensed by Cell Bank of Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences. Cells were cultured in RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum (FBS) in 95% O2 and 5% CO2 [15]. To study the protective effects of activation of α7nAChR on H2O2-induced HUVEC death, cells were seeded in 96-well plates (10,000 cells/well), and 3 wells were used for each treatment group.
Cell viability assay

Cell viability was evaluated by a non-radioactive cell counting kit-8 (CCK-8) assay as described previously [16]. To synchronize cells, cells were pre-incubated with 0.01% FBS for 8 hours (serum starvation). Then, cell were H₂O₂ (400 μM) or H₂O₂ (400 μM) plus PNU-282987 (3 and 10 μM) for 24 hours. Control cells were treated with PBS as vehicle control. After discarding the medium, cells were incubated with 10 μL of CCK-8 solution for 1 h at 37 °C. The optical density at 450 nm was analyzed in a microplate reader. Experiments were performed in duplicate.

LDH release assay

LDH release was determined by a lactate dehydrogenase (LDH) release assay using Promega CytoTox-ONE™ kit (Promega) [17]. In brief, 10⁴ cells were seeded in 96-well plates. Cells were pre-incubated with 0.1% FBS for 8 hours. Then, cell were H₂O₂ (400 μM) or H₂O₂ (400 μM) plus PNU-282987 (3 and 10 μM) for 6 hours. Control cells were treated with 0.05% (v/v) DMSO as vehicle control. The medium was transferred to a black fluorescence plate and incubated for 10 minutes with CytoTox-ONE™ reagent followed by stop solution. Fluorescence was measured at 560/590 nm [18].

Annexin V + PI assay

Annexin V + PI was performed as described previously [19]. Cells were treated with H₂O₂ (400 μM) or H₂O₂ plus PNU-282987 (10 μM) for 24 h. Then, 1 × 10⁴ cells were harvested and washed with Phosphate Buffered Saline (PBS). Cells were suspended in 500 μl binding buffer and then incubated for 5 min at room temperature in the dark with 5 μl Annexin-V-FITC and 10 μl PI additions. Flow cytometry analysis was performed in a FACS Calibur flow cytometry (BD Biosciences, San Jose, CA). Intact cells (Annexin V−/PI−) were discriminated from apoptotic cells (Annexin V+/PI−) and necrotic cells (Annexin V+/PI+) respectively [20].

Immunoblotting

Immunoblotting analyses of cell-extracts were performed as described previously [12, 21, 22]. Cells were lysed with RIPA buffer with protease inhibitor and protein phosphatase inhibitors. Protein fractions were separated in 10% SDS-PAGE, and transferred onto nitrocellulose membranes at 100V for 1~2 h. Nonspecific protein binding was blocked with 5% weight (w)/v nonfat milk and 0.1% v/v Tween-20 in Tris-buffered saline (TBS) followed by incubation with primary antibodies diluted in TBS containing 2% w/v bovine serum albumin and 0.1% v/v Tween-20. Then the membranes were incubated with IRDye-labeled secondary antibody and detected Odyssey system.

Immunofluorescence

Immunofluorescence was performed as described previously [23, 24]. HUVECs were seeded in confocal dishes approximately 24 h prior to imaging to ensure proper cell attachment. The cells were treated by H₂O₂ (100 μM) or H₂O₂ plus PNU-282987 (10 μM) for 4 hours and washed with cold PBS three times and fixed with 4% paraformaldehyde solution for 30 min. Then, cells were incubated with anti-AIF secondary antibody and detected Odyssey system.

Reactive oxygen species (ROS) assay

HUVECs seeded on 96 well/plates were treated with treated with H₂O₂ (100 μM) or H₂O₂ plus PNU-282987 (10 μM) for 6 h and then incubated with the dichloro-dihydro-fluorescein diacetate (DCFH-DA) probe for 30 minutes. The cells were washed 3 times by PBS. The fluorescence intensity was measured by fluorescence microscope at an excitation wavelength of 488 nm. All assays were performed in three individual experiments, each comprising no less than six visual field.

MDA and MnSOD activity assays

Intracellular MDA and MnSOD activity assays were performed as described previously [26-28]. HUVECs treated with treated with H₂O₂ (100 μM) or H₂O₂ plus PNU-282987 (10 μM) for 6 h were lysed by RAPI buffer. The MDA concentration and MnSOD activity of each sample were detected according to the manufacturer’s instruction using microplate reader respectively.
Fig. 1. Activation of α7nAChR promotes cell survival upon oxidant stress in endothelial cells. (A) Immunoblotting analysis showed that α7nAChR was expressed in brain tissue, RAW 264.7 cell line and HUVECs. (B) HUVECs were treated with H$_2$O$_2$ (400 µM) or H$_2$O$_2$ (400 µM) plus PNU-282987 (3 and 10 µM) for 6 hours and then cell viability was measured. *P < 0.05 vs. control (CTRL). #P < 0.05 vs. H$_2$O$_2$ alone. N =8. (C) HUVECs were treated with H$_2$O$_2$ (400 µM) or H$_2$O$_2$ (400 µM) plus PNU-282987 (3 and 10 µM) and then the LDH in cultured medium (LDH release) was measured. *P < 0.05 vs. control (CTRL). #P < 0.05 vs. H$_2$O$_2$ alone. N =8.

Statistical analysis

All of the statistical calculations were performed using the GraphPad Prism 5 software program. The data were expressed as mean ± SEM. Student’s t-test was used to compare two conditions, and a one-way ANOVA with Tukey correction was used for multiple comparisons [29]. Statistical significance was set at $P < 0.05$.

Results

Activation of α7nAChR promotes cell survival upon oxidant stress in endothelial cells

PNU-282987 is a well-established selective agonist of the α7nAChR [30, 31] and was used to activate α7nAChR in this study. Treatment of PNU-282987 (3 and 10 µM) had no effect on HUVECs viability and did not cause any cytotoxicity in HUVECs (Fig. 1B and 1C, left panels). However, treatment of PNU-282987 significantly blocked the H$_2$O$_2$-induced reduction of HUVECs viability in a dose-dependent manner (Fig. 1B, right panel). We also determined LDH release, a marker of membrane integrity. Treatment of PNU-282987 significantly decreased H$_2$O$_2$-induced LDH release in HUVECs in a dose-dependent manner (Fig. 1C, right panel).

Activation of α7nAChR decreases oxidant stress-induced apoptosis in endothelial cells

We next studied the effects of activation of α7nAChR on apoptosis of HUVECs. Annexin V + PI assay showed that H$_2$O$_2$ treatment increased the proportion of apoptotic cells (Annexin V+/PI−, Fig. 2A and 2B). Activation of α7nAChR by PNU-282987 markedly prevented this pro-
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Fig. 2. Activation of α7nAChR decreases oxidant stress-induced apoptosis in endothelial cells. (A) Representative images of flow cytometry with Annexin V + PI assay. (B) Quantitative analysis of the proportion of early apoptotic cell (Annexin V+/PI−). *P < 0.05 vs. blank. #P < 0.05 vs. H$_2$O$_2$ alone. N =3. (C) Quantitative analysis of the proportion of late apoptotic cell (Annexin V+/PI+). *P < 0.05 vs. blank. #P < 0.05 vs. H$_2$O$_2$ alone. N =3.

Fig. 3. Activation of α7nAChR reduces oxidant stress-induced apoptotic protein expression and inhibits apoptosis inducing factor (AIF) translocation in endothelial cells. (A) Representative immunoblotting image and quantitative analysis of bcl-2 and bax. *P < 0.05 vs. blank. #P < 0.05 vs. H$_2$O$_2$ alone. N =4. (B) Representative immunoblotting image and quantitative analysis of cleaved caspase-3. *P < 0.05 vs. blank. #P < 0.05 vs. H$_2$O$_2$ alone. N =4. (C) Representative immunofluorescence images obtained by confocal microscope showing the AIF translocation from cytoplasm into nucleus under H$_2$O$_2$ treatment and the inhibitory effect of PNU-282987 on AIF translocation. DAPI was used to stain nucleus. Yellow arrows indicate the nucleus translocation.

apoptotic effect of H$_2$O$_2$ (Fig. 2A and 2B). Moreover, activation of α7nAChR by PNU-282987 significantly attenuated the proportion of apoptotic cells (Annexin V+/PI−; Fig. 2A and 2C).

We also measured some molecular marker of apoptosis. As shown in Fig. 3A, the bcl-2/bax ratio, a key factor in the regulation of apoptosis, was decreased in H$_2$O$_2$-treated HUVECs but reversed by PNU-282987 treatment. Protein level of cleaved caspase-3, a well-characterized apoptosis molecular marker, was enhanced by H$_2$O$_2$ and partly blocked by activation of α7nAChR with PNU-282987 (Fig. 3B). Moreover, immunofluorescence assay of AIF showed that H$_2$O$_2$ induced AIF nuclear translocation, which was also prevented by PNU-
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282987 treatment (Fig. 3C). All these results indicate that activation of α7nAChR decreases oxidant stress-induced apoptosis in endothelial cells.

**Activation of α7nAChR depresses oxidant stress-induced increase of intracellular ROS in endothelial cells**

DCFH-DA assay demonstrated that H₂O₂ treatment triggered fluorescence in HUVECs, which was partly blocked by PNU-282987. *P < 0.05 vs. blank. *P < 0.05 vs. H₂O₂ alone. N =6. (B) The MDA levels in HUVECs. *P < 0.05 vs. blank. *P < 0.05 vs. H₂O₂ alone. N =6. (C) The MnSOD activity in HUVECs. *P < 0.05 vs. blank. *P < 0.05 vs. H₂O₂ alone. N =6.

VPO-1 is a novel heme-containing peroxidase catalyzing peroxidant reactions especially in cardiovascular system. H₂O₂ treatment significantly upregulated VPO-1 protein expression in HUVECs (Fig. 5). However, this upregulation of VPO-1 was prevented by activation of α7nAChR with PNU-282987 (Fig. 5). These results suggest that activation of α7nAChR suppressed the oxidant stress-induced VPO-1 upregulation.

To further explore which molecular mechanisms underlie the VPO-1 upregulation by H₂O₂ and downregulation by PNU-282987, we detected the phosphorylation of JNK signaling
pathway. H\textsubscript{2}O\textsubscript{2} treatment increased phosphorylation of JNK1/2 in HUVECs (Fig. 6A), which was largely blocked by PNU-282987 supplement (Fig. 6A). These synchronous changes raised the possibility that activation of α7nAChR might influence VPO-1 expression via JNK signaling pathway. To address this point, we blocked JNK signaling pathway using SP600125, a specific chemical inhibitor of JNK pathway. SP600125 attenuated the VPO-1 upregulation by H\textsubscript{2}O\textsubscript{2} treatment (Fig. 6B), indicating that the VPO-1 upregulation upon oxidant stress involves JNK signaling pathway. More importantly, SP600125 totally abolished the reduction of VPO-1 protein expression by PNU-282987 (Fig. 6B). These results point out that activation of α7nAChR reduces the oxidant stress-induced upregulation of VPO-1 through inhibiting JNK signaling pathway in endothelial cells.

*Methyllycaconitine blocked the cytoprotective effect of α7nAChR activation in endothelial cells*

At last, we used methyllycaconitine (MLA), a selective α7nAChR antagonist, to pretreat HUVECs for 2 hours, and then subjected the cells with H\textsubscript{2}O\textsubscript{2} or H\textsubscript{2}O\textsubscript{2} plus PNU-282987. MLA pretreatment totally blocked the protective effects of PNU-282987 on both cell viability (Fig. 7A) and LDH release (Fig. 7B).

**Discussion**

In the present study, we demonstrated that activation of α7nAChR using the selective agonist PNU-282987, promotes cell survival, decreases apoptosis and depresses the
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increase of ROS upon oxidant stress in endothelial cells. Moreover, we found that activation of α7nAChR reduced VPO-1 protein expression through inhibiting JNK1/2 phosphorylation. Pretreatment of α7nAChR antagonist MLA abolished the cytoprotective effect of PNU-282987. These data suggest that activation of α7nAChR could protect against oxidant stress-induced damage by suppressing VPO-1 in a JNK signaling pathway-dependent manner in endothelial cells.

The first important finding of our study is that activation of α7nAChR is protective on endothelial cells upon oxidant stress. An increasing body of evidence suggests that oxidant stress-caused endothelial dysfunction is involved in the pathogenesis of many cardiovascular diseases, including hypertension, hypercholesterolemia, atherosclerosis, diabetes, stroke and heart failure [1-4]. Oxidant stress is a well known cause of endothelial cell damage, and several lines of evidence suggest that it might influence the induction and signaling steps of endothelial cell apoptosis through different mechanisms [1-4, 32-35]. Previously, the protective effect of α7nAChR on cell survival has been documented in central nervous system [36-38]. Activation of α7nAChR prevented neuroblastoma cells death via inhibiting ROS in a concentration-dependent manner [36]. In astrocytoma cells, melatonin decreased LPS-induced nitrative and oxidative stress, and modulated LPS-induced mRNA expressions of α7nAChR and inflammatory cytokine genes [37]. In organotypic hippocampal cultures, activation of α7nAChR reduced cell death, reactive oxygen species production, and tumor necrosis factor release [38]. Our work is the first report about the protective effect of α7nAChR upon oxidant stress in endothelial cells, highlighting the potential therapeutic value of α7nAChR in cardiovascular disease. And, we speculate that the protective effect activation of α7nAChR may be not neural-specific.

The second important finding of our study is that activation of α7nAChR regulates VPO-1 expression. VPO-1 is a novel family member of peroxidases identified in cardiovascular system in 2008 [39]. VPO-1 is highly expressed in cardiomyocytes, endothelial cells and vascular smooth muscle cells [40-42], and thereby referred to vascular peroxidase (VPO). It is a glycosylated heme-peroxidase that is actively secreted into circulating plasma by vascular endothelial cells [39]. Moreover, VPO-1 mediates apolipoprotein E (ApoE) oxidation and impairs the clearance of plasma lipids [43]. Although the exact biological functions of VPO-1 remain largely unknown, it is believed that it is an enzyme that is downstream of NADPH oxidases (NOX) and functions to utilize NOX-derived hydrogen peroxide (H2O2) to produce hypochlorous acid (HOCl), a strong oxidant which greatly promotes oxidative...
stress in endothelial cells [44]. Consequently, VPO-1 is a critical promoter of endothelial oxidant stress. We found that activation of α7nAChR markedly reduced H$_2$O$_2$-induced VPO-1 expression in HUVECs. Previously, nicotine was reported to activate α7nAChR and attenuate inflammatory response in macrophages through HO-1 induction [38, 45]. Here, our results provide another interesting example of a critical regulatory effect of α7nAChR on intracellular ROS and ROS-related signaling pathways.

We showed that activation of α7nAChR attenuated the oxidant stress-induced phosphorylation of JNK. JNK signaling pathway is a key mediator of cell responses to environmental stimuli and a potent modulator in cell death mediated by ROS and nitrogen species [46]. Nicotine blocked JNK signaling in PC12 neuronal cells [47]. Treatment of endothelial cells with carbachol decreased phosphorylation of ERK1/2 and JNK through α7nAChR [48]. Suzuki et al. also demonstrated that the inhibition of TNF-α release by nicotine through α7nAChR was associated with the suppression of JNK signaling pathway [49]. Our result supported the notions that activation of α7nAChR is able to block JNK signaling activation. Notably, we also observed that the inhibitory effect of activation α7nAChR on VPO-1 was prevented by a chemical inhibitor of JNK1/2, suggesting that inhibitory effect of activation α7nAChR on VPO-1 is JNK signaling-dependent.

In conclusion, we demonstrate that activation of α7nAChR protects against oxidant stress-induced apoptosis and suppresses ROS level through downregulating VPO-1 in a JNK signaling-dependent manner in endothelial cells. Our results indicate that α7nAChR may be a therapeutic target for endothelial dysfunction and cardiovascular disease.

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Conflict of Interest

None.

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