

Nrf2 Upregulates ATP Binding Cassette Transporter Expression and Activity at the Blood–Brain and Blood–Spinal Cord Barriers

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Activation of nuclear factor E2-related factor-2 (Nrf2), a sensor of oxidative stress, is neuroprotective in animal models of cerebral ischemia, traumatic brain injury, subarachnoid hemorrhage, and spinal cord injury. We show here that Nrf2 activation with sulforaphane (SFN) *in vivo* or *in vitro* increases expression and transport activity of three ATP-driven drug efflux pumps at the blood–brain barrier [P-glycoprotein, ATP binding cassette b1 (Abcb1); multidrug resistance-associated protein-2 (Mrp2), Abcc2; and breast cancer resistance protein (Bcrp), Abcg2]. Dosing rats with SFN increased protein expression of all three transporters in brain capillaries and decreased by 50% brain accumulation of the P-glycoprotein substrate verapamil. Exposing rat or mouse brain capillaries to SFN increased P-glycoprotein, Bcrp, and Mrp2 transport activity and protein expression; SFN increased P-glycoprotein activity in mouse spinal cord capillaries. Inhibiting transcription or translation abolished upregulation of P-glycoprotein activity. No such effects were seen in brain capillaries from Nrf2-null mice, indicating Nrf2 dependence. Nrf2 signaled indirectly to increase transporter activity/expression. The p53 inhibitor pifithrin abolished the SFN-induced increase in transporter activity/expression, and the p53-activator nutlin-3 increased P-glycoprotein activity. SFN did not alter P-glycoprotein transport activity in brain and spinal cord capillaries from p53-null mice. Inhibitors of p38 MAPK and nuclear factor κ B (NF- κ B) blocked the effects of SFN and nutlin-3 on P-glycoprotein activity. These results implicate Nrf2, p53, and NF- κ B in the upregulation of P-glycoprotein, Bcrp, and Mrp2 at blood–CNS barriers. They imply that the barriers are tightened selectively (efflux transporter upregulation) by oxidative stress, providing increased neuroprotection, but also reduced penetration of many therapeutic drugs.

Key words: ABC transporters; blood-brain barrier; drug delivery; NF- κ B; P-glycoprotein; p53

Introduction

Nuclear factor E2-related factor-2 (Nrf2) is a redox-sensitive, ligand-activated transcription factor that plays a critical role in cellular defenses against oxidative and electrophilic stress (Cople et al., 2010; Cople, 2012). In the absence of oxidants and electrophiles, Nrf2 is inactive and retained in the cytoplasm bound to Kelch-like ECH-associated protein 1 (Keap1). After oxidation of sulfhydryl groups on Keap1, Nrf2 is released and translocates to the nucleus, binding to antioxidant-response elements (AREs) in the promoter regions of target genes. Nrf2 drives expression of key players in the electrophile/antioxidant re-

sponse, including genes that code for proteins that produce GSH, reduce reactive oxygen species (ROS), and metabolize xenobiotics (Cople, 2012).

Administering Nrf2 ligands is neuroprotective in animal models of cerebral ischemia (Alfieri et al., 2011), traumatic brain injury (TBI) (Yan et al., 2008; Dash et al., 2009), subarachnoid hemorrhage (Chen et al., 2011; Z. Wang et al., 2012), and spinal cord injury (SCI; Mao et al., 2012; X. Wang et al., 2012). In a mouse model of TBI, postinjury administration of sulforaphane (SFN), an Nrf2 ligand found in cruciferous vegetables, protects the blood–brain barrier, increasing expression of cytoprotective genes and preserving barrier integrity (Dash et al., 2009). Thus, Nrf2 has been proposed as a therapeutic target in stroke, TBI, and SCI.

Although the major focus of research on Nrf2 targets has been on detoxifying/antioxidant enzymes, several ATP binding cassette (ABC) transporters are Nrf2 targets. In barrier and excretory tissues, these transporters handle endogenous metabolites, xenobiotics (including therapeutic drugs), and xenobiotic metabolites. They contribute to xenobiotic defense mechanisms and are important determinants of xenobiotic uptake, distribution, and excretion. In hepatocytes, activating Nrf2 increases expression of multidrug resistance-associated protein 2 (Mrp2) to Mrp4

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(Abcc2–Abcc4) and possibly P-glycoprotein (Abcb1; Klaassen and Slitt, 2005; Maher et al., 2005; Aleksunes and Klaassen, 2012). Nrf2-null mice exhibit reduced hepatic expression of Mrp3 and Mrp4, and targeted deletion of Keap1 increases expression of Mrp1–Mrp5 and breast cancer resistance protein [Bcrp (Abcg2)].

The present study is concerned with the effects of Nrf2 activation on drug efflux transporters at the blood–brain and blood–spinal cord barriers. In both tissues, ABC transporters at the luminal, blood-facing, surface of capillary endothelial cells drive outward transport and prevent potentially neurotoxic xenobiotics from entering the CNS. Thus, these transporters limit CNS accumulation of many therapeutic drugs (Miller, 2010; Campos et al., 2012). Of these transporters, P-glycoprotein handles the widest array of xenobiotics. Moreover, the large number of therapeutic drugs transported by P-glycoprotein makes this transporter a primary obstacle to CNS pharmacotherapy. Increased P-glycoprotein expression at brain barriers further limits delivery of therapeutic drugs to the CNS (Miller, 2010).

We show here that activation of Nrf2 at the blood–brain and blood–spinal cord barriers increased expression and transport function of P-glycoprotein, Mrp2, and Bcrp. These Nrf2-driven increases in transporter expression/activity required p53, p38, and nuclear factor κ B (NF- κ B) to be active.

Materials and Methods

Materials. Mouse polyclonal P-glycoprotein antibody was obtained from Covance, and antibodies against MRP2 and BCRP were from Alexis Biochemicals. Heme oxygenase 1 (HO-1) antibody was purchased from Abcam. The fluorescent P-glycoprotein substrate [N- ϵ (4-nitrobenzofurazan-7-yl)-D-Lys⁸ cyclosporine A (NBD-CSA)] was custom-synthesized (Schramm et al., 1995). The fluorescent Mrp2 substrate sulforhodamine 101 free acid [Texas red (TR)] was purchased from Sigma-Aldrich, and the fluorescent Bcrp substrate BODIPY-prazosin was purchased from Life Technologies. PSC833 (3S,6S,9S,12R,15S,18S,21S,24S,30S,33S)-1,4,7,10,12,15,19,25,28-nonamethyl-33-[(E,2R)-2-methylhex-4-enoyl]-6,9,18,24-tetrakis(2-methylpropyl)-3,21,30-tri(propan-2-yl)-1,4,7,10,13,16,19,22,25,28,31-undecacyclo[tritiacontane-2,5,8,11,14,17,20,23,26,29,32-undecone], a specific inhibitor of P-glycoprotein, was kindly provided by Novartis. Mouse monoclonal β -actin antibody, Ficoll, SFN, *tert*-butylhydroquinone (*t*BHQ), and all other chemicals were obtained from Sigma-Aldrich. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) stock solution was prepared by RTI International. All reagents were of analytical grade or the best available pharmaceutical grade.

Animals. All experiments were performed in compliance with National Institutes of Health animal care and use guidelines and approved by the Animal Care and Use Committee of National Institute of Environmental Health Sciences (NIEHS). Male retired breeder Sprague Dawley rats (6–9 months; Taconic) were housed in temperature-controlled rooms under a 12 h light/dark cycle and were given *ad libitum* access to food and water. Nrf2 knock-out mice were provided by Dr. Steven Kleiberger at NIEHS; p53 knock-out mice were provided by Dr. Michael Fessler at NIEHS. Animals were killed by CO₂ inhalation, followed by decapitation. Brain capillaries were isolated and immediately used for

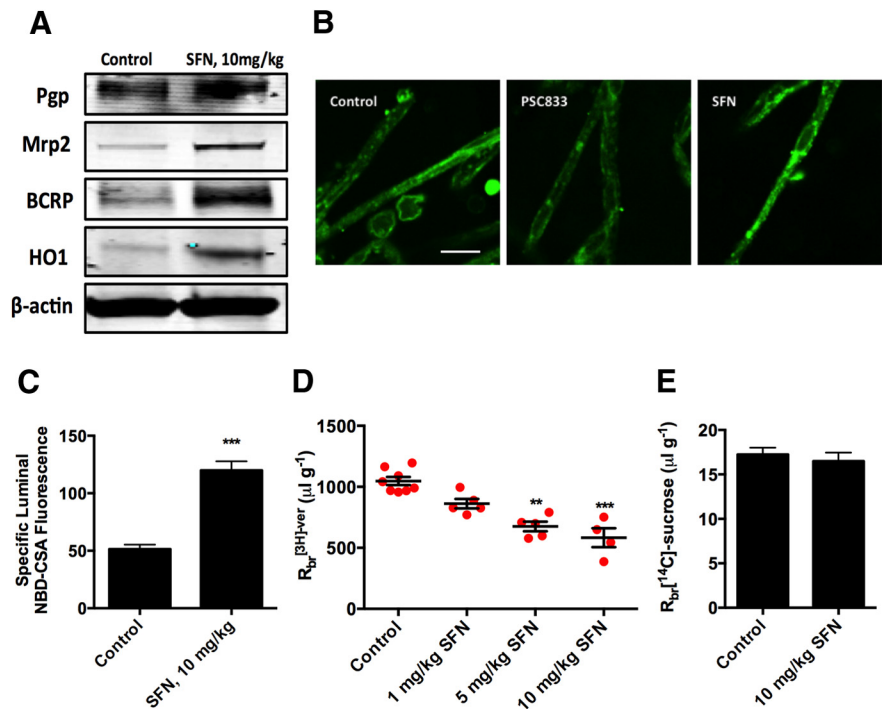


Figure 1. Effect of SFN dosing (1–10 mg/kg, *i.p.*, for 2 d) on ABC transporter expression and function at the rat blood–brain barrier. **A**, Western blots of brain capillary membranes (pooled tissue from 5 rats) showing that SFN dosing increases protein expression for three ABC transporters and the Nrf2-responsive gene HO-1. Pgp, P-glycoprotein. **B**, Representative confocal micrographs of brain capillaries from control and SFN-dosed rats (scale bar, 10 μ m). Capillaries were isolated from control and SFN-dosed rats and incubated with 2 μ M NBD-CSA without and with 5 μ M PSC833 (middle; *ex vivo* assay). **C**, Quantitation of specific NBD-CSA luminescence in capillaries isolated from control and SFN-dosed rats (*ex vivo* assay). Shown are mean \pm SEM for 8–12 capillaries from single preparations (pooled brains from 5 rats). *** p < 0.001, significantly higher than control. **D**, SFN dosing reduces brain accumulation of [³H]verapamil (P-glycoprotein substrate) as measured by *in situ* brain perfusion. Each point represents the value for a single rat. ** p < 0.01, significantly higher than control; *** p < 0.001, significantly higher than control. **E**, SFN dosing does not change brain accumulation of [¹⁴C]sucrose (tight junction permeability marker). Data are from five rats per group.

transport experiments; for Western blotting, capillary membranes were prepared and frozen for additional analysis.

Capillary isolation. Detailed procedures for capillary isolation were described previously (Miller et al., 2000; Hartz et al., 2004). Briefly, white matter, meninges, midbrain, choroid plexus, blood vessels, and olfactory lobes were removed from the brains under a dissecting microscope, and the brain tissue was homogenized. Tissue was kept in cold PBS (in mM: 2.7 KCl, 1.5 KH₂PO₄, 136.9 NaCl, 8.1 Na₂HPO₄, 1 CaCl₂, 0.5 MgCl₂, 5 D-glucose, and 1 sodium pyruvate) throughout the isolation procedure. An aliquot of 30% Ficoll was added to an equal volume of brain homogenate, and capillaries were separated from the parenchyma by centrifuging at 5800 \times *g* for 20 min. Capillary pellets were washed with 1% BSA in PBS and passed through a syringe column filled with glass beads. Capillaries bound to the glass beads were released by gentle agitation, washed with PBS, and used immediately.

Transport assay. Confocal microscopy-based transport assays with isolated rat brain capillaries were described previously (Hartz et al., 2004). All experiments were performed at room temperature in coverslip-bottomed imaging chambers filled with PBS. In general, rat or mouse brain capillaries were exposed to Nrf2 activator (SFN or *t*BHQ) without or with additional inhibitors. Fluorescent substrates, NBD-CSA for P-glycoprotein, TR for Mrp2, and BODIPY-prazosin for Bcrp (Hartz et al., 2004; Bauer et al., 2008b; Wang et al., 2010), were added, and luminal substrate accumulation was assessed 1 h later. In each experiment, a specific transport inhibitor [MK571 (3-[[3-[(E)-2-(7-chloroquinolin-2-yl) ethenyl]phenyl]-[3-(dimethylamino)-3-oxopropyl]sulfanyl]methyl] sulfanylpropanoate) for Mrp2 and Ko143 [*tert*-butyl 3-((3S,6S,12aS)-6-isobutyl-9-methoxy-1,4-dioxo-1,2,3,4,6,7,12,12a-octahydropyrazino[1',2':1,6]pyrido[3,4-b]indol-3-yl)propanoate) for Bcrp] was used to define the mediated component of luminal substrate accumulation (see Re-

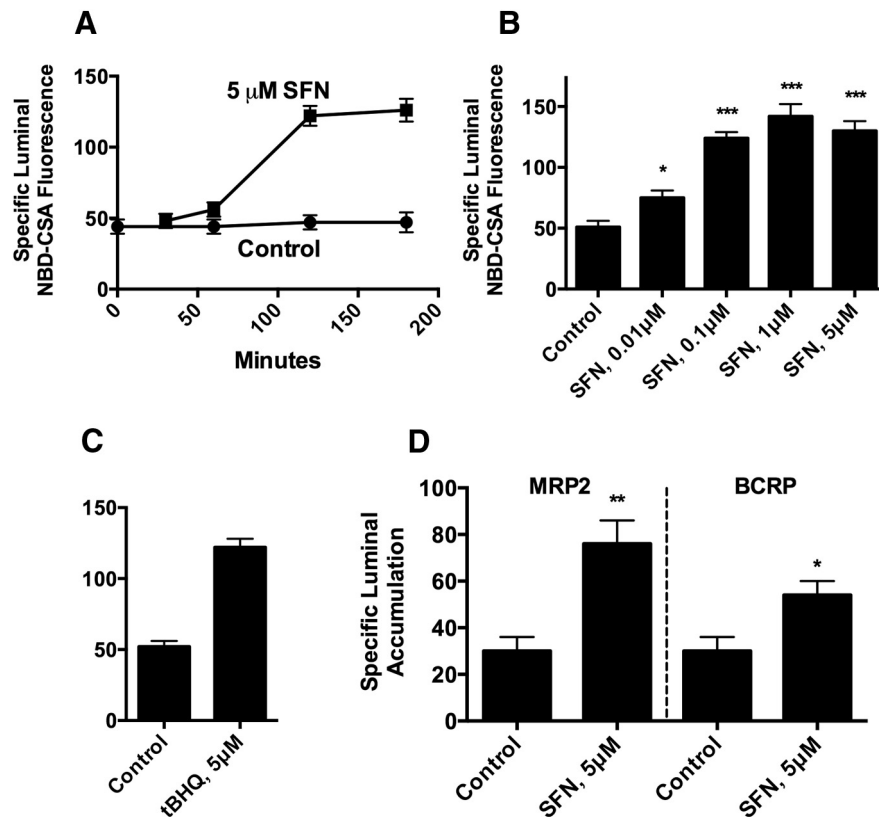


Figure 2. Effects of SFN exposure on ABC transporter transport activity in isolated rat brain capillaries (specific luminal accumulation of transporter-selective fluorescent drugs; see Results). **A**, Time course of SFN-induced increase of P-glycoprotein transport activity. Capillaries were incubated in medium with 5 μ M SFN for the time shown and then incubated for 1 additional hour with SFN and 2 μ M NBD-CSA. **B**, SFN dose response for P-glycoprotein activity. The exposure time is 4 h (3 h with SFN plus 1 h with SFN and 2 μ M NBD-CSA). **C**, Increased P-glycoprotein transport activity in brain capillaries exposed to tBHQ. **D**, SFN exposure increases MRP2 and Bcrp transport activity in brain capillaries. Shown are mean \pm SEM for 10–15 capillaries from single preparations (pooled brains from 8–10 rats). * p < 0.05, ** p < 0.01, *** p < 0.001, significantly higher than control.

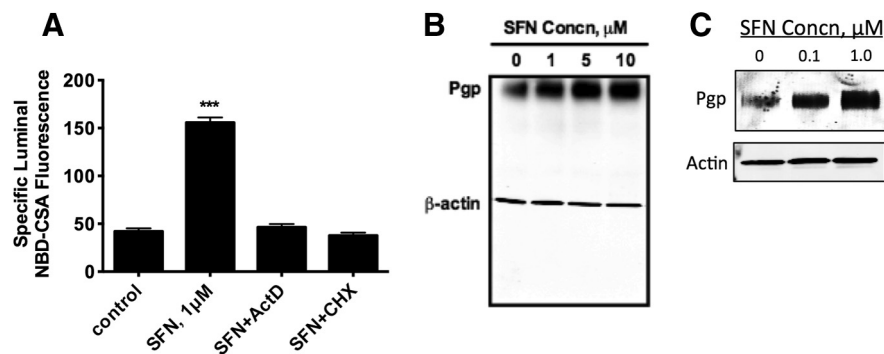


Figure 3. The SFN-induced increase in P-glycoprotein activity requires transcription and translation (rat brain capillaries). **A**, Inhibition of transcription [1 μ M actinomycin D (ActD)] or translation [100 μ g/ml cycloheximide (CHX)] abolishes the increase in transport activity caused by exposure to 5 μ M SFN. Shown are mean \pm SEM for 8–12 capillaries from a single preparation (pooled brains from 10 rats). *** p < 0.001, significantly higher than control. **B**, **C**, Western blots of brain capillary membranes showing that 4 h exposure of capillaries to SFN exposure increases P-glycoprotein (Pgp) expression in a concentration-dependent manner.

sults). To acquire images, the chamber containing the capillaries was mounted on the stage of a Zeiss model 510 inverted confocal laser scanning microscope and imaged through a 40 \times water-immersion objective (numeric aperture 1.2) using a 488 nm laser line for NBD-CSA and BODIPY-prazosin and a 543 nm laser line for TR. Images were saved to disk, and luminal fluorescence was quantitated by NIH ImageJ software as described previously (Miller et al., 2000). Data shown are for a single experiment that is representative of three to five replicates.

Western blots. Membranes were isolated from control and SFN-exposed capillaries as described previously (Hartz et al., 2004; Bauer et al., 2006). Membrane protein was assayed by the Bradford method. An aliquot of the membrane protein was mixed with NuPAGE 4 \times sample buffer (Invitrogen), loaded onto 4–12% Bis-Tris NuPAGE gel, electrophoresed, and then transferred to an Immobilon-FL membrane (Millipore). The membrane was blocked with Odyssey Blocking Buffer (Li-Cor Biosciences) at room temperature for 1 h and then immunoblotted with antibodies against P-glycoprotein. The membrane was stained with corresponding goat anti-mouse fluorescence dye IRDye 800 in PBS with 0.1% Tween 20 at room temperature for 45 min and then imaged using an Odyssey Infrared Imaging System (Li-Cor Biosciences). β -Actin (42 kDa; 1:2000) was used as a loading control.

Electrophoresis mobility shift assay. Nuclear protein extract was isolated from control and SFN-induced brain capillaries using an NE-PER kit (Pierce) and by following the instructions of the manufacturer. cDNA oligonucleotides [5'-CTA ATG GTG ACA AAG CAA CTT T-3' and 5'-AAA GTT GCT TTG TCA CCA TTA G-3' (ARE recognition sequence in bold); 5'-TAC AGA ACA TGT CTA AGC ATG CTG GGG ACT-3' and 5'-AGT CCC CAG CAT GCT TAG ACA TGT TCT GTA-3' (p53); and 5'-AGT TGA GGG GACTTT CCC AGG C-3' and G CCT GGG AAA GTC CCC TCA ACT-3' (NF- κ B)] were end labeled with IRDye680 fluorescent dyes by IDT and annealed at 100 $^{\circ}$ C for 5 min (B. Zhao et al., 2006; Wang et al., 2011). The electrophoresis mobility shift assay (EMSA) samples were prepared using Odyssey Infrared EMSA Buffer kit (Li-Cor Biosciences), with 5 μ g of nuclear protein extract used in each Nrf2/ARE binding reaction. The binding reaction was incubated at room temperature for 20 min, and the DNA–protein complexes were resolved on a pre-cast, 6% native polyacrylamide gel in 0.5 \times Tris Borate EDTA buffer. The gel was removed from the electrophoresis unit and directly imaged on the Odyssey Infrared Imaging System.

Immunostaining for NF- κ B. Isolated brain capillaries were exposed to SFN and other chemicals, fixed, permeabilized, and incubated with primary (rabbit polyclonal ab 16502; Abcam) and secondary [goat anti-rabbit IgG (H+L) Alexa Fluor 488; A11008; Life Technologies] antibodies as described previously (Wang et al., 2010). The primary antibody used recognizes p65, which translocates into the nucleus during NF- κ B activation. Immunostained capillaries were imaged with a Zeiss model 510 inverted confocal laser scanning microscope and imaged through a 40 \times water-immersion objective (numeric aperture 1.2) using a 488 nm laser line. Images

were saved to disk, and nuclear fluorescence was quantitated using NIH ImageJ software.

In situ brain perfusion. Brain perfusion was performed as described previously (Hawkins et al., 2010). Rats were anesthetized by intraperitoneal injection with 1 ml/kg ketamine mixture (in mg/ml: 79 ketamine, 3 zylazine, and 0.6 acepromazine) and administered heparin (10 kU/kg). The common carotid arteries were exposed by midline incision at the

neck and perfused with oxygenated Ringer's solution at 37°C (117 mM NaCl, 4.7 mM KCl, 0.8 mM MgSO₄, 24.8 mM NaHCO₃, 1.2 mM KH₂PO₄, 2.5 mM CaCl₂, 10 mM D-glucose, 39 g/L 170 kDa dextran, and 1 g/L BSA) at 3 ml/min. [¹⁴C]Sucrose (0.5 μCi/ml) or [³H]verapamil (0.1 μCi/ml) were infused into the circuit via syringe pump at 0.5 ml/min for 20 min. Samples of perfusate were collected from the cannulae at the end of each experiment. Brains were removed, stripped of meninges, midbrain, and choroid plexuses, and minced. Tissue and 100 μl perfusate samples were solubilized and counted (Hawkins et al., 2010). Results were expressed as the ratio of disintegrations per minutes in the brain/disintegrations per minutes in the perfusate (shown as R_{br} , μl/g).

Statistical analyses. Data are expressed as mean ± SEM. Statistical analyses of differences between groups was by one-way ANOVA (Newman–Keuls multiple comparison test) using Prism 5.0 software. Differences between two means were considered significant when $p < 0.05$.

Results

SFN upregulates ABC transporter expression *in vivo*

We dosed rats by intraperitoneal injection with 10 mg/kg SFN for 2 d and, on day 3, isolated brain capillaries for subsequent determination of ABC transporter expression and P-glycoprotein transport activity. Previous studies showed that this dosing protocol induces Nrf2 target enzymes in mouse brain and is neuroprotective in mice after ischemic stroke (J. Zhao et al., 2006). Figure 1A shows increased P-glycoprotein, Mrp2, and BCRP protein expression in brain capillaries from SFN-dosed rats. SFN dosing also increased expression of HO-1, a known Nrf2 target.

To assess transporter function, we assayed transport activity in brain capillaries isolated from control and SFN-dosed rats. Our laboratory established a method to measure the transport activity of ATP-driven efflux transporters in freshly isolated brain and spinal cord capillaries using fluorescent substrates, transporter-specific inhibitors, confocal microscopy, and digital image analysis (Hartz et al., 2004). For P-glycoprotein, specific transport activity is measured as PSC833-sensitive NBD-CSA accumulation in capillary lumens at steady state (60 min; Wang et al., 2010). Figure 1B shows representative confocal images of rat brain capillaries incubated to steady state in medium with 2 μM NBD-CSA without (control) and with 5 μM PSC833. Note the accumulation of fluorescence in the lumen of the control capillary and the substantial reduction (60–70%) in luminal fluorescence in the capillary exposed to 5 μM PSC833 (Fig. 1B). This concentration of inhibitor maximally reduces luminal NBD-CSA accumulation. Previous studies showed that luminal fluorescence remaining after PSC833 inhibition of NBD-CSA transport represents nonspecific accumulation, likely from diffusive entry and binding to cellular elements (Hartz et al., 2004). Recent experiments show that luminal NBD-CSA accumulation in brain and spinal cord capillaries from P-glycoprotein-null mice was the same as in capillaries from wild-type mice that were exposed to 5 μM PSC833 (Campos et al., 2012). For the present experiments using NBD-CSA as a P-glycoprotein substrate, results are expressed as PSC833-sensitive luminal fluorescence, which we take as a measure of specific P-glycoprotein transport activity. Figure 1B also shows increased NBD-CSA luminal fluorescence in brain capillaries isolated from rats that were dosed with 10 mg/kg SFN. Quantification of specific luminal fluorescence showed that P-glycoprotein transport activity was 150% higher than in brain capillaries from SFN-dosed rats than in capillaries from control rats (Fig. 1C).

We used *in situ* brain perfusion to measure the accumulation of [³H]verapamil in brains of control and SFN-dosed rats (1–10 mg/kg SFN for 2 d). We used altered [³H]verapamil accumulation in brain as a sensitive measure of changes in P-glycoprotein activity at the blood–brain barrier, with uptake decreasing with

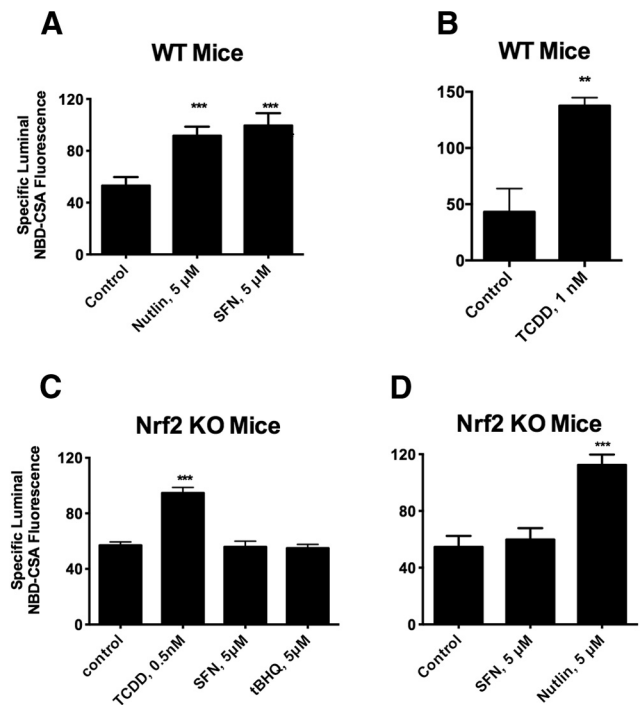


Figure 4. Effects of Nrf2 (SFN) and AhR (TCDD) ligands and p53 activation (nutlin-3) on P-glycoprotein transport activity in brain capillaries from wild-type (A, B) and Nrf2-null (C, D) mice. Shown are mean ± SEM for 8–12 capillaries from single preparations (pooled brains from 10 mice). ** $p < 0.01$, *** $p < 0.001$, significantly higher than control.

increased transporter activity and increasing with decreased activity (Wang et al., 2011; Cannon et al., 2012). As shown in Figure 1D, SFN dosing reduced brain [³H]verapamil accumulation in a dose-dependent manner. With 5 or 10 mg/kg SFN, accumulation decreased significantly, with 10 mg/kg reducing accumulation by ~50%. SFN had no effect on brain accumulation of [¹⁴C]sucrose, a sensitive measure of tight junction permeability (Fig. 1E). Thus, dosing rats with SFN increased blood–brain barrier expression of P-glycoprotein, Mrp2, and Bcrp, increased P-glycoprotein transport activity in isolated brain capillaries and reduced brain accumulation of a P-glycoprotein substrate.

SFN activation upregulates ABC transporters *in vitro*

To define the mechanism by which SFN alters activity and expression of ABC transporters at the blood–brain barrier, we examined SFN effects in isolated rat and mouse brain capillaries. We first exposed rat brain capillaries to 5 μM SFN for 1–4 h and then incubated them to steady state (1 additional hour) in medium with 2 μM NBD-CSA plus SFN. Figure 2A shows that SFN exposure increased specific luminal accumulation of NBD-CSA over 1–4 h. After 2–3 h of SFN exposure, P-glycoprotein transport activity had approximately tripled. For the remainder of the experiments presented here, capillaries were exposed for 3 h to ligands for Nrf2 and other ligand-activated transcription factors before fluorescent substrate was added to the medium for the 60 min transport assay (total exposure time, 4 h).

Exposing rat brain capillaries to SFN increased P-glycoprotein transport activity in a concentration-dependent manner, with concentrations as low as 0.01 μM SFN causing a significant increase and 1–5 μM SFN causing a maximal increase (Fig. 2B). With 1 or 5 μM SFN, transport activity was approximately three times that seen in control capillaries. Exposure to another Nrf2 ligand, tBHQ, increased transport activity to the same extent

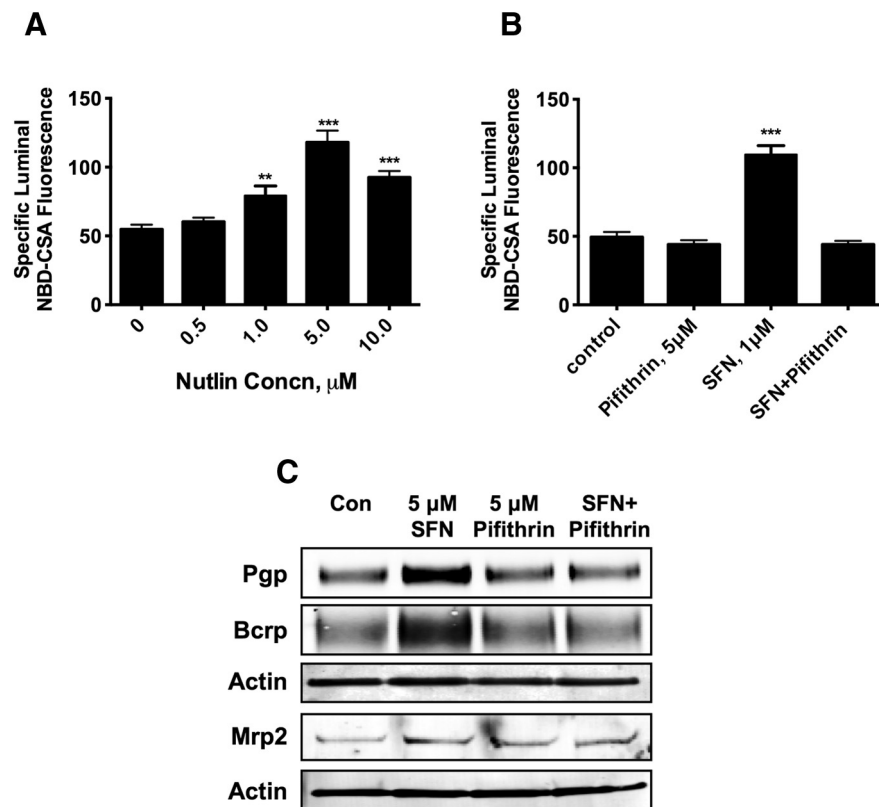


Figure 5. Involvement of p53 in SFN induction of ABC transporter expression and activity in rat brain capillaries. **A**, Nutlin-3, a p53 activator, increases P-glycoprotein activity in a concentration-dependent manner. **B**, Pifithrin, a p53 inhibitor, blocks the SFN-induced increase in P-glycoprotein activity. Shown are mean \pm SEM for 8–12 capillaries from single preparations (pooled brains from 10 rats). ** $p < 0.01$, *** $p < 0.001$, significantly higher than control. **C**, Western blots showing increased P-glycoprotein (Pgp), Bcrp, and Mrp2 expression in membranes from SFN-exposed capillaries. The increase in transporter expression is abolished when p53 is inhibited by pifithrin; pooled capillary membranes from 10 rats. Con, Control.

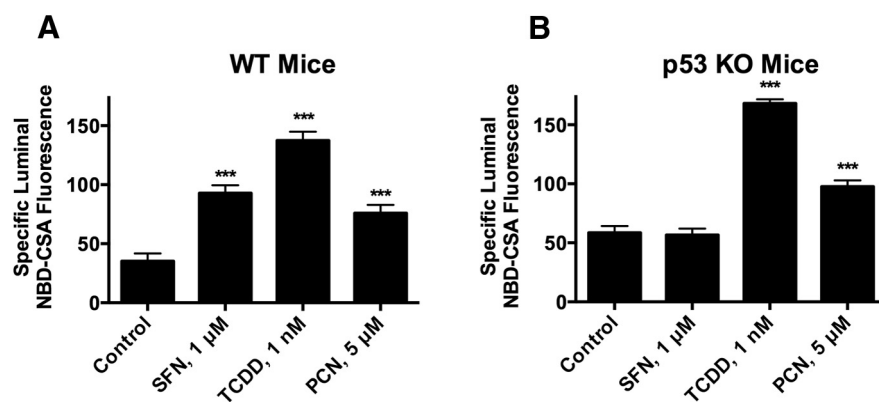


Figure 6. Effects of Nrf2 (SFN), AhR (TCDD), and PXR (PCN) ligands on P-glycoprotein transport activity in brain capillaries from wild-type (**A**) and p53-null (**B**) mice. Shown are mean \pm SEM for 8–12 capillaries from single preparations (pooled brains from 10 mice). *** $p < 0.001$, significantly higher than control.

as SFN (Fig. 2C). We also measured the effects of SFN exposure on the transport activity of two other ABC transporters, Mrp2 and Bcrp. Again, we used a confocal microscopy-based assay to measure accumulation of fluorescent substrates (TR for Mrp2 and BODIPY-prazosin for Bcrp) within rat brain capillary lumens; specific inhibitors used were MK571 for Mrp2 and Ko143 for Bcrp (Hartz et al., 2004; Bauer et al., 2008b; Wang et al., 2010). Figure 2D shows that 5 μM SFN significantly increased Mrp2 and Bcrp transport activity in brain capillaries.

The increase in P-glycoprotein transport activity caused by SFN exposure in brain capillaries was abolished by actinomycin D and cyclohexamide, indicating dependence on transcription and translation (Fig. 3A). We showed previously that neither actinomycin D nor cyclohexamide affected P-glycoprotein transport in brain capillaries (Wang et al., 2010). Consistent with translation of new transporter protein, Western blots of capillaries exposed to 0.1–10 μM SFN showed concentration-dependent increases in P-glycoprotein expression (Figs. 3B,C; expression data for Mrp2 and Bcrp in SFN-exposed capillaries are shown below).

No specific antagonist has been identified for Nrf2. To confirm the role of Nrf2 in the modulation of P-glycoprotein transport and expression, we isolated brain capillaries from wild-type (C57BL/6) and Nrf2 knock-out (C57BL/6 background) mice and exposed them to Nrf2 activators. In the capillaries from wild-type mice, 5 μM SFN significantly increased P-glycoprotein transport activity (Fig. 4A). In capillaries from Nrf2-null mice, neither SFN nor tBHQ increased transport activity (Fig. 4B). In capillaries from wild-type and Nrf2-null mice, P-glycoprotein transport activity was clearly inducible, because TCDD, an aryl hydrocarbon receptor (AhR) ligand, and nutlin-3, an activator of tumor suppressor p53, significantly increased transport activity (Fig. 4B–D). The TCDD effect is in agreement with our previous reports, showing that exposing rat brain capillaries to this AhR ligand up-regulated P-glycoprotein transport (Wang et al., 2011). The nutlin-3 effect is novel and is investigated in detail below.

Nrf2 signals through p53 and p38 MAPK

p53 is a tumor suppressor that affects cell fate decisions, including cell cycle arrest, apoptosis, differentiation, and stress resistance (Chen et al., 2012; Rotblat et al., 2012). The relationship between p53 and oxidative stress is complex, with p53 acting as a redox-sensitive transcription factor that can generate antioxidant or pro-oxidant responses (Vurusaner et al., 2012). Our initial experiments with mouse brain capillaries

indicated that activation of p53 by nutlin-3 increased P-glycoprotein transport activity (Fig. 4A). Additional experiments with rat brain capillaries showed that exposure to 1–10 μM nutlin-3 for 3 h increased P-glycoprotein transport activity in a concentration-dependent manner (Fig. 5A). We used three strategies to determine whether p53 was involved in Nrf2 signaling to ABC transporters. First, we pretreated rat brain capillaries with 5 μM pifithrin, a reversible inhibitor of p53-dependent gene transcription, and then exposed them to 1 μM SFN. Pifithrin blocked the ability of SFN to

increase P-glycoprotein transport activity (Fig. 5B). Second, we pretreated rat brain capillaries with pifithrin, exposed them to 5 μ M SFN for 3 h, and analyzed ABC transporter expression in capillary membranes by Western blots. Figure 5C shows clearly that SFN increased expression of P-glycoprotein and Bcrp and possibly Mrp2; pifithrin blocked the increases in protein expression.

Third, we isolated brain capillaries from wild-type (C57BL/6) and p53-null mice and measured changes in P-glycoprotein transport activity after exposure to SFN, TCDD (AhR ligand), and pregnenolone 16 α -carbonitrile [PCN; pregnane X receptor (PXR) ligand]. We showed previously that ligand activation of AhR and PXR increases P-glycoprotein transport activity and protein expression in rodent brain capillaries (Bauer et al., 2004; Wang et al., 2011). Figure 6A shows that, in capillaries from wild-type mice, all three ligands increased P-glycoprotein transport activity. Although transport activity in capillaries from p53-null mice increased with exposure to TCDD and PCN, no such increase was seen with exposure to SFN (Fig. 6B). Thus, functional p53 is required for Nrf2 to upregulate P-glycoprotein, Mrp2, and Bcrp at the blood-brain barrier.

To further map the chain of events connecting Nrf2 activation to increased P-glycoprotein function, we tested whether specific inhibitors of protein kinase signaling blocked the ability of SFN to increase P-glycoprotein transport activity in rat brain capillaries. Pretreating capillaries with inhibitors of MEK [U0126 (1,4-diamino-2,3-dicyano-1,4-bis(*o*-aminophenylmercapto)butadiene)] or phosphatidylinositol 3-kinase/Akt (wortmannin), did not affect SFN action (data not shown). In contrast, pretreatment with a specific p38 MAPK inhibitor, SB203580 [4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)-1*H*-imidazole], abolished the effect of SFN on P-glycoprotein transport (Fig. 7A). Preliminary experiments with another p38 MAPK inhibitor confirm this finding (data not shown). These findings are consistent with a previous report showing that Nrf2 signals through p38 MAPK to increase HO-1 expression (Paine et al., 2010). To determine whether p38 MAPK signaling was downstream of p53 in the Nrf2 pathway, we exposed rat brain capillaries to both nutlin-3 and SB203580. As shown in Figure 7B, SB203580 abolished the effect of nutlin-3 on transport, indicating that p38 MAPK was downstream of p53.

Signaling through NF- κ B

NF- κ B is a transcription factor that was implicated in responses to a number of cellular stressors, including inflammatory, genotoxic, and oxidative stress. In rat brain capillaries, inhibition of NF- κ B abolishes the increases in P-glycoprotein activity and protein expression caused by exposure to TNF- α and excess glutamate (Bauer et al., 2004, 2008a). Here, we used *N*-[2-(4-phenoxyphenyl)ethyl]-4,6-quinazolidinediamine (QNZ), an NF- κ B inhibitor that blocks activation, and SN50, a cell-permeable peptide

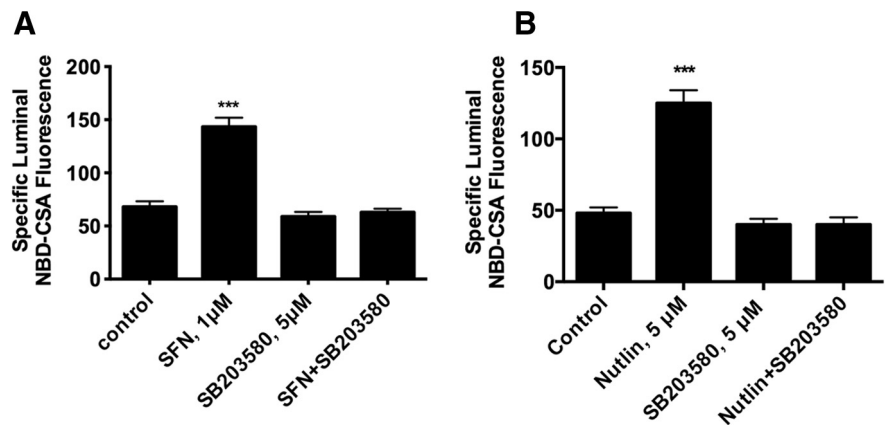


Figure 7. Effects of a p38 MAPK inhibitor (SB203580) on the increase in P-glycoprotein transport activity caused by exposure to SFN (A) and nutlin-3 (B). Shown are mean \pm SEM for 10–12 capillaries from single preparations (pooled brains from 6 rats). *** p < 0.001, significantly higher than control.

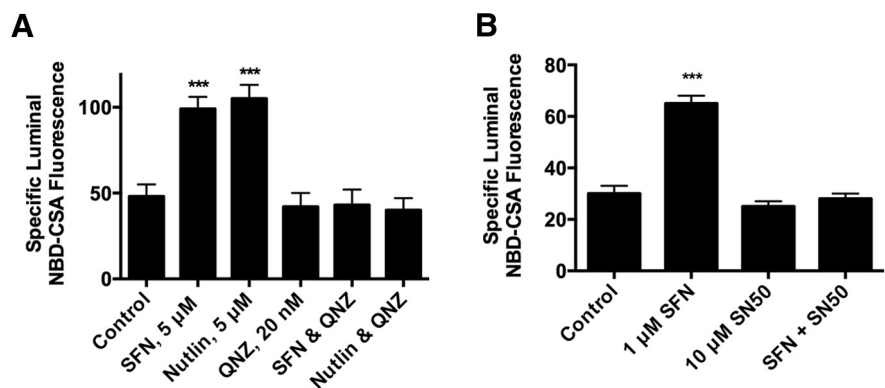


Figure 8. A, An NF- κ B inhibitor (QNZ) blocks the increases in P-glycoprotein transport activity caused by exposure to SFN and nutlin-3. B, A second NF- κ B inhibitor (SN50) blocks the increases in P-glycoprotein transport activity caused by exposure to SFN. Shown are mean \pm SEM for 8–12 capillaries from single preparations (pooled brains from 6 rats). *** p < 0.001, significantly higher than control.

inhibitor that blocks nuclear translocation, to determine whether NF- κ B was involved in Nrf2- and p53-driven increases in P-glycoprotein activity in rat brain capillaries. As shown in Figure 8, A and B, QNZ abolished the effects of SFN and nutlin-3 on transport activity; SN50 abolished the effects of SFN on transport activity.

EMSA provides a means of determining whether specific transcription factors move into the nucleus and are capable of binding to added DNA probes encoding consensus promoter binding sites for the proteins. We exposed rat brain capillaries to 5 μ M SFN, isolated rat brain capillary nuclear proteins, and measured binding of Nrf2, p53, and NF- κ B to cognate fluorescent DNA probes. In each case, SFN exposure of capillaries increased probe mass in gels, indicative of transcription factor binding to the probe (Figure 9A). Thus, in brain capillaries, SFN exposure results in nuclear translocation of Nrf2, p53, and NF- κ B.

We immunostained isolated rat brain capillaries to confirm SFN-induced, nuclear translocation of the NF- κ B component p65, a measure of NF- κ B activation. Figure 9B shows representative transmitted light and confocal fluorescence images of capillaries immunostained for p65. The white arrows indicate nuclei for which fluorescence intensity profiles were measured. In this experiment, capillaries were fixed after 30 min exposure to 5 μ M SFN without and with inhibitors (20 nM QNZ or 10 μ M SN50). Preliminary experiments showed that nuclear fluorescence intensity increased maximally at that time. Note first that individual pixel fluorescence intensities across the representative nucleus from the SFN-exposed

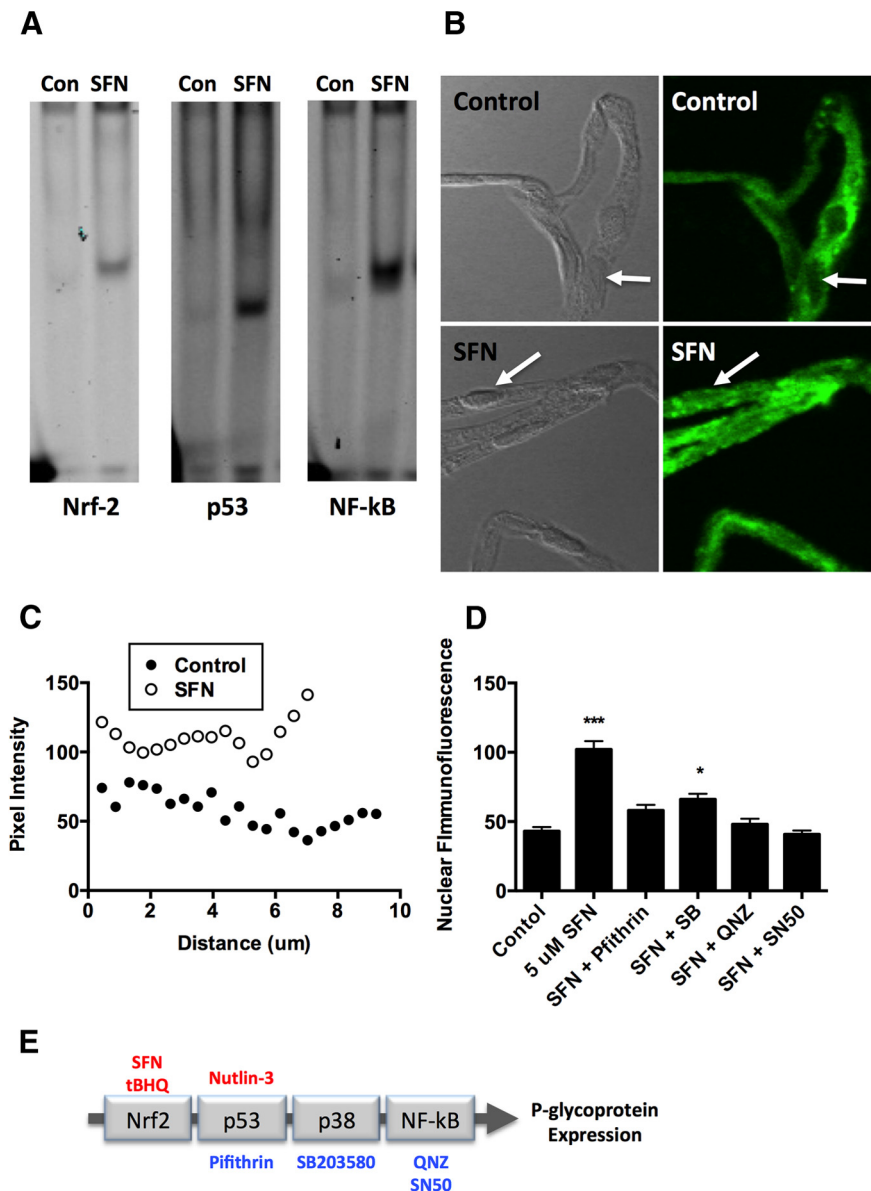


Figure 9. *A*, EMSAs show nuclear translocation of Nrf2, p53, and NF- κ B in response to exposure of rat brain capillaries to 5 μ M SFN for 4 h. Con, Control. *B*, Immunostaining for p65 shows nuclear translocation of NF- κ B after 30 min exposure to 5 μ M SFN. Arrows indicate nuclei selected for pixel intensity profile analysis. *C*, Pixel intensity profiles for immunostained (p65) nuclei in control and SFN-exposed capillaries. There is no overlap in the intensity profiles. *D*, Mean nuclear pixel intensities in immunostained (p65) capillaries. SFN exposure more than doubles pixel intensity and inhibitors of p53, p38, and NF- κ B activation and translocation essentially block this increase. Shown are mean \pm SEM for 40–60 nuclei (15–25 capillaries) from two capillary preparations. * $p < 0.05$, *** $p < 0.001$, significantly higher than control. *E*, The sequence of events through which Nrf2 ligands increase P-glycoprotein expression. Activators are shown in red, and inhibitors are in blue.

capillary were higher than in the nucleus from the control capillary (Fig. 9C). Measurements of average nuclear fluorescence intensities showed at least a doubling after SFN exposure (Fig. 9D). Importantly, both NF- κ B inhibitors, QNZ and SN50, blocked this increase, as did inhibitors of p53 (pifithrin) and p38 (SB203580; Fig. 9D).

Together, our findings for isolated brain capillaries show that Nrf2 requires p53, p38, and NF- κ B to upregulate ABC transporter expression at the blood–brain barrier. They also indicate that NF- κ B is essential for upregulation of transport activity when p53 is activated independently of Nrf2. These results define a sequence of events leading to increased transporter expression in rodent brain capillaries exposed to SFN (Fig. 9E).

Nrf2 signaling in spinal cord capillaries

We demonstrated recently that ABC transporter function and regulation could be studied in spinal cord capillaries isolated from rats and mice using the same procedures as with brain capillaries (Campos et al., 2012). As in brain capillaries, we used PSC833-dependent steady-state luminal accumulation of NBD-CSA as a measure of specific P-glycoprotein transport activity in spinal cord capillaries. Figure 10A shows that exposing spinal cord capillaries from wild-type mice to 5 μ M SFN, 1 nM TCDD, or 5 μ M PCN for 3 h nearly doubled transport activity. The TCDD and PXR results confirm previous findings in rat spinal cord capillaries (Campos et al., 2012). The SFN result indicates that, as in brain capillaries (above), activation of Nrf2 increased P-glycoprotein transport activity. In spinal cord capillaries from p53-null mice, exposure to TCDD and PCN increased transport activity, but SFN had no effect (Fig. 10B). Thus, as with brain capillaries from rats and mice (see above), SFN increased P-glycoprotein transport activity in mouse spinal cord capillaries, and those increases were p53 dependent.

Discussion

Together, Nrf2 and its cytoplasmic partner Keap1 function as a redox/electrophile sensor, driving a concerted cellular response to oxidative and electrophilic stress. Activation of Nrf2 is protective in a number of animal models of tissue injury, including ozone and arsenic toxicity in lung, acetaminophen hepatotoxicity, and diabetic nephropathy (Aleksunes and Manautou, 2007; Cho and Kleeberger, 2007; Li et al., 2012). In the CNS, preconditioning with Nrf2 ligands is neuroprotective in animal models of cerebral ischemia, TBI, subarachnoid hemorrhage, and SCI (Yan et al., 2008; Dash et al., 2009; Alfieri et al., 2011; Chen et al., 2011; Mao et al., 2012; X. Wang et al., 2012; Wang et al., 2013). Recent phase 3 clinical trials show that dimethyl fumarate, an Nrf2 ligand (Linker et al., 2011), reduced relapse rates and improved neuroradiologic outcomes in patients with relapsing–remitting multiple sclerosis (Fox et al., 2012; Gold et al., 2012). Given the potential for Nrf2 activation during treatment of CNS disease, it is important to understand the full range of consequences.

Limited information for peripheral tissues indicates that upregulation of xenobiotic efflux transporter expression is part of the Nrf2 response, e.g., in liver, multiple Mrps are Nrf2 targets (Klaassen and Slitt, 2005; Maher et al., 2005; Aleksunes and Klaassen, 2012). In the present study, we show that activation of Nrf2 at the rat and mouse

blood–brain barriers increased protein expression of P-glycoprotein, Bcrp, and Mrp2, three ABC transporters that function as xenobiotic efflux pumps. Increased transporter expression and transport activity occurred after *in vivo* or *in vitro* exposure to the Nrf2 ligand SFN. In rats, SFN dosing (5–10 mg/kg) decreased by 40–50% brain accumulation of verapamil, a drug that is a P-glycoprotein substrate. This is approximately the same magnitude decrease in brain verapamil accumulation seen previously when P-glycoprotein expression increased two to three times through the action of the xenobiotic-activated nuclear receptors constitutive androstane receptor and AhR (Wang et al., 2010; Wang et al., 2011). Experiments with isolated brain capillaries established that SFN acted through Nrf2 (lack of SFN effect in capillaries from Nrf2-null mice) and that the increase in P-glycoprotein transport activity was abolished when transcription or translation was inhibited. We found similar increases in P-glycoprotein transport activity in mouse spinal cord capillaries exposed to SFN, indicating that, in both CNS barrier tissues, P-glycoprotein is Nrf2 responsive.

More than 2000 genes are Nrf2 targets, although the pattern of Nrf2 effects is tissue specific (Copple et al., 2010; Copple, 2012). Certainly, the full extent of the response to Nrf2 activation at the blood–brain and blood–spinal cord barriers has been only partially defined. We show here increased expression of three ABC transporters and HO-1 in brain capillaries from SFN-exposed rats. Previously, J. Zhao et al. (2006) found increased expression of the Nrf2 targets HO-1, GST α 3, and NAD(P)H quinone oxidoreductase in brain capillaries from SFN-exposed mice. Additional consequences of Nrf2 activation at these CNS barriers remain to be explored.

Presumably, increased efflux transport activity at CNS barriers is needed to remove products of ROS detoxification, e.g., GSH conjugates, formed as a result of increased enzyme activity. Indeed, previous work in cell models of the blood–brain barrier, in isolated brain capillaries, and *in vivo* showed that both exposure to hydrogen peroxide and depletion of glutathione increases P-glycoprotein expression (Felix and Barrand, 2002; Nwaozuzu et al., 2003). Given the present results, it is likely that Nrf2 activation was involved.

P-glycoprotein and Bcrp are recognized as major impediments to CNS pharmacotherapy. Because they are critical gatekeepers for many CNS-acting drugs, they can substantially limit the ability of drugs to access sites of action in the CNS. For both transporters, increased expression at the blood–brain barrier further restricts drug uptake into the brain (Miller, 2010). Recent studies indicate that, for some classes of drugs, e.g., several tyrosine kinase inhibitors used as chemotherapeutics, these two transporters work together (Agarwal et al., 2011). Both transporters are expressed at the blood–spinal cord barrier (Campos et al., 2012); the extent to which they work together in that tissue is not clear. In view of the present findings for both CNS barriers, we suggest caution when intentionally activating Nrf2 for neuroprotection, because increased drug efflux transporter expression and possibly drug metabolizing enzyme activity would impair subsequent CNS pharmacotherapy.

The present study also discloses a novel sequence of events that drives Nrf2-induced increases in expression of P-glycoprotein and Bcrp (and possibly Mrp2) in rat and mouse brain and spinal cord capillaries (Fig. 8B). Inhibition of p53, p38 MAPK, or NF- κ B abolished the SFN-induced increases in ABC transporter activity/expres-

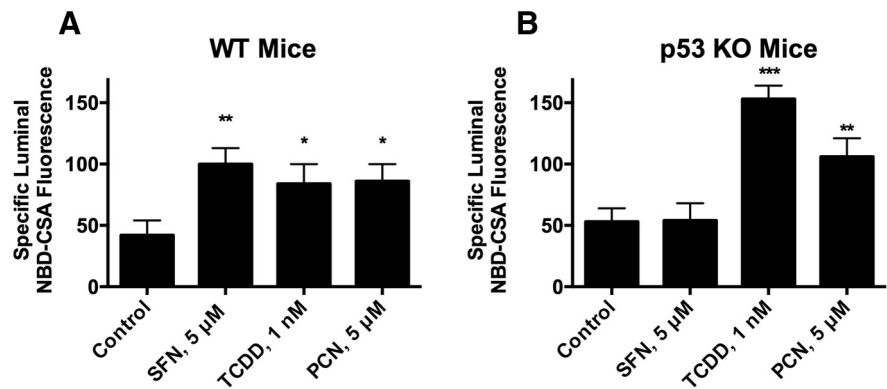


Figure 10. Effects of Nrf2 (SFN), AhR (TCDD), and PXR (PCN) ligands on P-glycoprotein transport activity in spinal cord capillaries from wild-type (A) and p53-null (B) mice. Shown are mean \pm SEM for 10–12 capillaries from single preparations (pooled brains from 10 mice). * p < 0.05, ** p < 0.01, *** p < 0.001, significantly higher than control.

sion. Moreover, in isolated rat brain capillaries, the p53 inhibitor pifithrin blocked SFN-induced increases in transporter activity and protein expression. Conversely, the p53 activator nutlin-3 increased transport activity of P-glycoprotein. Consistent with those results, SFN exposure did not increase P-glycoprotein transport activity in brain and spinal cord capillaries from p53-null mice. In addition, inhibition of either p38 MAPK or NF- κ B blocked the effects of SFN and nutlin-3 on transporter activity. Thus, in brain barrier tissues, activation of p53, p38, and NF- κ B was required for Nrf2 to signal increased transporter expression and activity.

In response to oxidative/electrophilic stress, Nrf2 can both signal and function as a transcription factor (Copple et al., 2010; Copple, 2012). Both p38 MAPK and NF- κ B are known downstream targets of Nrf2 signaling (Wakabayashi et al., 2010; Schneider and Krämer, 2011; Copple, 2012). The novel combination disclosed here involves Nrf2 and p53. In cell models, depending on the expression level, p53 activation can be pro-oxidant or antioxidant (Vurusaner et al., 2012). However, there are no reports showing p53 as a target of Nrf2 signaling. In fact, several studies show Nrf2 and p53 to be mutually antagonistic. Clearly, this is not the case for regulation of ABC transporter expression at the blood–brain and blood–spinal cord barriers.

Although our experiments show that Nrf2, p53, p38, and NF- κ B work in concert to change transporter expression, it is not clear how this happens. On the one hand, Nrf2, p53, and p38 MAPK could be the backbone of a cytoplasmic signaling pathway that leads to NF- κ B translocation and increased transporter expression. On the other hand, Nrf2, p53, and NF- κ B could be required to be present in the nucleus before transporter expression increases. The present results provide some guidance in this matter. In rat brain capillaries exposed to SFN, EMSA shows that Nrf2, p53, and NF- κ B all translocate to the nucleus; immunostaining confirms this for NF- κ B and demonstrates the dependence of nuclear translocation on p53 and p38. However, in capillaries from Nrf2-null mice, the p53 activator nutlin-3 still increased P-glycoprotein transport activity, and inhibition of either p38 or NF- κ B abolished the p53-induced increases in activity. Thus, although SFN exposure leads to nuclear translocation of Nrf2, p53, and NF- κ B, p53 does not require nuclear Nrf2 to increase P-glycoprotein expression, implying that Nrf2 signals to p53, which in turn activates p38. At present, it is not clear how Nrf2 activates p53 nor whether p53 is needed in the nucleus to support the action of NF- κ B.

In summary, we show here that activating the oxidative/electrophilic stress sensor Nrf2 at the blood–brain and blood–spinal cord barrier increases protein expression and transport activity of three

ABC transporters that can function as xenobiotic efflux pumps. In both barrier tissues, Nrf2 signals indirectly through p53, p38 MAPK, and NF- κ B to increase transporter expression. Given the large number of therapeutic drugs handled by these efflux transporters, one would expect reduced CNS access and thus reduced drug efficacy when brain and spinal cord endothelial cells undergo oxidative stress or when Nrf2 is activated for neuroprotection.

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