# Role of ALK5/SMAD2/3 signaling in the regulation of NOX expression in cerebral ischemia/reperfusion injury

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**Abstract.** Nicotinamide adenine dinucleotide phosphate oxidase (NOX)-derived reactive oxygen species (ROS) serve an important role in cerebral ischemia/reperfusion (I/R) injury. However, the mechanism by which ROS generation is regulated has not yet been fully elucidated. The present study aimed to explore the role of transforming growth factor-β signaling in ROS generation. Sprague Dawley rats were subjected to I/R injury and PC-12 cells were transfected with small interfering RNA against activin receptor-like kinase (ALK)5 or hypoxia/reoxygenation (H/R). The results indicated that I/R or H/R significantly increased ALK5 expression, SMAD2/3 phosphorylation and NOX2/4 expression and activity, concomitant with ROS generation and apoptosis. In addition, ALK5 knockdown significantly reversed changes induced by H/R treatment in PC-12 cells. These results suggest that ALK5/SMAD2/3 signaling serves a key role in oxidative stress. To the best of our knowledge, this is the first study to demonstrate that ALK5/SMAD2/3 activation is associated with the regulation of NOX2/4 expression and exacerbates I/R injury.

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*Abbreviations:* I/R, ischemia/reperfusion; NOX, nicotinamide adenine dinucleotide phosphate oxidase; ROS, reactive oxygen species; H/R, hypoxia/reoxygenation; ALK5, activin receptor-like kinase 5; TGF-β, transformation growth factor-β

*Key words:* cerebral ischemia/reperfusion injury, activin receptor-like kinase 5/SMAD2/3 signaling, nicotinamide adenine dinucleotide phosphate oxidase 2, nicotinamide adenine dinucleotide phosphate oxidase 4, reactive oxygen species

## Introduction

Ischemic stroke remains the one of the leading neurological diseases, with high morbidity and mortality worldwide (1). The main pathological processes of ischemic stroke include excitotoxicity, inflammatory response and oxidative stress, which ultimately lead to irreversible neuronal injury (2). Among these, oxidative stress is the most widespread process during ischemia/reperfusion (I/R) injury and describes a major difficulty for clinical treatment (3,4). However, the detailed mechanism of oxidative stress has not yet been fully elucidated. Previous studies have demonstrated that nicotinamide adenine dinucleotide phosphate (NADPH) oxidases (NOX) are the primary enzymes responsible for reactive oxygen species (ROS) generation and seven NOXs have been identified in mammals, including NOX1-5, dual oxidase (DUOX)-1 and DUOX-2 (5). A previous study identified that NOX2 and NOX4 are the major NOXs responsible for brain tissue ROS production in a rat ischemic stroke model (6). Numerous factors are reported to be associated with the regulation of NOX expression, including the transcription factor, nuclear factor (NF)-κB and myosin light chain (7). However, the detailed mechanism by which NOX expression is regulated has not yet been fully elucidated.

Transforming growth factor-β (TGF-β) is associated with numerous biological processes, including cell growth, differentiation, migration, survival, adhesion and apoptosis (8), in addition to serving an important role in tumor suppression (9). Through interacting with TGF-β receptors (serine/threonine kinases), TGF-β triggers a cascade of events, including activation of the extracellular signal-regulated kinase signaling pathway and mechanistic target of rapamycin-mediated protein synthesis (10). These processes usually involve phosphorylation of SMAD2/3 and activin receptor-like kinase (ALK)5 (10). Emerging evidence has demonstrated that TGF-β signaling is crucial in the pathogenesis of several central nervous system (CNS) disorders, including neurodegenerative disorders (11). Previous studies have demonstrated that increased TGF-β levels are associated with cerebral ischemic injury (12,13). These results suggest that TGF-β signaling may serve an important function in ischemic stroke. Although

previous studies have demonstrated that TGF-β signaling is associated with ROS production (14), the association between ALK5/SMAD2/3 signaling and oxidative stress in ischemic stroke remains unclear.

The present study aimed to investigate the role of ALK5/SMAD2/3 signaling in oxidative stress. Using an I/R injury rat model, PC-12 cell hypoxia/reperfusion (H/R) model and ALK5 silencing, it was identified that ALK5/SMAD2/3 signaling was associated with the regulation of NOX2/4 expression. To the best of our knowledge, this is the first study to explore the role of ALK5/SMAD2/3 signaling in NOX-mediated oxidative stress in I/R injury, which may provide a novel target for ischemic stroke therapy.

## Materials and methods

*Animal experiments*. A total of 16 male Sprague Dawley rats (age, 9 weeks; weight, 250-300 g) were purchased from Hunan SJA Laboratory Animal Co., Ltd. (Changsha, China). Animals were allowed to accommodate to 27˚C, regular atmosphere, 12-h light/dark cycle, with free to food and water for 1 week. Prior to experiments, rats fasted for 24 h, with free access to water only. The study was performed according to the Guide for the Care and Use of Laboratory Animals, published by the National Institutes of Health (15) and experiments were approved by the Hunan Normal University Veterinary Medicine Animal Care and Use Committee (Changsha, China).

The rat model of I/R injury was established as previously described (16). Briefly, the middle cerebral artery of the rats was occluded for 2 h with subsequent reperfusion for 24 h. The body temperature of the rats was maintained at ~37˚C throughout the procedure. Animals from the sham group, without I/R injury, underwent the same procedure, except that the occluding filament was inserted 7 mm above the carotid bifurcation instead.

The animals were randomly allocated to two groups (n=8 per group): The sham group, which underwent surgical procedures without occlusion of the middle cerebral artery and the I/R group, which was subjected to 2 h of ischemia followed by 24 h of reperfusion. At the end of reperfusion, all animals were sacrificed prior to obtaining brain tissues, which were collected to assess cell apoptosis, mRNA and protein expression.

*Terminal deoxynucleotidyl-transferase-mediated dUTP nick end labeling (TUNEL) staining*. Cellular apoptosis in the brain tissue from different treatment groups was analyzed by TUNEL assay according to the manufacturer's instructions of a commercial kit (Colorimetric TUNEL Apoptosis Assay kit; C1091; Beyotime Institute of Biotechnology, Shanghai, China). Briefly, brains were embedded into paraffin and fixed with 10% formaldehyde at 27˚C for 20 min. Following, brains were cut into 5-$\mu$m sections and incubated with 1X biotin-labeled deoxyuridine triphosphate (Beyotime Institute of Biotechnology, Haimen, China) at 37˚C for 1 h. Samples were incubated with streptavidin-horseradish peroxidase (HRP) at 37˚C for 30 min and 0.05% 3'-diaminobenzidine development solutions at 25˚C for 10 min in sequential order. Brain slides were examined microscopically (light microscope; magnification, x200) and imaged with a high-resolution digital camera (Nikon Eclipse 80i; Nikon Corporation, Tokyo, Japan).

*Cell culture*. PC-12 cell line was purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; HyClone; GE Healthcare, Chicago, IL, USA) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific, Inc., Waltham, MA, USA) and penicillin (100 U/ml) and streptomycin (0.1 mg/ml) and maintained in 95% air and 5% $CO_2$ at 37˚C. Cells were subcultured and seeded into 6- or 24-well plates at $10^5$ cells/well for transfection experiments. Following transfection, cells were digested with 0.2% trypsinogen and collected for mRNA or protein analysis.

*Cell model of H/R*. To establish an H/R model, PC-12 cells were subjected to 5 h of hypoxia ($N_2/CO_2$, 95:5) in preconditioned hypoxic medium (serum-free DMEM without glucose or sodium pyruvate, which was incubated at 37˚C under hypoxic conditions for 2 h), followed by 20 h of reoxygenation (5% $CO_2$). Hypoxic medium was replaced with fresh medium upon switching to reoxygenation.

Cells were divided into three groups (n=6 wells/group): H/R group, which was subjected to 5 h of hypoxia followed by 20 h of rexoygenation; +negative control (NC) small interfering (si)RNA group, where cells were transfected with negative control siRNA and +ALK5 siRNA group, where cells were transfected with siRNA against ALK5 prior to H/R. At the end of reoxygenation, cells were collected to assess apoptosis, ROS production and mRNA and protein expression.

*siRNA knockdown experiments*. siRNA against ALK5 (sense, 5'-CAUAUUGCUGCAACCAGGATT-3'; antisense, 5'-UCCUGGUUGCAGCAAUAUGTT-3') was designed and synthesized by Sangon Biotech Co., Ltd., (Shanghai, China). Prior to the experiment, mixtures of Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) and 80 nM ALK5 or NC siRNA (sense, 5'-UUCUCCGAACGUGUC ACGUTT-3'; antisense, 5'-ACGUGACACGUUCGGAGA ATT-3') were prepared. Mixtures were added into 24-well cell plates for 6 h for transfection at 30˚C. Following, cells were incubated in a $CO_2$ incubator at 37˚C for 24 h prior to gene expression measurement, according to the manufacturer's protocol.

*Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)*. RT-qPCR was used to analyze ALK5, NOX2 and NOX4 mRNA levels in brain tissue. Total RNA was extracted using TRIzol reagent (Takara Biotechnology Co., Ltd., Dalian, China) and concentration and purity of RNA were determined spectrophotometrically. A total of 200 ng RNA from each sample was used for RT, which was performed according to the manufacturer's protocol of the RT kit (DRR037A; Takara Biotechnology Co., Ltd.). qPCR was performed to determine ALK5, NOX2 and NOX4 mRNA expression levels, using SYBR Premix Ex Taq (Takara Biotechnology Co., Ltd.) and an ABI 7300 system (Applied Biosystems; Thermo Fisher Scientific, Inc.). qPCR primers for ALK5, NOX2, NOX4 and β-actin are presented in Table I. Data analysis was performed

Table I. Primers for reverse transcription-quantitative polymerase chain reactions.

| Gene | Forward primer (5'-3') | Reverse primer (5'-3') | Product size (bp) |
|---|---|---|---|
| NOX2 | ACAAGGTTTATGACGATGAGCC | TTGAGCAACACGCACTGGAA | 174 |
| NOX4 | CTGACAGGTGTCTGCATGGT | ACTTCAACAAGCCACCCGAA | 160 |
| ALK5 | TTGTTGAGGAGAAGCTGAGGC | CACTGTAATGCCTTCGCCCC | 154 |
| β-actin | CCCATCTATGAGGGTTACGC | TTTAATGTCACGCACGATTTC | 150 |

NOX, nicotinamide adenine dinucleotide phosphate oxidase; ALK5, activin receptor-like kinase 5.

using the $2^{-\Delta\Delta Cq}$ method using ABI 7300 software (v2.4; Thermo Fisher Scientific, Inc.) (17). Relative expression of ALK5, NOX2 and NOX4 mRNA was normalized against β-actin mRNA.

*Hoechst staining and lactate dehydrogenase (LDH) release*. Cellular apoptosis in PC-12 cells of different treatment groups was analyzed by Hoechst assay (Beyotime Institute of Biotechnology). Briefly, cells were incubated at 25˚C with Hoechst 33258 solutions (Beyotime Institute of Biotechnology) for 20 min. Images were examined microscopically (light microscope; magnification, x200) and photographed by a high-resolution digital camera (Nikon Eclipse 80i; Nikon Corporation).

Cell toxicity of PC-12 was assessed by LDH release using a microplate reader. The procedure was performed according to the manufacturer's protocol of a commercial LDH release kit (C0016; Beyotime Institute of Biotechnology, Shanghai, China).

*Western blotting*. Total protein from each sample was extracted using the method described by Zhang *et al* (6). Proteins (40 $\mu$g) were separated on 10% SDS-PAGE gels and transferred to polyvinylidene fluoride membranes. Membranes were blocked with 5% milk at 25˚C for 2 h and membranes incubated with 1:2,000 diluted rabbit anti-phosphorylated (p)-SMAD2/3 (sc-517575; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), NOX2 (sc-130543; Santa Cruz Biotechnology, Inc.), NOX4 (sc-30141; Santa Cruz Biotechnology, Inc.), ALK5 (sc-101574; Santa Cruz Biotechnology, Inc.), caspase-3 (sc-7272; Santa Cruz Biotechnology, Inc.) and mouse anti-β-actin (1:2,000; sc-47778; Santa Cruz Biotechnology, Inc.) at 4˚C for 16 h followed by incubation with HRP-goat anti-mouse IgG (1:2,000; A0216; Beyotime Institute of Biotechnology) or anti-rabbit IgG (1:2,000; A0208; Beyotime Institute of Biotechnology). Bands were detected using an enhanced chemiluminescence kit (GE Healthcare, Chicago, IL, USA) and a Molecular Imager ChemiDoc XRS system (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Densitometric analysis was conducted with ImageJ 1.43 (National Institutes of Health, Bethesda, MD, USA).

*NOX and caspase-3 activity*. NOX activity was measured using a commercial NADPH oxidase activity quantification kit (GMS50096.1, Shanghai Genmed Pharmaceutical Technology Co., Ltd., Shanghai, China) following the manufacturer's protocol. Briefly, cells were centrifuged at 12,000 x g for 30 min at 4˚C and the supernatant of the cell lysates was incubated with oxidized cytochrome c in a quartz cuvette at 30˚C for 3 min, NOX substrate (NADPH) was added to the reaction mixture and incubated at 30˚C for a further 15 min. The change of absorbance at 550 nm was monitored. NOX activity was estimated by calculating the cytochrome c reduction per min.

Measurements of caspase-3 activity were performed according to the manufacturer's protocol of a commercial Caspase-3 Activity Assay kit (Beyotime Institute of Biotechnology). Briefly, cell lysate (10 $\mu$l) was mixed with working solution containing caspase-3 substrate (90 $\mu$l, Ac-DEVD-pNA) and the mixture was incubated at 37˚C for 60 min. The absorbance was recorded at 405 nm. Enzyme activity was recorded as U/g protein, where 1 U was defined as the amount of enzyme required to react with 1 nmol of Ac-DEVD-pNA per h at 37˚C.

*Determination of ROS levels*. Intracellular ROS levels were determined with 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA), a cell-permeable indicator of ROS (Beyotime Institute of Biotechnology). DCFH-DA is non-fluorescent until cleavage of the acetate groups by intracellular ROS. Briefly, PC-12 cells ($10^7$ cells/well) were washed with PBS and incubated with DCFH-DA (10 $\mu$M) at 37˚C for 20 min. ROS-mediated fluorescence was observed under a fluorescence microscope, with excitation set to 502 nm and emission at 523 nm. Results are expressed using arbitrary units.

*Statistical analysis*. SPSS software (version 11; SPSS, Inc., Chicago, IL, USA) was used for statistical analysis. Data are presented as mean ± standard error of the mean. Differences among multiple groups were analyzed by analysis of variance with Bonferroni's multiple comparison test. Student's t-test was used to compare two groups. $P<0.05$ was considered to indicate a statistically significant difference.

## Results

*I/R injury induces ROS production and apoptosis in brain tissues*. To explore the mechanism for clinical I/R injury, a rat I/R model was established that simulated ischemic stroke. ROS level, caspase-3 enzyme activity and expression were determined and apoptosis was detected by TUNEL staining, in order to evaluate oxidative stress injury. As indicated in Fig. 1A, tissues with I/R injury exhibited higher ROS production compared with the sham group. This suggested that these

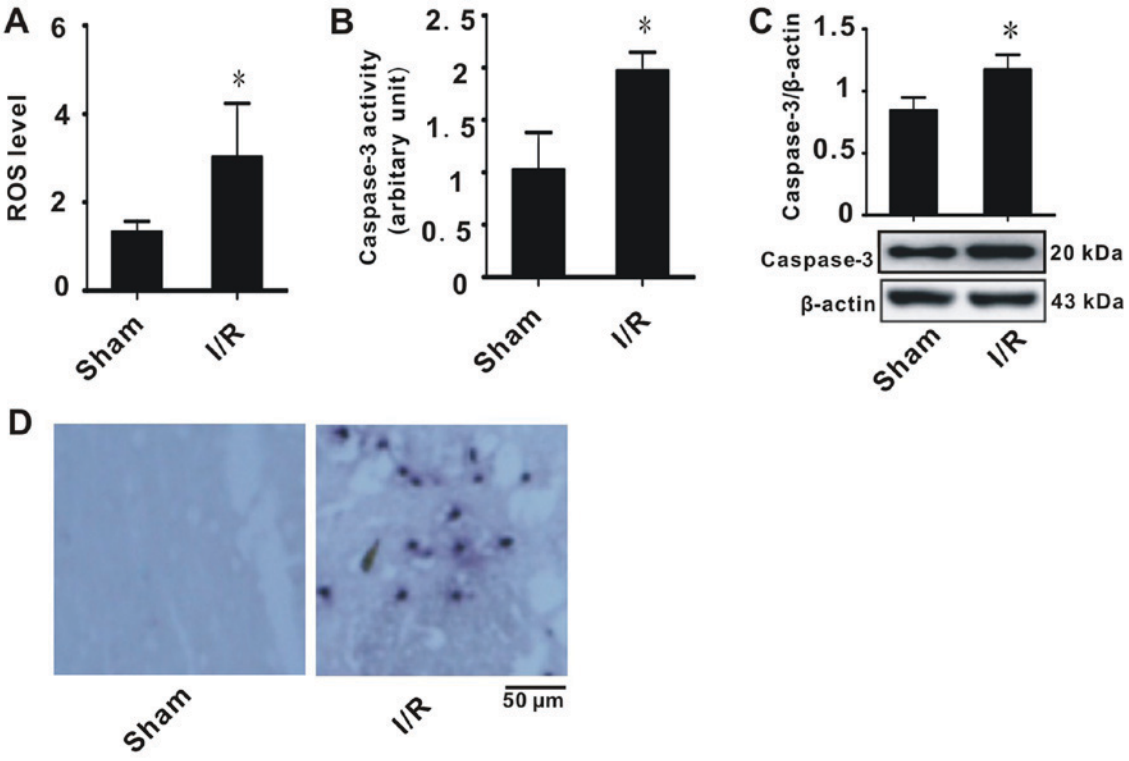


Figure 1. I/R injury induces ROS production and apoptosis in brain tissues. (A) ROS level (n=8). Caspase-3 (B) activity and (C) protein expression (n=8). (D) Representative images of terminal deoxynucleotidyl-transferase-mediated dUTP nick end labeling-stained brain tissue from each group. Data were evaluated by student's t-test. *P<0.05 vs. Sham. I/R, ischemia/reperfusion; ROS, reactive oxygen species.

tissues suffered from oxidative stress. In addition, it was indicated that I/R significantly increased caspase-3 expression and activity compared with the sham group (Fig. 1B and C). TUNEL staining indicated that tissues with I/R had significantly increased apoptosis compared with the sham group (Fig. 1D). These results suggested that the rats exhibited evident brain injury.

*I/R injury affects ALK5, p-SMAD2/3, NOX2 and NOX4 expression*. As ALK5/p-SMAD2/3 signaling regulates gene expression and NOX2/4 activity is the main source of cellular ROS, effects of I/R injury on ALK5, p-SMAD2/3, NOX2 and NOX4 mRNA and protein expression were determined, in addition to measuring the total NOX activity. The results indicated that I/R injury significantly increased ALK5 mRNA and protein expression (Fig. 2A and B) and phosphorylation of SMAD2/3 (Fig. 2C-E). It was observed that I/R injury significantly increased NOX2/4 expression and activity (Fig. 2F-J). These data indicated that NOX2/4 expression was associated with ALK5/p-SMAD2/3 expression.

*ALK5 knockdown affects ALK5, p-SMAD2/3, NOX2 and NOX4 expression in PC-12 cells*. The association between ALK5/p-SMAD2/3 and NOX2/4 was assessed. An H/R model was constructed *in vitro* to simulate I/R injury. Cells were treated with siRNA against ALK5 or NC siRNA. The results indicated that ALK5 siRNA significantly decreased ALK5 mRNA and protein expression, indicating that ALK5 was knocked down successfully (Fig. 3A and B). Effects of ALK5 knockdown on phosphorylation of SMAD2/3 and expression of NOX2/4 were evaluated. Cells treated with ALK5 siRNA exhibited a significantly lower SMAD2/3 phosphorylation level compared with cells treated with H/R alone (Fig. 3C-E). ALK5 knockdown significantly decreased NOX2/4 mRNA and protein expression and total NOX enzyme activity, when compared with the H/R group (Fig. 3F-J). These results suggested that ALK5/p-SMAD2/3 signaling serves a key function in the regulation of NOX2/4 expression and in oxidative stress injury.

*ALK5 knockdown affects ROS generation and apoptosis in PC-12 cells*. To further determine whether ALK5 knockdown affects ROS generation in PC-12 cells, the total ROS level was measured. It was revealed that cells treated with ALK5 siRNA had significantly decreased ROS levels when compared with the H/R group (Fig. 4A). Expression and enzyme activity of caspase-3, a mediator for apoptosis, were also measured. It was identified that ALK5 knockdown significantly decreased caspase-3 expression and enzyme activity (Fig. 4B and C). Hoechst staining indicated that ALK5 knockdown decreased apoptosis in PC-12 cells when compared with the H/R or NC siRNA groups (Fig. 4D). Consistent with the results for ROS generation, caspase-3 activity and Hoechst staining, LDH release was significantly decreased in PC-12 cells treated with ALK5 siRNA when compared with the H/R group (Fig. 4E). These results indicated that ALK5 knockdown inhibited apoptosis of PC-12 cells and that the underlying mechanism was associated with the inhibition of ROS generation.

## Discussion

The results of the present study demonstrated that ALK5/p-SMAD2/3 signaling was associated with oxidative stress

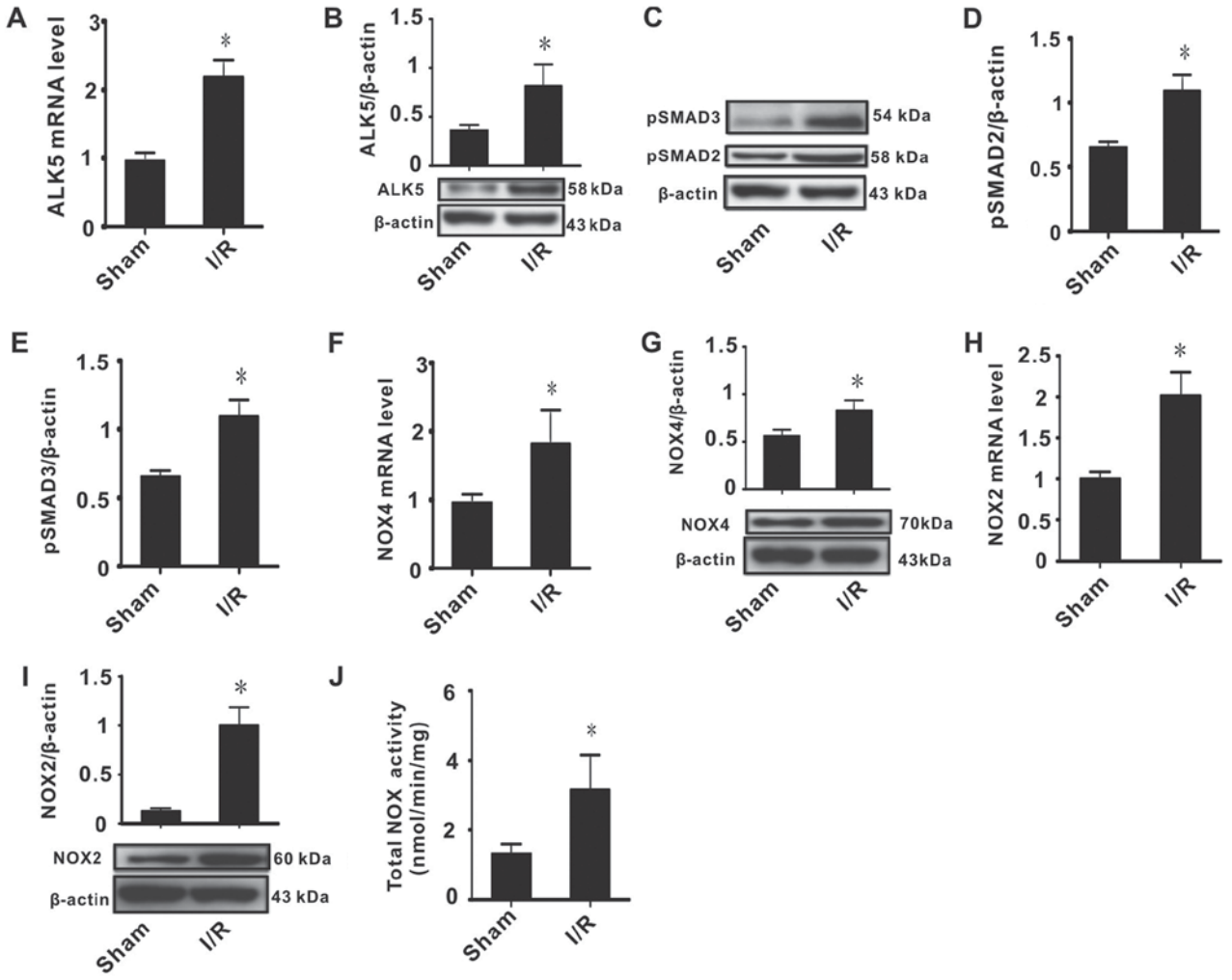


Figure 2. I/R injury affects ALK5, p-SMAD2/3, NOX2 and NOX4 expression. ALK5 (A) mRNA and (B) protein levels. (C) Representative western blots for p-SMAD2/3 expression. Ratio of (D) p-SMAD2 and (E) p-SMAD3 to β-actin. NOX4 (F) mRNA and (G) protein levels. NOX2 (H) mRNA and (I) protein levels. (J) Total NOX activity. Data were evaluated using student's t-test. *P<0.05 vs. Sham. I/R, ischemia/reperfusion; ALK5, activin receptor-like kinase 5; p-, phosphorylated; NOX, nicotinamide adenine dinucleotide phosphate oxidase.

following I/R injury. It was identified that increased oxidative stress was associated with increased NOX2/4 and that NOX2/4 expression was associated with ALK5 expression and phosphorylation of SMAD2/3. ALK5 knockdown significantly alleviated the damage to PC-12 cells induced by H/R treatment. H/R contributed to increased LDH release and ROS, which are primarily produced by NOX2/4 in mammalian neuronal cells (18). However, siRNA against ALK5 significantly suppressed NOX2/4 expression and decreased phosphorylation of SMAD2/3. The present study indicated that activation of ALK5/p-SMAD2/3 signaling exacerbates the extent of brain injury in I/R by upregulating NOX2/4 expression.

Oxidative stress is associated with the pathogenesis of a range of neurodegenerative disorders, including Alzheimer's disease, Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis, multiple sclerosis and ischemic stroke (5,18,19). Excessive ROS generation, including superoxide anions, hydrogen peroxide and hydroxyl radicals, is one of the main causes for mitochondrial DNA mutations, mitochondrial respiratory chain damage, membrane permeability alteration and $Ca^{2+}$ dyshomeostasis (20). Previous studies have reported that ROS are generated endogenously from molecular oxygen by cellular oxidases, including mono- and dioxygenases of the mitochondrial electron chain transport system, peroxidases, NOX, nitric oxide synthases, cytochrome 450, cyclooxygenases, lipooxygenases and xanthine oxidases (5,21). Although seven subtypes of NOXs have been identified in rat tissues, including NOX1-5, DUOX1 and DUOX2, NOX2 and NOX4 were demonstrated to be the major contributors to brain tissue ROS production (6). In a previous study, it has been identified that NOX2/4 expression is increased in brain tissues following I/R injury (6). Consistent with this, NOX inhibition or knockout has been demonstrated to significantly alleviate oxidative stress and protect against I/R injury, which demonstrates that NOX2/4 serve key functions in cerebral ischemic stroke (22).

Numerous factors, including transcription factors (NF-κB, activator protein-1, signal transducer and activator of transcription 1/3 and CCAAT/enhancer binding protein), nuclear receptors (peroxisome proliferator-activated receptor α) and epigenetic regulators (histone acetylation and microRNAs), are associated with the regulation of NOX expression (23). Previously, the tumor suppressor TGF-β was also demonstrated to be a key factor in ROS generation (24,25). It has previously been identified that TGF-β may induce ROS generation through

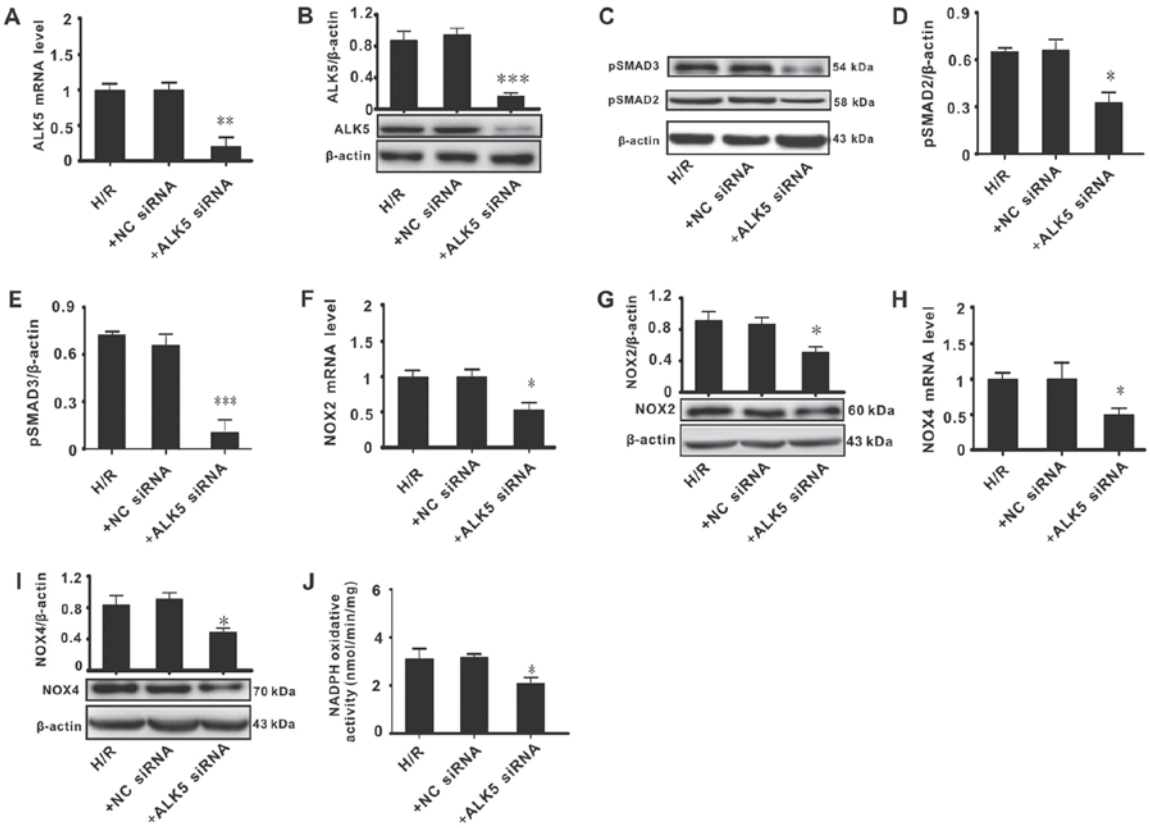


Figure 3. ALK5 knockdown affects ALK5, p-SMAD2/3, NOX2 and NOX4 expression in PC-12 cells. ALK5 (A) mRNA and (B) protein levels. (C) Representative western blots for p-SMAD2/3 expression. Ratio of (D) p-SMAD2 and (E) p-SMAD3 to β-actin. NOX2 (F) mRNA and (G) protein levels. NOX4 (H) mRNA and (I) protein levels. (J) Total NOX activity. Data were evaluated using analysis of variance and Bonferroni's post hoc test. $^{*}$P<0.05, $^{**}$P<0.01, $^{***}$P<0.001 vs. H/R. ALK5, activin receptor-like kinase 5; p-, phosphorylated; NOX, nicotinamide adenine dinucleotide phosphate oxidase; H/R, 5 h hypoxia/20 h reoxygenation; +NC siRNA, negative control small interfering RNA; +ALK5 siRNA, siRNA against ALK5.

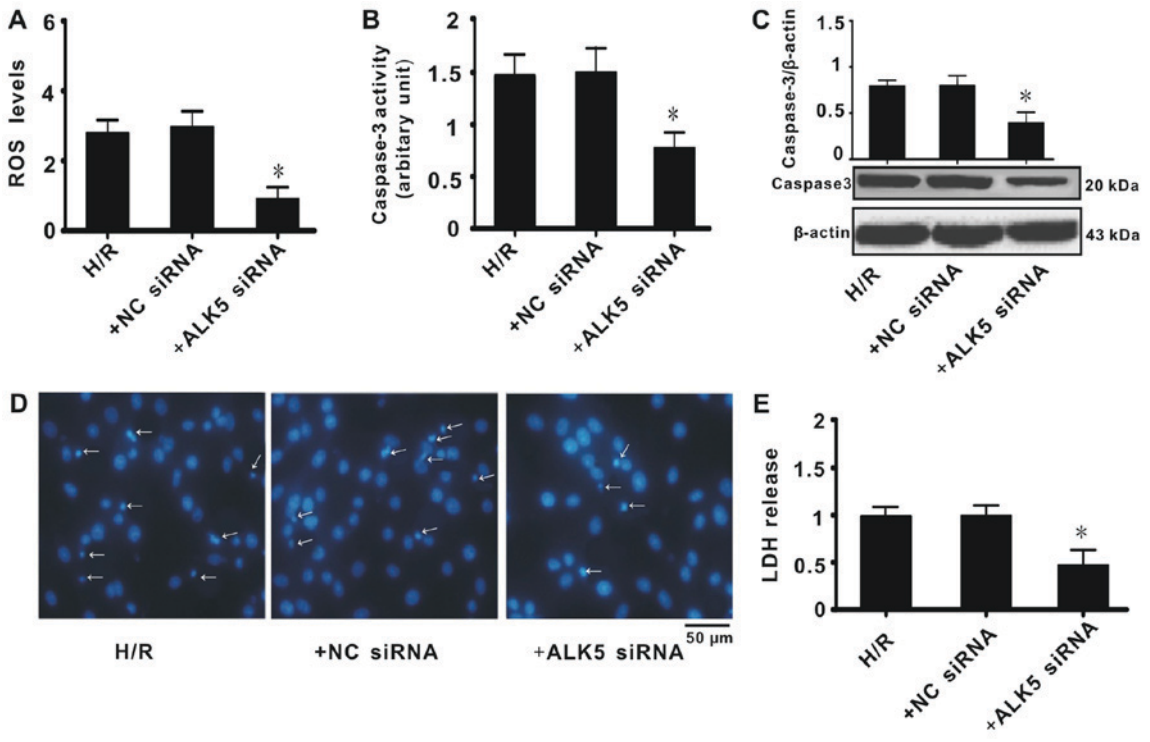


Figure 4. ALK5 knockdown affects ROS generation and apoptosis in PC-12 cells. (A) ROS level. Caspase-3 (B) activity and (C) protein expression. (D) Representative images of Hoechst staining; arrows indicate apoptotic cells. (E) LDH release. Data were evaluated using analysis of variance and Bonferroni's post hoc test. $^{*}$P<0.05 vs. H/R. ALK5, activin receptor-like kinase 5; ROS, reactive oxygen species; LDH, lactate dehydrogenase; H/R, 5 h hypoxia/20 h reoxygenation; +NC siRNA, negative control small interfering RNA; +ALK5 siRNA, small interfering RNA against ALK5.

the TGF-β/ALK5/SMAD2/3, mitogen-activated protein kinase or c-Jun N-terminal kinase pathways (13). The canonical TGF-β signaling pathway includes TGF-βs, TGF-β receptors (including ALK1 and ALK5) and SMAD1-8 (10). For ALK5, the TGF-β signaling is as follows: TGF-β binds ALK5 to form a complex exhibiting kinase activity; the activated kinase phosphorylates downstream substrates, SMAD2/3, aided by SMAD4; p-SMAD2/3 translocates to the nucleus and promotes gene expression (8,10). Emerging evidence has demonstrated that TGF-β signaling is critical in the pathogenesis of several CNS disorders, including neurodegenerative disorders and ischemic stroke (11,12). In the present study, it was identified that ALK5/SMAD2/3 signaling was associated with NOX2/4 expression and ROS generation following I/R injury. Similarly, previous studies have identified that increased TGF-β levels were associated with cerebral ischemic injury (12). It is speculated that ALK5/SMAD2/3 signaling serves an important role in ischemic stroke by regulating NOX2/4 expression and ROS generation.

Excessive ROS are harmful to cells and body due to their damage to biological macromolecules, including alcohol dehydrogenase 2 (26,27). Therefore, inhibiting the production of ROS is an effective method for treatment of oxidative stress injuries and explains the neuroprotective role of NOX inhibitors (28), including gp91ds-tat and apocynin (29,30). These results suggest that NOX2/4 are potential targets for antioxidant therapy. Considering the role of the ALK5/SMAD2/3 signaling pathway in the regulation of NOX2/4 expression, inhibiting ALK5/SMAD2/3 signaling may be an effective therapy for reducing oxidative stress. In summary, the present study demonstrated that the ALK5/SMAD2/3/NOX pathway serves a key function in I/R injury and provides a potential novel target for ischemic stroke antioxidant treatment.

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**Availability of data and materials**

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

**Authors' contributions**

ZL and APW contributed to the acquisition of data. XMD, GHH, MLZ and ZBY contributed to the design of the experiments and drafting of the manuscript. All authors have read and approved the manuscript.

**Ethics approval and consent to participate**

The use of animals in the present study was approved by the Hunan Normal University Veterinary Medicine Animal Care and Use Committee.

**Patient consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

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