Brain mitochondria from rats treated with sulforaphane are resistant to redox-regulated permeability transition

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Abstract Oxidative stress promotes Ca^{2+}-dependent opening of the mitochondrial inner membrane permeability transition pore (PTP), causing bioenergetic failure and subsequent cell death in many paradigms, including those related to acute brain injury. One approach to preconditioning against oxidative stress is pharmacologic activation of the Nrf2/ARE pathway of antioxidant gene expression by agents such as sulforaphane (SFP). This study tested the hypothesis that administration of SFP to normal rats increases resistance of isolated brain mitochondria to redox-sensitive PTP opening. SFP or DMSO vehicle was administered intraperitoneally to adult male rats at 10 mg/kg 40 h prior to isolation of non-synaptic brain mitochondria. Mitochondria were suspended in medium containing a respiratory substrate and were exposed to an addition of Ca^{2+} below the threshold for PTP opening. Subsequent addition of tert-butyl hydroperoxide (tBOOH) resulted in a cyclosporin A-inhibitable release of accumulated Ca^{2+} into the medium, as monitored by an increase in fluorescence of Calcium Green 5N within the medium, and was preceded by a decrease in the autofluorescence of mitochondrial NAD(P)H. SFP treatment significantly reduced the rate of tBOOH-induced Ca^{2+} release but did not affect NAD(P)H oxidation or inhibit PTP opening induced by the addition of phenylarsine oxide, a direct sulfhydryl oxidizing agent. SFP treatment had no effect on respiration by brain mitochondria and had no effect on PTP opening or respiration when added directly to isolated mitochondria. We conclude that SFP confers resistance of brain mitochondria to redox-regulated PTP opening, which could contribute to neuroprotection observed with SFP.

Keywords Nrf2 · Calcium · Peroxide · Pyridine nucleotide · Oxidation/reduction

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Introduction

Mitochondrial sensitivity to oxidative stress is strongly implicated in the pathophysiology of many diseases and disorders, including those affecting the central nervous system (Fiskum et al. 1999; Starkov et al. 2004; Niizuma et al. 2009; Navarro and Boveris 2009). Mitochondrial targets of oxidative stress include metabolic enzymes, proteins involved in electron transport and oxidative phosphorylation, DNA and RNA, membrane lipids, and a Ca^{2+}-activated, non-selective pore in the inner membrane known as the permeability transition pore (PTP). Opening of the normally quiescent PTP results in transmembrane equilibration of small ions and molecules of up to approximately 1,500 Da (Halestrap et al. 2002; Rasola and Bernardi 2007; Lemasters et al. 2009), therefore causing membrane depolarization and uncoupling of oxidative phosphorylation. PTP opening also results in release of mitochondrial metabolites, including pyridine nucleotides and glutathione, which are necessary for energy metabolism and defense against oxidative stress. Cyclophilin D is at this juncture the only mitochondrial protein unequivocally associated with the PTP and appears responsible for mediating both Ca^{2+}-

induced pore opening and inhibition of pore opening by the cyclophilin drug, cyclosporin A. Cyclophilin D knock-out mice are relatively resistant to ischemia/reperfusion injury to both the heart and brain (Baines et al. 2005;Schinzel et al. 2005), illustrating the importance of PTP opening in pathophysiology.

While abnormally high intramitochondrial Ca^{2+} is the primary stimulus for PTP opening, oxidative stress greatly increases the sensitivity of PTP opening to Ca^{2+} (Akao et al. 2003). Oxidative stress promotes PTP opening either by direct oxidation of mitochondrial proteins and possibly lipids by reactive O_2 and nitrogen species, e.g., superoxide and nitric oxide, or by causing an oxidized shift in the mitochondrial redox state, such as occurs during metabolism of peroxides by the glutathione peroxidase/reductase system (Petrosillo et al. 2009; Navet et al. 2006; Petronilli et al. 2009; Kowaltowski et al. 2000). This oxidized shift in redox state can be monitored through measurements of NAD(P)H autofluorescence (Duchen 1992) and indirectly results in PTP opening through impaired reduction of oxidized macromolecules (Catisti and Vercesi 1999).

The levels of gene products that are responsible for both detoxification of reactive O_2 species and for the reducing power that drives their detoxification are controlled through transcriptional regulation employing antioxidant response elements (ARE) that interact with transcriptional activating factors such as Nrf2 (Thimmulappa et al. 2002). One mechanism by which the Nrf2/ARE pathway of antioxidant- and other cytoprotective-gene expression is activated is oxidation of critical cysteine sulfhydryl groups located on KEAP1, a cytoplasmic Nrf2 binding protein. Upon oxidation of KEAP1, Nrf2 is released and translocates to the nucleus where it binds to AREs (Jaiswal 2004).

Sulforaphane (SFP), an isothiocyanate derived from a glucosinolate found in cruciferous vegetables, e.g., broccoli, forms mixed disulfide bonds with KEAP1, and is a well-studied pharmacologic activator of Nrf2-mediated gene expression (Zhang et al. 1992; Kensler et al. 2000). SFP demonstrates neuroprotection in several rat models of acute brain injury, e.g., stroke (Zhao et al. 2006) and head trauma (Zhao et al. 2005), in which evidence for mitochondrial PTP involvement exists (Okonkwo and Povlishock 1999; Kristian and Siesjo 1998). Although effects of SFP on mitochondrial PTP have not been reported, rats fed a broccoli-enriched diet exhibit significant increases in aortic smooth muscle mitochondrial proteins that could influence PTP opening, including thioredoxin, thioredoxin reductase, glutathione reductase, glutathione, and superoxide disumutase and catalase enzyme activities (Mukherjee et al. 2008). This study tested the hypothesis that PTP opening by isolated brain mitochondria is inhibited by treatment of rats with sulforaphane.

Materials and methods

Chemicals and reagents

R,S-Sulforaphane was purchased from LKT Laboratories, Inc (St. Paul, MN). Mannitol, sucrose, EGTA, HEPES, Tris, potassium phosphate dibasic (K_2HPO_4), magnesium chloride ($MgCl_2$), malate, glutamate, oligomycin, carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP), adenosine 5′-diphosphate (ADP), bovine serum albumin (BSA), percoll, succinate, rotenone, calcium, dimethylsulfoxide (DMSO) and *tert*-butyl hydrogen peroxide were obtained from Sigma–Aldrich (St. Louis, MO, USA). Ultra-pure potassium chloride was obtained from EM Sciences (Fort Washington, PA, USA).

Treatment of rats with sulforaphane

All experimental procedures were approved by the Institutional Animal Care and Use Committee and conducted in accordance with the recommendations provided in the Guide for the Care and Use of Laboratory Animals. Male 300 g Fischer 344 rats (Charles River) were injected intraperitoneally with sulforaphane at 10 mg/kg in 200 µL solution containing 40% DMSO and 60% isotonic saline. Animals received DMSO plus saline as the vehicle control.

Isolation of non-synaptic brain mitochondria

Rats were euthanized by decapitation and their forebrains rapidly removed, chopped and homogenized in ice-cold isolation buffer (225 mM mannitol, 25 mM sucrose, 10 mM Hepes, 1 mM EGTA, pH 7.4, at 4°C). The homogenate was centrifuged at $1,330 g$ for 3 min. The pellets were re-suspended and re-centrifuged at 1,330 g for 3 min. The combined supernatants were centrifuged at $21,200 g$ for 10 min. The pellets were re-suspended in 15% Percoll and layered on a 40%/23% Percoll gradient. The tubes containing the Percoll gradients were then centrifuged at $31,700 g$ for 10 min. The mitochondrial fraction located at the interface between the 40% and 23% layers was removed, diluted with isolation buffer and centrifuged at $16,700 g$ for 10 min. The purified mitochondrial pellet was re-suspended in isolation buffer and de-fatted bovine serum albumin was added to the suspension at 10 mg/mL. After a final centrifugation at 9,000 g for 10 min, the mitochondria were re-suspended in approximately 20 µL of isolation medium not containing EGTA or BSA. Mitochondrial protein concentrations were measured using a Lowry DC kit (Bio-Rad, Hercules, CA, USA) with bovine serum albumin used as concentration standards.

Mitochondrial oxygen consumption

The respiratory activities of isolated brain mitochondria were measured polarimetrically with a Clark-type oxygen electrode apparatus (Hansatech Instruments, Norfolk, England). Mitochondria were suspended at a protein concentration of 0.5 mg/ml in buffer containing 125 mM KCl, 20 mM HEPES, 2 mM K_2HPO_4, 0.01 mM EGTA, 1 mM $MgCl_2$ (pH 7.0) at 37 °C, plus glutamate (5 mM) and malate (0.1 mM) or succinate (5 mM) plus the electron transport chain complex 1 inhibitor, rotenone (4 μM). Addition of ADP (0.5 mM) was used to initiate State 3 (phosphorylating) respiration. Oligomycin (2.5 μg/ml), an inhibitor of the mitochondrial ATP synthase, was used to induce State 4 (resting) respiration. The use of oligomycin to obtain the rate of respiration limited by the inner membrane proton permeability is necessary when magnesium is present as even small contamination of isolated mitochondria with Mg-dependent ATPases, e.g., the plasmalemmal sodium pump, can result in turnover of mitochondrially-generated ATP, resulting in an artificially high rate of State 4 respiration in the absence of oligomycin. Maximal respiration was initiated with the addition of the protonophore uncoupler, FCCP (54 nM). Rates of oxygen consumption are expressed as nmol O_2/mg mitochondrial protein/min). The respiratory control ratio (RCR) is defined as the rate of ADP-stimulated oxygen consumption (State 3) divided by the rate of respiration determined in the presence of oligomycin (State 4).

Measurements of mitochondrial Ca^{2+}-uptake and release and pyridine nucleotide redox state

Mitochondria were suspended in a cuvette at a protein concentration of 0.5 mg/ml in 2 ml of 125 mM KCl, 2 mM K_2HPO_4, 1 mM $MgCl_2$, 20 mM Tris-HCl, 5 mM succinate, and 4 μM rotenone, pH 7.4 at 30°C. The use of the electron transport chain complex II substrate succinate in the presence of the complex I inhibitor rotenone allows for the mitochondrial pyridine nucleotide redox state (NAD(P) H/NAD(P)$^+$) to be varied between totally reduced (no added pro-oxidant) to oxidized (after addition of pro-oxidants), independent of mitochondrial respiration and membrane potential. The medium free Ca^{2+} was measured fluorimetrically in the presence of the Ca^{2+}-sensitive fluorescent dye Calcium Green 5N (0.1 μM), using excitation and emission wavelengths of 506 nm and 532 nm. Autofluorescence of reduced mitochondrial pyridine nucleotides (NADH plus NADPH) was measured simultaneously using excitation and emission wavelengths of 350 nm and 460 nm. Fluorescence measurements were performed on a Hitachi 2500 spectrofluorimeter, equipped with magnetic stirring and cuvette temperature control. Fluorescent wavelength settings cycled every 2 s for medium Ca^{2+} and NAD(P)H measurements.

Mitochondria were added to the cuvette and allowed to equilibrate, bioenergetically, for 2 min, as reflected by steady-state Calcium Green 5N and NAD(P)H fluorescence. $CaCl_2$ was then added to the medium at a level that the mitochondria could completely accumulate with only a very slow rate of subsequent spontaneous release. At 200 s after the addition of Ca^{2+}, either tert-butyl hydroperoxide or phenylarsine oxide were added as pro-oxidant inducers of PTP opening, as reflected by the release of accumulated Ca^{2+}. In some experiments, the PTP inhibitor, cyclosporin A (1 μM), was present in the medium in which mitochondria were suspended. PTP opening was quantified by measuring the rate of increase in Calcium Green 5N fluorescence after the addition of pro-oxidants. The effect of these pro-oxidants on the pyridine nucleotide redox state was quantified by measuring the extent to which NAD(P)H autofluorescence was lost after their addition.

Immunoblot analysis of cyclophilin D

Isolated mitochondria were lysed in RIPA buffer containing a cocktail of protease and phosphatase inhibitors (Calbiochem). Equal amounts of mitochondrial protein (5 μg) from each sample were separated by SDS-PAGE (4–12% Bis-Tris gels) (Invitrogen) and transferred to PVDF membranes (Invitrogen), and then incubated with primary antibodies (overnight at 4 °C) with the mouse monoclonal antibodies anti-cyclophilin D antibody 1:500,000 *(Mitosciences, Cat. #MSA04)* anti-VDAC antibody 1:500,000 *(Mitosciences, Cat. #MSA03)*. The membranes were then washed with PBST and incubated for 1 h at room T in HRP-conjugated anti-mouse antibodies *(Millipore Cat. # 12-349)* at 1:2000 dilution for 1 h at room T. The washed blots were then treated with enhanced chemiluminiscence detection reagent *(Amersham Bioscience, UK)*. Densitometric analysis of the protein bands was performed using the Image J software.

Data analysis

Data are expressed as means ± S.E.M. of n different experiments. Differences between data obtained from mitochondria obtained from rats treated with sulforaphane or drug vehicle were assessed by students *t*-test. For data that are not normally distributed, the Mann-Whitney rank sum test was used. $P < 0.05$ was considered to be statistically significant.

Results

Inhibition of peroxide-induced brain mitochondrial PTP opening by treatment of rats with sulforaphane

Figure 1a provides the results from one typical experiment where pro-oxidant-induced PTP opening was evaluated by monitoring the increase in Calcium Green 5N fluorescence that occurs during release of accumulated mitochondrial Ca^{2+} into the medium. Pilot experiments were first performed to establish a level of added $CaCl_2$ that when added alone would not induce PTP opening but when followed by the pro-oxidant, tert-butyl hydroperoxide (tBOOH), would induce a measurable rise in Calcium Green 5N fluorescence. The immediate increase in fluorescence that occurred upon addition of 50 μM Ca^{2+} (100 nmol/mg protein) was followed by a rapid decrease, with mitochondrial Ca^{2+} uptake reaching completion within 20 s. In the absence of further additions, the fluorescence remained relatively constant, but increased slowly over the following 300 s (Fig. 1a, Line 1 and 2), using mitochondria isolated from either SFP- or drug vehicle-treated rats. Addition of tBOOH (250 μM) at 200 s after the addition of Ca^{2+} resulted in an immediate rise in Calcium Green 5N fluorescence, due to release of accumulated mitochondrial Ca^{2+}, which was slower for mitochondria from SFP-treated rats (Fig. 1a, Line 3 and 5). The presence of SFP in the mitochondrial suspensions at concentrations up to 100 μM had no effect on either Ca^{2+} uptake or release (not shown). The presence of the PTP inhibitor, cyclosporin A, virtually eliminated the tBOOH-induced Ca^{2+} release (Fig. 1a, Line 4). The Ca^{2+} release induced by tBOOH was incomplete, as indicated by the fact that the subsequent addition of the Ca^{2+} ionophore, ionomycin, caused a large additional increase in Calcium Green 5N fluorescence (Fig. 1a, Line 3).

Comparisons were made between the initial slope of the tBOOH-induced increase in Calcium Green 5N fluorescence for mitochondria isolated from 9 rats that had been injected intraperitoneally 40 h earlier with SFP and 7 rats that were injected 40 h earlier with the saline/DMSO vehicle. Figure 2a demonstrates that there was a significant, approximately 50% slower rate of tBOOH-induced Ca^{2+} release by mitochondria from the SFP-treated rats. There was no difference, however, between the background release-rate for mitochondria from the two animal groups (n=4/group).

Previous studies indicate that tBOOH induces opening of the PTP indirectly by oxidation of protein sulfhydryl groups due to glutathione oxidation during tBOOH metabolism by glutathione peroxidase and reductase. This shift in

Line:Treatment	Time (sec)		
	0	100	200
1:Vehicle	Ca^{2+}	---	---
2:SFP	Ca^{2+}	---	---
3:Vehicle	Ca^{2+}	tBOOH	IONO
4:Vehicle	Ca^{2+}/CsA	tBOOH	---
5:SFP	Ca^{2+}	tBOOH	---
6:Vehicle	Ca^{2+}	PhAsO	

Line:Treatment	Time (sec)	
	0	100
1:Vehicle	Ca^{2+}	---
2:SFP	Ca^{2+}	---
3:Vehicle	Ca^{2+}	tBOOH
4:SFP	Ca^{2+}	tBOOH
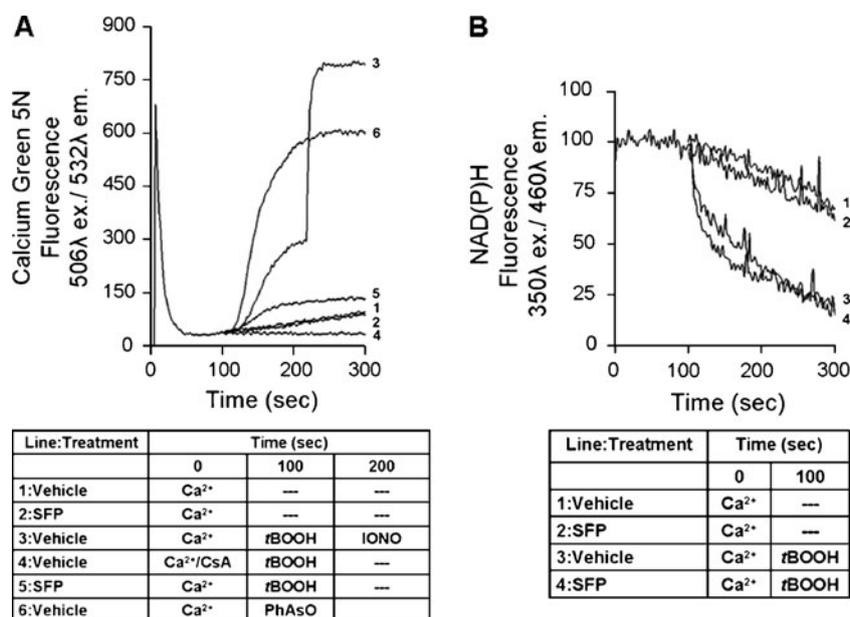
Fig. 1 Inhibition of peroxide-induced permeability transition pore (PTP) opening by systemic sulforaphane administration. **a** Mitochondrial Ca^{2+} uptake and release was measured by monitoring the fluorescence of Calcium Green 5N in the medium. At time zero, 50 μM $CaCl_2$ was added to a suspension of non-synaptic brain mitochondria (0.5 mg/ml) in medium containing succinate as the respiratory substrate in the presence of rotenone, an inhibitor of complex I of the electron transport chain. Spontaneous release of accumulated Ca^{2+} was very slow and not affected by treatment of rats with SFP (Lines 1, 2). The subsequent addition of 250 μM tert-butyl hydroperoxide (tBOOH) (Lines 3, 5) or 30 μM phenylarsine oxide (PhAsO; Line 6) induced PTP opening and release of Ca^{2+}, which was inhibitable by the presence of 1 μM cyclosporin A (Line 4). Release of Ca^{2+} by tBOOH was incomplete compared to that observed after subsequent addition of 3 μM ionomycin (Line 3). **b** Mitochondrial pyridine nucleotide redox state was measured by monitoring the autofluorescence of NAD(P)H in the absence (Lines 1, 2) or presence of tBOOH (Lines 3, 4), with no apparent effects of SFP treatment

A

B
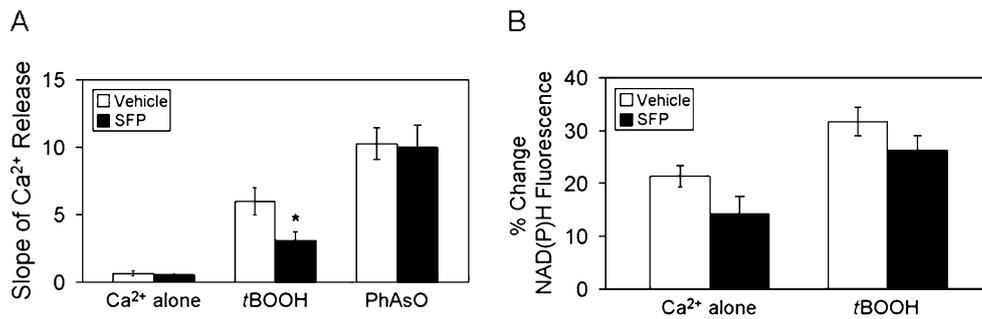
Fig. 2 Effects of systemic sulforaphane administration on peroxide-induced brain mitochondrial calcium release and pyridine nucleotide oxidation. **a** Comparisons of the initial rise in Calcium Green 5 N fluorescence after the addition of 250 μM tert-butyl hydroperoxide (tBOOH), 30 μM phenylarsine oxide (PhAsO) or no addition (Ca^{2+} alone) for brain mitochondria isolated from rats treated 40 h earlier with either sulforaphane (SFP; 10 mg/kg) or drug vehicle. * $p<0.05$, $n=9$. **b** Comparisons of the extent to which NAD(P)H autofluorescence decreased after the addition of tBOOH or no addition to suspensions of mitochondria from SFP- or drug vehicle-treated rats

mitochondrial redox state can be continuously monitored by following the autofluorescence of NAD(P)H which is lost during NAD(P)H oxidation by glutathione reductase. We therefore followed NAD(P)H redox state along with the medium free Ca^{2+} concentration to determine if mitochondria from SFP-treated rats are relatively resistant to tBOOH-induced oxidation or are resistant to PTP opening caused by NAD(P)H oxidation. As shown in Fig. 1b, Line 3 and 4, addition of tBOOH to the mitochondrial suspensions caused a rapid loss of NAD(P)H autofluorescence for mitochondria from both SFP- and vehicle-treated rats, compared to the slow, modest decline in fluorescence that occurred in the absence of tBOOH. The extent to which NAD(P)H was oxidized by tBOOH was not significantly different for mitochondria from the two animal groups (Fig. 2b).

Sulforaphane treatment does not protect against PTP opening induced by direct sulfhydryl group oxidation

Experiments were performed where PTP opening was induced by exposure of isolated brain mitochondria to phenylarsine oxide, a direct sulfhydryl group oxidizing agent (Kowaltowski et al. 2000), to determine if treatment of rats with SFP protects against this direct oxidation or just against indirect oxidation induced by peroxide metabolism. Figure 1a, Line 6, demonstrates that phenylarsine oxide induces a greater rate and extent of Ca^{2+} release than that induced by tBOOH. Comparison of the release rate for mitochondria from SFP- and vehicle-treated rats shown in Fig. 2a indicates no difference between these groups ($n=4$/group).

Mitochondrial respiration is unaffected by treatment of rats with sulforaphane

Sensitivity of mitochondria to PTP opening can be influenced by many factors, including normal mitochondrial bioenergetic

activities, e.g., rates of respiration (Mirandola et al. 2010). We therefore compared rates of ADP-stimulated State 3 respiration, oligomycin induced State 4 respiration, and the respiratory control ratios calculated from these rates for mitochondria isolated from rats treated with sulforaphane and drug vehicle. As shown in Table 1, there were no significant differences between these rates of respiration and respiratory control ratios for brain mitochondria respiring on NADH-dependent oxidizable substrates, malate plus glutamate, from SFP- and drug vehicle-treated animals. In addition, one experiment was performed to compare rates of respiration on succinate in the presence of rotenone. Similar values were obtained from mitochondria from the SFP- and vehicle-treated rats (State 3=164 vs 194 nmoles O$_2$/min/mg).

In addition to determining if treatment of rats with SFP affects respiration by isolated brain mitochondria, we also tested for any direct effects of SFP on mitochondria isolated from the brains of normal animals. The presence of SFP at concentrations up to 100 μM had no discernible effect on either State 3 or 4 respiration (not shown), indicating that SFP exhibits no mitochondrial toxicity even at levels that are an order of magnitude higher than the maximal that could be reached within the brain or other tissues at doses that have been used for neuroprotection. Additional experi-

Table 1 Respiratory characteristics of non-synaptic brain mitochondria isolated from sulforaphane and drug vehicle-treated rats. ADP-stimulated (State 3) and oligmycin-induced (State 4) respiration was measured in medium containing 5 mM malate plus 5 mM glutamate as oxidizable substrates, as described in Materials and Methods. The respiratory control ratio (RCR) is State 3/State 4. Values represent the means ± S.E. for $n=4$ animals per group

Respiration parameters	DMSO	SFP
State 3	134±18	168±13
State 4	27±2	26±2.7
RCR	5±0.8	6.5±0.3

ments tested the effects of phenylarsine oxide on mitochondrial respiration and found no effects at the concentration used to induce PTP in mitochondria pre-loaded with Ca^{2+} (not shown).

Mitochondrial cyclophilin D immunoreactivity is unaffected by treatment of rats with sulforaphane

Cyclophilin D is the primary mitochondrial protein whose level is known to affect sensitivity to PTP opening. We therefore used immunoblots for cyclophilin D from mitochondrial protein extracts to determine if differences exist in brain mitochondria from SFP- and vehicle-treated rats. A representative immunoblot for cyclophilin D and the mitochondrial voltage dependent anion channel (VDAC) is shown in Fig. 3a. Densitometric ratios for cyclophilin D/VDAC for 7 vehicle-treated rats and 7 SFP-treated rats are shown in Fig. 3b. There was no significant difference in these ratios for the two groups, although the ratios tended to be lower in mitochondria from animals treated with SFP.

Discussion

The primary conclusion reached from these experiments is that intraperitoneal injection of rats with a non-toxic level of sulforaphane results in resistance of isolated nonsynaptic brain mitochondria to peroxide-induced PTP opening measured 40 h later. The potential significance of this conclusion is that if such resistance also occurs to mitochondria as they exist within the brain, treatment of animals with SFP could protect against PTP opening and its metabolically catastrophic consequences that are known to occur in acute brain injury, e.g., caused by ischemia or trauma, and even in neurodegenerative diseases.

Our experiments provide some insight into the mechanisms by which SFP confers resistance to peroxide-induced release of accumulated Ca^{2+}. The complete inhibition of tBOOH-induced Ca^{2+} release by cyclosporin A verifies that

the SFP is affecting PTP opening rather than some other mechanism of release, which can occur particularly with brain mitochondria, depending on the experimental conditions (Andreyev et al. 1998). Previous in vitro experiments comparing mitochondria from wild-type and Bcl2 overexpressing cell lines demonstrated that Bcl2 protects against tBOOH-induced PTP opening exclusively by protecting against oxidation of NAD(P)H and its redox partners (Kowaltowski et al. 2000). This mechanism is not responsible for SFP induced resistance to peroxide-triggered PTP opening, as indicated by no significant difference between the extent of NAD(P)H oxidation for mitochondria from SFP- and vehicle treated rats. This finding is important since ingestion of cruciferous vegetables, from which SFP is derived, can increase the level of Bcl2, at least in the heart (Mukherjee et al. 2010). SFP treatment also does not affect rates of respiration by isolated mitochondria and does not have any direct affects on mitochondrial respiration or PTP opening when added to mitochondrial suspensions at concentrations up to 100 μM. These findings argue against any generalized effects of SFP on mitochondrial bioenergetics, which could affect PTP opening. The level of cyclophilin D immunoreactivity is also not affected by SFP treatment, indicating that the expression of this well-established regulator of PTP activity is not the basis of the SFP effect. It is possible, however, that the relative redox state of cysteine sulfhydryls present on this protein or other PTP affiliated proteins, e.g., the adenine nucleotide translocase, could be the key to understanding PTP resistance conferred by systemic administration of SFP (Linard et al. 2009; Costantini et al. 2000). While the NAD(P)H-based reducing power appears unaffected by SFP treatment, the mitochondrial components that utilize this power to inhibit PTP opening may be the target of SFP. The finding that SFP administration did not inhibit PTP opening caused by exposure of mitochondria to phenylarsine oxide indicates that SFP does not affect sensitivity of proteins to oxidation but rather affects the process whereby peroxide metabolism elicits oxidative stress and subsequently protein oxidation. Thus experiments are in progress to determine if levels of mitochondrial glutathione, thioredoxin, or thioredoxin are increased by SFP treatment, as they are affected in other tissues at either the cellular or mitochondrial levels (Crane et al. 2009; Angeloni et al. 2009; Vauzour et al. 2010).

Future determination of which mitochondrial protein or proteins mediate the inhibition of redox-regulated PTP opening by systemic SFP administration will help test the hypothesis that the primary action of SFP is to activate the Nrf2 pathway of cytoprotective gene expression. Numerous studies, including our own with primary cultures of neurons and astrocytes (Danilov et al. 2009; Siebert et al. 2009; Soane et al. 2010), have provided evidence in favor of this
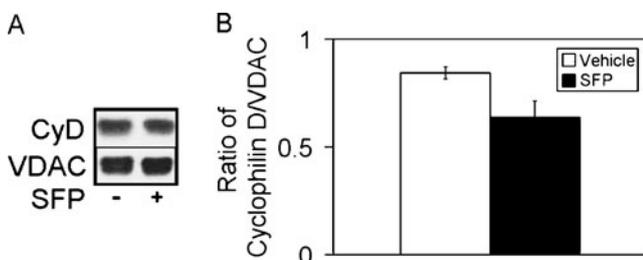
Fig. 3 Cyclophilin D immunoreactivity in non-synaptic brain mitochondria from sulforaphane and drug vehicle-treated rats. **a** Sample immunoblots for cyclophilin D (CyD) and the voltage gated anion channel (VDAC) for mitochondria isolated from the brain of a rat treated 40 h earlier with sulforaphane (SFP) or drug vehicle. **b** Densitometric ratios of mitochondrial CyD/VDAC for $n=7$ animals/group

primary mechanism for SFP (Dinkova-Kostova and Talalay 2008); however, at a dose approximately 10 times higher than that used in our experiments, SFP may exert additional effects, e.g., inhibition of histone deacetylase (Myzak et al. 2006). Irrespective of the specific steps by which SFP confers resistance of mitochondria to peroxide-induced PTP opening, this effect could contribute importantly to the cytoprotection observed by SFP in both in vitro and in vivo models of cell injury and death for the brain, heart, and other vital organs.

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