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EGFR mediates astragaloside IV-induced Nrf2 activation to protect cortical neurons against *in vitro* ischemia/reperfusion damages

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ABSTRACT

In this study, we tested the potential role of astragaloside IV (AS-IV) against oxygen and glucose deprivation/re-oxygenation (OGD/R)-induced damages in murine cortical neurons, and studied the associated signaling mechanisms. AS-IV exerted significant neuroprotective effects against OGD/R by reducing reactive oxygen species (ROS) accumulation, thereby attenuating oxidative stress and neuronal cell death. We found that AS-IV treatment in cortical neurons resulted in NF-E2-related factor 2 (Nrf2) signaling activation, evidenced by Nrf2 Ser-40 phosphorylation, and its nuclear localization, as well as transcription of antioxidant-responsive element (ARE)-regulated genes: *heme oxygenase-1* (*HO-1*), *NAD(P)H:quinone oxidoreductase 1* (*NQO-1*) and *sulphiredoxin 1 (SRXN-1)*. Knockdown of Nrf2 through lentiviral shRNAs prevented AS-IV-induced ARE genes transcription, and abolished its anti-oxidant and neuroprotective activities. Further, we discovered that AS-IV stimulated heparin-binding-epidermal growth factor (HB-EGF) release to trans-activate epidermal growth factor receptor (EGFR) in cortical neurons. Blockage or silencing EGFR prevented Nrf2 activation by AS-IV, thus inhibiting AS-IV-mediated anti-oxidant and neuroprotective activities against OGD/R. In summary, AS-IV protects cortical neurons against OGD/R damages through activating of EGFR-Nrf2 signaling.

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1. Introduction

Ischemic stroke is an important cause of mortality in China and the most common reason of disability around the world [1,2]. A significant amount of reactive reactive oxygen species (ROS) is generated in cerebral tissues with ischemia-reperfusion, causing severe oxidative damages on cerebral neurons [3,4]. ROS suppression becomes an important way to prevent ischemic injury [3,4].

Astragalosides are the major active component extracted from the root of *Astragalus membranaceus*, a traditional Chinese medicine (TCM) also termed as "Huangqi". Recent studies have demonstrated the anti-inflammatory, anti-oxidative, anti-cancer and immuno-regulatory activities of astragalosides [5–7]. Astragaloside IV(AS-IV) is a saponin purified from *A. membranaceus*, which has showed beneficial effects in several disease models [8]. The potential role of AS-IV against ischemic-reperfusion-induced neuronal injuries has not extensively studied.

The antioxidant-responsive element (ARE) is a cis-acting regulatory element in promoter regions of many important antioxidant genes [9]. Nuclear factor-E2-related factor 2 (Nrf2), as a transcription factor, regulates the basal and inducible expression of numerous anti-oxidant genes through binding to ARE [10]. Studies have shown that activation of Nrf2/ARE pathway could increase nuclear localization of Nrf2, and induce the expression of the Nrf2/ARE-dependent genes, such as *heme oxygenase-1 (HO-1)* and *NAD(P)H:quinone oxidoreductase 1 (NQO-*

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1), to lessen cerebral ischemic injuries [10]. Hence, this work studied the effect of AS-IV on Nrf2/ARE signaling activation in primary cortical neurons, and focused on the underlying signaling mechanisms.

2. Materials and methods

2.1. Chemicals, reagents and antibodies

AS-IV, purchased from Sigma Chemical Co. (St. Louis, MO), was dissolved in dimethyl sulfoxide (DMSO, Sigma) and stored at −20 °C. The final concentration of DMSO in the culture media was less than 0.1%. 1,2-dithiole-3-thione (D3T) sulforaphane (SFN) and tert-butylhydroquinone (t-BHQ) were also purchased from Sigma. AG-1478 was purchased from Calbiochem (Darmstadt, Germany). Anti-EGFR, p-EGFR (Tyr-1045), p-EGFR (Tyr-1068), anti-Nrf2, anti-Lamin-B and anti-Tubulin antibodies were purchased from Cellular Signaling Technology (Beverly, MA). P-Nrf2 antibody was purchased from Abcam (Cambridge, MA).

2.2. Primary cortical neuronal cultures

Neocortical cultures of neurons from mouse embryos at 14–15 d were prepared, the dissected tissues were minced separately into small pieces and digested with trypsin (0.125%) for 5 min, before culture medium (containing 10% FBS) was added. The mixture was subjected to centrifugation at 500 rpm for 5 min. The cells were resuspended in DMEM supplemented with 10% FBS and plated onto poly-L-lysine-coated plates for 4 h at 37 °C. After cells attached, the medium was replaced with neuronal culture medium consisting of serum-free neurobasal medium supplemented with 2% B27, 0.5 mM glutamine, followed by re-incubation for 7–8 d for maturation of cortical neurons, with half of the medium being changed every 2 d.

2.3. Oxygen and glucose deprivation/re-oxygenation (OGD/R)

For OGD, neuron cultures were placed in a hypoxia chamber containing an atmosphere of <0.2% O_2, 5% CO_2, 95% N_2, >90% humidity, and 37 °C. Within the chamber, the medium was removed and replaced with oxygen/glucose-free balanced salt solution (BSS: 143 mM of Na, 5.5 mM of K, 1.8 mM of Ca_2, 1.8 mM of Mg, 125 mM of Cl, 26 mM of HCO_3, 1 mM of PO_4, and 0.8 mM of SO_4, pH 7.4, <0.1% O_2). After OGD of 180 min, cultures were taken out of the chamber and transferred to the regular cell culture incubator for re-oxygenation of indicated time.

2.4. Measurement of ROS

Intracellular ROS were detected by fluorescent probe 2, 7-dichlorofluorescein diacetate (DCFH-DA, Molecular Probes, Eugene, OR). DCFH-DA is converted by intracellular esterases, which is oxidized into the highly fluorescent dichlorofluorescein (DCF) in the presence of a proper oxidant. Neurons were incubated with DCFH-DA (5 μM) at 37 °C in the dark for 10 min and then washed with PBS. The DCF fluorescence intensity was quantified using a fluorescence microplate reader (SpectraMax M5, Molecular Devices, Sunnyvale, CA) at an excitation of 485 nm and an emission of 538 nm.

2.5. Cell viability study

MTT (10 μl, 0.5 mg/ml, sigma) was added to each culture well. After incubation at 37 °C for 4 h, the formazan crystals were dissolved by addition of 100 μl DMSO, and the plates were shaken vigorously to ensure complete solubilization. Formazan absorbance was assessed at 570 nm by microplate reader.

2.6. Lactate dehydrogenase (LDH) assay

LDH is a cytoplasmic enzyme sequestered inside viable cells with intact plasma membranes, and is released from cells with damaged membranes (e.g. necrotic cells). Therefore, the amount of LDH released from cells into medium indicates the level of toxicity. After treatment, the medium was collected and assayed for LDH activity, using a LDH detection kit from Roche Applied Science (Indianapolis, IN). % LDH release = LDH released in conditioned medium/(LDH released in conditioned medium + LDH in cell lysates)* 100%, and it was utilized as a quantitative measurement of neuronal death.

2.7. Fragmented DNA detection by ELISA (apoptosis-ELISA assay)

Nucleosomal DNA fragmentation is one of the biological markers for apoptosis. Fragmented DNA was assessed as previously described [11], by measuring DNA associated with nucleosomal histones using a specific two-site ELISA with an anti-histone primary antibody and a secondary anti-DNA antibody according to the manufacturer's instructions (Roche).

2.8. Western blots

After treatment, aliquots of 20 μg of proteins were separated by 10% SDS-PAGE gel and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA). After blocking with 10% non-fat dry milk for 1 h, membranes were incubated with described antibodies overnight at 4 °C, followed by incubation with secondary antibodies for 1 h at room temperature. The blots were visualized with enhanced chemiluminescence (ECL). The nuclei of cultured neurons were isolated by the nuclei Isolation kit purchased from Sigma (Shanghai, China), based on the instructions provided.

2.9. Real-time polymerase chain reaction

Total RNA was extracted using Trizol reagents (Invitrogen, Carlsbad, CA), which was DNase-treated and purified by using the RNeasy Mini Kit (Qiagen, Valencia, CA). Then cDNA was synthesized using an Omniscript RT kit (Qiagen, Valencia, CA) following the manufacturer's instructions. Real-time PCR was performed in a Stepone Real-Time PCR instrument (Applied Biosystems, Foster City, CA) with the SYBR Green RT-PCR system using Fast-Plus EvaGreen® qPCR Master Mix kit (Biotium, Hayward, CA). Relative expression level for each target gene was normalized to the housekeeping gene β-actin. A negative control with non-genetic material was included to eliminate nonspecific reactions or contamination. Each sample was tested in triplicate and the $2^{-\Delta\Delta CT}$ method was used to analyze the relative transcription data [11]. The following primer sequences were utilized: for mouse *β-actin*: sense, *CCTGACCGAGCGTGGCTACAGC*; antisense, *AGCCTCCAGGGCATCGGAC*; For mouse *Nrf2*: sense, *TCTCCTCGCTGGAAAAAGAA*; antisense, *AATGTGCTGGCTGTGCTTTA*. For mouse *HO-1*: sense, *TCCCAGACACCGCTCCTCCAG*; antisense, *GGATTTGGGGCTGCTGGTTTC*. For mouse *NQO-1*: sense, *GGAAGCTGCAGACCTGGTGA*; antisense, *CCTTTCAGAATGGCTGGCA*. For mouse *sulfiredoxin 1 (SRNX1)*: sense, *CCCACTGGACCAACTTCTGT*; antisense, *GTGGCTAGCTCAGACCAAGG*. All the primer sequences were from published literatures.

2.10. Lentiviral shRNA and infection

Lentivirus-packed non-targeted scramble shRNA and lentiviral shRNA targeting mouse epidermal growth factor receptor (EGFR) were purchased from Santa Cruz. Two different lentiviral shRNA constructs targeting non-overlapping mouse *Nrf2* cDNA sequence were obtained from Santa Cruz (*Nrf2-shRNA-1*) and Sigma (*Nrf2-shRNA-2*), respectively. Cortical neurons were infected with above lentivirus shRNA in the presence of 10 μg/ml polybrene for 12 h. Uninfected cells were eliminated by exposure to 0.5 μg/ml puromycin for 48 h.

2.11. Heparin-binding-epidermal growth factor (HB-EGF) ELISA

HB-EGF ELISA was performed using a murine HB-EGF ELISA kit (R&D Systems), according to the manufacturer's instructions. The amount of HB-EGF protein in the conditional medium was normalized to the standard.

2.12. Data analysis

Data were shown as mean ± standard deviation (SD). Statistical analyses were performed using one-way ANOVA analysis followed by Bonferroni post hoc tests for multiple comparisons tests. Paired Student's t-tests were used for comparisons of two groups only. Differences were considered significant when $p < 0.05$.

3. Results

3.1. Pre-treatment of AS-IV protects cortical neurons from OGD/R

The oxygen and glucose deprivation/re-oxygenation (OGD/R) model has been widely applied in cultured neurons to simulate brain ischemia/reperfusion. This *in vitro* model was utilized to study the neuroprotective effects of AS-IV. Results showed that pre-treatment of AS-IV (10–50 μM, for 6 h) significantly inhibited OGD/R-induced neuron viability loss, detected by reduction of MTT OD (Fig. 1A). AS-IV alone at the concentration of 1–50 μM had no significant effect on neuron survival (Fig. 1A). Of the concentrations tested, AS-IV at 50 μM showed highest efficiency in protecting neurons from OGD/R (Fig. 1A). Note that AS-IV at higher concentrations (100–300 μM) alone could inhibit neuron survival (Data not shown). The neuroprotective effect of AS-IV was further tested, and OGD/R-induced neuron cell death (detected by LDH release assay, Fig. 1B) and cell apoptosis (detected by apoptosis-ELISA assay, Fig. 1C) were both dramatically inhibited by AS-IV pre-treatment. Thus, AS-IV pre-treatment protects cortical neurons from OGD/R damages.

3.2. AS-IV induces mRNA expression of ARE-regulated genes and inhibits OGD/R-induced ROS accumulation

This study was performed to test the effect of AS-IV on Nrf2 signaling. Activated Nrf2 translocates to nuclei, where it activates ARE to promote transcription of ARE-regulated genes. Several of these genes have been discovered, including *HO-1*, *NQO-1* and *SRXN-1* among others [12]. We thus examined mRNA expression of ARE-dependent genes in AS-IV-stimulated neurons. Real-time PCR results demonstrated that AS-IV treatment dose-dependently increased mRNA expression of *HO-1* (Fig. 2A), *NQO-1* (Fig. 2B), and *SRXN-1* (Fig. 2C). We next compared AS-IV's activity *vs.* several known Nrf2 activators. As shown in Fig. 2D and E, Nrf2 activators including 2-dithiole-3-thione (D3T), tert-butylhydroquinone (t-BHQ) and sulforaphane (SFN) [12] all induced *HO-1* and *NQO-1* mRNA expression in cortical neurons. The activity of AS-IV was stronger than t-BHQ, but was weaker than D3T or SFN (all 50 μM) (Fig. 2D and E). Meanwhile, above Nrf2 activators and AS-IV inhibited OGD-R-induced ROS production (Fig. 2F) and neuronal damages (Fig. 2G and H) (see similar studies [13,14]). AS-IV-mediated anti-oxidant activity (Fig. 2F) and neuroprotection (Fig. 2G and H) were comparable to same concentration of SFN, weaker than D3T, but were more potent than t-BHQ. Thus, AS-IV induces mRNA expression of ARE-regulated genes, while suppressing OGD/R-induced ROS accumulation in cortical neurons.

3.3. Nrf2 is required for AS-IV-mediated anti-oxidant and neuroprotective activities against OGD/R

To test the role of Nrf2 in AS-IV-mediated neuroprotective effects, lentiviral shRNA method was applied to knockdown Nrf2 in cortical neurons. As shown in Fig. 3A, both protein and mRNA expressions of Nrf2 were significantly downregulated by targeted-lentiviral shRNAs (Fig. 3A). Two lentiviral packed shRNAs targeting non-overlapping cDNA sequence of Nrf2 were utilized (Fig. 3A). AS-IV-induced mRNA expressions of *HO-1* (Fig. 3B), *NQO-1* (Fig. 3C), and *SRXN-1* (Fig. 3D) were significantly inhibited by Nrf2-shRNA knockdown. As a result, the ROS scavenging ability of AS-IV against OGD/R was dramatically inhibited (Fig. 3E). Meanwhile, AS-IV-mediated neuroprotective effects were alleviated in neurons with Nrf2-knockdown, evidenced by increased cell viability

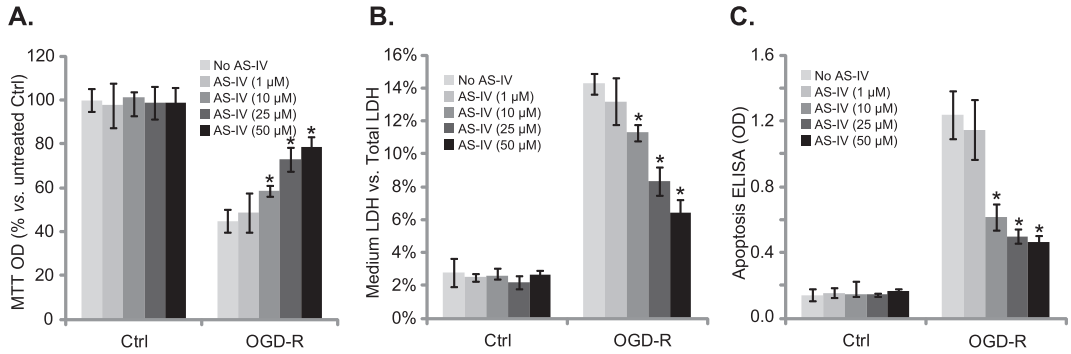

Fig. 1. Pre-treatment of AS-IV protects cortical neurons from OGD/R-Primary murine cortical neurons, pretreated with indicated concentration of AS-IV for 6 h, were subjected to OGD (180 min), followed by 24 h of reperfusion (adding back oxygen and glucose, OGD-R), cell viability was tested by MTT assay (A), cell death and apoptosis were tested by LDH release assay (B) and Histone DNA Apoptosis-ELISA assay (C), respectively. Control cells were maintained in regular culture medium (termed as "Ctrl"). For each assay, n = 5. Experiments in this and following figures were repeated three times, and similar results were obtained. Data were expressed as means ± SD (For all figures). *$p < 0.05$ *vs.* OGD/R only group.

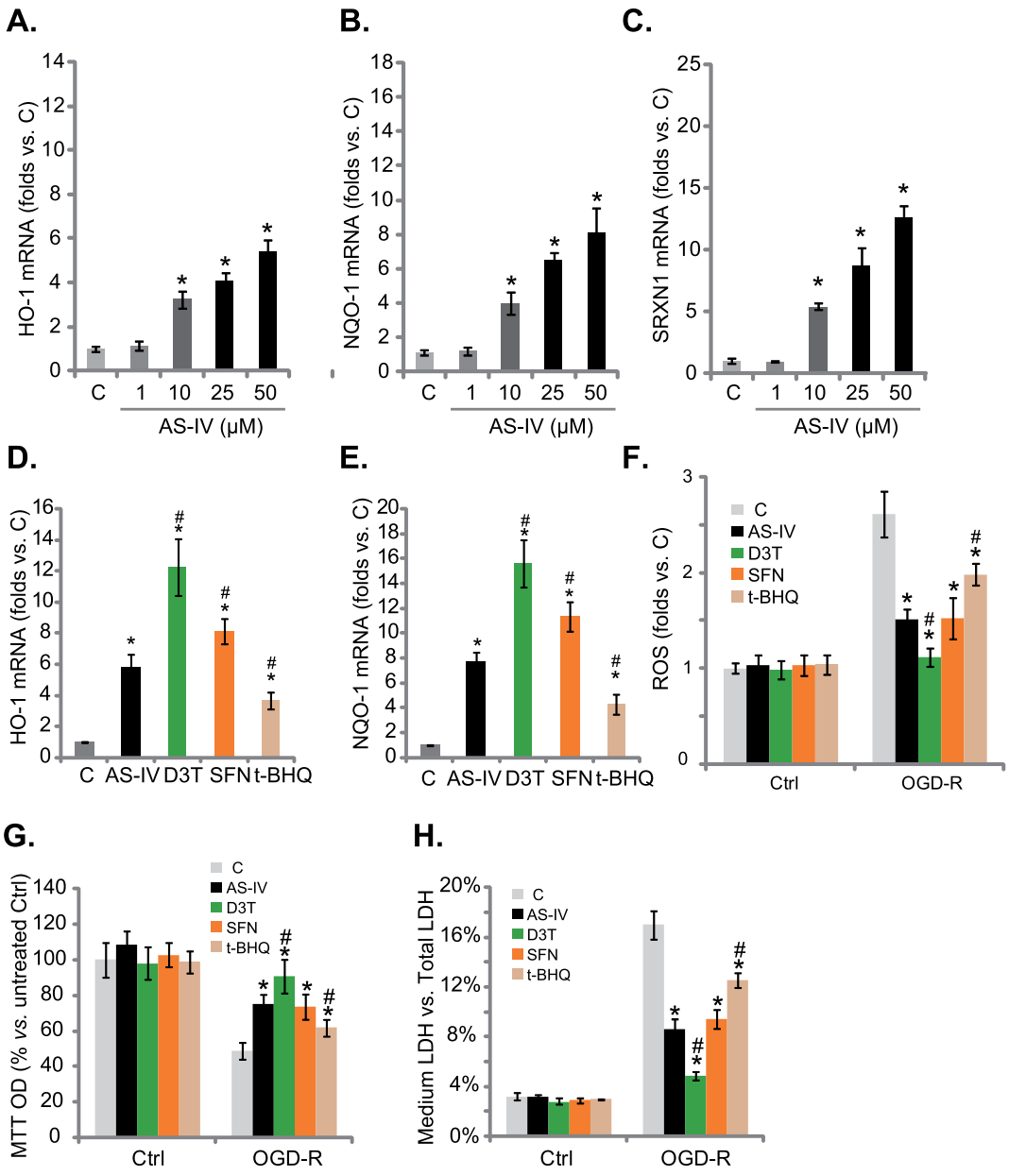

Fig. 2. AS-IV induces transcription of ARE-regulated genes and inhibits OGD/R-induced ROS accumulation-Relative mRNA expression (vs. untreated control, "C") of *HO-1* (A), *NQO-1* (B) and *SRXN-1* (C) in murine cortical neurons stimulated with indicated concentration of AS-IV (1–50 μM). Relative mRNA expression (vs. "C") of *HO-1* (D) and *NQO-1* (E) in murine cortical neurons stimulated with AS-IV (50 μM), 2-dithiole-3-thione (D3T, 50 μM), sulforaphane (SFN, 50 μM) or tert-butylhydroquinone (t-BHQ, 50 μM) for 6 h. Primary murine cortical neurons, pretreated with AS-IV (50 μM), 2-dithiole-3-thione (D3T, 50 μM), sulforaphane (SFN, 50 μM) or t-BHQ (50 μM) for 6 h, were subjected to OGD (180 min), followed reperfusion (OGD-R), after 6 h, intracellular ROS level was tested by FACS assay (F); after 24 h, cell viability was tested by MTT assay (G) and cell death was tested by LDH release assay (H). For each assay, n = 5. *p < 0.05 vs. "C" (A–E). *p < 0.05 vs. OGD/R only group (F–H). #p < 0.05 vs. OGD/R + D3T group (F–H).

reduction (Fig. 3F) and apoptosis (Fig. 3G). Together, these results suggest that Nrf2 is required for AS-IV-mediated transcription of ARE-genes, ROS scavenging and neuroprotective activities.

3.4. *EGFR trans-activation mediates AS-IV-induced Nrf2 activation and neuroprotection*

Next, we studied the potential upstream signaling for Nrf2 activation by AS-IV in primary neurons. The epidermal growth factor (EGF)-EGFR signaling network is among one of the best-characterized signaling systems [15]. Besides being activated by its ligands, EGFR could also be activated indirectly by a number of agents (EGFR "trans-activation"). As shown in Fig. 4A, AS-IV treatment in cortical neurons induced EGFR trans-activation, evidenced by EGFR phosphorylation at Tyr-1045 and Tyr-1068, which was blocked by EGFR kinase inhibitor AG-1478 and by EGFR-shRNA knockdown (Fig. 4A). ELISA results showed that the content of HB-EGF in the conditioned medium of AS-IV-stimulated cortical neurons was significantly increased (Fig. 4B–C). The effect of AS-IV on HB-EGF release was both dose- and time-dependent (Fig. 4B–C). Significantly, AG-1478 or EGFR-shRNA knockdown also inhibited AS-IV-induced Nrf2 phosphorylation at Ser-40 (Fig. 4A), a key cite that is critical for its activation and Kelch-like ECH-associated protein 1 (Keap1) disassociation [16]. As a result, AS-IV-induced nuclear localization of Nrf2 (both p- and regular) was also prevented by EGFR inhibition (Fig. 4D), meanwhile mRNA expressions

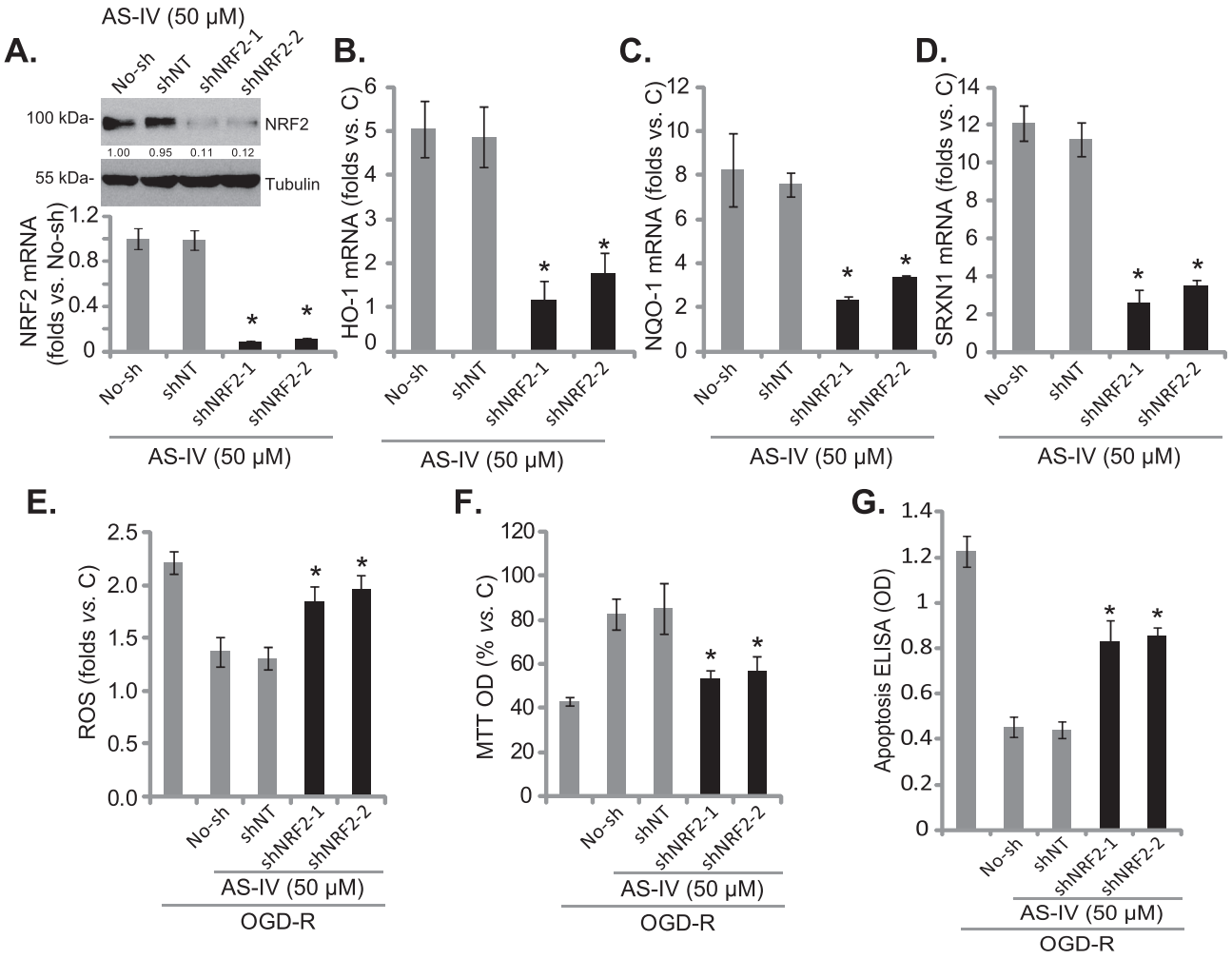

Fig. 3. Nrf2 is required for AS-IV-mediated anti-oxidant and neuroprotective activities against OGD/R-Primary cortical neurons were infected with lentiviral particles encoding non-targeted shRNA (sh-NT), Nrf2 shRNA-1 (shNRF2-1) or Nrf2 shRNA-2 (shNRF2-2) for 12 h, followed by puromycin selection. These cells and the control non-infected cells were stimulated with AS-IV (50 μM, for 6 h), Nrf2 and tubulin protein expressions were tested by Western blots (A, upper panel), relative mRNA expressions (vs. untreated control, "C") of *Nrf2* (A, lower panel), *HO-1* (B), *NQO-1* (C) and *SRXN-1* (D) were tested by real-time PCR. Above cells, pretreated with AS-IV (50 μM, for 6 h), were subjected to OGD followed reperfusion, intracellular ROS content was tested by FACS assay (E) 6 h after reperfusion, while cell viability (MTT assay, F) and apoptosis (apoptosis-ELISA assay, F) were tested 24 h after reperfusion. "C" stands for untreated control. For each assay, n = 5. *$p < 0.05$ vs. shNT group.

of ARE-dependent genes (*HO-1* and *SRXN-1*) by AS-IV were also inhibited (Fig. 4E—F). More importantly, as shown in Fig. 4G—H, AS-IV-mediated anti-oxidant and neuroprotective activities against OGD-R were also alleviated by EGFR inhibition or silencing. Thus, EGFR trans-activation mediates AS-IV-induced Nrf2 activation to protect neurons against OGD/R.

4. Discussions

Nrf2 regulates transcriptional activation of anti-oxidant genes through binging to ARE [17]. In this study, we found that SA-IV treatment in murine cortical neurons induced Nrf2 Ser-40 phosphorylation and nuclear localization, promoted transcription of ARE-dependent genes (*HO-1*, *NQO-1* and *SRXN-1*), while inhibiting ROS accumulation by OGD/R, such effects were inhibited by Nrf2 shRNA-knockdown. SA-IV-mediated neuroprotective effects against OGD/R were also alleviated with Nrf2 knockdown. Thus, activation of Nrf2 signaling might be the key mediator of SA-IV-induced anti-oxidant and neuroprotective effects in cortical neurons.

EGFR signaling pathway regulates cell survival and many important cellular functions in a variety of ways [18]. Several EGFR ligands, including EGF, amphiregulin, and transforming growth factor α(TGF-α) have been indentified to directly bind to EGFR [18], the latter recruits adaptor proteins to activate downstream signaling pathways, which promote cell survival [18]. EGFR could also be trans-activated by various other stimuli [18,19], a process termed as "trans-activation". Here, we propose that AS-IV-induced Nrf2 activation also depends on EGFR trans-activation. Our evidences include that HB-EGF content was increased in the conditioned medium of AS-IV-treated neurons, and EGFR was thus trans-activated. EGFR inhibition (by AG-1478) or silencing (by lentiviral shRNA) prevented AS-IV-induced Nrf2 activation (Nrf2 phosphorylation, its nuclear accumulation and ARE-gene transcription). Significantly, the anti-oxidant and neuroprotective activities against OGD-R of AS-IV were also attenuated by EGFR inhibition or silencing. Thus, we indentified a novel function following EGFR trans-activation by AS-IV: mediating Nrf2 signaling activation and inhibiting OGD-R-induced neuron damages. Our results are consistent with other findings showing that EGFR could mediate Nrf2 signaling activation by several other stimuli [20—22].

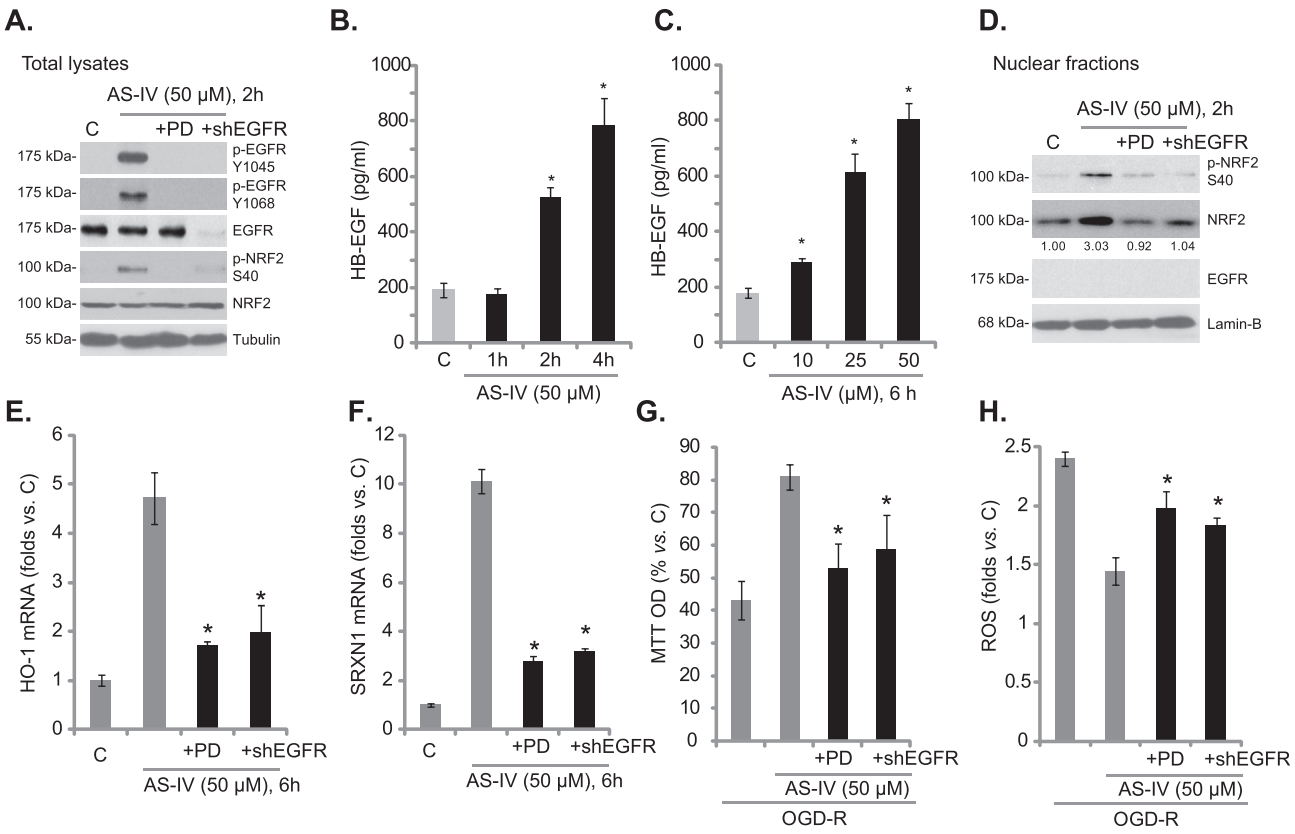

Fig. 4. EGFR trans-activation mediates AS-IV-induced Nrf2 activation and neuroprotection-Primary cortical neurons, infected with or without lentiviral particles encoding EGFR-shRNA (shEGFR), were pretreated with AG-1478 (1 μM) for 1 h, followed by AS-IV (50 μM) stimulation for indicated time, listed proteins in total (A) or nuclear lysates (D) were tested by Western blots, *HO-1* (E) and *SRXN-1* (F) mRNA expressions were tested by real-time PCR (vs. untreated control, "C"). HB-EGF content in conditioned medium of murine cortical neurons stimulated with indicated AS-IV was tested by ELISA assay (B–C). Above cells (with or without EGFR shRNA), pretreated with AS-IV (50 μM, for 6 h) or plus AG-1478 (1 μM, 6 h), were subjected to OGD for 180 min followed reperfusion (OGD/R), relative cell viability (vs. untreated control or "C", G) was tested 24 h after reperfusion, relative ROS level (vs. untreated control or "C", H) was tested 6 h after reperfusion. For each assay, n = 5. *p < 0.05 vs. untreated control ("C") (B–C). *p < 0.05 vs. AS-IV only group (E–H).

Studies have shown that phosphorylation of Nrf2 at Ser-40 is a critical signaling event leading to ARE-mediated cellular antioxidant response [16]. Ser-40 phosphorylation is required for Nrf2 separating from Keap-1 and its nuclear translocation [16]. Here we found that SA-IV-induced Nrf2 Ser-40 phosphorylation was blocked by EGFR inhibition or silencing, thus EGFR activation is required for AS-IV-induced Nrf2 Ser-40 phosphorylation and subsequent activation. It is likely that EGFR regulates Nrf2 phosphorylation indirectly (see Refs. [20–22]). As a matter of fact, protein kinase C (PKC) is recognized as the upstream kinase for Nrf2 Ser-40 phosphorylation, and PKC is the major downstream signal cascade activated by EGFR [23,24]. The detailed signaling mechanisms of Nrf2 activation by AS-IV or EGFR need further investigations.

In summary, our results unveiled that SA-IV protects cortical neurons against OGD-R injuries by activation of Nrf2 signaling. EGFR trans-activation is found to be responsible for SA-IV-induced Nrf2 activation, as well as its neuroprotective effects. Thus, SA-IV may exert a therapeutic effect on ischemia stroke.

Conflict of interests

Authors have no conflict of interests.

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