Transcription Factor Nrf2 Protects Renal Dopamine D1 Receptor Function During Oxidative Stress

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Abstract—The renal dopaminergic system plays a significant role in controlling sodium excretion and blood pressure (BP). Overwhelming evidence shows that oxidative stress downregulates renal dopamine receptors (D1R), and antioxidant supplementation protects D1R function. However, the mechanisms for benefits of antioxidants in protecting D1R function are unknown. We investigated the role of nuclear factor E2−related factor 2 (Nrf2), a redox-sensitive transcription factor, in reducing oxidative stress, protecting renal D1R function and lowering BP in rats. Male Sprague-Dawley rats were treated with L-buthionine-sulfoximine (BSO) and sulforaphane for 4 weeks. Rats treated with BSO exhibited significant increase in oxidative stress and BP. BSO treatment reduced renal D1R expression and abolished SKF38393 (a D1R agonist)-induced Na/K-ATPase and Na/H-exchanger (NHE3) inhibition. Also, in these rats, SKF38393 failed to promote sodium excretion. BSO caused an increase in nuclear factor-κB expression, a modest nuclear translocation of Nrf2 and a moderate activation of phase II antioxidant enzymes. Treatment of rats with sulforaphane alone induced modest activation of Nrf2 and phase II antioxidant enzymes, although having no effect on BP, redox status, or D1R function. However, sulforaphane prevented oxidative stress, protected D1R function, and abrogated hypertension in BSO-treated rats. In these animals, sulforaphane, whereas attenuating nuclear factor-κB activation, caused a robust stimulation of Nrf2 and phase II antioxidant enzyme pathway. In conclusion, oxidative stress via nuclear factor-κB activation downregulated D1R function causing a decrease in sodium excretion, which contributed to an increase in BP. Sulforaphane via activation of Nrf2-phase II antioxidant enzyme pathway mitigated oxidative stress and nuclear factor-κB activation, preserved D1R function, and prevented hypertension. (Hypertension. 2013;62:00-00.)

Key Words: hypertension ■ Na/K-ATPase ■ NHE3 ■ nuclear factor-κB ■ sulforaphane

Renal dopamine maintains sodium homeostasis and regulates blood pressure (BP) by promoting sodium excretion especially during sodium replete conditions.1,3 The actions of dopamine are mediated via dopamine receptors, which are coupled to G proteins and consist of D1-like receptors (D1 and D5) that stimulate adenylyl cyclase activity and D2-like receptors (D2, D3, and D4) that inhibit adenylyl cyclase.1,3 Each receptor has been implicated in the regulation of renal sodium transport and BP control.1,3 These receptors are expressed along the renal tubule, often overlapping in sites, such as the proximal tubule, thick ascending limb, and collecting duct.1,4 In the mammalian kidney, dopamine serves as a major regulator of salt and water reabsorption by inhibiting proximal tubular solute and water transport, mediated by the inhibition of various sodium transporters, such as apical NHE3, chloride-bicarbonate exchanger and Na-P cotransport, and basolateral Na/K-ATPase and Na-HCO3 cotransport.4,9

Of all the dopamine receptor subtypes, D1R is most widely studied because it relates to its role in the regulation of renal sodium homeostasis and BP.1,9 A defect in dopamine D1R function in the kidney leads to an impairment in the ability of dopamine to promote sodium excretion, which may eventually lead to sodium retention and development of hypertension.1,9 An alteration in renal D1R function has been reported in essential hypertension, Dahl salt-sensitive hypertension, and in obesity and age-associated hypertension.10-13 Although the exact mechanisms for impaired D1R function are not fully understood, it is reported that oxidative stress plays an important role in D1R dysfunction and development of hypertension.14-16 Oxidative stress causes D1R hyperphosphorylation, an important post translational modification, which regulates D1R signaling and generally contributes to its desensitization.14,15 It has been shown that oxidative stress increases the activity of various serine/threonine protein kinases, which lead to D1R hyperphosphorylation.6 These kinases include the G protein–coupled receptor kinases and protein kinase C.17,18 Previously, we have shown that oxidative stress increased activity and membranous translocation of protein kinase C and G protein–coupled receptor kinases, which phosphorylated and desensitized the proximal tubular D1R in various oxidative stress–related hypertensive animal models.19 Antioxidant treatment of these animals restored D1R function and reduced BP. It is reported that, in addition to direct free radical scavenging, antioxidants can induce redox-sensitive transcription factors, which in turn can activate endogenous antioxidant enzymes and reduce oxidative stress.20 Of particular importance is the nuclear factor E2−related factor 2 (Nrf2), a transcription factor, which can be induced by various antioxidants.21,22 Once
activated, Nr2 translocates to nucleus and increases the expression of phase II antioxidant enzymes, which in turn reduce the oxidative stress. We have previously reported that in rats, L-buthionine-sulfoximine (BSO)-induced oxidative stress caused D1R dysfunction and hypertension, both of which can be mitigated by concomitant treatment with antioxidant tempol. However, the mechanisms for these beneficial effects of tempol on D1R function and BP are not yet known. Therefore, the present study was designed to test the hypothesis that sulfophane, an antioxidant, induces Nr2 that via phase II antioxidant enzyme activation decreases oxidative stress, restores D1R function, and lowers BP in BSO-treated rats.

Methods

Adult male Sprague-Dawley rats (200–250 g) were purchased from Harlan (Indianapolis, IN) and acclimatized for a week before the initiation of antioxidant/antioxidant treatment. The animals were divided into the following 4 groups: (1) V (vehicle), rats kept on tap water; (2) BSO, rats provided with 30 mmol/L BSO; (3) S, rats provided with 10 µmol/L sulfophane; and (4) BSO+S, rats provided with both BSO and sulfophane. All the experiments were approved by the Institutional Animal Care and Use Committee.

Urinary 8-isoprostane was measured by radioimmunoassay kit (Cayman, Ann Arbor, MI), and malondialdehyde was determined by the method of Mihara and Uchiyama. Conscious BP of rats was measured by radiotelemetry (DSI, Minneapolis, MN) as detailed before. Briefly, anesthesia was induced by 5% isoflurane and main-anesthesia was maintained at 2.0% to 2.5% isoflurane throughout the surgery. Abdominal aorta was exposed by making a midline abdominal incision, and an arterial vein was catheterized for infusing saline or SKF38393. A midline abdominal incision was made, the aorta was cannulated and secured with tissue adhesive (Vetbond from 3M, St Paul, MN). The body of the transmitter was sutured to the abdominal musculature, and animals were allowed to recover for 7 to 10 days before the recording of BP. BP was measured throughout the treatment, and daily recording of individual rats was averaged.

Renal Function

Rats were anesthetized with Inactin (100 mg/kg IP) and the left jugular vein was catheterized for infusing saline or SKF38393. A midline abdominal incision was performed, and the left ureter was catheterized to collect urine samples. The effect of SKF38393 on sodium excretion was determined by following a previously detailed protocol, which consisted of a 45-minute stabilization period after the surgery followed by 5 consecutive 30-minute collection periods: 2 basal (saline alone was infused), 1 SKF38393 (1 µg/k per minute), and 2 recovery (saline alone). Urine and plasma were analyzed by atomic absorption spectroscopy (PerkinElmer, Waltham, MA) to determine urinary sodium excretion and fractional excretion of sodium.

Proximal Tubule Preparation

Renal proximal tubules were prepared as detailed previously. Rats were anesthetized with sodium pentobarbital (50 mg/kg IP) and, after a midline abdominal incision was made, the aorta was cannulated below the kidney, and in situ digestion was accomplished by perfusing the kidneys with iso-osmotic collagenase and hyaluronidase (Sigma, St Louis, MO). Proximal tubules were purified and enriched by using density gradient centrifugation with 25% Ficoll in Krebs-Hanseleit buffer. An intact proximal tubular band over Ficoll was collected and cold washed 3x with Krebs-Hanseleit buffer. Protein concentration was determined by using a BCA kit with BSA as standard. Cell fractionation, separation of cytosol, membrane, and whole cell lysate were performed by routine laboratory methods as detailed elsewhere.

Assay of Sodium Transporters

Na/K-ATPase and NHE3 activity were determined as detailed previously. Briefly, proximal tubules isolated from 4 experimental groups (V, BSO, S, and BSO+S) were incubated with 1 µmol/L of SKF38393 at 37°C for 15 minutes without or with D1R antagonist SCH23390 (1 µmol/L). The tubules were lysed by flash-freezing, and Na/K-ATPase activity was determined by incubating tubular homogenates (1 mg of protein/mL) with ATP in the absence and presence of 1 mmol/L of ouabain at 37°C. NHE3 activity was determined by measurement of 5-(N-methyl-N-isobutyl)-amiloride sensitive 22Na+ uptake in brush border membrane vesicles. As detailed previously, following the treatment of proximal tubules, from all the experimental groups, with D1R agonist and antagonist, tubules were used to prepare brush border membrane vesicles; 22Na+ uptake was used to measure NHE3 activity. It is worth mentioning that BSO or sulfophane was not present during D1R agonist/antagonist treatment.

Immunoblotting

Proximal tubular cell homogenate, membrane, and cytosol proteins were solubilized in Laemml buffer, separated on SDS-PAGE, and transferred to nitrocellulose membrane. The nitrocellulose membranes were blocked with 5% BSA and incubated with antibodies against D1R (D1R, Millipore, Cat# MAB5290), nuclear factor-kB (NF-kB; Cell Signaling, Cat# 3034), Nrf2 (Cell Signaling, Cat# 8882), and GAPDH (Millipore, Cat# MAB374) followed by incubation with horseradish peroxidase-conjugated secondary antibodies. The blots were analyzed as detailed previously.

Assay of Phase II Antioxidant Enzymes

NAD(P)quinone oxidoreductase 1 (NQO1; rat assay kit from Usen Life Sciences, Cat# E80969Ra), glutathione-S-transferase (GST; assay kit from Cayman, Cat# 703302), and heme oxygenase (H0; 1; assay kit from Enzo Life Sciences, Cat# AD1-EKS-800) were measured by using commercially available kits as per manufacturer's protocol.

Statistics Analysis

Differences between the means were evaluated using unpaired Student's t test or 2-way ANOVA with Bonferroni posttest, as appropriate. P<0.05 was considered statistically significant.

Results

Animals treated with BSO for 4 weeks exhibited oxidative stress as evidenced by increased urinary 8-isoprostane (pg/mL; V, 76 ±4±3.2; BSO, 128±6±6.5, P<0.05) and renal malondialdehyde levels (nmol/mg protein; V, 0.45±0±0.04; BSO, 0.83±0±0.9; P<0.05, and these animals also had a significant increase in BP compared with vehicle treatment (Figure 1). Supplementation of sulfophane to BSO-treated rats prevented the increase in oxidative stress (isoprostane: S, 68±3±±4.1; BSO+S, 82±0±±3.2 and malondialdehyde: S, 0.41±0±±0.0; BSO+S, 0.51±0±±0.06) and BP (Figure 1). Both sulfophane and BSO had no effect on food and water intake or gain in body weight (data not shown) during the course of treatment.

Effect of BSO and Sulfaphane on Renal D1R Expression

BSO treatment reduced renal D1R protein expression and D1/D5R numbers as measured by [3H]SCH23390 binding (Figure 2A and 2B). The decrease in D1R protein expression was observed both in cell homogenate (data not shown) and membranes (Figure 2A), indicating a downregulation of receptors. Sulfophane prevented the D1R downregulation and decrease in ligand binding in BSO-treated rats (Figure 2A and 2B). Renal D1R expression was comparable in rats kept on tap water (vehicle) and those supplemented with sulfophane, indicating that sulfophane has no direct effect on receptor sequestration (Figure 2A and 2B).
Effect of BSO and Sulforaphane on SKF38393-Induced Na/K-ATPase and NHE3 Inhibition

Incubation of proximal tubules with D1R agonist SKF38393 (1 μmol/L) reduced Na/K-ATPase and NHE3 (22Na uptake) activity in vehicle-treated rats (Figure 3A and 3B). However, SKF38393 failed to inhibit these sodium transporters in BSO-treated rats (Figure 3A and 3B). Sulforaphane, per se, had no effect on D1R response but it rescued the SKF38393-induced inhibition of sodium transporters in BSO-treated rats (Figure 3A and 3B). Incubation of proximal tubules with D1R antagonist SCH23390 abolished SKF38393-induced inhibition of N/K-ATPase and NHE3 (Figure 3A and 3B), indicating that SKF38393-mediated inhibition of these sodium transporters is D1R specific.

Effect of BSO and Sulforaphane on SKF38393-Induced Natriuresis

SKF38393 infusion caused a significant increase in urinary sodium excretion and fractional excretion of sodium in vehicle- and sulforaphane-treated rats (Figure 4A and 4B). However, SKF38393 failed to increase urinary sodium excretion or fractional excretion of sodium in BSO-treated rats (Figure 4A and 4B). Supplementation of sulforaphane restored the natriuresis in BSO-treated rats (Figure 4A and 4B).

Effect of BSO and Sulforaphane on Transcription Factors NF-κB and Nrf2

BSO treatment significantly increased nuclear NF-κB expression, whereas having a moderate impact on nuclear Nrf2 in
renal proximal tubules (Figure 5A and 5B). Sulforaphane alone had no effect on NF-κB but caused a modest increase in Nrf2 expression (Figure 5A and 5B). Concomitant treatment of rats with BSO and sulforaphane prevented NF-κB activation, while causing a robust increase in Nrf2 activation (Figure 5A and 5B). Interestingly, BSO and sulforaphane did not increase the cytosolic Nrf2 expression (Figure 5C); in fact there was a decrease in cytosolic Nrf2 protein in sulforaphane and BSO plus sulforaphane–treated rats, indicating a translocation of Nrf2 from cytosol to nucleus (Figure 5C). The failure to see a decrease in cytosolic Nrf2 protein in rats treated with BSO alone could be attributable to the fact that only moderate activation of Nrf2 was observed in these animals.

**Effect of BSO and Sulforaphane on Phase II Antioxidant Enzymes**

Similar to Nrf2 activation, both sulforaphane and BSO individually caused a moderate increase in phase II antioxidant enzymes NQO1, GST, and HO-1 (Figure 6A through 6C). However, there was a marked increase in these enzymes when rats were treated with both BSO and sulforaphane (Figure 6A through 6C).

**Discussion**

In this study, we showed that sulforaphane activated Nrf2, which led to stimulation of phase II antioxidant enzymes NQO1, GST, and HO-1. The activation of Nrf2–phase II pathway reduced oxidative stress, prevented NF-κB activation, preserved renal D1R function, and prevented an increase in BP in BSO-treated rats. In the absence of sulforaphane, BSO treatment of SD rats increased oxidative stress, activated NF-κB, and reduced renal D1R expression. These factors led to the failure of SKF38393 to inhibit renal tubular sodium transporters (Na/K-ATPase and NHE3) or to promote sodium excretion, thus causing an increase in BP.

Oxidative stress is an independent risk factor for the development of hypertension. Although, the evidences that relate oxidative stress to hypertension are overwhelming, it is not clear how oxidative stress leads to this relationship. We and others have shown that oxidative stress can attenuate renal D1R functionality, which could disrupt renal sodium regulation and lead to hypertension. Dopamine via renal D1R activation regulates sodium excretion especially in sodium replete conditions. This is supported by the findings in various animal models of hypertension where D1R dysfunction has been shown to be an important contributor to the development of hypertension. As an example, in spontaneously hypertensive rats, a genetic model of hypertension, the development of hypertension segregates with a genetic defect in D1R, whereas the hyperactive characteristic of these animals is independent of renal D1R function. In various other animal models, such as Dahl salt-sensitive rats, obese Zucker rats, and animals made hypertensive by exogenous administration of pharmacological compounds, a strong link between D1R dysfunction and hypertension is observed. In addition, mice lacking D1R also show a...
significant increase in BP. Interestingly, in rats, oxidative stress seems to be an underlying factor for the dopamine receptor dysfunction and, in mice, the lack of dopamine receptor leads to an increase in oxidative stress. These data underline an important regulatory pattern between oxidative stress and D1R; an increase in oxidative stress suppresses dopamine receptor function, whereas lack of a functioning dopamine receptor disrupts the redox status of a cell. Here, we tried to investigate the role of redox-sensitive transcription factors because it relates to the effect of oxidative stress on renal D1R function and the beneficial effects of antioxidants in protecting D1R function and, thus, mitigating oxidative stress–associated hypertension.

Treatment of Sprague-Dawley rats with BSO, a pro-oxidant compound that induces oxidative stress by attenuating glutathione synthesis, increased oxidative stress and activated a pro-oxidant transcription factor NF-κB. An increase in oxidative stress and activation of NF-κB caused renal D1R downregulation as evidenced by a decrease in D1R protein content and receptor numbers. Supplementing BSO-treated rats with sulforaphane reduced oxidative stress, mitigated NF-κB activation, and prevented D1R downregulation. These data are consistent with previous studies from others and our own laboratory showing that antioxidant treatment can rescue D1R function in both animal models exhibiting oxidative stress or cells in culture exposed to oxidants. The exact mechanism for the involvement of NF-κB is not clear; however, it has been shown that NF-κB can transcriptionally upregulate serine threonine kinase, such as protein kinase C and G protein–regulated kinases. It is well established that serine/threonine kinase can both downregulate and uncouple D1R from G protein by serine hyperphosphorylation. Therefore, it is plausible that D1R downregulation could involve NF-κB–induced activation of protein kinases, and sulforaphane via inhibition of NF-κB rescues D1R expression.

The functional consequence of D1R downregulation is reflected not only by the failure of D1R agonist SKF38393 to inhibit sodium transporters Na+/K-ATPase and NHE3 but also more importantly by the inability of SKF38393 to increase sodium excretion in BSO-treated rats. Therefore, it is conceivable that the failure of renal D1R to regulate sodium excretion could be an important contributor to the increase in BP observed in these animals. Interestingly, sulforaphane that restored D1R expression and function also mitigated hypertension, thus, further supporting a strong relationship among oxidative stress, renal D1R dysfunction, and BP in experimental animals.

To identify the mechanisms responsible for sulforaphane-mediated mitigation of NF-κB activation and protection of renal D1R function, we investigated the role of another redox-sensitive transcription factor Nrf2. It is shown that under normal redox status, Nrf2 is mostly bound to a cytosolic protein Keap 1 and, thus, plays a minor role in gene regulation.

However, stimulatory factors that include oxidants or antioxidants, such as sulforaphane, can dissociate the Nrf2-Keap 1 complex. The stimulated Nrf2 migrates/translocates to nucleus and activates a battery of phase II antioxidant enzymes, such as NQO1, GST, and HO-1, which in turn help to normalize the redox status of the cell. In the present study, we found that BSO and sulforaphane caused a modest Nrf2 nuclear translocation, which was reflected by a moderate increase in the activities NQO1, GST, and HO-1. However, the concomitant treatment of BSO and sulforaphane caused a robust activation of both Nrf2 and phase II antioxidant enzymes, which in turn could be responsible for a decrease in oxidative stress in these animals. Although a decrease in oxidative stress, per se, could mitigate NF-κB activity, however, it has been shown that Nrf2 activation can also directly suppress NF-κB function. Therefore, we suggest that activation of Nrf2-phase II antioxidant enzyme pathway in response to sulforaphane could, both directly and indirectly, attenuate NF-κB activation, protect D1R function and, thus, mitigate hypertension during oxidative stress.

Taken together, our data show that oxidative stress via NF-κB downregulates D1R causing failure of dopamine to inhibit sodium transporters or induce natriuresis, which could lead to a decrease in sodium excretion and hypertension. Sulforaphane, via activation of Nrf2-phase II enzyme pathway, reduces oxidative stress, mitigates NF-κB activation, protects D1R function and, thus, ameliorates hypertension during oxidative stress.

Limitations

This study identifies a link among oxidative stress, redox-sensitive transcription factors, renal D1R function, and hypertension. Because rats were used to perform the study, it is not possible to control the function of these molecules independently. Therefore, the present data do not indentify whether protection of renal D1R by Nrf2 activation was attributable to mere stimulation of phase II antioxidant enzymes and subsequent decrease in oxidative stress or whether a direct interaction at protein or gene level is involved between these 2 molecules. Also, the mechanism responsible for upregulation of NF-κB in BSO-treated rats and normalization of this transcription factor in response to sulforaphane need to be identified. However, it is plausible that the mitigation of oxidative stress by sulforaphane could be a major contributor to the normalization of NF-κB because oxidative stress is a known stimulant for NF-κB. It is worth mentioning that there are reports to suggest that Nrf2 can directly suppress NF-κB at transcription level. Also, the mechanism responsible for sulforaphane-mediated Nrf2 activation was not identified because these studies are beyond the scope of this article.

Perspective

Our studies show that oxidative stress activates both pro-oxidant and antioxidant transcription factors. The activation of pro-oxidant transcription factor NF-κB could contribute to D1R downregulation, which leads to decreased sodium excretion in response to SKF38393. The defective D1R signaling and sodium handing could be contributing factors to the development of hypertension. Oxidative stress, per se, can activate Nrf2-phase II antioxidant pathway, a pivotal defensive system that plays an important role in mitigating oxidative during health and disease. However, our data show that an exogenous compound like sulforaphane, which is a potent Nrf2 inducer, is required to cause a robust activation of the Nrf2-phase II antioxidant pathway. Once fully activated, Nrf2-phase II antioxidant signaling can mitigate oxidative stress, suppress NF-κB activation, preserve renal D1R function, and prevent development of oxidative stress–associated hypertension.

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Disclosures
None.

References

Novelty and Significance

What Is New?
- Identification of redox signaling factors that directly regulate renal D1R signaling during oxidative stress.
- The observation that the antioxidant sulfaphenax exerts an antihypertensive action during oxidative stress by activating nuclear factor E2–related factor 2–phase II antioxidant enzyme pathway.

What Is Relevant?
- The study identifies a mechanism by which renal D1R function can be protected to attenuate development of hypertension during oxidative stress.
- It is possible that neutriceuticals that activate phase II antioxidant enzymes via nuclear factor E2–related factor 2 can serve as potential therapeutic agents in the treatment of hypertension and other renovascular disorders associated with oxidative stress.

Summary
Oxidative stress activates nuclear factor–κB that downregulates renal D1R function, leading to diminished SKF38393–induced Na+K-ATPase and NHE3 inhibition, resulting in sodium retention and hypertension. Antioxidant sulfaphenax activates nuclear factor E2 related factor 2, which causes activation of phase II antioxidant enzymes and a decrease in oxidative stress and nuclear factor–κB activation, leading to normalization of D1R signaling and subsequent lowering of blood pressure.
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