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ORIGINAL ARTICLE

Acute antioxidant and cytoprotective effects of sulforaphane in brain endothelial cells and astrocytes during inflammation and excitotoxicity

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Abstract

Sulforaphane (SFN), a bioactive phytochemical isothiocyanate, has a wide spectrum of cytoprotective effects that involve induction of antioxidant genes. Nongenomic antioxidant effects of SFN have not been investigated. Brain oxidative stress during inflammation and excitotoxicity leads to neurovascular injury. We tested the hypothesis that SNF exhibits acute antioxidant effects and prevents neurovascular injury during oxidative stress. In primary cultures of cerebral microvascular endothelial cells (CMVEC) and cortical astrocytes from the newborn pig brain, a pro-inflammatory cytokine TNF-α and an excitotoxic glutamate elevate reactive oxygen species (ROS) and cause cell death by apoptosis. Nox4 NADPH oxidase is the main Nox isoform in CMVEC and cortical astrocytes that is acutely activated by TNF-α and glutamate leading to ROS-mediated cell death by apoptosis. The Nox4 inhibitor GKT137831 blocked NADPH oxidase activity and overall ROS elevation, and prevented apoptosis of CMVEC and astrocytes exposed to TNF-α and glutamate, supporting the leading role of Nox4 in the neurovascular injury. Synthetic SFN (10^{-11}-10^{-6} mol/L) inhibited NADPH oxidase activity and reduced overall ROS production in CMVEC and astrocytes within 1-hour exposure to TNF-α and glutamate. Furthermore, in the presence of SFN, the ability of TNF-α and glutamate to produce apoptosis in CMVEC and cortical astrocytes was completely prevented. Overall, SFN at low concentrations exhibits antioxidant and antiapoptotic effects in cerebral endothelial cells and cortical astrocytes via a via a nongenomic mechanism that involves inhibition of Nox4 NADPH oxidase activity. SFN may prevent cerebrovascular injury during brain oxidative stress caused by inflammation and glutamate excitotoxicity.

KEYWORDS

antioxidants, apoptosis, astrocytes, endothelial cells, excitotoxicity, inflammation, isothiocyanates, neurovascular unit, newborn pigs, oxidative stress, primary cells, sulforaphane

1 | INTRODUCTION

Cerebrovascular disease, a consequence of brain oxidative stress, frequently leads to sustained neurological complications. Endothelial and astrocyte components of the neurovascular unit contribute to cerebral blood flow regulation, blood-brain barrier (BBB) integrity, and glutamate clearance from the brain's extracellular space. Thus, maintaining the neurovascular functionality during the oxidative stress conditions is a key component in neuroprotection. NADPH oxidase (Nox family) is a potent enzymatic source of superoxide ($O_2^{\bullet-}$) generation in the brain which is rapidly activated during epileptic seizures, inflammation, ischemia, and hypoxia.[1-13] Our previous studies found that the Nox inhibitors diphenylene iodonium (DPI) and apocynin and the superoxide scavenger tiron reduced oxidative stress and prevented apoptosis in cerebral vascular endothelial cells exposed to inflammatory and excitotoxic stimuli.[3,4] Among several Nox isoforms (Nox1, Nox2, and Nox4) expressed in cerebral endothelium, constitutively active Nox4 has been identified as the primary source of inflammation-induced oxidative stress leading to neurovascular injury.[3,4,14,15] Nox4 gene-depleted cerebral endothelial cells did not respond to pro-inflammatory conditions ROS elevation or apoptosis.[4] In vivo, systemic administration of apocynin and tiron to epileptic newborn pigs greatly attenuated ROS production by cerebral vessels and cortical astrocytes, and improved postictal cerebrovascular functionality.[16] Overall, NADPH oxidase is a promising target for the pharmacological therapy of neonatal cerebrovascular disease developed after epileptic seizures, asphyxia/hypoxia, and inflammation.

Sulforaphane (SFN), a member of the isothiocyanate family characterized by the common -N = C = S group, is formed via the myrosinase-catalyzed conversion of glucoraphanin contained in broccoli, Brussels sprouts, and other cruciferous vegetables.[17,18] SFN exhibits a wide spectrum of neuroprotective and cardioprotective effects that have been linked to gene targeting and upregulation of over 50 genes involved in phase II detoxification and antioxidant defense, including glutathione system, oxidative phosphorylation, catalase, and inducible heme oxygenase 1 (HO-1) via the Nrf2-mediated gene targeting mechanism.[19-26] Remarkably, acute nongenomic antioxidant effects of SFN remain largely unexplored.

In this study, we tested the hypothesis that SFN exhibits acute antioxidant and cytoprotective effects against oxidative stress-induced cerebral vascular injury. Through our findings, we provide the first experimental evidence that SFN at low concentrations has acute antioxidant and antiapoptotic cytoprotective effects that are based on the ability of this compound to inhibit Nox4 NADPH oxidase activity in cerebral vascular endothelial cells and astrocytes during basal, inflammatory, and excitotoxic conditions.

2 | MATERIALS AND METHODS

2.1 | Materials

Cell culture reagents were purchased from GE Healthcare Life Sciences. Matrigel was obtained from Corning. Human TNF-α was from R&D Systems. CellROX and dihydroethidium were from Life Technologies (Thermo Fisher Scientific). GKT137831 was purchased from MedKoo Biosciences, Inc. D, L-sulforaphane and all other reagents were from Sigma.

2.2 | Animals

Newborn piglets (1-5 days old, 1.5-2.5 kg, either sex) were purchased from a commercial breeder. The Animal Care and Use Committee of the University of Tennessee Health Science Center reviewed and approved all procedures involving animals.

2.3 | Isolation and primary cultures of cerebral microvascular endothelial cells and astrocytes

All procedures were described in detail previously.[3,10,11] The brain cortex was dissected from ketamine/acepromazine/xylazine anesthetized newborn pigs and homogenized in M199. Cerebral microvessels (300-60 μm) were collected by consecutive filtration of the brain cortex homogenate through 300- and 60-μm nylon mesh filters. Cerebral micriovascular endothelial cells (CMVEC) were dislodged by collagenase-dispase and were separated by the Percoll density gradient centrifugation. CMVEC were plated on Matrigel-coated surfaces and cultured in DMEM supplemented with 20% fetal bovine serum (FBS), 30 μg/mL endothelial cell growth supplement, 1 U/mL heparin, and antibiotic/antimycotic mixture for 5-6 days until confluent. Cortical astrocytes collected from the vessel-free brain parenchyma were cultured on 75-mL Costar flasks in astrocyte-supporting media (DMEM with 10 ng/mL epidermal growth factor, 20% FBS, and antibiotic/antimycotic mixture) for 10-14 days as described elsewhere.[10,11,27] For experiments involving detection of ROS and apoptosis, the confluent astrocytes were replated on 12-well Costar culture plates and grown for additional 5-6 days. All experiments were performed in separately cultured CMVEC and astrocytes following an overnight exposure to DMEM containing 0.1% FBS to achieve quiescence.

2.4 | ROS detection in intact cells

Intracellular ROS level in neurovascular cells was determined by the fluorogenic probe CellROX Deep Red oxidative stress reagent (Invitrogen) and by dihydroethidium (DHE). These cell-permeable oxidant-sensitive probes produce strong fluorescence signal upon oxidation that could be measured using fluorescence microscopy and spectroscopy. Cortical astrocytes were exposed to pro-oxidants TNF-α (30 ng/mL) or glutamate (2 mmol/L) in the absence or presence of SFN (1-5 μmol/L) or $O_2^{\bullet-}$ scavenger tiron (1 mmol/L) for 1 hour at 37°C. CellROX (5 μmol/L) was added for last 30 minutes of the incubation.[28] The cells were washed twice with PBS and fixed with 3.7% formaldehyde at room temperature

for 15 minutes. Coverslips were briefly rinsed with water, air-dried, and mounted on microscopy slides using an anti-fade mounting medium (Vectashield). The red fluorescence emitted by oxidized CellROX was visualized by fluorescence microscopy (adsorption/emission maxima, ~644/665 nm).[28] Acquired images taken using equal exposure time, sensitivity and resolution (10 images per each treatment) were analyzed using the ImageJ biomaging software.[29] At least five distinct areas with labeled cells on each image were measured, and the cell-free background was subtracted from the average value for each treatment. As an alternative method of quantitative ROS detection, we used DHE fluorescence spectroscopy as described previously.[3,4] CMVEC and cortical astrocytes were incubated with pro-oxidants TNF-α (30 ng/mL) or glutamate (2 mmol/L) in the absence and presence of SFN (1-5 μmol/L) for 60 minutes at 37°C. To evaluate the contribution of NADPH oxidase to overall ROS production, we used common Nox inhibitors DPI (10 μmol/L) and apocynin (0.5 mmol/L), along with the superoxide scavenger tiron (1 mmol/L). A novel selective Nox4 inhibitor GKT137831 (20 μmol/L)[30,31] was additionally used to probe for Nox4 contribution to overall ROS production by CMVEC and astrocytes. DHE (20 μmol/L) was added to the incubation media for the last 20 minutes. The products of DHE oxidation intercalate into nuclear DNA and emit red fluorescence. Pooled cells were washed twice with ice-cold Dulbecco's phosphate buffered saline, and lysed by sonication. Cell lysates were cleared by centrifugation and transferred to Falcon 96-well black plates. Fluorescent products of DHE oxidation (Ox-DHE) 2-hydroxyethidium and ethidium (excitation/emission maxima, 485/590 nm) were measured by the Synergy HT multi-mode microplate reader (BioTek Instruments) and normalized to the protein amount measured by the bicinchoninic acid method (Pierce).

2.5 | NADPH oxidase activity

NADPH oxidase activity was measured by NADPH-enhanced $O_2^{\bullet -}$ production in neurovascular cell homogenates using the lucigenin-enhanced luminescence assay as described previously.[3,4,32] Confluent quiescent CMVEC and astrocytes were disrupted by sonication in 5 mL of ice-cold buffer (20 mmol/L KH_2PO_4, 1 mmol/L EGTA) containing protease inhibitors. Nox activity was measured in a reaction mixture containing 50 mmol/L KH_2PO_4 (pH 7.0), 1 mmol/L EGTA, 150 mmol/L sucrose, and protease inhibitors (total volume, 300 μL). DPI (10 μmol/L), apocynin (0.5 mmol/L), and Nox4 inhibitor GKT137831 were used to evaluate the specificity of NADPH oxidase activity detection. Cell preparations (~100 μg protein) were incubated with the substrate (NADPH, 100 μmol/L) in the absence or presence of TNF-α (15 ng/m), glutamate (0.2 mmol/L), and SFN (5 μmol/L) for 60 minutes at 37°C. Lucigenin (50 μmol/L), a chemiluminescent detector of $O_2^{\bullet -}$ production was added to the reaction mixture for the last 20 minutes of the incubation. Lucigenin luminescence was measured by a Synergy HT microplate reader (BioTek Instruments) and normalized to the protein amount (Pierce).

2.6 | Apoptosis detection by DNA fragmentation and loss of cell contacts

DNA fragmentation and loss of cell-cell and cell-matrix contacts are the key events in apoptosis in CMVEC[3,33] and cortical astrocytes.[10,11] DNA fragmentation was detected by formation of cytoplasmic histone-associated DNA fragments using the cell death detection kit ELISA PLUS (Roche Applied Science).[3] CMVEC and astrocytes were treated with TNF-α (30 ng/mL) or glutamate (2 mmol/L) in the absence or presence of SFN (1-5 μmol/L) or tiron (1 mmol/L) for 3 hours (CMVEC[3]) and 5 hours (astrocytes[10,11]). Cell media were collected for counting of dislodged cells due to loss of cell contacts during apoptosis. Adherent cells were used for quantification of apoptotic DNA fragments (mono- and oligonucleosomes) by ELISA. Immobilized histone-complexed DNA fragments were developed with 2,2'-azino-di(3-ethylbenzthiazolin-sulfonate), and the absorbance at 405 nm was measured by the Synergy HT multi-mode microplate reader (BioTek Instruments).

2.7 | Statistical analysis

Data are presented as means ± SE of absolute values or percent of control. Data were analyzed by analysis of variance (ANOVA) for independent measurements. A level of $P < .05$ was considered significant.

3 | RESULTS

3.1 | Sulforaphane exhibits antioxidant properties in the neurovascular cells

Pro-inflammatory cytokine TNF-α and excitotoxic glutamate are pro-oxidants that rapidly increase ROS formation in primary CMVEC and cortical astrocytes from newborn pigs.[3,4,10,11,27] We investigated whether SFN exhibits antioxidant properties in a model of acute oxidative stress in the neurovascular unit. We applied the fluorescence microscopy approach using a novel cell-permeable fluorogenic probe CellROX Deep Red Reagent that exhibits bright red fluorescence upon oxidation in live cells.[28] Enhanced CellROX fluorescent signals were observed in cortical astrocytes exposed to TNF-α (30 ng/mL; Figure 1A) or glutamate (2 mmol/L; Figure 1C) for 1 hour at 37°C. Quantification of the fluorescence intensity detected a ~2-fold ROS elevation in the presence of TNF-α (Figure 1B) and glutamate (Figure 1D). Remarkably, SFN (1 μmol/L) blocked TNF-α and glutamate-enhanced CellROX signals as efficiently as a potent superoxide scavenger tiron (1 mmol/L) (Figure 1A-D).

We also detected ROS formation in neurovascular cells using an oxidant-sensitive fluorescent probe DHE as in our previous studies.[3,4] CMVEC and cortical astrocytes exposed to TNF-α (30 ng/mL) and glutamate (2 mmol/L) for 1 hour at 37°C exhibited a 2-3-fold ROS

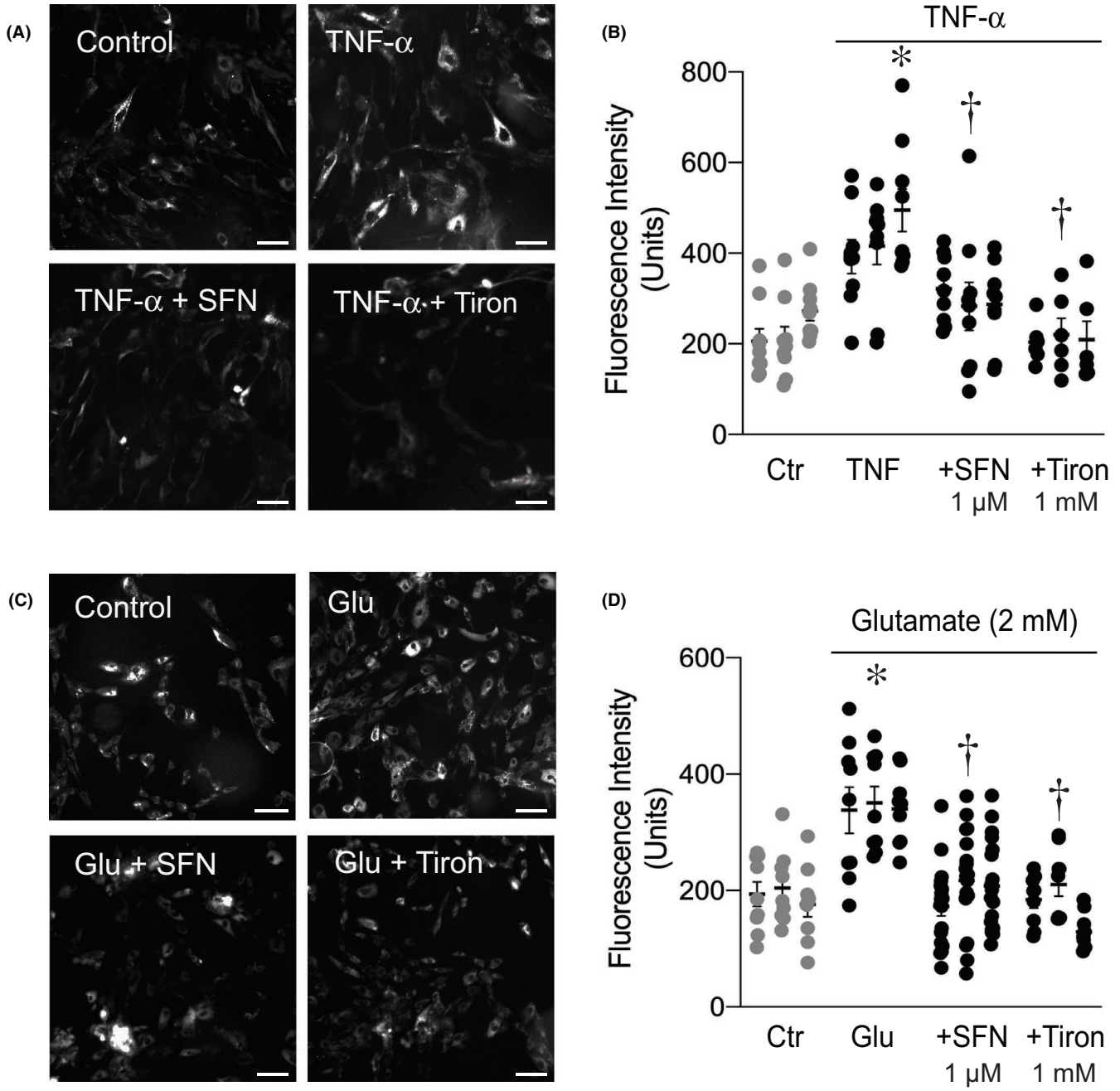

FIGURE 1 Fluorescence microscopy detection of reactive oxygen species (ROS) in cortical astrocytes using the fluorogenic probe CellROX Deep Red Reagent. Cultured cortical astrocytes from newborn piglets were untreated (Control, Ctr) or treated with TNF-α (30 ng/mL; A, B) or glutamate (2 mmol/L; C, D) for 1 h at 37°C in the absence or presence of sulforaphane (1 μmol/L) or superoxide scavenger tiron (1 mmol/L). CellROS Deep Red Reagent (5 μmol/L) was used to detect ROS production. The red fluorescence emitted by oxidized CellROX was examined by fluorescence microscopy. Images (10 microscope fields/treatment) were analyzed using the ImageJ software. A, C: representative images. B, D: fluorescence intensity normalized to the background (data represent the average of 3 independent experiments). Bar, 100 μm. Values are means ± SE. $^{*}P < .05$ compared with the control baseline value. $^{\dagger}P < .05$ compared with TNF-α or glutamate alone

elevation that was greatly inhibited by tiron (1 mmol/L) (Figure 2A,B). Common Nox inhibitors apocynin (0.5 mmol/L) and DPI (10 μmol/L), and a novel Nox4 inhibitor GKT137831 (20 μmol/L) blocked ROS elevation (Figure 2A,B) suggesting that Nox4 NADPH oxidase is the major source of oxidative stress in neurovascular cells exposed to TNF-α and glutamate. SFN (1 and 5 μm) greatly reduced or completely blocked ROS elevation caused by TNF-α and glutamate in both CMVEC (Figure 2A) and astrocytes (Figure 2B). These data show that SFN exhibits potent antioxidant properties in the neurovascular unit that are comparable to the effects of common antioxidants and selective Nox inhibitors.

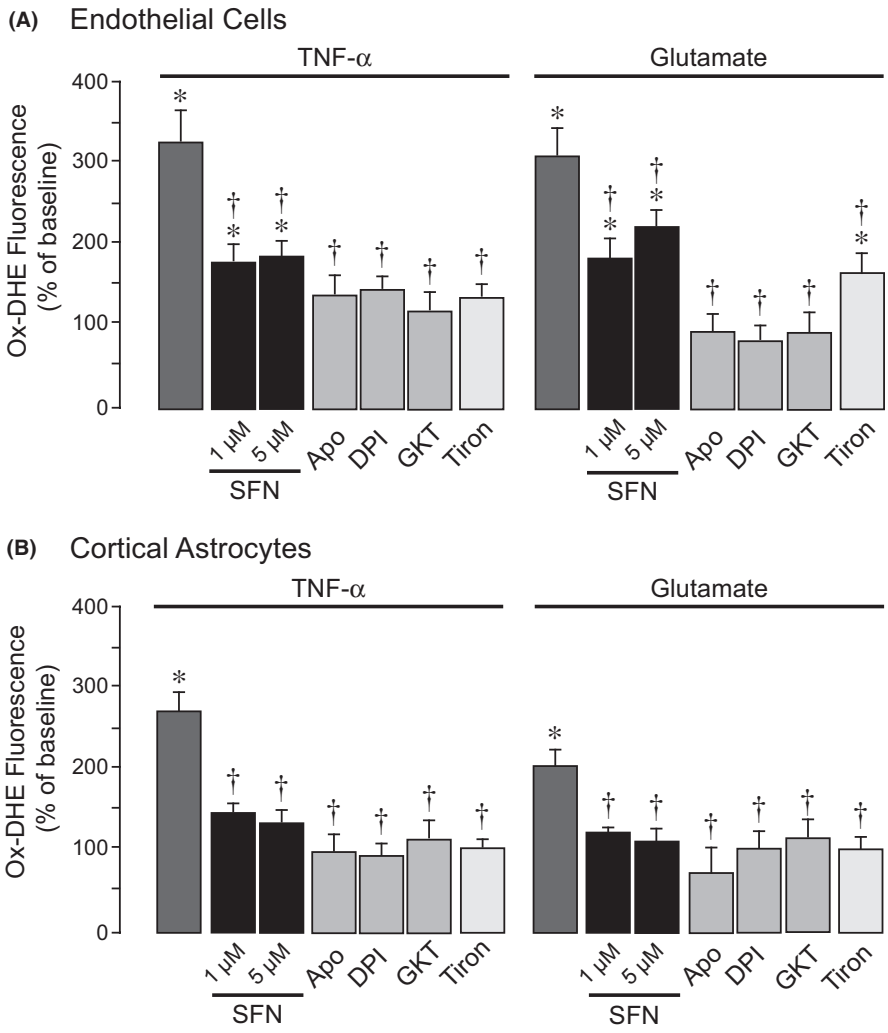

FIGURE 2 Sulforaphane inhibits production of reactive oxygen species (ROS) in cerebral microvascular endothelial cells (CMVEC, A) and cortical astrocytes (B) exposed to TNF-α and glutamate. Primary CMVEC and astrocytes from newborn piglets were treated with TNF-α (30 ng/mL, 1 h) or glutamate (2 mmol/L, 1 h) in the absence or presence of sulforaphane (1 and 5 μmol/L), NADPH oxidase inhibitors apocynin (Apo, 0.5 mmol/L) and DPI (10 μmol/L), Nox4 inhibitor GKT 137 831 (20 μmol/L), and superoxide scavenger tiron (1 mmol/L). ROS production was measured by accumulation of oxidized fluorescent products of DHE (Ox-DHE) and expressed as % of baseline values. Data represent the average of 10 independent experiments. Values are means ± SE. $^{*}P < .05$ compared with the baseline value. $^{\dagger}P < .05$ compared with TNFα or glutamate alone

3.2 | Sulforaphane inhibits NADPH oxidase activity in the neurovascular cells

The endogenous substrate availability is a limiting factor in the enzyme activity in intact cells. Thus, we detected NADPH oxidase activity in neurovascular cells as a NADPH-enhanced DPI-inhibited $O_2^{\bullet -}$ production using a superoxide-sensitive chemiluminescent probe lucigenin as described previously.[3,4] During the control conditions, NADPH-enhanced $O_2^{\bullet -}$ production by CMVEC (Figure 3A) and astrocytes (Figure 3B) was greatly reduced by DPI (5 μmol/L) (Figure 3A, B) suggesting the assay specifically detects NADPH oxidase activity. TNF-α (15 ng/mL) and glutamate (0.2 mmol/L) acutely elevated DPI-inhibited NADPH oxidase activity in both CMVEC and astrocytes (Figure 3A,B). The Nox4-selective inhibitor GKT137831 (20 μmol/L) greatly reduced NADPH oxidase activity in CMVEC and astrocytes during control, TNF-α and glutamate-stimulated conditions (Figure 3A,B). These data confirm our previous findings in Nox4-siRNA-downregulated endothelial cells that Nox4 largely contributes to total enzyme activity.[3,4] SFN, as efficiently as GKT137831, inhibited NADPH oxidase activity in CMVEC (Figure 3A) and cortical astrocytes (Figure 3B) during control and stimulated conditions. Next, we detected the dose-dependent effects of SFN on NADPH oxidase activity. SFN at a wide concentration range 10^{-11}-10^{-6} mol/L exhibited significant inhibitory effects on NADPH oxidase activity in endothelial cells and astrocytes during control, TNF-α and glutamate-stimulated conditions (Figure 4). These data indicate that SFN at low nano- and micromolar concentrations inhibits NADPH oxidase activity in neurovascular cells during control, inflammatory, and excitotoxic conditions.

3.3 | Acute antiapoptotic effects of sulforaphane during oxidative stress

Oxidative stress during inflammatory and excitotoxic conditions leads to neurovascular injury by apoptosis. DNA fragmentation, loss of cell-cell contacts, and cell dislodgement from the matrix are key markers of apoptosis in the neurovascular unit.[3] In our study, CMVEC and cortical astrocytes responded to TNF-α (30 ng/mL) and glutamate (2 mmol/L) by DNA fragmentation (Figure 5A,B) and cell detachment (Figure 6A,B) as detected after 3-5 hours of the exposure. Tiron (1 mmol/L) prevented apoptotic indices including DNA fragmentation (Figure 5A,B) and cell detachment (Figure 5A,B), indicating that oxidative stress is the main cause of apoptosis in the neurovascular unit. SFN (1-5 μmol/L)

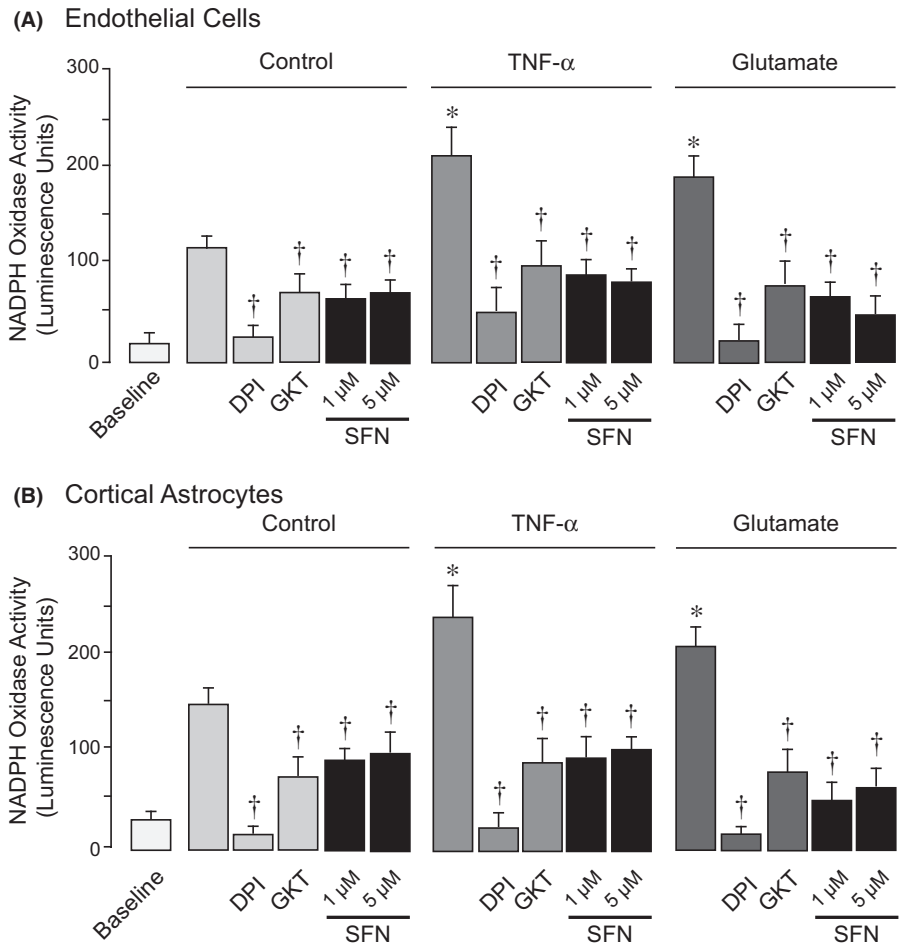

FIGURE 3 Sulforaphane inhibits NADPH oxidase activity in cerebral microvascular endothelial cells (CMVEC, A) and cortical astrocytes (B). Cell homogenates of CMVEC and astrocytes were untreated (Control) or treated with TNF-α (15 ng/mL) or glutamate (0.2 mmol/L) in the absence or presence of NADPH oxidase inhibitor DPI (5 μmol/L), Nox4 inhibitor GKT137831 (20 μmol/L), or sulforaphane (SFN, 1 and 5 μmol/L) for 1 h at 37°C. NADPH oxidase activity was determined as $O_2^{\bullet -}$ production from NADPH measured by enhanced lucigenin luminescence. NADPH (100 μmol/L) was included under all experimental conditions, except for the baseline. Data represent the average of eight independent experiments. Values are means ± SE. $^*P < .05$ compared with the corresponding control values. $^\dagger P < .05$ compared with TNFα or glutamate alone

greatly reduced or completely prevented DNA fragmentation and loss of cell contacts caused by TNF-α and glutamate in CMVEC and cortical astrocytes (Figures 5A,B and 6A,B). These findings demonstrate that SFN exhibits remarkable cytoprotective properties and promotes neurovascular cell survival during acute oxidative stress.

4 | DISCUSSION

NADPH oxidase is the major contributor to oxidative stress in the brain during inflammation, epileptic seizures, and ischemia. NADPH oxidase activation in primary cultures of cerebral vascular endothelial cells and cortical astrocytes exposed to pro-inflammatory cytokine TNF-α and excitotoxic glutamate leads to neurovascular cell death by apoptosis. Our major novel finding is that, the natural isothiocyanate sulforaphane, at low nano- and micro-molar concentrations, exhibits antioxidant effects in the neurovascular unit via a nongenomic mechanism that involves inhibition of NADPH oxidase catalytic activity and prevents oxidative stress-induced neurovascular injury during inflammatory and excitotoxic conditions.

Oxidative stress is an important target for pharmacological intervention. Novel approaches to cerebral vascular protection during oxidative stress are essential for improving long-term outcomes of neonatal cerebral vascular disease. Our previous data using siRNA-mediated Nox1, Nox2, and Nox4 selective gene silencing demonstrated that Nox4 NADPH oxidase is the major ROS-generating system in the cerebral vascular system of newborn pigs that is rapidly activated via a posttranslational mechanism during inflammatory and excitotoxic conditions thus leading to cell death by apoptosis.[3,4] In the present study, we have confirmed these data using a novel Nox4 selective inhibitor GKT137831 that reduced ROS elevation caused by exposing CMVEC and cortical astrocytes to TNF-α and glutamate. Overall, Nox4 NADPH oxidase appears to be a promising pharmacological target for preventing cerebrovascular dysfunction caused by oxidative stress conditions.

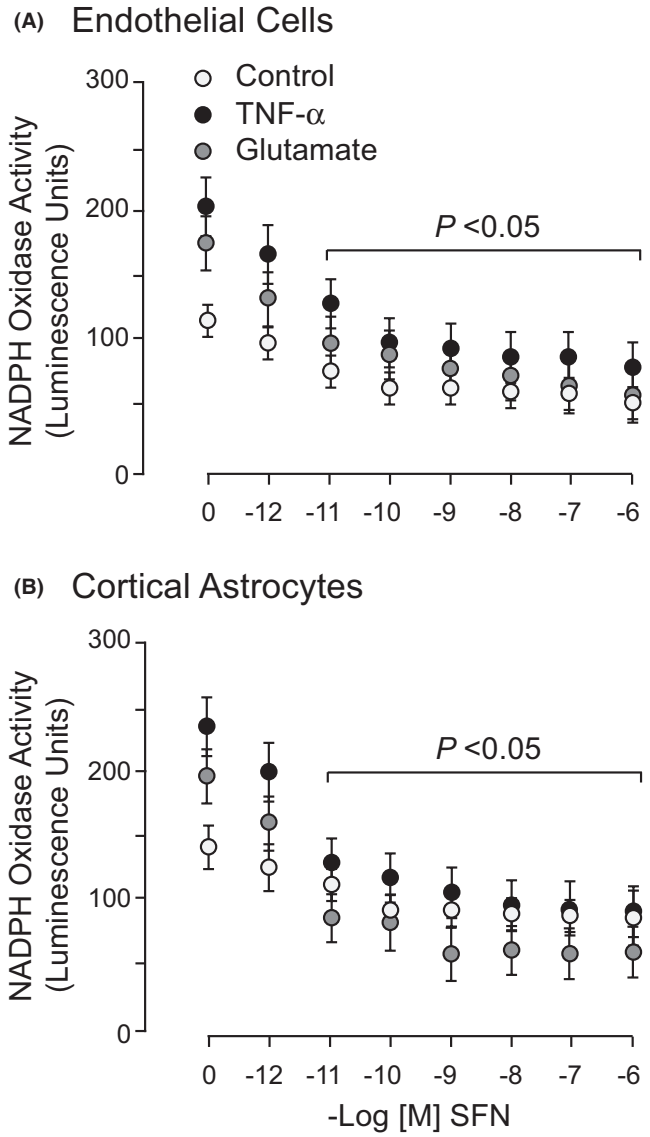

FIGURE 4 Dose-response effects of sulforaphane on NADPH oxidase activity in cerebral microvascular endothelial cells (CMVEC, A) and cortical astrocytes (B). Cell homogenates of CMVEC and astrocytes were untreated (Control) or treated with TNF-α (15 ng/mL) or glutamate (0.2 mmol/L) in the absence or presence of sulforaphane (SFN, 10^{-12}-10^{-6} mol/L) for 1 h at 37°C. NADPH oxidase activity was determined as $O_2^{\bullet -}$ production from NADPH measured by enhanced lucigenin luminescence. NADPH (100 μmol/L) was included under all experimental conditions. Data represent the average of six independent experiments. Values are means ± SE. *$P < .05$ compared with the corresponding control values

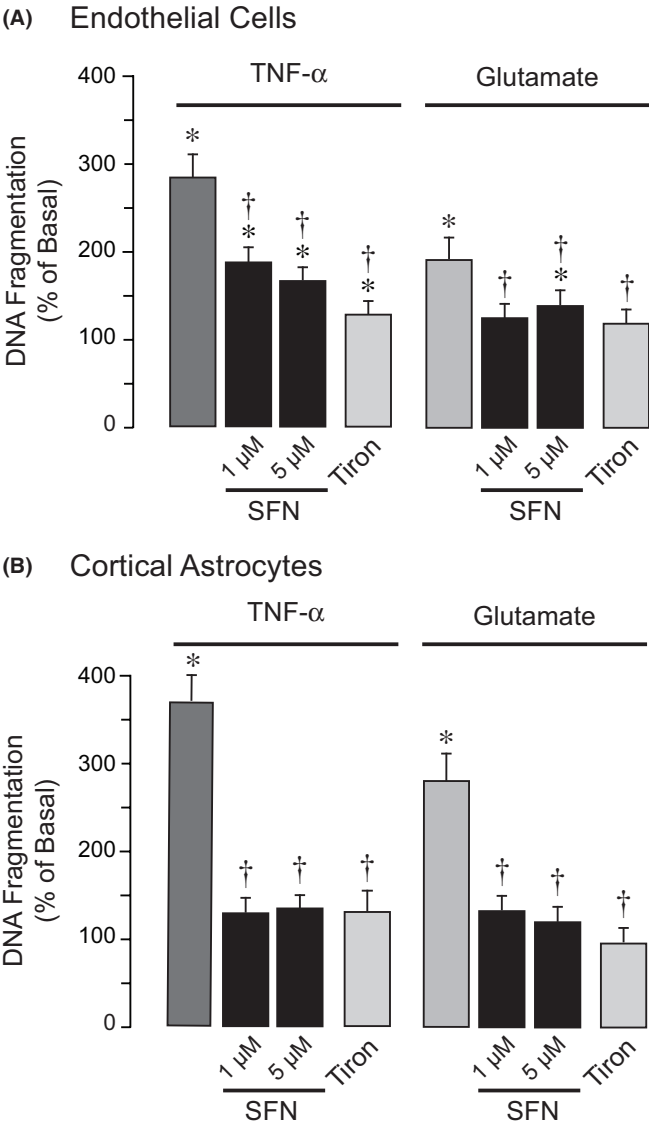

FIGURE 5 Sulforaphane prevents apoptosis in cerebral microvascular endothelial cells (CMVEC, A) and cortical astrocytes (B) caused by oxidative stress. Cultured CMVEC (A) and cortical astrocytes (B) from newborn piglets were treated with pro-oxidants TNF-α (30 ng/mL) or glutamate (2 mmol/L) for 3 h (CMVEC) or 5 h (astrocytes) at 37°C in the absence or presence of sulforaphane (1 and 5 μmol/L) or antioxidant tiron (1 mmol/L). DNA fragmentation, the key event of apoptosis, was detected by ELISA and expressed as % of baseline values. Data represent the average of six independent experiments. Values are means ± SE. *$P < .05$ compared with the baseline value. †$P < .05$ compared with TNFα or glutamate alone

SFN [4-(methylsulfinyl)butyl isothiocyanate] and other isothiocyanates have been widely recognized as gene-targeting indirect antioxidants due to their ability to modulate the cellular redox status by transcriptional activation of phase II antioxidant enzymes/proteins (glutathione S-transferase, quinone reductase, glucuronosyltransferase, NAD(P)H/quinone oxidoreductase1, and heme oxygenase-1) via the Nrf2-mediated gene targeting mechanism.[17,18,20,21,23,26,34-38] The gene-targeting mechanism involves SFN binding to thiol groups of the cytoplasmic actin-bound protein Keap1 that forms the complex with the transcription factor Nrf2, thus allowing Nrf2 to migrate to the nucleus and activate the antioxidant response element of Phase II cytoprotective genes and accelerate their transcription.

We provide first evidence of immediate nongenomic antioxidant effects of SFN in the neurovascular cells. SFN at low nano- and micro-molar concentrations exhibits acute antioxidant and cytoprotective effects in the in vitro neurovascular model of oxidative stress.

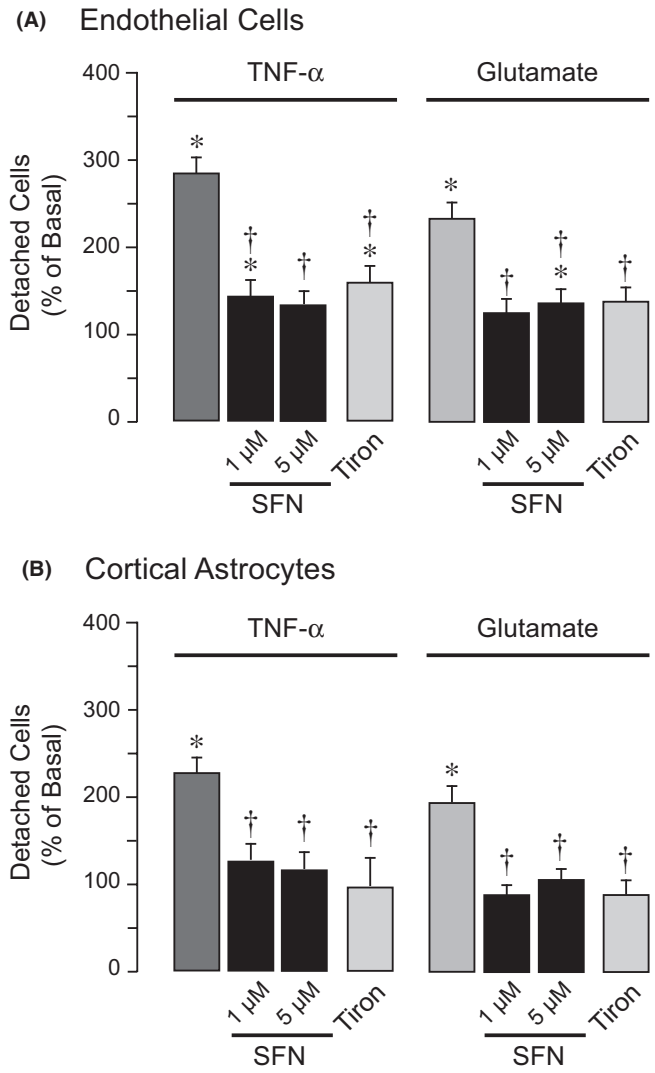

FIGURE 6 Sulforaphane prevents the loss of cell-matrix contacts in cerebral microvascular endothelial cells (CMVEC, A) and cortical astrocytes (B) caused by oxidative stress. Primary CMVEC and cortical astrocytes from newborn piglets were treated with pro-oxidants TNF-α (30 ng/mL) or glutamate (2 mmol/L) for 3 h (CMVEC) or 5 h (astrocytes) at 37°C in the absence or presence of sulforaphane (1 and 5 μmol/L) or antioxidant tiron (1 mmol/L). Cell detachment due to loss of cell contacts was expressed as % of baseline values. Data represent the average of six independent experiments. Values are means ± SE. $^{*}P < .05$ compared with the baseline value. $^{\dagger}P < .05$ compared with TNFα or glutamate alone

Oxidative stress caused by neonatal brain disease, including inflammation/infection, epileptic seizures, and asphyxia/hypoxia leads to cerebral vascular injury, dysregulation of cerebral blood flow, loss of blood-brain barrier integrity, and, finally, to neurodegeneration. Antioxidant-based approaches using brain-permeable antioxidants are needed to preserve the structural and functional integrity of the neurovascular unit. Previously, we demonstrated that SFN penetrates the blood-brain barrier following systemic administration to newborn pigs[16] and is, therefore, a potential neurovascular cytoprotective compound.

Endothelial cells and astrocytes are the main components of the neurovascular unit that are most vulnerable to oxidative stress injury.[3,4,10,11,27] We used primary cultures of CMVEC and cortical astrocytes from newborn pigs exposed to pro-inflammatory cytokine TNF-α or excitotoxic neurotransmitter glutamate as the in vitro model of acute neurovascular injury caused by inflammatory and excitotoxic conditions. CMVEC and astrocytes acutely responded to TNF-α and glutamate by a surge of ROS leading to apoptotic death within 3 hours of exposure. Nox4 NADPH oxidase activation by TNF-α and glutamate accounts for oxidative stress and apoptosis of neurovascular cells. Common NADPH oxidase inhibitors DPI and apocynin, as well as novel Nox4 inhibitor GKT137831 block ROS elevation caused by TNF-α and glutamate in neurovascular cells. These pharmacological data confirms and expands our previous findings that used siRNA-based genomic approach to demonstrate that Nox4 is the major source of oxidative stress in cerebral endothelial cells exposed to inflammatory conditions.[3,4] In the presence of SFN, NADPH oxidase activation, ROS formation, DNA fragmentation, and loss of cell contacts caused by TNF-α and glutamate in CMVEC and astrocytes were completely prevented. SFN at low nano- and micro-molar concentrations acts as efficiently as GKT137831 for blocking ROS elevation in response to TNF-α and glutamate, suggesting that Nox4 is a main target for SNF inhibition in CMVEC and astrocytes. Overall, our findings demonstrate that SFN exhibits acute antioxidant cytoprotective effects in neurovascular cells by inhibiting Nox4 NADPH oxidase activation.

There are several potential mechanisms by which SFN may inhibit NADPH oxidase activity. First, isothiocyanates may bind to functional cysteine thiol groups in various enzymes and proteins, thus modulating their activities. Acute inhibitory effects of SFN on AMPK/mTOR activities have been shown in primary murine hepatocytes,[39] human aortic vascular smooth muscle cells,[40] and the human osteosarcoma cell line.[41] Therefore, a posttranslational modification of the Nox4 catalytic subunit by SFN leading to the enzyme inhibition needs to be considered. The regulation of Nox4 activity is not well understood.[2,42-47] Unlike the Nox1 and Nox2 catalytic subunits, Nox4 is constitutively active, and does not require cytosolic proteins for full activation. The role of the membrane constituent p22(phox) in Nox4 activation has not been well established. There is no evidence that Nox4 is activated via PKC-, serine/threonine-, and/or Ca^{2+}-mediated phosphorylation.[46] This is strongly supported by our finding that the inhibitors of Akt, ERK1/2, or p38MAPK-dependent phosphorylation did not reduce the Nox4-mediated ROS elevation in response to TNF-α in porcine cerebral endothelial cells.[4] Overall, it is unlikely that SFN inhibits Nox4 activity via a phosphorylation-targeted mechanism.

Second, ROS scavenging potencies of isothiocyanates can contribute to the antioxidant effects of SFN in neurovascular cells. Recent reports suggest that, due to the presence of a carbon-bound reduced sulfur atom, some isothiocyanates may exhibit direct antioxidant activity by neutralizing ROS in the cell.[17,34] Thus, the ROS scavenging effects of SFN may account for its ability to reduce ROS

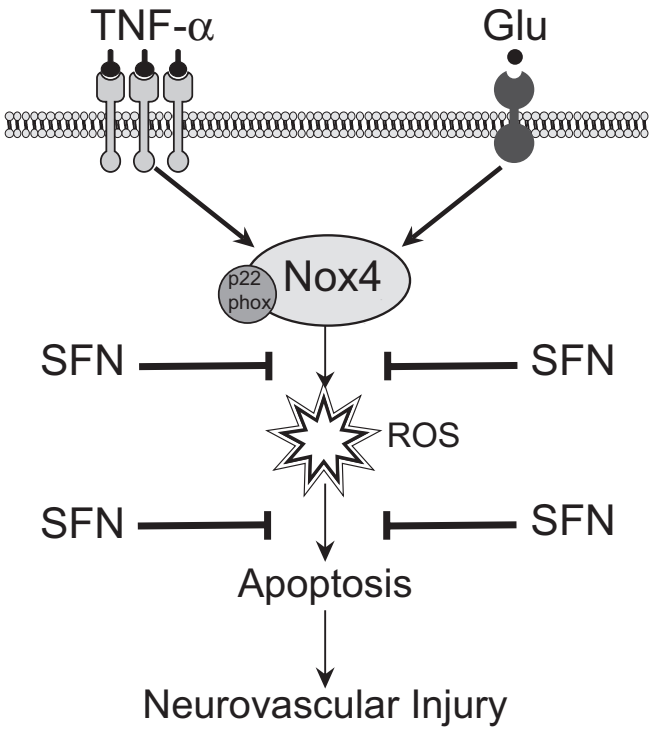

FIGURE 7 The mechanism of acute antioxidant and cytoprotective effects of sulforaphane in neurovascular cells. Nox4, the main NADPH oxidase isoform in cerebral vascular endothelial cells and astrocytes, is activated by pro-inflammatory cytokine TNF-α and glutamate, leading to oxidative stress and cell death by apoptosis. Sulforaphane, a cell-permeable isothiocyanate, inhibits Nox4 activity in cerebral endothelial cells and astrocytes, reduces ROS production, and prevents oxidative stress-induced neurovascular cell injury during inflammation and excitotoxicity

species produced via NADPH oxidase activation during inflammatory and excitotoxic conditions.

Third, the possibility that H_2S, an antioxidant gaseous mediator,[48-51] may play a part in the mechanism of SFN cytoprotection cannot be ignored. The isothiocyanate moiety has been recognized as a potential H_2S-releasing group. However, we conducted H_2S measurements and provided solid evidence that SFN is not a spontaneous H_2S donor molecule.[16] Importantly, we have collected extensive in vivo and in vitro evidence that SFN rapidly activates the endogenous production of H_2S in the live brain and in the brain parenchyma via the metabolism of cysteine by cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE).[16] Both enzymes are highly expressed in cortical astrocytes and cerebral vascular endothelial cells and account for H_2S production by the brain.[50] The inhibitory effect of endogenous H_2S and H_2S donors on Nox4 NADPH oxidase activity has been reported in animal models of oxidative stress disease.[52,53] These findings may indicate that endogenous H_2S contributes to SFN-elicited antioxidant neurovascular cytoprotection during oxidative stress. However, considering that SFN (10^{-11}-10^{-6} mol/L) is far more effective in providing antioxidant cytoprotection as compared to H_2S (10^{-5}-10^{-4} mol/L),[48,49,51] we suggest that the contribution of the H_2S-dependent antioxidant mechanism, although likely, does not fully account for the antioxidant properties of SFN to protect neurovascular cells from oxidative stress injury.

We also cannot exclude that, in addition to NADPH oxidase, other sources of ROS can be targeted by SFN. Mitochondrial electron chain complexes I and II contribute to a burst of pro-apoptotic $O_2^{\bullet-}$ generated by CMVEC exposed to TNF-α and glutamate.[3,4,9] It has been reported that SFN may modulate the production of mitochondrial ROS. Cell-specific distinct mitochondrial effects of SFN may include mitochondrial damage and apoptosis in cancer cells and, conversely, mitoprotection in normal cells.[21,53-55] However, it appears that in the neurovascular model of oxidative stress caused by inflammation and excitotoxicity, the mitochondrial involvement is limited compared to major role of NADPH oxidase.

Overall, we have provided the first evidence in native neurovascular cells that SFN has acute nongenomic antioxidant and cytoprotective effects that are based on the ability of this compound to inhibit Nox4 NADPH oxidase activity during basal, inflammatory, and excitotoxic conditions (Figure 7). Importantly, SFN is a cell-permeable compound that can penetrate noncompromised blood-brain barrier during systemic administration. We propose that SFN may provide a potent pharmacological tool to prevent deleterious changes in cerebrovascular structure and function and to preserve blood-brain barrier integrity thus promoting vascular neuroprotection during brain oxidative stress caused by inflammation, epilepsy, ischemia, and hypoxia.

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CONFLICT OF INTEREST

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

AUTHORS' CONTRIBUTIONS

Parfenova, Liu, and Chandaka participated in research design. Liu, Chandaka, and Zhang conducted experiments. Parfenova, Liu, Chandaka, and Zhang performed data analysis. Parfenova, Liu, Chandaka, and Zhang wrote or contributed to the writing of the manuscript.

DATA AVAILABILITY STATEMENT

Data sharing not applicable to this article as no datasets were generated or analyzed during the current study. The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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