Kidney

Role of Nuclear Factor Erythroid 2–Related Factor 2 in the Oxidative Stress–Dependent Hypertension Associated With the Depletion of DJ-1

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Abstract—Renal dopamine 2 receptor dysfunction is associated with oxidative stress and high blood pressure (BP). We have reported that DJ-1, an oxidative stress response protein, is positively regulated by dopamine 2 receptor in the kidney. The transcription factor nuclear factor erythroid 2–related factor 2 (Nrf2) regulates the expression of several antioxidant genes. We tested the hypothesis that Nrf2 is involved in the renal DJ-1–mediated inhibition of reactive oxygen species production. We have reported that silencing dopamine 2 receptor in mouse renal proximal tubule cells decreases the expression of DJ-1. We now report that silencing DJ-1 or dopamine 2 receptor in mouse proximal tubule cells and mouse kidneys decreases Nrf2 expression and activity and increases reactive oxygen species production; BP is also increased in mice in which renal DJ-1 or dopamine 2 receptor is silenced. DJ-1−/− mice have decreased renal Nrf2 expression and activity and increased nitrotyrosine levels and BP. Silencing Nrf2 in mouse proximal tubule cells does not alter the expression of DJ-1 or dopamine 2 receptor, indicating that Nrf2 is downstream of dopamine 2 receptor and DJ-1. An Nrf2 inducer, bardoxolone, normalizes the systolic BP and renal malondialdehyde levels in DJ-1−/− mice without affecting them in their wild-type littermates. Because Nrf2 ubiquitination is increased in DJ-1−/− mice, we conclude that the protective effect of DJ-1 on renal oxidative stress is mediated, in part, by preventing Nrf2 degradation. Moreover, renal dopamine 2 receptor and DJ-1 are necessary for normal Nrf2 activity to keep a normal redox balance and BP.

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Key Words: dopamine receptors ▪ kidney ▪ oxidative stress

Essential hypertension is associated with increased positive sodium balance and oxidative stress. Renal dopamine plays an important role in the normal regulation of sodium balance and systemic blood pressure (BP).1–4 Indeed, hypertension is associated with decreased renal dopamine production and receptor function.1–4 Dopamine receptors are classified into two families: D1-like receptors (D1R and D5R) and D2-like receptors (D2R, D3R, and D4R). Deletion of any of the dopamine receptor genes in mice results in increased BP by mechanisms that are dopamine receptor subtype specific.4 In particular, D1−/− and D2−/− mice have hypertension that is associated with oxidative stress.5–7

Dopamine and D2R agonists have antioxidant activity that may be responsible for their neuroprotective effects.8,9 We have reported that the antioxidant property of D1R is mediated, in part, by positive regulation of several antioxidant proteins, including heme oxygenase-2,4,3 paraoxonase-2,10 and DJ-1.11 DJ-1 (aka Park 7), initially identified as an oncogene and an autosomal recessive gene in Parkinson disease, is widely expressed in the body, including the brain, heart, kidney, and liver.12 DJ-1 is a peroxiredoxin that exerts a protective role against oxidative stress in several diseases.13–15 DJ-1 may be responsible for the neuroprotection afforded by D1R.16 We have reported that the antioxidant effect of D1R is dependent, in part, on DJ-1 expression/activity in the kidney.11 Renal silencing of DJ-1 in the mouse increases reactive oxygen species (ROS) production and BP.

Nuclear factor erythroid 2–related factor 2 (Nrf2) is a transcription factor that regulates the expression of several antioxidant genes. Nrf2, together with small Maf proteins, binds to antioxidant response element in the regulatory regions of target genes.17 Nrf2 has also been reported to inhibit the development and progression of several diseases affecting the kidney.18 DJ-1 may be involved in the regulation of Nrf219–23 but it is not known if the protective effect of DJ-1 on renal oxidative stress is dependent on Nrf2. Therefore, we tested the hypothesis that Nrf2 is involved in the antioxidant activity of DJ-1 in the kidney.

Materials and Methods

The experimental procedures are detailed in the online-only Data Supplement.
Results

**DJ-1 Colocalizes With Nrf2 in the Renal Proximal Tubule**

Immunofluorescence microscopy of mouse kidney sections showed that DJ-1 is expressed in the brush border and cytoplasm, whereas Nrf2 is expressed in the brush border, cytoplasm, and nucleus (Figure 1). Double immunofluorescence microscopy (Figure 1, merge) revealed that Nrf2 colocalized with DJ-1 in the cytoplasm and brush border of renal tubules.

**Silencing Drd2 Decreases DJ-1 Expression, Nrf2 Expression and Activity, and Increases ROS production**

To determine whether Nrf2 is involved in the antioxidant effect of DJ-1 and D_2R in the kidney, we studied the kidneys of mice that underwent renal-selective silencing of Drd2. Renal-selective silencing of Drd2 decreased D_2R expression (48±2%) and increased the systolic BP (ΔSBP: 19±8%), in agreement with the increased BP of D_2R−/− mice.5,11

Renal DJ-1 expression was decreased by −45±2%, Nrf2 by −22±2%, and glutathione S-transferase (GST) by −63±6%, whereas NADH quinone oxidoreductase (NQO1) expression was not affected (Figure 2A). Drd2 silencing in mouse renal proximal tubule (RPT) cells decreased Nrf2 promoter activity (−53±2%; Figure 2B) and increased ROS production (+146±43%; Figure 2C). These results confirm our report that DJ-1 expression and ROS production are regulated by the D_2R in the kidney and that D_2R regulates renal Nrf2 expression activity.

**Renal Silencing of DJ-1 Decreases Renal Nrf2 Expression and Activity and Results in Oxidative Stress–Dependent Hypertension**

We have reported that the renal silencing of DJ-1 decreased DJ-1 expression (30±6%) and increased BP (ΔBP: 18±3%).11 We now show in these mice that the renal silencing of DJ-1 also decreased the protein expression of Nrf2 (57±6%), and its target genes NQO1 (46±11%) and GST (28±7%), but not the protein expression of D_2R (Figure 3A). We also determined whether silencing DJ-1 with DJ-1 small interfering RNA affects D_2R protein expression in mouse RPT cells. We found that DJ-1 depletion decreased the expression of DJ-1 (−75±15%) but did not affect the protein expression of D_2R (Figure 3B), which reflects our in vivo data. Moreover, DJ-1 silencing decreased the expression of Nrf2 (63±11%), its target genes, NQO1 (75±15%) and GST (61±8%), and Nrf2 promoter activity (58±1%; Figure 3C), but increased ROS production (206±15%; Figure 3D). These results suggest that DJ-1 maintains normal renal redox balance by positively regulating Nrf2 expression and function, and that D_2R is upstream of DJ-1 in this pathway.

To determine whether the antioxidant action of DJ-1 is exerted via Nrf2, we extended our studies in DJ-1−/− mice. The absence of DJ-1 expression in DJ-1−/− mice was confirmed by immunoblotting (Figure 4A). The systolic BP was increased in DJ-1−/− mice by 31±8%, relative to their wild-type littermates (Figure 4B). Immunoblots showed that DJ-1−/− mice had decreased renal expression of Nrf2 (47±7%), NQO1 (19±3%), and GST (51±12%), but D_2R and Keap1 expression were not affected (Figure 3A and 3B). NADPH oxidase activity was also not altered but nitro-tyrosine protein was increased by 77±31% in the kidney of DJ-1−/− mice, indicating that DJ-1 negatively regulates oxidative stress independent of NADPH oxidase activity (Figure 4B). The latter effect of DJ-1 is independent of D_2R because germline deletion of D_2R is associated with increased renal expression Nox1, Nox2, and Nox4.5

To confirm the role of Nrf2 in the increased oxidative stress and BP associated with renal DJ-1 depletion, DJ-1−/− mice were treated with bardoxolone, a Nrf2 inducer (Figure 4C). Bardoxolone increased renal Nrf2 expression in both wild-type (118±17%) and DJ-1−/− mice (112±49%), relative to their vehicle-treated controls, and normalized the increased BP (before treatment 118±3.9%; after treatment 95±3.97% versus WT) and renal malondialdehyde production (before treatment 140±6%:after treatment 76±9% versus WT) in DJ-1−/− mice. Thus, the hypertension in DJ-1−/− mice is mediated by oxidative stress, in part, by the downregulation of Nrf2 expression. The fact that bardoxolone increased Nrf2 expression in wild-type mice but did not decrease their normal malondialdehyde levels and BP could be taken to indicate that under normal conditions, Nrf2 has no effect on oxygen radical formation or BP.

**Renal Silencing of Nrf2 Increases BP and Silencing Nrf2 Decreases NQO1 and GST But Not D_2R and DJ-1 Expression in Mouse RPT Cells**

We, next, determined whether Nrf2 directly participated in the DJ-1 and D_2R-mediated regulation of renal ROS production and BP. We found that renal-selective silencing of Nrf2 that decreased Nrf2 expression (−51±9%), increased BP (+12±6.3 mg Hg; Figure S1A in the online-only Data Supplement). In mouse RPT cells, Nrf2 small interfering RNA that decreased Nrf2 expression (34±4%) also decreased the expression of its target genes NQO1 (29±1%) and GST (25±7%). However, silencing of Nrf2 did not alter the expression of D_2R and DJ-1,
and decreased ROS production (18±3%), as expected,11 but with quinpirole increased the expression of DJ-1 (22±5%), the D2R agonist quinpirole. We found that stimulation of D2R of Nrf2 and its target genes, we quantified the mRNA expression stimulation was responsible for the increase in the expression of Nrf2, NQO1, and GST, or Nrf2 promoter activity in mouse RPT cells (Figure S2A–S2C), indicating that D2R and DJ-1 can decrease ROS production. Mouse RPT cells were studied 72 hours after transfection with Drd2-siRNA or nonsilencing siRNA. Nrf2 promoter activity was measured in whole RPT cell homogenates using dichlorofluorescin diacetate and corrected for protein concentration. Data are expressed as mean±SE; n=6 per group. "P<0.05 vs nonsilencing siRNA, t test.

We have reported that in mouse RPT cells, D2R stimulation with quinpirole, a D2R agonist, increased DJ-1 expression that was partially blocked by a selective D2R antagonist.11 To determine whether the increase in DJ-1 expression with D2R stimulation was responsible for the increase in the expression of Nrf2 and its target genes, we quantified the mRNA expression of Nrf2 and target genes in mouse RPT cells treated with the D2R agonist quinpirole. We found that stimulation of DJ-1 with quinpirole increased the expression of DJ-1 (22±5%), and decreased ROS production (18±3%), as expected,11 but did not modify the mRNA expression of Nrf2, NQO1, and GST, or Nrf2 promoter activity in mouse RPT cells (Figure S2A–S2C), indicating that D2R and DJ-1 can decrease ROS production in mouse RPT cells independent of Nrf2.

Depletion of DJ-1 Increases Ubiquitination of Nrf2

The preceding studies showed that silencing DJ-1 downregulated Nrf2 expression and function in the kidney. DJ-1 degradation is regulated by Nrf2 ubiquitination.24 Therefore, we determined whether renal DJ-1 could increase the stability of Nrf2 protein, by reducing its ubiquitination and presumably inhibiting its degradation, via the proteasomal pathway. We immunoprecipitated Nrf2 from kidney homogenates of DJ-1−/− mice and wild-type littermates using rabbit Nrf2 antibody and immunoblotted the immunoprecipitates with mouse monoclonal ubiquitin antibody. The amount of ubiquitinated Nrf2 was increased in DJ-1−/− mice compared with their wild-type littermates (Figure 5A). This result was corroborated by immunoprecipitating kidney homogenates with ubiquitin antibody and immunoblotting the immunoprecipitates with Nrf2 antibody (Figure 5B). In the kidneys of DJ-1−/− mice, Nrf2 ubiquitination was increased (438±161%), suggesting that the decrease in Nrf2 expression in DJ-1−/− mice may be the result of increased Nrf2 degradation of ubiquitinated Nrf2, via the proteasomal pathway.25

Discussion

We have recently reported that disruption of the gene Dnd2 in mice results in hypertension that is associated with increased ROS production,3,10 and that the D2R-mediated negative regulation of ROS production is, in part, because of its positive regulation of DJ-1 expression.11 We now report that D2R and DJ-1 are necessary for the normal function of Nrf2 and that DJ-1 prevents its ubiquitination and presumably its proteasomal degradation. Because Drd2 silencing decreases both DJ-1 and Nrf2 expressions, whereas silencing DJ-1 decreases the expression of Nrf2 but not D2R, and silencing Nrf2 does not decrease the expression of either D2R or DJ-1, D2R is upstream of both DJ-1 and Nrf2, and DJ-1 is upstream of Nrf2, in the regulation of renal ROS production and BP (Figure 6).

We have reported that silencing of DJ-1 expression in mouse RPT cells and kidney increases Nox4 expression and NAPDH oxidase activity.11 However, NAPDH oxidase activity is not increased in DJ-1−/− mice, indicating the activation of a compensatory mechanism with germline deletion of DJ-1 that remains to be identified. DJ-1−/− mice have a 2-fold increase in the mitochondrial H2O2 in the brain14 and increased sensitivity to stroke-induced neural injury.15 Our data show that the renal production of nitro-tyrosine is increased in DJ-1−/− mice that may be the result of an increase in peroxynitrite production, because peroxynitrite is formed by the interaction between NO and superoxide.26

DJ-1 positively regulates Nrf2 expression in hepatocyte-derived carcinoma, corneal endothelial cells,19,20 and human lung epithelial cells in conditions associated with oxidative stress.21 In addition, DJ-1 protects human neuroblastoma cells and astrocytes from oxidative stress via Nrf2.22,23 However, in neurons, Nrf2 can be activated independent of DJ-1,27 suggesting that DJ-1–mediated regulation of Nrf2 could also be tissue specific.

The potential role for Nrf2 on BP regulation is not well defined. There were no differences in BP between

Figure 2. Effect of renal-selective Drd2 silencing on renal DJ-1, nuclear factor erythroid 2–related factor 2 (Nrf2), NADH quinone oxidoreductase (NQO1), and glutathione S-transferase (GST) expression, Nrf2 promoter activity, and reactive oxygen species (ROS) production. A, Renal-selective Drd2 silencing in vivo. Renal cortical Drd2 was silenced as described in Materials and Methods. Systolic blood pressure was measured (Cardiomax II) from the aorta, via the femoral artery, under pentobarbital anesthesia. mRNA expressions of dopamine receptor D2 (Drd2), DJ-1, Nrf2, NQO1, and GST were quantified by quantitative reverse transcriptase polymerase chain reaction in mouse renal cortex. GAPDH was used for normalization of the data. Data are expressed as mean±SE; n=3 to 4 per group. *P<0.05 vs nonsilencing small interfering RNA (siRNA), t test. B, Renal proximal tubule (RPT) cell Nrf2 promoter activity. Mouse RPT cells were studied 72 hours after transfection with Drd2-siRNA or nonsilencing siRNA. Nrf2 promoter activity was measured by a reporter assay (Qiagen). Data are expressed as mean±SE; n=6 per group. *P<0.05 vs nonsilencing siRNA, t test. C, RPT cell ROS production. Mouse RPT cells were studied 72 hours after transfection with Drd2-siRNA or nonsilencing siRNA. ROS production was measured in whole RPT cell homogenates using dichlorofluorescin diacetate and corrected for protein concentration. Data are expressed as mean±SE; n=6 per group. *P<0.05 vs nonsilencing siRNA, t test.
expressions of Nrf2, NQO1, and GST were quantified by qRT-PCR. Data are expressed as mean±SE; n=3 per group, *P<0.05 vs nonsilencing small interfering RNA (siRNA), t test.

The inability of D2R to increase Nrf2 activity, in spite of the fact that both D2R and DJ-1 decrease ROS production, may at first glance contradict the apparent effect of D2R on renal expression of DJ-1, NQO1, and GST observed in previous studies.11 However, the stimulation of D2R with quinpirole did not affect Nrf2 expression and activity and the expression of its targets genes (Figure S2). These occurred in spite of the fact that silencing of D2R decreased Nrf2 expression after silencing small interfering RNA treatment, in spite of the ability of bardoxolone to normalize ROS production and BP of DJ-1−/− mice.

As indicated above, the hypertension of D2R−/− mice is decreased.11 The renal-selective silencing of Nrf2 via the renal subcapsular infusion of Nrf2-small interfering RNA increases BP. This in contrast to the normal BP of mice with germline deletion of Nrf2.28 It is possible that germline deletion of Nrf2 results in expression of genes, during development, or in non-renal tissues, that may compensate for the loss of Nrf2. Therefore, the stimulation of D2R in mouse RPT cells increased DJ-1 expression and decreased ROS production, confirming our previous report.11 However, the stimulation of D2R with quinpirole did not affect Nrf2 expression and activity and the expression of its targets genes (Figure S2). These occurred in spite of the fact that silencing of D2R or DJ-1 increases ROS production and decreases Nrf2 expression and Nrf2 promoter activity (current study). Nevertheless, part of the oxidative stress induced by D2R silencing may be because of the downregulation of Nrf2 because D2R and DJ-1 are necessary for normal Nrf2 function. Antioxidants regulated by Nrf2, other than D2R and DJ-1, such as HO-1 could be involved in the antioxidant mechanism of DJ-1 and D2R. However, we have reported that the antioxidant effect of D2R is not related to HO-1 but rather, in part, because of heme oxygenase-2.5 Therefore, D2R stimulation increases the expression of some antioxidants such as heme oxygenase-2, PON2,10 and DJ-1, independently of Nrf2 (Figure 6).

As indicated above, the hypertension of D2−/− mice is associated with oxidative stress10,11 and the renal expression of DJ-1 in D2−/− is decreased.11 The renal-selective silencing of Dnd2 in
mice increases BP; renal-selective silencing of Drd2 in mice also increases ROS production and decreases DJ-1 and Nrf2 expression and Nrf2 promoter activity and the expression of one of the target genes of Nrf2, GST. However, in contrast to the decreased expression of Nqo1, when DJ-1 or Nrf2 is silenced, NQO1 expression is not downregulated when Drd2 is silenced. The failure of NQO1 to be decreased when Drd2 is silenced may be related to nuclear factor (NF)-κB, which is upregulated when Drd2 is silenced; NF-κB can increase NQO1 expression.

Banday et al have reported that oxidative stress, via NF-κB activation, impairs renal D1R expression and function. The activation of Nrf2-phase II enzyme pathway, directly and indirectly decreases NF-κB activation, causing a decrease in oxidative stress and restoration of D1R expression and function. However, the renal D1R expression is not altered by the oxidative stress provoked by the downregulation of DJ-1 and Nrf2 (current results). Thus, the effect of oxidative stress on the expression of dopamine receptors may be subtype specific. D1R function could be impaired when D1R is silenced because of NF-κB activation. However, renal D1R expression is not decreased in Drd2 knockout mice (unpublished studies). Reports of D1-like receptor binding in the striatum of Drd2 knockout mice are not consistent, but the concomitant absence of D1R and D1R signaling is lethal in mice.

In conclusion, Nrf2 mediates, in part, the antioxidant effects of D2R and DJ-1 in the kidney and the oxidative stress–dependent hypertension associated with renal-selective depletion of D2R or DJ-1. Germline deletion of D2R can result in salt-sensitive hypertension that may be mouse strain dependent. The role of sodium balance in the hypertension associated with DJ-1 deficiency remains to be determined.

Figure 4. Effect of germ-line disruption of DJ-1 in mice on the expressions of renal dopamine receptor D2 (D2R), nuclear factor erythroid 2–related factor 2 (Nrf2), NADH quinone oxidoreductase (NQO1), glutathione S-transferase (GST), and nitro-tyrosine, and blood pressure. Effect of bardoxolone treatment on renal malondialdehyde (MDA) and blood pressure in DJ-1−/− mice.

Figure 5. Nuclear factor erythroid 2–related factor 2 (Nrf2) ubiquitination in DJ-1−/− mice.
Further studies are needed to determine whether modulation of renal DJ-1 and Nrf2 functions is a therapeutic approach in hypertension.

**Perspectives**

Our results show that D$_2$R and DJ-1 are necessary for normal Nrf2 activity in the kidney. Renal selective silencing of D$_2$R or DJ-1 in mice increases ROS production and BP that are mediated, in part, by increased Nrf2 degradation. Further studies are needed to determine whether modulation of renal DJ-1 and Nrf2 functions is a therapeutic approach in hypertension.

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**Disclosures**

None.

**References**


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Short title: DJ-1 regulates Nrf2 degradation in the kidney

ROLE OF NRF2 IN THE OXIDATIVE STRESS-DEPENDENT HYPERTENSION ASSOCIATED WITH THE DEPLETION OF DJ-1


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SUPPLEMENTAL MATERIAL

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Materials and methods.

Immunofluorescence and confocal analysis
Thin sections (3 μm) of formalin-fixed paraffin-embedded mouse kidneys were deparaffinized in xylene and rehydrated with step-down concentrations of ethanol. DJ-1 was visualized using a polyclonal mouse anti-DJ-1 antibody (Santa Cruz Biotech), followed by Alexa Fluor 488-goat anti-mouse IgG antibody (Molecular Probes). Nrf2 was visualized using a polyclonal rabbit anti-Nrf2 antibody (Santa Cruz), followed by Alexa Fluor 568-goat anti-rabbit IgG antibody (Molecular Probes). As a negative control, the primary antibodies were replaced with normal rabbit serum at an appropriate dilution. Colocalization of DJ-1 and Nrf2 was identified by the yellow color in the merged images.

DJ-1 deficient (DJ-1−/−) Mice
The original F2 hybrid strain (129/SvXC57BL/6J, Oregon Health Sciences University) that contained the mutated DJ-1 allele (DJ-1−/−) was backcrossed to wild-type C57BL/6J for >20 generations and genotyped. All mice strains were obtained from Jackson Laboratory (Bar Harbor, ME) and were bred in the Animal Care Facility of the University of Maryland School of Medicine. DJ-1−/− male mice and wild-type littermates (DJ-1+/+) were studied at 6 to 8 months of age. All studies were approved by the Animal Care and Use Committee of the University of Maryland School of Medicine. Systolic blood pressures were measured (Cardiomax II) from the aorta, via the femoral artery, under pentobarbital anesthesia (50 mg/kg IP). Blood pressures were recorded 1 hour after the induction of anesthesia and when the blood pressures were stable. The mice were euthanized (pentobarbital 100 mg/kg) at the conclusion of the study. The organs were harvested and flash-frozen, prior to their preparation for specific studies.

Acute renal-selective downregulation of Drd2 and DJ-1.
Renal cortical Drd2 or DJ-1 was silenced by the renal subcapsular infusion of specific siRNAs, via an osmotic minipump. In brief, adult male C57BL/6J mice were uninephrectomized one week prior to the implantation of osmotic minipumps. For implantation of the minipumps, the mice were anesthetized with pentobarbital (50 mg/kg body weight, intraperitoneally). Osmotic minipumps (ALZET® Osmotic Pump, 100 µl; flow rate 0.5 µl/hr. for 7 days) were filled with previously validated Drd2- or DJ-1- specific siRNA (delivery rate, 3 μg/day) or non-silencing siRNA as control. The siRNAs were dissolved in transfection reagent (TransIT® In Vivo Gene Delivery System, Mirus) under sterile conditions. The minipumps were fitted with polyethylene delivery tubings (Alzet #0007701) and the tip of the tubing was inserted within the subcapsular space of the remaining kidney. Surgical glue was applied at the puncture site to hold the tube in place and prevent extrarenal leakage. The osmotic pump was sutured to the abdominal wall to prevent excessive movement of the pump (10,11).

Cell culture
Undifferentiated mouse RPT cells were cultured from progenitor kidney cells (kindly supplied by Dr. Ulrich Hopfer, Case Western Reserve University, School of Medicine), isolated from mouse embryo kidneys, following the procedure described by Woost et al (42). Differentiated
cells were cultured to 60-70% confluence, transfected using hyperfect (Qiagen) with nonsilencing siRNA (30 nmol/L, Qiagen), DJ-1-siRNA (30 nmol/L, Qiagen), Drd2-siRNA (30 nmol/L, Qiagen), or Nrf2-siRNA (70 nmol/L, Qiagen), and studied 72 hours later. In additional experiments, the cells were cultured to 90-95% confluence, serum-starved for 2 hours and treated for 24 hours with 1 μmol/L quinpirole (D2R agonist, Sigma-Aldrich).

**Immunoblotting**

Mouse kidney homogenates and cell lysates were subjected to immunoblotting, as reported previously (5,11). The primary antibodies used were polyclonal rabbit anti-DJ-1 (NOVUS, #NB300-270), polyclonal rabbit anti-D2R (Millipore, #AB5084P), polyclonal rabbit anti-Nrf2 (Santa Cruz,sc-722), polyclonal goat anti-NQO1 (Santa Cruz,sc-16464), polyclonal rabbit-GST (Cell Signaling, #2622), polyclonal rabbit anti-nitro-tyroxine (Cell Signaling, #9691S), polyclonal rabbit anti-Keap1(Santa Cruz,sc-15246) and monoclonal mouse anti-GAPDH (Millipore,#MAB374). The densitometry values were corrected by the expression of GAPDH.

**Detection of nitrotyrosine by Western blot.**

Mouse kidney homogenates were immunblotted as reported previously (5,11), using a nitrotyrosine antibody to assess the nitro-tyrosine abundance in DJ-1/- mice. To compare the nitro-tyrosine production between D-1/- mice and wild-type littermates, densitometric quantification of all the bands present in the immnublot was performed for each sample. The results of the quantification are shown as integrated values (area × density of the band), corrected for GAPDH density, and expressed as percent of the mean value of wild-type mice.(43,44,45,46)

**Malondialdehyde (MDA)**

MDA was quantified in kidney tissue homogenates in PBS using a commercial Kit (Cell Biolabs, Inc). All assays were performed in duplicate.

**Nrf2 promoter activity**

The Nrf2 promoter activity was measured by a Cignal reporter assay kit (QIAGEN Company). Mouse RPT cells were cotransfected with a mixture of two plasmid constructs, inducible Nrf2 transcription factor responsive element driving the expression of firefly luciferase and constitutively expressing renilla luciferase. Luciferase expression was measured using Dual-Glo luciferase assay system (Dual-Glo luciferase assay system, Promega Corporation). Nrf2 promoter activity was calculated using the luciferase expression; firefly luminescence was normalized to renilla luminescence in each samples.

**Detection of ROS**

Intracellular ROS were assayed by measuring the oxidation of 2′, 7′-dichlorofluorescein diacetate (DCFDA, Molecular Probes). Briefly, cells were incubated with fresh DCFDA (10 μM) in medium for 30 min at 37°C. DCFDA fluorescence was measured using a microplate reader in 96-well plates at an excitation wavelength of 485 nm and emission wavelength of 530 nm. ROS production was expressed in arbitrary units (AU), corrected for protein concentration (AU/per mg protein).
**Determination of NADPH Oxidase Activity**

NADPH oxidase activity (light units per milligram of protein) was determined by measuring NADPH-induced chemiluminescence in the presence of lucigenin (5 μmol/L) and NADPH (100 μmol/L; ICN Biomedicals) in total cell lysates of kidney tissue; Data were normalized by protein concentration (10,11).

**Treatment with bardoxolone.**

Male DJ-1/− mice and male wild-type littermates were injected intraperitoneally for 2 weeks with bardoxolone methyl (CDDO-Me, 10 μmol/L) (0.03 mg/kg, 150μl 3x/wk) or vehicle (47). Blood pressure was measured, as described in the expanded methods (online supplemental material) that also contain descriptions of other methods, including immunofluorescence imaging and measurement of ROS.

**Co-immunoprecipitation**

Renal tissues from male DJ-1/− mice and wild-type littermates were lysed using RIPA buffer. Equal amounts of renal lysates (500 μg of protein) were mixed with polyclonal mouse ubiquitin antibody (Millipore), normal rabbit IgG (Santa Cruz Biotechnology) as negative control, or rabbit polyclonal Nrf2 antibody (Santa Cruz), as positive control. The immune complexes were pelleted out, and the bound proteins were eluted using 30μl of Laemmli buffer. The samples were subjected to immunoblotting and probed with Nrf2 antibody. The experiment was repeated under the same conditions but immunoprecipitated with Nrf2 antibody and immunoblotted with ubiquitin antibody.

**Statistical Analyses**

Data are presented as mean ± SEM. Comparisons between two groups used the Student’s t-test. One-way factorial ANOVA, followed by post-hoc analysis using the Holm-Sidak multiple comparison test, was used to assess significant differences among three or more groups. P<0.05 was considered statistically significant.


Online supplement figures S1 and S2.

Figure S1. Effect of silencing Nrf2 on the protein expressions of Nrf2, NQO1, GST, DJ-1, and D2R and ROS production in mouse RPT cells. (A) Renal-selective Nrf2 silencing in vivo. Renal cortical Nrf2 was silenced as described in materials and methods. Systolic blood pressure was measured (Cardiomax II) from the aorta, via the femoral artery, under pentobarbital anesthesia. Renal cortical homogenates were immunoblotted using a Nrf2 antibody. Actin was used for normalization of the data. Data are expressed as mean ± S.E. n=4/group. *P< 0.05, vs. non-silencing siRNA, t-test. (B) Nrf2, NQO1, GST, DJ-1, and D2R expressions. Mouse RPT cells were studied 72 hours after transfection with Nrf2-specific siRNA or non-silencing siRNA. Total cell lysates were immunoblotted using Nrf2, NQO1, GST, DJ-1, and D2R polyclonal antibodies. Data were normalized by GAPDH. Data are expressed as mean ± S.E. n = 3/group. *P< 0.05 vs. non-silencing siRNA, t-test. (C) ROS production. ROS production was measured using DCFDA, corrected for protein concentration. Data are expressed as mean ± S.E., n = 5/group. *P< 0.05 vs. non-silencing siRNA, t-test.

Figure S2. Effect of D2R stimulation on DJ-1, Nrf2, NQO1, and GST protein expression, Nrf2 promoter activity, and ROS production. (A) DJ-1 protein, and Nrf2, NQO1, and GST mRNA expression. Mouse RPT cells were treated with the D2R/D3R agonist quinpirole (1 μmol/L, 24 h). Total cell lysates were immunoblotted using a DJ-1 antibody. The mRNA
expressions of Nrf2, NQO1, and GST were quantified by qRT-PCR. Data were normalized by GAPDH. Data are expressed as mean ± S.E. n 3-4/group, *P< 0.05 vs. vehicle-treated cells, t-test. (B) Nrf2 promoter activity. The Nrf2 promoter activity was measured by a reporter assay (Qiagen). Data are expressed as mean ± S.E. n= 3-4/group,*P< 0.05 vs. vehicle-treated cells, t-test. (C) ROS production. ROS production was measured using DCFDA and corrected for protein concentration. Data are expressed as mean ± S.E. n = 5/group, *P< 0.05 vs. vehicle-treated cells, t-test.