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Astragaloside IV and ferulic acid synergistically promote neurite outgrowth through Nrf2 activation

Yini Liang[a,1], Yu Zou[a,1], Chengu Niu[b], Yingcai Niu[a,*]

[a] The Institute of Medicine, Qiqihar Medical University, Qiqihar 161006, China
[b] Department of Hematology, The First Affiliated Hospital, Harbin Medical University, Harbin 150001, China

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ABSTRACT

Recently, nuclear factor (erythroid-derived 2)-like 2 (Nrf2) have nuclear localization and nuclear exclusion signals and shuttle between the cytoplasm and the nucleus. Thus, we hypothesised that astragaloside IV (AS-IV) induction nuclear import of Nrf2 and ferulic acid (FA) inhibition nuclear export of Nrf2 contribute to synergistic antioxidant effects of combination of FA and AS-IV (FA/AS-IV). Here, we have demonstrated that FA/AS-IV enhances neurite outgrowth of PC12 cells challenged with lead acetate (PbAc) *via* antioxidant properties in a synergistic manner. Concomitantly, FA/AS-IV significantly promotes Nrf2 activation and induces "phase-II" enzymes during PbAc toxicity, compared with either FA or AS-IV alone. Interestingly, FA but not AS-IV activates the extracellular signal-regulated kinases 1 and 2 (ERK1/2), leading to an increase in both *de novo* synthesis of Nrf2 and nuclear import of Nrf2. Simultaneously, AS-IV but not FA suppresses Fyn phosphorylation *via* Akt-mediated inhibition of GSK-3β, which inhibited nuclear export of Nrf2. Importantly, dual activation of both ERK1/2 and Akt by FA/AS-IV in PC12 cells challenged with PbAc is mediated by independent mechanisms, which are supported by pharmacological inhibitors. Collectively, these results support the notion that the FA/AS-IV may be promising in therapy for lead developmental neurotoxicity. This combination deserves further study *in vivo*.

1. Introduction

The brain development is a very complex phenomenon which involves cell proliferation, migration, differentiation, maturation and synaptogenesis (Jiang and Nardelli, 2016). Lead, environmentally abundant heavy-metal pollutant, is a developmental neurotoxin that causes neurological alterations in offspring of gestationally exposed mothers, even in the absence of symptoms in the mother (Zhu et al., 2016a,b). Recent findings have indicated that prenatal lead intoxication inhibits neurite outgrowth, a critical aspect of neuronal development (Hu et al., 2008).

Oxidative stress in the developing central nervous system has been inferred as one of the important mechanism related to lead's neurotoxicity (Assi et al., 2016). The neurons are vulnerable to oxidative insults because the major part of neuronal cell membrane is polyunsaturated fatty acid, an substrate for reactive oxygen species (Wang et al., 2017). One of the effective ways to prevent cellular damage after lead exposure is using antioxidants (Kasperczyk et al., 2017). The nuclear factor (erythroid-derived 2)-like 2 (Nrf2) is a master regulator of a battery of phase II detoxification genes containing antioxidant response element (ARE) in their promoters (Buendia et al., 2016). Under basal conditions, Nrf2 is tethered in the cytoplasm by Keap1 (Djordjevic et al., 2015). In response to pharmacological and food-derived agents with antioxidant properties, Nrf2 accumulates and translocates to the nucleus where it activates phase II detoxifying/antioxidant enzymes (Qin and Hou, 2016). The available evidence, albeit limited, is highly suggestive that Nrf2 promotes neurite outgrowth (Yang et al., 2015).

Glycogen synthase kinase-3 (GSK-3) is involved in diverse physiological processes, including neuronal development (Jung et al., 2016). Activated Akt inactivates GSK-3β by phosphorylating its Ser9 residue (Lu et al., 2016a,b). Ser9-phosphorylation of GSK-3β decrease nuclear accumulation of Fyn, which relieves nuclear exclusion and degradation

Abbreviations: ARE, antioxidant response element; AS-I, astragaloside IV; CI, combination index; DN, dominant-negative; ERK1/2, extracellular signal-regulated kinases 1 and 2; FA, ferulic acid; GCLc, glutamate cysteine ligase catalytic subunit; GSK-3β, glycogen synthase kinase-3β; HO-1, heme oxygenase 1; LPO, lipid peroxidation; MAPK, mitogen-activated protein kinase; Nrf2, nuclear factor erythroid 2-related factor 2; PbAc, lead acetate; ROS, reactive oxygen species

* Corresponding author at: 333 BuKui Street, JianHua District, Qiqihar, The Institute of Medicine, Qiqihar Medical University, Qiqihar, 161006, China.
E-mail address: nyc1968@126.com (Y. Niu).

[1] These authors contributed equally to this work.

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of Nrf2 (Dai et al., 2017). Accumulating evidences indicate that ARE-dependent gene activation by compounds with antioxidant properties is mediated by mitogen-activated protein kinases (MAPKs) (Chaiprasongsuk et al., 2017). Activation of the c-Jun N-terminal kinases 1 (JNK1) and extracellular signal-regulated kinases 2 (ERK2) pathways appears to enhance nuclear localization of Nrf2 while the activation of the p38 mitogen-activated protein kinase ($p38^{MAPK}$) pathway seems to inhibit Nrf2 nuclear import in response to antioxidant (Lu et al., 2016a,b).

Natural products have been an important source for drug discovery (Zhang et al., 2017). It has been reported that Ferulic acid (FA) improves auditory function, Enhances cognitive function, and exerts neuroprotection *via* its antioxidant properties by activation of Nrf2/HO-1 signaling pathway (Fetoni et al., 2010; Mhillaj et al., 2018; Catino et al., 2016). We previously showed that plant-derived antioxidants such as astragaloside IV (AS-IV) or FA inhibits lead acetate (PbAc)-induced inhibition of neurite outgrowth by inducing accumulation of Nrf2 in the nucleus by a variety of mechanisms *in vitro* (Yu et al., 2017, 2016a,b). The complexity of the molecular mechanisms for Nrf2 shuttling between the cytoplasm and nucleus requires multi-target intervention. Does AS-IV acts synergistically with FA to induce nuclear translocation of Nrf2, if so, what are roles of ERK1/2 and GSK-3β pathways in the synergistic action?

On the basis of our previous findings, we hypothesize that AS-IV-induced Nrf2 nuclear import through an ERK1/2-dependent mechanism and FA-mediated inhibition of Nrf2 nuclear export *via* GSK-3β-dependent pathways contributes to synergistic antioxidant effect by combinations of AS-IV and FA (FA/AS-IV). Results in this report support our hypothesis.

2. Material and methods

2.1. Cell culture and treatments

Both SH-SY5Y and PC12 cells were obtained from the Shanghai Institute of Cell Biology (Shanghai, China) and maintained at 37 °C in 5% CO_2 in DMEM medium, supplemented with 10% FBS, 1% antibiotic and antimycotic solution, and murine nerve growth factor (50 ng/mL; Promega, Madison, WI; # G5141) in a CO_2 atmosphere. In some experiments, PC12 cells were pretreated with pharmacological inhibitors including PD98059 (10 μM; ERK1/2 inhibitor; Sigma-Aldrich, St. Louis, MO; #P215), SB203580 (10 μM; p38-MAPK inhibitor; Sigma; #S8307), SP600125 (10 μM; JNK inhibitor; Sigma; #S5567), LiCl (30 mM; GSK-3β inhibitor; Sigma; #746460), LY294002(10 μM ; PI3K inhibitor; Sigma; #L9908), Zn-PP IX (10 μM; HO-1 inhibitor; Strem Chemicals, Bischeim, France; #691500), and DPI (10 μM; NADPH oxidase inhibitor; Sigma; #D2926) for 30 min and then treated with FA (Purity ≥98.0%; 1 μM; Nanjing Jingzhu Bio-Technology Co, Nanjing, China; #JZ131210 A) and/or AS-IV (Purity ≥98.0%; 0.3 μM; Nanjing Jingzhu Bio-Technology Co; # JZ140508) in the presence PbAc (25 μM; Tianjin Basifu chemical co, Tianjin, China; #520) for a further 24 h to evaluate whether these pathways play a role in combining FA with AS-IV for synergism. Combinatory effects were examined with the CalcuSyn software (Biosoft, Cambridge, UK) and reactive oxygen species (ROS) analyses.

2.2. Measurement of oxidative stress

Intracellular ROS were determined by an oxidation-sensitive fluorescent probe, DCF-DA, supplied with OxiSelect™ Intracellular ROS Assay Kit purchased from Cell Biolabs Inc. (San Diego, CA; # STA-342). In brief, PC12 cells were rinsed in PBS, incubated with DCFH-DA diluted in PBS for 30 min at 37 °C. Fluorescence was determined at 480 nm excitation and 530 nm emission using a microplate reader (Safire2, Tecan Group Ltd, Maennedorf, Switzerland). GSH was determined by the Glutathione Assay Kit obtained from Cayman Chemical (Ann Arbor, MI; # 703002), which utilizes the enzyme glutathione reductase to measure total GSH by the spectrophotometric method. Lipid peroxidation (LPO) levels were spectrophotometrically determined with ferrous ion oxidation-xylenol orange method using the Lipid Hydroperoxide Assay kit (Cayman Chemical; #705002). Oxidative-stress-related biomarker assays were performed as per the procedure recommended by the manufacturer.

2.3. Neurite outgrowth assay

Neurite outgrowth was assessed by a fluorometric plate reader (Safire2) at 483/525 nm for cell viability indicator and 554/567 nm for cell membrane stain using the Neurite Outgrowth Staining Kit (Life Technologies Corporation (Carlsbad, CA; # A15001) according to the protocol of the manufacturer and described previously (Yu et al., 2016a,b).

2.4. Analyses of cell growth

Cell viability were determined by the MTS assay using CellTiter 96® Aqueous Non-Radioactive Cell Proliferation Assay kit (Promega; # G5421). MTS reagent (20 μl) is added directly in the incubation media and incubated at 37 °C for an hour. Absorbance at 490 nm was then measured with an ELX800 microplate reader (BioTek Instruments Inc).

2.5. Protein concentrations assay

Total cellular protein and membrane protein were extracted using M-PER® Mammalian Protein Extraction Reagent (Pierce, Rockford, lL; #78501) and Mem-PER™ Plus Membrane Protein Extraction Kit (Pierce; #89842), respectively. Protein content was measured using the BCA protein assay kit from Pierce (#23227). Results were expressed as membrane-to-total protein ratio in parallel with the surface/volume ratio.

2.6. Real-time PCR

Total RNA was obtained from PC12 cells with the Trlzol Reagent (Invitrogene, Carlsbad, CA; #15596-026). Complementary DNA (cDNA) was prepared using total RNA and random hexamer primers following the recommendations of primeScript™ RT reagent kit (Takara Biotechnology, Dalian, China; #RR037 A). Quantitative PCR of the cDNA was performed in an Agilent Stratagene M × 3005 P (Agilent Stratagene, CA) with a SYBR® premix Ex Taq™ (Takara; # RR420 A). The primers used in real-time PCR were as follows: 5′-CCC AGC ACA TCC AGA CAG AC-3′ (Nrf2 forward) and 5′-TAT CCA GGG CAA GCG ACT C-3′ (Nrf2 reverse); 5′-TGC TCG CAT GAA CAC TCT G -3′ (HO-1 forward) and 5′-TCC TCT GTC AGC AGT GCC T-3′ (HO-1 reverse); 5′-AGA CAA AAC ACA GTT GGA GCA G-3′ (GCLm forward) and 5′-CAG TCA AAT CTG GTG GCA TC -3′(GCLm reverse); 5′-GGC AAG ATA CCT TTA TGA CCA GTT-3′(GCLc forward) and 5′-TGC AGC ACT CAA AGC CAT AA-3′(GCLc reverse); 5′-GAC AAC TTT GGC ATC GTG GA-3′ (GAPDH forward) and 5′-ATG CAG GGA TGA TGT TCT GG-3′(GAPDH reverse). GAPDH was used as housekeeping gene. Data were analyzed according to the $2^{\Delta\Delta Ct}$ method (Yu et al., 2017).

2.7. Western blot analysis

Cellular cytoplasmic and nuclear fractions were prepared by using Nuclear and Cytoplasmic Protein Extraction kit (Beyotime Biotechnology, Haimen, China; #P0028) as per the protocol of the manufacturer. Proteins were fractionated by using 10% SDS-PAGE, and then transferred to nitrocellulose membranes, and blotted with the indicated primary antibody followed by peroxidase-conjugated secondary antibodies. Immunoreactive bands were visualized using the chemiluminescence reagent ECL (Thermo; #34079). Antibodies were

purchased from the following vendors: Antibodies against p-GSK-3β (Ser9) (Cell Signaling Technology, Beverly, MA; #5558; dilution: 1/1000), Erk1/2 (Cell Signaling #9102; dilution: 1/1000), p-Akt (Ser473) (Cell Signaling #9271; dilution: 1/1000), GAPDH (Cell Signaling #2118; dilution: 1/1000), Lamin B1 (Cell Signaling #12586; dilution: 1/1000), p-ERK 1/2 (Thr202/Tyr204) (Santa Cruz Biotechnology, Santa Cruz, CA; sc-16982; dilution: 1/500), HO-1 (Santa Cruz #sc-1796; dilution: 1/500), Nrf2 (Santa Cruz #sc-722; dilution: 1/500), Akt1 (Santa Cruz #sc-5298; dilution: 1/500), p-Fyn (Thr 12) (Santa Cruz #sc-377555; dilution: 1/500), GSK-3β (Santa Cruz #sc-8257; dilution: 1/500), Fyn (Santa Cruz #sc-434; dilution: 1/500), and GCLc (Abcam, Cambridge, UK; #ab55435; dilution: 1/1000) and GCLm (Abcam #ab153967; dilution: 1/1000).

2.8. Transcription factor activation assay

The MAPK activated transcription factors c-Myc, ATF-2, MEF2, c-Jun, and STAT1 in the nuclear extracts from SH-SY5Y cells were measured using the TransAM™ MAPK Family Transcription Factor Assay Kit (Active Motif, Carlsbad, CA; #47296) according to the protocol of the manufacturer. After adding indicated primary antibody and the HRP substrate, the colorimetric change was measured with an ELX800 microplate reader (BioTek Instruments Inc., Vermont) at 450nm.

2.9. Kinase assay

Akt and ERK1/2 kinase activity assays were performed using non-radioactive kits manufactured by Cell Signaling Technology (#9840 and #9800) as per the manufacturer's instructions. Briefly, after the indicated treatment, the PC12 cells were lysed, and then cell lysates were immunoprecipitated with either an immobilized anti-Akt antibody or with an immobilized phospho-p44/442 MAPK antibody. The immobilized precipitated enzymes were used for kinase assays using either Elk1 fusion protein (for p44/42 MAP) or GSK-3 fusion protein (for Akt) followed by Western blot analysis with phospho-Elk-1 (Ser383) antibody or phospho-GSK-3α/β (Ser21/9) antibody that allow detection and quantitation of phosphorylated substrates.

2.10. Luciferase reporter assay

Semiconfluent PC12 cells were transiently transfected with pGL4.37[luc2P/ARE/ Hygro] (Promega; #E3641), an ARE-containing luciferase reporter plasmid, using the FuGENE® 6 transfection reagent (#E2691) according to the manufacturer's instructions (Roche, Indianapolis, IN). Cells were co-transfected with *renilla* luciferase reporter plasmid pRL-TK (Promega; #E224 A) to control Transfection efficienc. Total cell extracts were analyzed for luciferase activity using a Dual-Glo® Luciferase Assay System (Promega; #E2920) as per the procedure recommended by the manufacturer.

2.11. Statistics

Data are expressed as means ± SD of at least three independent experiments. Differences between groups were compared using one-way analysis of Variance (ANOVA) associated with Student-Newman-Keuls tests. Statistical significance was accepted for P values of < 0.05.

3. Results

3.1. FA/AS-IV synergistically promotes neurite outgrowth of PC12 cells challenged with PbAc via antioxidant properties

Previously, we showed that both FA and AS-IV individually protect against PbAc-induced inhibition of neurite outgrowth through antioxidant activity (Yu et al., 2017, 2016a,b). However, whether FA combined with AS-IV exhibits antioxidant properties in a synergistic manner remains unclear. Calculation of combination index (CI) shows that at a constant concentration ratio of 10:3 of FA and AS-IV synergistically inhibits PbAc-triggered ROS generation. The highest synergy index of 0.346 reached when 0.3 μM AS-IV was combined with 1 μM FA (Table 1 and Fig. 1A). Therefore, these concentrations were used so as not to mask any effects seen with the combination in subsequent experiments. As illustrated in Fig. 1B, PbAc-induced increase in LPO level was significantly attenuated by the FA/AS-IV as compared with FA or AS-IV alone, which is consistent with ROS results. GSH is a small intracellular thiol molecule which is considered as a non-enzymatic antioxidant. As shown in Fig. 1C, the FA/AS-IV appears to enhance the GSH levels, compared with either FA or AS-IV alone.

Table 1
Combination index for FA and AS-IV on ROS production in PC12 cells exposed to PbAc.

FA (μM)	AS-IV (μM)	CI	Fa	Description
0.25	0.075	0.696	0.962	synergism
0.5	0.15	0.424	0.865	synergism
1.0	0.3	0.346	0.693	synergism
2.0	0.6	0.612	0.662	synergism
4.0	1.2	0.961	0.596	additive

Combination index (CI) was calculated using CompuSyn software. Interpretation of CI values: CI = 0.9–1.1, CI < 0.9, and CI > 1.1 indicates additive, synergism and antagonism, respectively. Fa, fraction affected.

Since antioxidant therapy can offset developmental neurotoxicity induced by PbAc (Sepehri and Ganji, 2016), next we tested if FA/AS-IV enhances neurite outgrowth. As shown in Fig. 1D, neurite outgrowth of PC12 cells treated with FA/AS-IV was significantly stimulated compared with either treatment alone. RNA interference-imposed knockdown of Nrf2 (Santa Cruz # sc-156128-SH) partially but significantly eliminates FA/AS-IV-promoting neurite outgrowth. Fluorescence imaging also corroborated these findings (Supplementary Fig. 1). As shown by BCA protein analyses (Fig. 1E), compared with PbAc-challenged PC12 cells, FA or FA/AS-IV elicited a significant increase in the membrane/total protein ratio, a parameter of the membrane complexity, in PbAc-challenged PC12 cells. Membrane/total protein ratio also showed an upward trend in response to AS-IV treatment, however this did not reach statistical significance. Neuroprotection by FA/AS-IV were also confirmed by MTS assay in primary dissociated hippocampal neurons and obtained similar results from neurite outgrowth assay (Fig. 1F). FA/AS-IV attenuated PbAc-triggered ROS generation in primary dissociated hippocampal neurons as compared with FA or AS-IV alone (Fig. 1G).

3.2. FA/AS-IV synergistically promotes nuclear translocation of Nrf2 during PbAc toxicity

Because Nrf2 is known to be at the center of cellular antioxidant defence system (Done and Traustadóttir, 2016), we were prompted to investigate whether FA/AS-IV was capable of activating the Nrf2-ARE antioxidant system in PC12 cells challenged with PbAc. As shown by Western blot analyses (Fig. 2A and B), FA, AS-IV, and the combination induced Nrf2 nuclear localization, and the combination was more efficacious than either FA or AS-IV alone. PCR results showed that FA alone or in combination with AS-IV up-regulated Nrf2 mRNA expression in PbAc-challenged PC12 cells, while AS-IV alone apparently did affect Nrf2 mRNA expression (Fig. 2C). These observations were verified by Nrf2-mediated ARE transactivation based ARE-driven luciferase reporter gene assay (Fig. 2D). Among ARE-driven phase-II enzymes, glutamate-cysteine ligase and HO-1 have attracted special attention because of their therapeutic effects against developmental neurotoxicity (Ye et al., 2016). Glutamate cysteine ligase catalytic subunit (GCLc) possesses all the catalytic activity of GCL, whereas glutamate cysteine ligase catalytic modifier subunit (GCLm) serves to optimize the catalytic properties of GCL holoenzyme. As shown by Western blot

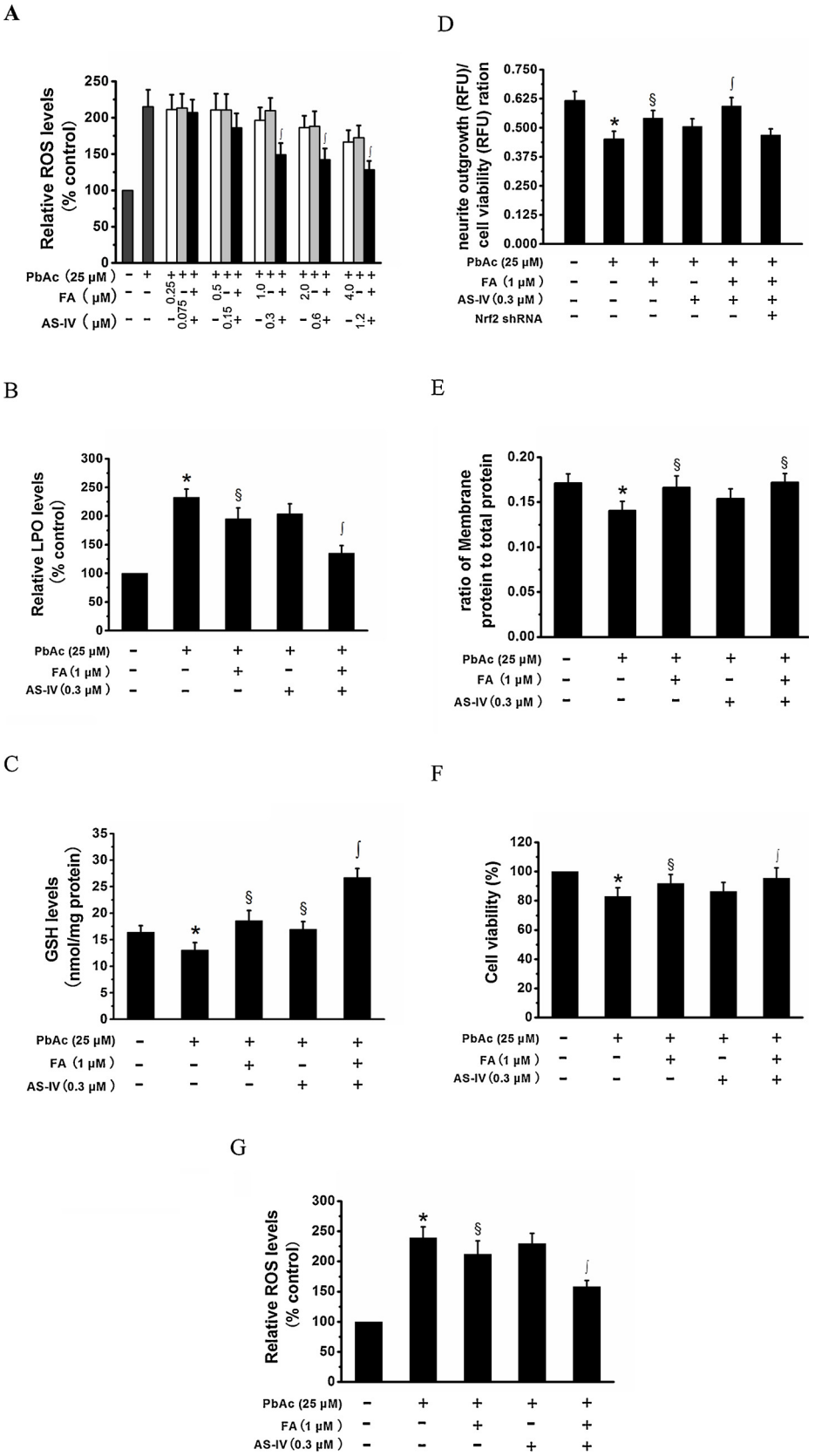

Fig. 1. Antioxidant properties contribute to FA/AS-IV potentiation of neurite outgrowth in PC12 cells challenged with PbAc. A, PC12 cells were treated with varying concentrations of FA or/and AS-IV at a fixed ratio (10:3), followed by challenge with PbAc. Intracellular ROS generation was measured using the dichlorofluorescein assay. B, PC12 cells were treated with FA and/or AS-IV followed by challenge with PbAc for 24 h. Intracellular LPO was determined by the redox reactions with ferrous ions using colorimetric method. C, GSH level was determined indirectly by reduction of 5,5′-dithiobis(2-nitrobenzoic acid) using GSH-dependent glutathione reductase. D, neurite outgrowth is monitored *via* bright orange-red staining of outer cell membrane surfaces and green fluorescence staining of cell viability. E, protein content was evaluated using the BCA assay for calculating membrane-to-total protein ratio. F, primary dissociated hippocampal neurons were prepared from embryonic day 18 rat fetuses. MTS assays of cell proliferation. G, primary dissociated hippocampal neurons were prepared from embryonic day 18 rat fetuses. Primary cultured rat hippocampal neurons were treated with FA and/or AS-IV followed by challenge with PbAc for 24 h. Intracellular ROS generation was measured using the dichlorofluorescein assay. Data are means ± SD, $n = 3$; *, $p < 0.05$ *vs.* control; §, $p < 0.05$ *vs.* PbAc challenge; ∫, $p < 0.05$ *vs.* FA or AS-IV alone.

(Fig. 3A–C) and real-time PCR assays (Fig. 3D and E), the FA/AS-IV significantly up-regulated the expression of HO-1, GCLc and GCLm gene expression at both the protein and mRNA levels, compared with the treatment of FA or AS-IV alone. HO-1 inhibitor zinc protoporphyrin (Zn-PP IX; Strem Chemicals, Bischeim, France) significantly reduced both neurite outgrowth promoting and ROS scavenging effects of FA/AS-IV in PbAc-challenged PC12 cells but did not abolish it completely, suggesting that HO-1 plays an important, but not exclusive role, those

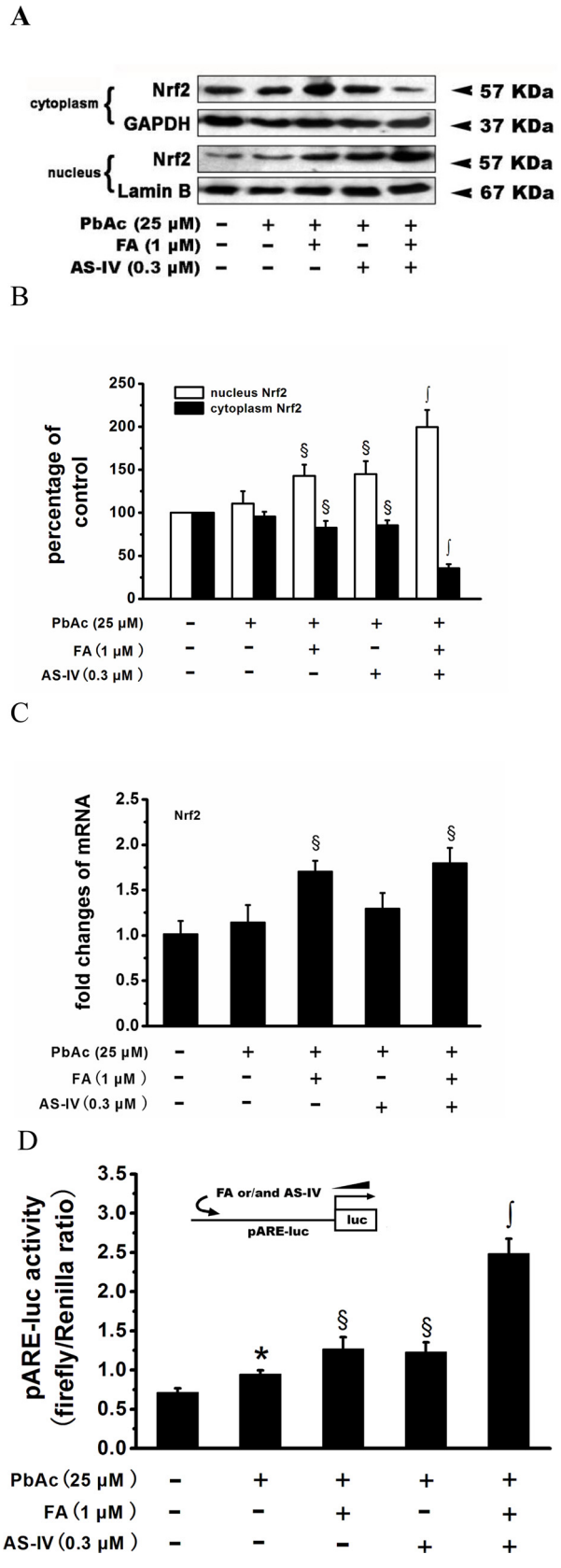

Fig. 2. FA and/or AS-IV activates Nrf2 antioxidant pathways in PbAc-challenged PC12 cells. PC12 cells were treated with FA and/or AS-IV followed by challenge with PbAc for 24 h. A, cytoplasmic and nuclear Nrf2 expression were analyzed by western blot, and GAPDH or Lamin B served as loading control. B, densitometric quantitation expressed as the relative cytoplasmic and nuclear Nrf2 normalized to GAPDH or Lamin B. C, total RNA was isolated and used to prepare cDNA. Nrf2 message was quantified by RT-PCR, and normalized to the levels of GAPDH. D, pGL4.37[luc2P/ARE/ Hygro] and pRL-TK plasmid were transiently co-transfected into PC12 cells. After the indicated treatment, whole-cell extracts were prepared and then analysed for luciferase activity. Data are means ± SD, $n = 3$; *, $p < 0.05$ *vs.* control; §, $p < 0.05$ *vs.* PbAc challenge; ∫, $p < 0.05$ *vs.* FA or AS-IV alone.

effects (Fig. 3F).

3.3. FA, but not AS-IV, activates the ERK1/2 upstream of Nrf2

Nrf2 *de novo* synthesis and/or stabilization has been reported to be controlled by MAPK pathways (Jeong et al., 2016). As shown by MAPK family transcription factor ELISA analyses (Fig. 4A), FA but not **AS-IV** dramatically increases ATF-2, c-Jun, and c-Myc DNA binding activity in PbAc-challenged PC12 cells, but no significant change in DNA binding activity of MEF2 and STAT1 were observed. To address the role of individual MAPK pathways in Nrf2 gene expression regulated by FA/AS-IV, we examined the effects of various dominant-negative (DN) MAPK mutants. As shown in the Western blot analysis in Fig. 4B and C, DN-ERK2 (Cell Biolabs Inc; #ADV-113) inhibited accumulation of Nrf2 in the nucleus induced by FA/ AS-IV while DN-JNK1 (Cell Biolabs Inc; #ADV-115) or DN-p38α (Cell Biolabs Inc; #ADV-105) failed to reverse the Nrf2 nuclear localization.

Next, we tested if FA/AS-IV activates ERK1/2 in PC12 cells exposed to PbAc. As shown by Western blot analysis (Fig. 4D and E), FA induced phosphorylation (activation) of ERK1/2 in PbAc-challenged PC12 cells. AS-IV did not significantly modify ERK1/2 phosphorylation in the absence or the presence of FA. Consistent with Western blot analysis of phospho-ERK1/2, the activity of ERK1/2 detected by a nonradioactive kinase assay with ElK1 as a substrate showed similar results in response to FA, AS-IV, and the combination treatment (Fig. 4F and G). ARE-driven luciferase reporter gene assay showed that blocking ERK1/2 by PD98059 significantly reduced ARE-controlled luciferase activity induced by FA/AS-IV in PbAc-challenged PC12 cells but did not abolish it completely (Fig. 4H).

3.4. AS-IV, but not FA, suppresses Fyn phosphorylation via Akt-mediated inhibition of GSK-3β activity

The sub-cellular location of Nrf2 is mainly determined by the balance between its nuclear import and export (Niture et al., 2014). The main signalling route responsible for nuclear export of Nrf2 is the classical Fyn pathway (Mathur et al., 2016). Our Western blot studies revealed that AS-IV, but not FA, reduces constitutive activation (phosphorylation) of Fyn, while FA did not enhance the inhibitory effects of AS-IV. LiCl or LY294002 abrogated FA/AS-IV-mediated inhibition of Fyn phosphorylation in PbAc-challenged PC12 cells (Fig. 5A and B). Given that GSK-3β acts upstream of Fyn kinase in regulation of nuclear export of Nrf2 (Jain and Jaiswal, 2007), we next investigated whether FA/AS-IV inhibits active (that is, dephosphorylated) GSK-3β. As illustrated in Fig. 5C and D, the data from the Western blot analysis indicated that AS-IV, but not FA, increased phosphorylated (that is, inactive) GSK-3β. LY294002 abolished FA/AS-IV-induced GSK-3β phosphorylation, whereas Fyn shRNA (Santa Cruz #sc-29321-SH) failed to do so, indicating that Akt is located upstream of GSK-3β inactivation. Next, we examined the effect of FA/AS-IV on Akt kinase activity. An *in vitro* kinase assay using GSK-3 as a substrate revealed that AS-IV, but not FA, increased Akt kinase activity, which was not affected by co-treatment with FA (Fig. 5E and F). Western blot of Akt phosphorylation

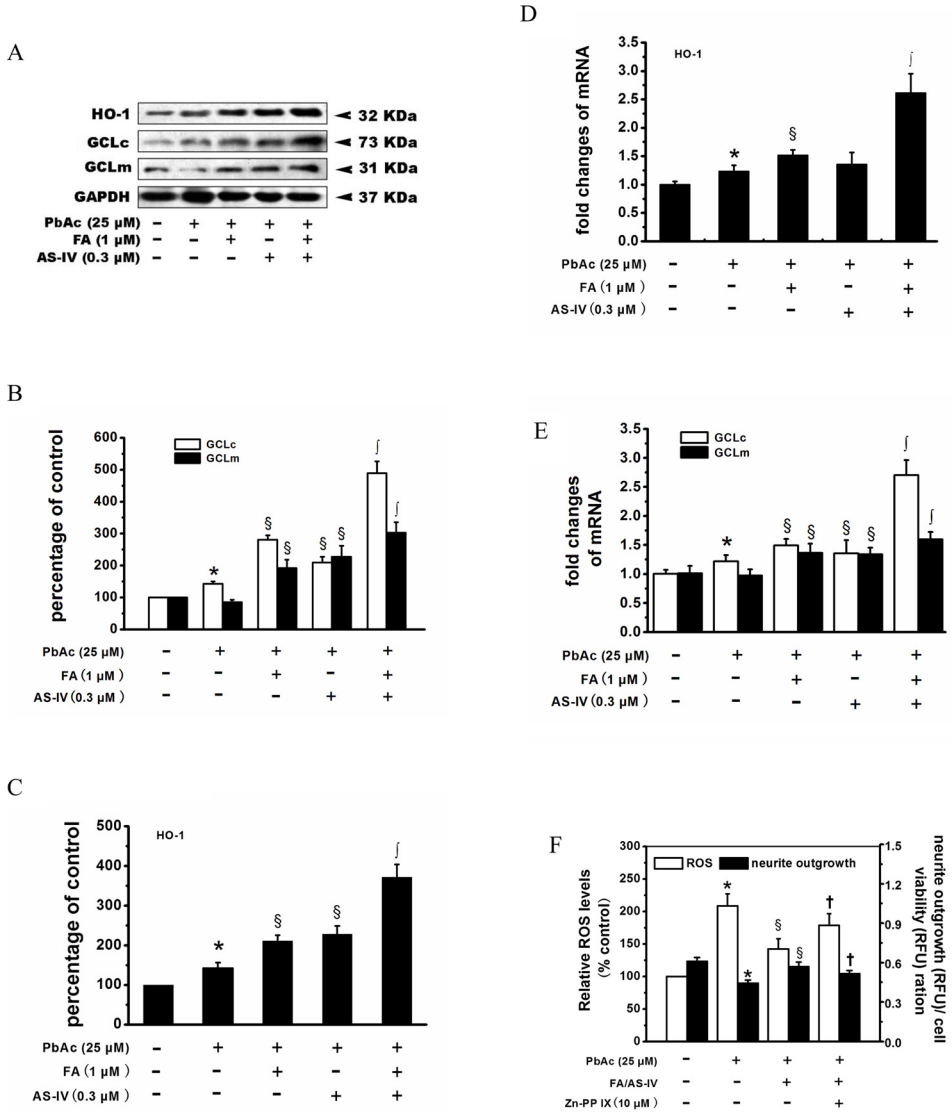

Fig. 3. FA and/or AS-IV induces phase II antioxidants/detoxification enzymes in PbAc-challenged PC12 cells. PC12 cells were treated with FA and/or AS-IV followed by challenge with PbAc for 24 h. A, protein expression of HO-1, GCLc, and GCLm were analyzed by western blot, and GAPDH served as loading control. B, densitometric quantitation expressed as the relative GCLc (open bars) and GCLm (solid bars) normalized to GAPDH. C, densitometric quantitation expressed as the relative HO-1 normalized to GAPDH. D, total RNA was isolated and used to prepare cDNA. HO-1 message was quantified by RT-PCR, and normalized to the levels of GAPDH. E, total RNA was isolated and used to prepare cDNA. GCLc (open bars) and GCLm (solid bars) message was quantified by RT-PCR, and normalized to the levels of GAPDH. F, PC12 cells were treated with FA/AS-IV followed by challenge with PbAc for 24 h in the presence (+) or absence (-) of Zn-PP IX. Intracellular ROS generation was measured using the dichlorofluorescein assay. Neurite outgrowth is monitored *via* bright orange-red staining of outer cell membrane surfaces and green fluorescence staining of cell viability. Data are means ± SD, $n = 3$; *, $p < 0.05$ *vs.* control; §, $p < 0.05$ *vs.* PbAc challenge; ʃ, $p < 0.05$ *vs.* FA or AS-IV alone; †, $p < 0.05$ *vs.* FA + AS-IV.

revealed similar results. Fyn shRNA or LiCl did not significantly modify Akt phosphorylation in PbAc-challenged PC12 cells (Fig. 5G and H).

3.5. Dual activation of both ERK1/2 and Akt by FA/AS-IV are mediated by independent mechanisms

Considering that ERK1/2, Akt, and GSK-3β signaling molecules are intracellular key components of Nrf2 nuclear/cytoplasmic shuttling pathway, we were interested in mapping the chronology between them by using pharmacological inhibitors. As depicted in Fig. 6A and B, treatment with either LY294002 or LiCl failed to change the stimulatory effect of FA/AS-IV on ERK1/2 phosphorylation in PC12 cells challenged with PbAc. Likewise, this stimulatory effect of FA/AS-IV was not reversed by ROS inhibitor diphenyleneiodonium (DPI) pre-treatment. Furthermore, the ERK1/2 inhibitor PD98059 had no effect on FA/AS-IV-induced Akt phosphorylation. The NADPH oxidase inhibitor DPI did not diminish FA/AS-IV-mediated Akt phosphorylation in PC12 cells, indicating that FA/AS-IV-induced Akt phosphorylation is located upstream of ROS scavenging (Fig. 6C and D). However, our study has limitations and future experiments are required to confirm the role of ROS by using the potent antioxidant N-acetylcysteine. Interestingly, PD98059 or LY294002 pre-treatment only partially attenuated FA/AS-IV-induced increase in ARE-driven luciferase activity, while PD98059 plus LY294002 abolish it completely (Fig. 6E). In contrast to the partial block of FA/AS-IV-mediated ROS-scavenging *via* blockade of either pathway alone in PC12 cells challenged with PbAc, the simultaneous inhibition of ERK1/2 and Akt pathways completely reverse ROS-scavenging effect of FA/AS-IV (Fig. 6F). These results support the notion that ERK1/2 and Akt are two separate and independent pathways involving Nrf2 nuclear accumulation.

4. Discussion

The major new finding from the present study is that FA and AS-IV synergistically attenuate PbAc-induced inhibition of neurite outgrowth *via* its antioxidant properties *in vitro*. Furthermore, we report that induction of Nrf2 nuclear translocation is required for FA/AS-IV-mediated ROS-Scavenging through two separate events: 1) FA enhances ERK1/2-dependent nuclear import of Nrf2; 2) AS-IV suppresses Fyn-dependent nuclear export of Nrf2 *via* Akt-GSK-3β pathway. This conclusion is supported by Akt and/or ERK1/2 inhibitors blocking FA/AS-IV-mediated induction of ARE-driven luciferase activity. While we acknowledge that *in vitro* models may not be clinically relevant, our observations produced testable hypotheses for *in vivo* studies of synergistic neuroprotective effect of FA and AS-IV.

The developing brain is extremely sensitive to lead, even at concentrations, it is generally considered safe in mature brain (Gąssowska et al., 2016). The common chelating agents have many side effects and

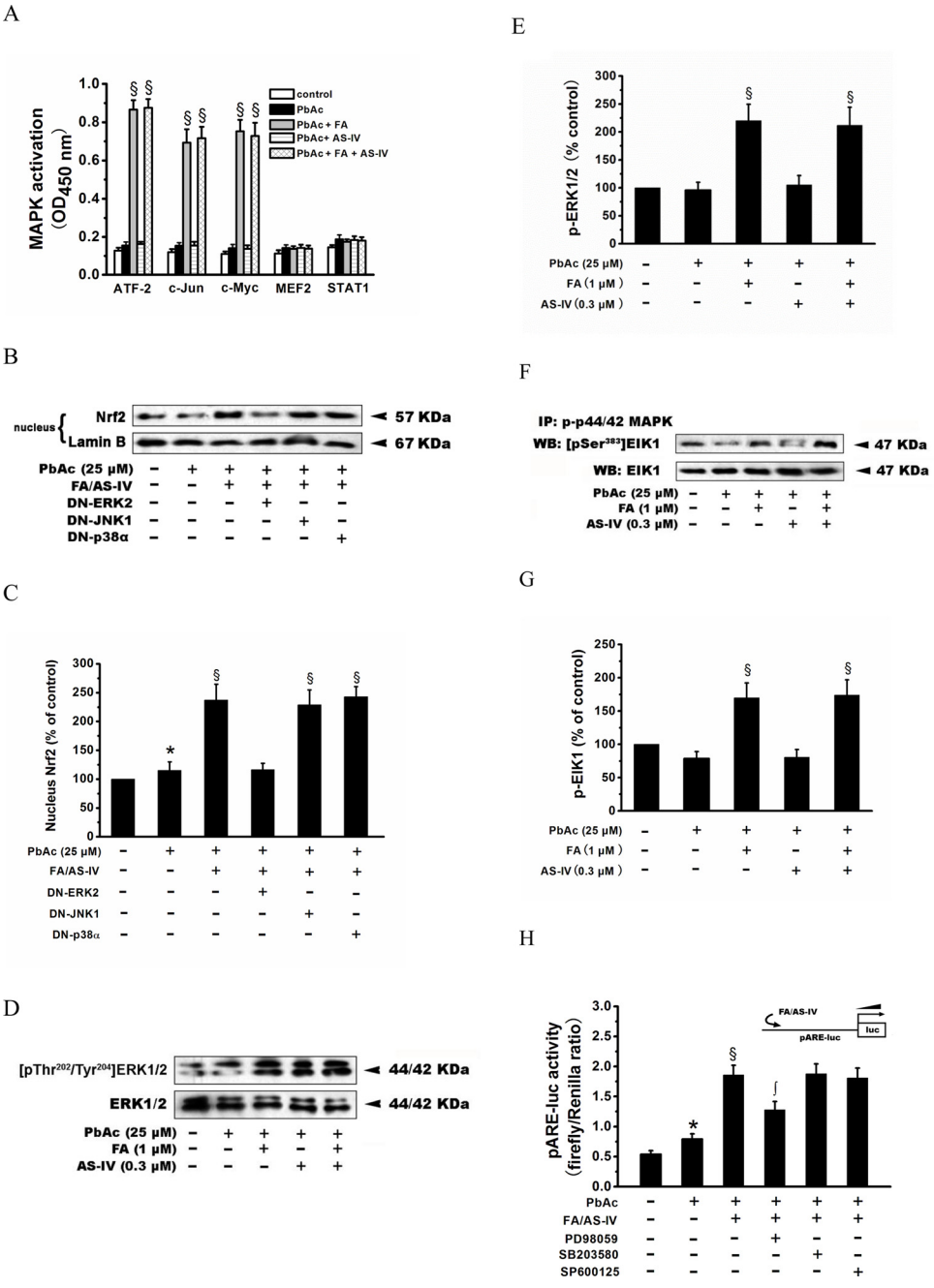

Fig. 4. FA, but not AS-IV, activates the ERK1/2 in PbAc-challenged PC12 cells. A, SH-SY5Y cells were treated with FA and/or AS-IV followed by challenge with PbAc for 24 h. Activated form of the MAPK-regulated transcription factors were spectrophotometrically determined by ELISA-based method. The histogram represents the activation levels of c-Myc, ATF-2, MEF2, c-Jun, and STAT1 in the nuclear extracts. B, PC12 cells were transiently transfected with DN mutants of DN-p38α, JNK1, or ERK2. Transfected cells were treated with FA/AS-IV followed by challenge with PbAc for 24 h. Protein expression of nuclear Nrf2 was analyzed by western blot, and Lamin B served as loading control. C, densitometric quantitation expressed as the relative nuclear Nrf2 normalized to Lamin B. D, PC12 cells were treated with FA and/or AS-IV followed by challenge with PbAc for 24 h. Phosphorylation of ERK1/2 was analyzed by western blot, and total ERK1/2 served as loading controls. E, densitometric quantitation expressed as the relative p-ERK1/2 normalized to total ERK1/2. F, ERK1/2 kinase activity was measured using a specific immunoprecipitation with immobilized phospho-p44/442 MAPK antibody, then an *in vitro* kinase assay of Elk1 fusion protein was performed. G, densitometric quantitation expressed as the relative p-EIK1 normalized to total EIK1. H, pGL4.37[luc2P/ARE/ Hygro] and pRL-TK plasmid were transiently co-transfected into PC12 cells. After the indicated treatment, whole-cell extracts were prepared and then analysed for luciferase activity. Data are means ± SD, $n = 3$; *, $p < 0.05$ *vs.* control; §, $p < 0.05$ *vs.* PbAc challenge; ∫, $p < 0.05$ *vs.* FA/AS-IV treatment.

adverse events and are incapable of ameliorating lead-induced neurotoxicity (Smith and Strupp, 2013). New efforts are being directed towards the study of antioxidants as an alternative approach to prevent it (Krempaská et al., 2016). The *in vitro* PC12 cell model chosen for our experiments can assess the direct effects of FA/AS-IV on neurite outgrowth in the absence of many of the confounding variables found in animal studies, such as stress, nutrition or confounds involving maternal factors. Indeed, a novel finding of the present study is that FA and AS-IV synergistically protects against PbAc-induced inhibition of neurite outgrowth. It has already been demonstrated that FA or AS-IV exert anti-oxidative and/or neuroprotection actions *in vitro*, but these effects often require high concentrations which would likely be impossible to sustain *in vivo*. More interestingly, the current study showed that a combination of FA and AS-IV allow for use of significantly lower doses of FA and AS-IV, on account of a synergism.

Many pieces of evidence suggest that oxidative stress-induced inhibition of neurite outgrowth may be involved in some of the pathologies associated with lead developmental neurotoxicity (He et al., 2017). Epidemiologic studies showed significant positive associations between oxidative stress markers and blood lead levels in workers with long-term high lead exposure (Dobrakowski et al., 2017). Although antioxidant therapy is unlikely to provide a panacea against lead developmental neurotoxicity, a number of antioxidants are necessary to counteract its harmful effects (Pal et al., 2015). Plants produce a variety of antioxidant components to protect their structures against oxidative stress produced during photosynthesis (Nascimento et al., 2016). Indeed, an overwhelming body of evidence indicates that various plant-derived components revealing their potent antioxidant properties (Beaven et al., 2016). To our knowledge, this is the first study to show that FA/AS-IV exerts greater antioxidative actions than either agent alone in PbAc-challenged PC12 cells.

Antioxidants therapy for lead developmental neurotoxicity holds great promise. However, results so far have been rather disappointing and most clinical studies have failed due to various reasons (Rendón-

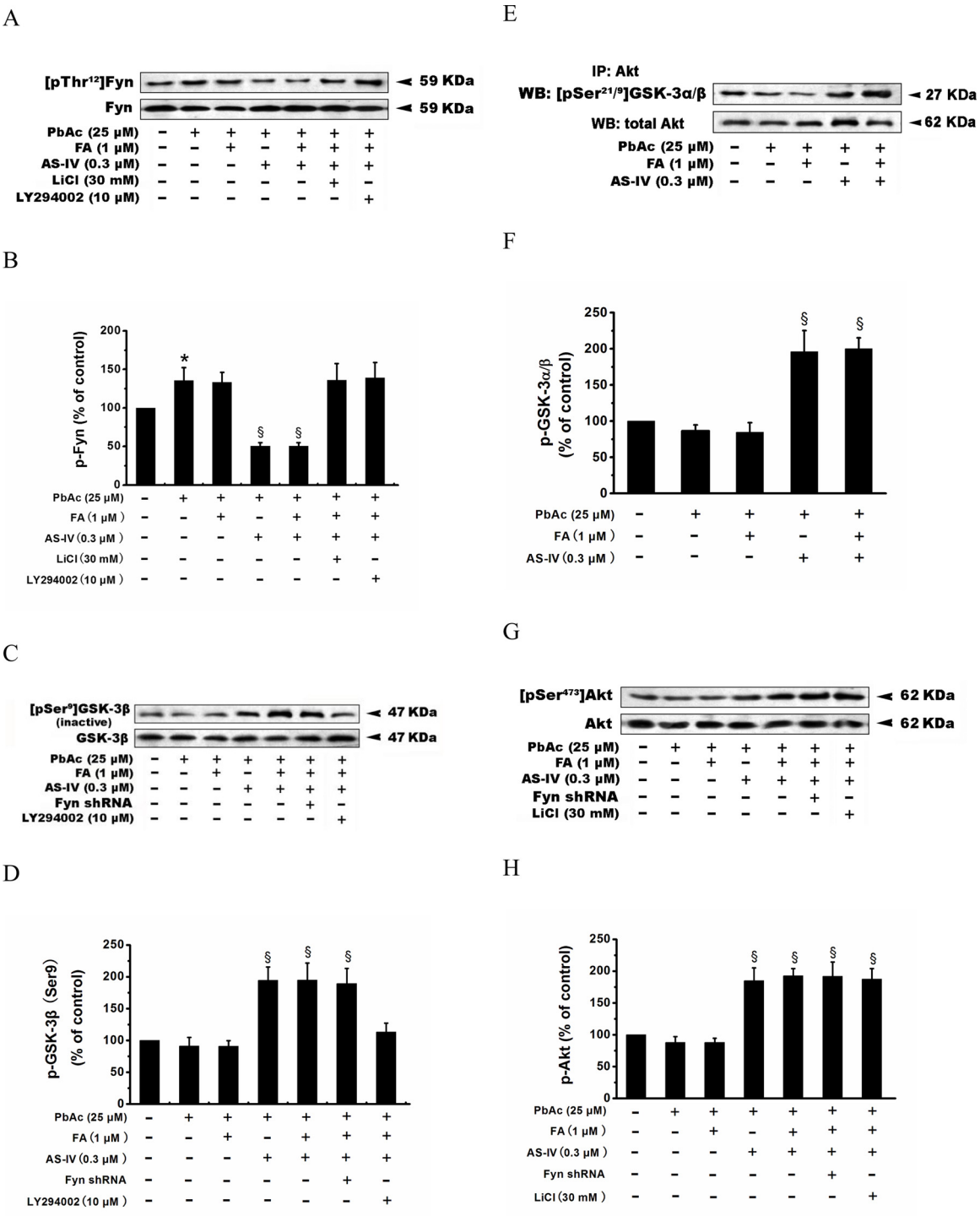

Fig. 5. AS-IV, but not FA, suppresses Akt/GSK-3β/Fyn pathway in PbAc-challenged PC12 cells. A, PC12 cells were pre-treated with FA and/or AS-IV followed by challenge with PbAc for 24 h in the presence (+) or absence (-) of LY294002 or LiCl. Phosphorylation of Fyn was analyzed by western blot, and total Fyn served as loading control. B, densitometric quantitation expressed as the relative p-Fyn normalized to total Fyn. C, the Fyn shRNA was transiently transfected into PC12 cells. Transfected cells were treated with FA and/or AS-IV followed by challenge with PbAc for 24 h in the presence (+) or absence (-) of LY294002. Phosphorylation of GSK-3β was analyzed by western blot, and total GSK-3β served as loading controls. D, densitometric quantitation expressed as the relative p-GSK-3β normalized to total GSK-3β. E, Akt kinase activity was a measured using a specific immunoprecipitation with an immobilized phospho-Akt rabbit antibody, then an *in vitro* kinase assay of GSK-3 fusion protein was performed. F, densitometric quantitation expressed as the relative p-GSK-3α/β normalized to total Akt. G, Phosphorylation of Akt was analyzed by western blot, and total Akt served as loading control. H, densitometric quantitation expressed as the relative p-Akt normalized to total Akt. Data are means ± SD, $n = 3$; *, $p < 0.05$ *vs.* control; §, $p < 0.05$ *vs.* PbAc challenge.

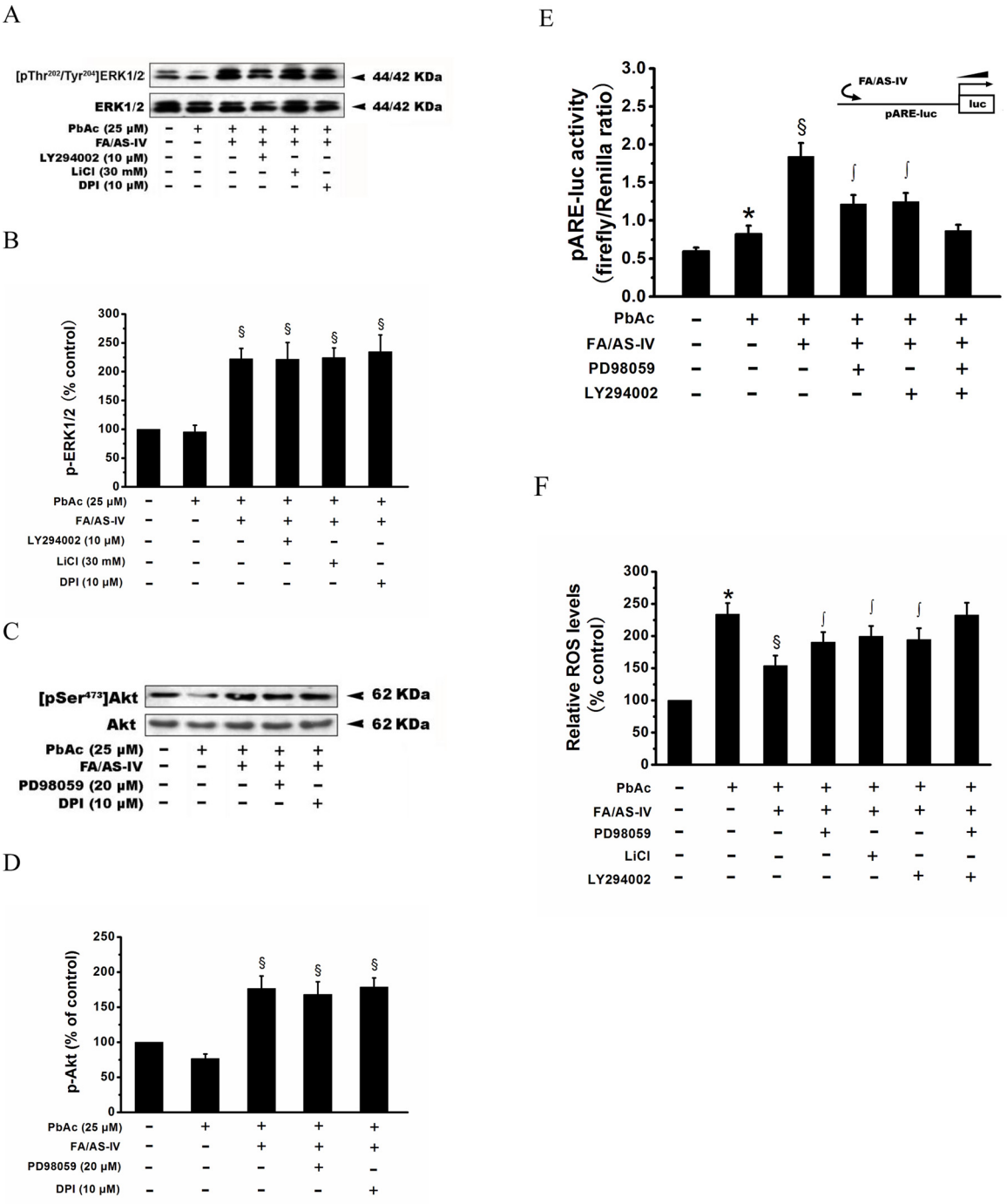

Fig. 6. FA/AS-IV activates both ERK1/2 and Akt pathways *via* independent mechanisms. PC12 cells were pre-treated with FA/AS-IV followed by challenge with PbAc for 24 h in the presence (+) or absence (-) of PD98059, LiCl, LY294002, or DPI. A, phosphorylation of ERK1/2 was analyzed by western blot, and total ERK1/2 served as loading control. B, densitometric quantitation expressed as the relative p-ERK1/2 normalized to total ERK1/2. C, phosphorylation of Akt was analyzed by western blot, and total Akt served as loading control. D, densitometric quantitation expressed as the relative p-Akt normalized to total Akt. E, pGL4.37[luc2P/ARE/Hygro] and pRL-TK plasmid were transiently co-transfected into PC12 cells. After the indicated treatment, whole-cell extracts were prepared and then analysed for luciferase activity. F, intracellular ROS generation was measured using the dichlorofluorescein assay. Data are means ± SD, $n = 3$; *, $p < 0.05$ *vs.* control; §, $p < 0.05$ *vs.* PbAc challenge; ∫, $p < 0.05$ *vs.* FA/AS-IV treatment.

Ramírez et al., 2014). One possible explanation is that supplementation of one antioxidant in too big dosage might therefore be harmful. Another possible explanation is that many antioxidants (such as Vitamin C or GSH) are also highly dependent on other antioxidants in order for them to be active. A strategy based on the inhibition of one signal pathway of nuclear localization and nuclear exclusion signals in control of Nrf2 have failed to protect against lead developmental neurotoxicity (Danhof, 2016). Therefore, we speculate that simultaneous activation of nuclear import signals and inhibition of nuclear export signals may provide more effective antioxidant strategy. It is interesting that although both FA and AS-IV individually protect against PbAc-induced inhibition of neurite outgrowth, when combined, AS-IV enhanced the potency of the neuroprotective action of FA. This finding suggests that FA/AS-IV may work through different mechanism to induce accumulation of Nrf2 in the nucleus.

The elevated expression of “phase-II” enzyme genes are considered

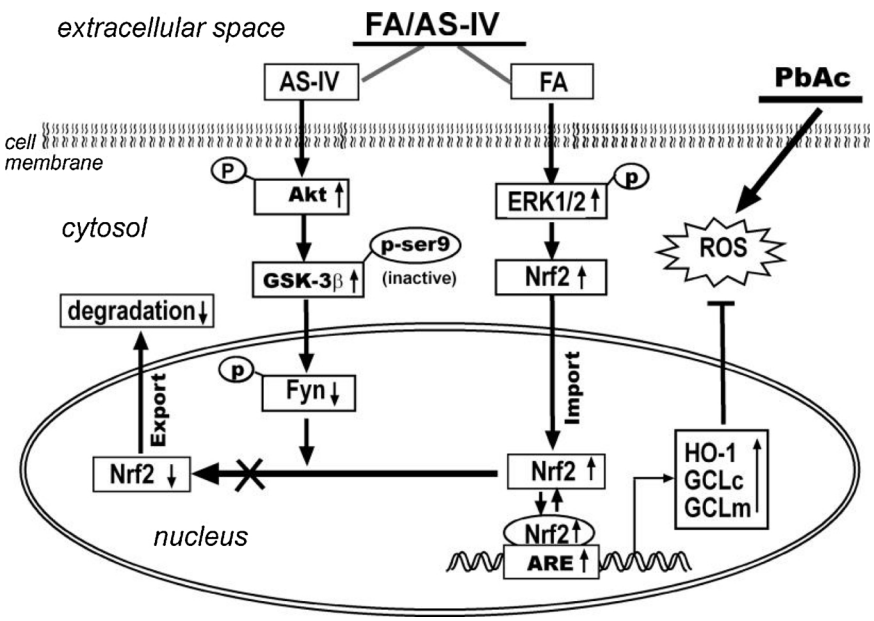

Fig. 7. Putative mechanisms whereby FA/AS-IV induces accumulation of Nrf2 in the nucleus. AS-IV increases Akt and GSK-3β phosphorylation and inhibiting Fyn-mediated Nrf2 nuclear export and degradation. FA activates ERK1/2, leading to the *de novo* synthesis of Nrf2 and its nuclear translocation. As a result, FA/AS-IV synergistically promotes nuclear localization of Nrf2 and attenuates oxidative stress *in vitro*. See text for details.

to be an adaptive response to adverse conditions (Cui et al., 2016). Heme oxygenase is a hemeproteins involved in the regulation of several functions in the nervous system. Increased oxidative stress serve in the brain as a trigger for the induction of HO-1 and Biliverdin reductase-A which enhance cellular antioxidant defenses to counteract oxidative damage (Mancuso et al., 2006; Barone et al., 2011). In the present study, up-regulation of GCLc, GCLm, and HO-1 expression at levels of transcription and translation represent a potential mechanism of cellular defense against lead, showing that developing neurons have the limited ability to cope with oxidative stress. The concentration of lead used in this study was 25 μM, which is relatively lower compared with those used in our previous studies (50 μM) (Li et al., 2014), although this is consistent with adaptive response to lead challenge. Several recent studies demonstrate that sustained exposure to high levels of PbAc leads to a loss of adaptive response (Hossain et al., 2016). This discrepancy might result from differences concentrations of lead. FA/AS-IV substantially enhances GCLc, GCLm, and HO-1 expression at levels of transcription and translation in PbAc-challenged PC12 cells. However, it has been quite difficult to define the role of GSH in the neuroprotection afforded by FA/AS-IV owing to GSH's pleiotropic effects on neuron. In the present study, HO-1, GCLc, GCLm, and GSH have been shown as several events for FA/AS-IV with antioxidant properties. HO-1 is the rate-limiting enzyme in the bilirubin production. The cascade elicited by bilirubin through ERK1/2 is cytoprotective (Mancuso et al., 2008). The bilirubin concentration in the supernatants of PC12 cells treated with and without lead and FA/AS-IV were not measured since there is no commercially available supernatants assay, and improved kits will be required for further analysis in the supernatants of the *in vitro* culture medium. Our results showed that PbAc decreased the content of GSH, but increased the expression of HO-1 proteins in PC12 cells. The effect of AS-IV on GSH levels in PbAc-challenged PC12 cells was much more pronounced than on HO-1 proteins expression.

Nrf2 is a master regulator for the induction of antioxidative genes, which is critical in defending cells against environmental insults (Deshmukh et al., 2017). The level of Nrf2 accumulation in the nucleus is mainly determined by its nuclear import and export (Kaspar et al., 2009). Recent findings, including ours, have showed that Nrf2 is a druggable target in neurons for lead developmental neurotoxicity (Johnson and Johnson, 2015; Yu et al., 2017, 2016a,b). Thus, compounds that regulate the Nrf2 pathway can render neuroprotection to lead toxicity through induction of GCL as well as HO-1 to prevent accumulation of ROS (Peng et al., 2017). Here we show that the mystery underlying the mechanisms of AS-IV-induced the increased nuclear accumulation of Nrf2 but not at transcript levels may be related to inhibition of its nuclear exclusion. In contrast, FA induced Nrf2 expression at levels of transcription and translation, leading translocation of Nrf2 into the nucleus.

The results presented herein indicated that PbAc alone did not significantly decrease active (phosphorylated) ERK1/2, indicating that the lead-induced developmental neurotoxicity do not result from ERK1/2 inactivation. A potential mechanism underlying FA-mediated *de novo* synthesis of Nrf2 and its nuclear translocation may be FA-evoked ERK1/2 phosphorylation. Furthermore, the SB203580 and SP600125 did not modify FA-induced Nrf2 nuclear translocation, thus ruling out p38 MAPK and JNK contributions. Taken together, these data suggest that ERK1/2 is necessary but not sufficient for accumulation of Nrf2 in the nucleus. We observed that pre-treatment of PbAc-challenged PC12 cells with PD98059 partially prevented the increase in ARE transactivation induced by FA/AS-IV, strongly suggesting a role of ERK1/2 in this event. Recent studies have pointed to the involvement of MEK1 in FA activation of ERK1/2 in Schwann cells. Although speculative at this time, it is plausible that the FA-mediated ERK1/2 activation may lies in induction of MEK1 phosphorylation in PbAc-challenged PC12 cells (Zhu et al., 2016a,b).

It was reported that activation of GSK-3β is an upstream events that resulted in the phosphorylation of Fyn (Shang et al., 2015). GSK-3β is negatively regulated by phosphorylation of Ser9 (Li et al., 2012). Our results indicate that AS-IV-induced activation of Akt caused GSK-3β phosphorylation at Ser9 and Fyn dephosphorylation in PbAc-challenged PC12 cells. These data suggest that Akt is essential, but not sufficient, for Nrf2 nuclear translocation and that one or more pathway in addition to Akt are also involved. We show that LY294002 or LiCl completely abrogates Fyn dephosphorylation in response to AS-IV, supporting the concept that GSK-3β is an obligate upstream mediator of Fyn dephosphorylation. Furthermore, although LY294002, a PI3K non-specific inhibitor, was used in this study, it may be that the use of Akt-specific inhibitor MK-2206 or Akt shRNA would be more convincing. It has been reported that AS-IV activates phosphoinositide 3-kinases, an important regulator of Akt activation, in liver tissues from rats (Jia et al., 2014). Although we do not provide direct evidence, the speculation is that AS-IV induced phosphorylation of Akt by regulating phosphoinositide 3-kinases activity under our experimental conditions. Akt and ERK1/2 pathways play, as expected, additive/synergistic roles in the nuclear accumulation of Nrf2 induced by FA/AS-IV in PbAc-challenged PC12

cells. Akt and p-ERK1/2 phosphorylation levels did not change by the addition of a PD98059 and LY294002, respectively, suggesting that the activation of the Akt and ERK1/2 signaling pathways by FA/AS-IV is mediated by independent mechanisms.

Our results, however, must be explained in the context of certain limitations: 1) the *in vivo* system is multi-factorial, and cell culture models may not be clinically relevant; 2) a comprehensive analysis of gene expression profiles is required to further the understanding of the mechanism underlying synergy of FA and AS-IV; 3) the roles of any other enzymes in the removal of ROS requires additional studies. 4) given that primary neurons form synapses, thus incorporating significant neuromodulatory and trophic inputs, primary neurons are more physiologically relevant than immortalized cell lines. 5) further studies are necessary to evaluate neurite outgrowth promoting and ROS scavenging effects of FA and/or AS-IV in cells under normal conditions.

To conclude, AS-IV suppresses Fyn phosphorylation *via* the Akt-dependent GSK-3β inactivation with the involvement of Nrf2 nuclear exclusion. FA induces ERK1/2 activation, and this result in translocation of Nrf2 to the nucleus. A model of sequential events behind FA/AS-IV-induced accumulation of Nrf2 in the nucleus in PbAc-challenged PC12 cells is proposed in Fig. 7.

5. Conclusion

It bears emphasis that the underlying molecular mechanisms are more complex than it is described here. We cannot rule out possible involvement of other phase II enzymes induced by FA/AS-IV, which serve as a defense system against oxidative stress. Our results cautiously suggest multi-targeting strategy with compound combinations aimed at boosting their antioxidant defences against lead developmental neurotoxicity.

Conflict of interest statement

None declared.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.mad.2019.04.002.

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