Role of Renal DJ-1 in the Pathogenesis of Hypertension Associated With Increased Reactive Oxygen Species Production

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Abstract—The D₂ dopamine receptor (D₂R) is important in the pathogenesis of essential hypertension. We have already reported that systemic deletion of the D₂R gene in mice results in reactive oxygen species (ROS)-dependent hypertension, suggesting that the D₂R has antioxidant effects. However, the mechanism of this effect is unknown. DJ-1 is a protein that has antioxidant properties. D₂R and DJ-1 are expressed in the mouse kidney and colocalize and cofunction in mouse renal proximal tubule cells. We hypothesized that D₂Rs regulate renal ROS production in the kidney through regulation of DJ-1 expression or function. Heterozygous D₂R⁻/⁻ mice have increased blood pressure, urinary 8-isoprostanes, and renal Nox 4 expression, but decreased renal DJ-1 expression. Silencing D₂R expression in mouse renal proximal tubule cells increases ROS production and decreases the expression of DJ-1. Conversely, treatment of these cells with a D₂R agonist increases DJ-1 expression and decreases Nox 4 expression and NADPH oxidase activity, effects that are partially blocked by a D₂R antagonist. Silencing DJ-1 expression in mouse renal proximal tubule cells increases ROS production and Nox 4 expression. Selective renal DJ-1 silencing by the subcapsular infusion of DJ-1 siRNA in mice increases blood pressure, renal Nox 4 expression, and NADPH oxidase activity. These results suggest that the inhibitory effects of D₂R on renal ROS production are at least, in part, mediated by a positive regulation of DJ-1 expression/function and that DJ-1 may have a role in the prevention of hypertension associated with increased ROS production. *(Hypertension. 2012;59:00-00.)*

Key Words: dopamine D2 receptor ■ DJ-1 ■ hypertension ■ kidney ■ oxidative stress

Dopamine synthesized in the kidney has an important role in the regulation of fluid and electrolyte balance and systemic blood pressure.¹⁻³ Dopamine exerts its actions via 2 families of G-protein-coupled receptors D1-like receptors (D₁R and D₅R) and D₂-like receptors (D₂R, D₃R, and D₄R). Several lines of evidence show that an intact dopaminergic system is necessary to maintain normal blood pressure and that genetic hypertension is associated with alterations in dopamine production and receptor function.¹⁻⁴

Deletion of any dopamine receptor in mice results in increased blood pressure by mechanisms that are receptor-dependent. In particular, mice lacking the D₂R gene have reactive oxygen species (ROS)-dependent hypertension.⁴ Moreover, dopamine and D₂R agonists have been shown to have antioxidant activity.⁵⁻⁶ D₂R agonists have free radical scavenging and antioxidant activities both in vitro and in vivo.⁵⁻⁶ In vitro and in vivo studies have also shown that the protective effects of the D₂R are abolished in the presence of D₂R antagonists, indicating receptor specificity.⁷⁻⁸ However, the mechanisms involved in the antioxidant effects of the D₂Rs are not known.

DJ-1 (also known as Park 7) is a protein originally described as an oncogene.⁹ It is present in most rodent and human tissues, such as the brain, heart, kidney, liver, pancreas, and skeletal muscle.⁹ DJ-1 was also identified as an autosomal-recessive gene of Parkinson disease. DJ-1 is a multifunctional oxidative stress response protein that defends cells against ROS and mitochondrial damage.¹⁰ Its protective role against oxidative stress has been demonstrated in several pathological disease models both in vitro and in vivo.¹¹⁻¹⁴ However, the physiological role of the DJ-1 in the kidney is unknown.

We hypothesized that DJ-1 is involved in the antioxidant activity of renal D₂R. In this study, we found that D₂R physically interacts with DJ-1 and regulates the expression of the DJ-1 in the kidney. Our in vitro and in vivo data show that the inhibitory effect of D₂R on renal ROS production is at least, in part, mediated by regulating DJ-1 expression and function. These findings indicate an essential role of DJ-1 in the increase in blood pressure associated with oxidative stress.

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Materials and Methods

D2R-Deficient Mice

The original F2 hybrid strain (129/SvxC57BL/6J; Oregon Health Sciences University, Portland, OR) that contained the mutated Drd2 allele (D2<sup>−/−</sup>) was backcrossed to wild-type C57BL/6J for >20 generations and genotyped. All mice were bred in the Animal Care Facility of the Children’s Research Institute, Children’s National Medical Center. D2<sup>−/−</sup> mice and wild-type littermates (D2<sup>+/+</sup>) were studied at 6 to 8 months of age; D2<sup>−/−</sup> mice were used similar to D2<sup>−/−</sup> mice, they have high blood pressure<sup>14</sup> and increased oxidative stress but they do not have increased aldosterone production as do D2<sup>−/−</sup> mice.<sup>13</sup> We wanted to study the role of DJ-1 and D2R on renal oxidative stress without the confounding effect of increased aldosterone levels. All studies were approved by the Animal Care and Use Committee of the Children’s Research Institute/Children’s National Medical Center. Mice were housed in metabolic cages the day before blood pressure measurement for collection of 24-hour urine samples. Systolic blood pressures were measured (Cardiomax II; Columbus Instruments, Columbus, OH) from the aorta, via the femoral artery, under pentobarbital anesthesia (50 mg/kg intraperitoneally). 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 before their preparation for specific studies.

Acute Renal-Specific Downregulation of DJ-1

Renal cortical DJ-1 was silenced by the subcapsular infusion of DJ-1-specific siRNA via an osmotic minipump. In brief, adult male C57BL/6J mice were uninephrectomized 1 week before 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, Alzet, Sandy, UT) were filled with previously validated DJ-1-specific siRNA (delivery rate 3 µg/d) or nonsilencing siRNA as control. The siRNAs were dissolved in an in vivo transfection reagent (TransIT In Vivo Gene Delivery System; Mirus, Madison, WI) under sterile conditions. The minipumps were fitted with polyelectrolyte delivery tubings (Alzet 0007T01) and the tip of the tubing was inserted within the subcapsular space of the remaining kidney. Surgical glue (Histoacryl, B Braun, Tutmoss, Germany) was applied at the puncture site to hold the tube in place and prevent extraneous leakage. The osmotic pump was sutured to the abdominal wall to prevent puncture site to hold the tube in place and prevent extrarenal leakage.

Determination of NADPH Oxidase Activity

NADPH oxidase activity was determined by measuring NADPH-induced chemiluminescence in the presence of lucigenin (5 µmol/L; Invitrogen, Carlsbad, CA) and NADPH (100 µmol/L; ICN Biomedicals, Irvine, CA).<sup>15</sup> The specificity of the NADPH-dependent superoxide anion production was verified by treatment with diphenylene iodium (Sigma Aldrich, St Louis, MO).

Cell Culture

Undifferentiated mouse renal proximal tubule cells were cultured from progenitor kidney cells (kindly supplied by Dr Ulrich Hopfer, Case Western Reserve University, School of Medicine, Cleveland, OH) isolated from mouse embryo kidneys, following the procedure described by Woost et al.<sup>17</sup> Differentiated cells were cultured to 60% to 70% confluence and transfected using Hyperfect (Qiagen, Germantown, MD) with nonsilencing siRNA (30 nmol/L; Qiagen) or Drd2 siRNA (30 nmol/L; Qiagen) and studied after 72 hours. In additional experiments, cells cultured to 90% to 95% confluence were serum-starved for 2 hours and treated for 24 hours with 1 µmol/L quinpirole (D2R/D3R agonist; Sigma-Aldrich) or 1 µmol/L quinpirole plus 1 µmol/L L-741,262 (selective D2R antagonist; Sigma-Aldrich).<sup>18,19</sup>

Immunofluorescence and Confocal Analysis

Thin sections (3 µm) of formalin-fixed paraffin-embedded mouse kidney 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, Santa Cruz, CA), followed by Alexa Fluor 568 goat antimouse IgG antibody (Molecular Probes, Eugene, OR). D2R was visualized using a polyclonal rabbit anti-D2R antibody (Millipore, Billerica, MA), followed by Alexa Fluor 568 goat antimouse IgG antibody (Molecular Probes). As a negative control, the primary antibodies were replaced with normal rabbit serum at an appropriate dilution. Colocalization of D2R and DJ-1 was identified by the yellow color in the merged images.

Immunoblotting

Mouse kidney homogenates and cell lysates were subjected to immunoblotting, as reported previously.<sup>4,15,16</sup> The primary antibodies used were polyclonal rabbit anti-DJ-1 (NB300-270, Novus, Littleton, CO), polyclonal rabbit anti-D2R (AB5084P, Millipore), polyclonal rabbit anti-Nox4 (318701, Epitomics, Burlingame, CA), and monoclonal mouse anti-GAPDH (MAB374, Millipore). The densitometry values were corrected by the expression of GAPDH.

Detection of ROS

Intracellular ROS were assayed through the oxidation of 2',7'-dichlorofluorescein diacetate (Molecular Probes). Briefly, cells were incubated with fresh dichlorofluorescein diacetate (10 µmol/L) in medium for 30 minutes at 37°C. Dichlorofluorescein diacetate 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 corrected for protein concentration (arbitrary units/µg protein). All assays were performed in duplicate.

Comunoprecipitation

Serum-starved mouse renal proximal tubule cells were lysed using RIPA lysis buffer. Equal amounts of cell lysates (500 µg of protein) were mixed with polyclonal rabbit anti-D2R antibody (Millipore) or normal rabbit IgG (Santa Cruz Biotech) as negative control, or polyclonal rabbit anti-DJ-1 antibody (Novus) as positive control. The immune complexes were pelleted out, and the bound proteins were eluted using 30 µL of Laemmli buffer. The samples were subjected to immunoblotting and probed with the rabbit anti-DJ-1 antibody.

Statistical Analysis

Data are mean±SEM. Comparisons between 2 groups used the Student t test. One-way analysis of variance was followed by post hoc analysis using the Holm-Sidak multiple comparison test to assess significant differences among ≥3 groups. P<0.05 was considered statistically significant.

Results

DJ-1 Is Expressed in the Mouse Kidney and Physically Interacts With D2R

DJ-1 is expressed in the mouse kidney as shown by immunofluorescence staining and a specific band in Western blots (Figures 1 and 2). In the mouse renal cortex, DJ-1 is expressed mainly in the brush border and the cytosol of proximal tubule cells with minimal expression in distal tubule cells. In the proximal tubule, DJ-1 partially colocalizes with the D2R (Figure 1A). Furthermore, DJ-1 and D2R physically interact in the mouse kidney, as shown by the bands corresponding to the molecular size of DJ-1 in the mouse kidney.
samples after immunoprecipitation with an anti-D2R antibody and immunoblotting with an anti-DJ-1 antibody (Figure 1B).

Expression of DJ-1 Is Increased in Mice With Decreased D2R Expression

To determine the role of DJ-1 in the antioxidant effect of D2R, we studied mice with heterozygous deletion of D2R allele (D2R+/−). As mentioned, D2R−/− mice were used to avoid the confounding effect of increased aldosterone because they have high blood pressure and increased oxidative stress but urinary aldosterone excretion in D2R−/− mice is similar to those in wild-type littermates (4.3 ± 1.5 vs 5.2 ± 1.3 ng/d, respectively) and lower than those in D2R+/− mice, indicating that increased oxidative stress in D2R−/− mice is independent of aldosterone. The expression of D2R in the renal cortex of D2R−/− mice is decreased to 75% relative to wild-type littermates. This is in agreement with previous reports indicating that in the brain the mutated Drd2 allele is dominant-negative. Both systolic and diastolic blood pressures, measured under anesthesia, were increased in D2R−/− mice compared to their wild-type littermates (systolic, 126 ± 6 vs 92 ± 4 mm Hg; diastolic, 97 ± 5 vs 60 ± 3 mm Hg; Figure 2), in agreement with our previous reports. In D2R−/− mice, the urinary excretion of 8-isoprostane was increased by 71% and similar to homozygous D2R−/− mice. Also in agreement with our previous results in D2R−/− mice, the urinary excretion of 8-isoprostane was increased by 71% and the renal cortical expression of the NADPH-oxidase subunit Nox 4 was increased by 125% in D2R−/− mice. In contrast the DJ-1 expression in the renal cortex was decreased by 30% in D2R−/− mice, suggesting that D2R may regulate DJ-1 expression.

Silencing the D2R in Mouse Renal Proximal Tubule Cells Decreases DJ-1 Expression and Increases NADPH Oxidase Activity

To confirm the results obtained in D2R−/− mice, we silenced D2R expression in mouse renal proximal tubule cells using D2R siRNA. D2R expression was decreased by 70%, whereas DJ-1 expression was decreased by 60%. This was associated with an increase (180%) in ROS production (Figure 3A).

D2R Stimulation Increases DJ-1 Expression and Decreases Nox4 Expression and NADPH Oxidase Activity in Mouse Renal Proximal Tubule Cells

In mouse renal proximal tubule cells, treatment with the D2R agonist quinpirole increased DJ-1 expression by ~40%. This effect was partially blocked by a selective D2R antagonist. The treatment also modestly decreased NADPH oxidase activity (15%) and Nox4 expression (21%). These data...
suggest that D2R may have a role in regulating DJ-1 expression that may affect Nox4 expression and NADPH oxidase activity (Figure 3B). The greater effect of D2R silencing than D2R stimulation on ROS production, Nox4 expression, and NADPH oxidase activity could be related to difference in degree of change in DJ-1 expression (70% vs 40%).

Silencing DJ-1 Expression Increases ROS Production and Nox4 Expression in Mouse Renal Proximal Tubule Cells
To determine if part of the antioxidant effect of the D2R is through regulation of DJ-1 expression/function, we silenced DJ-1 expression in mouse renal proximal tubule cells using DJ-1 siRNA. The treatment decreased DJ-1 expression by 60% and increased ROS production by 70%, as well as the expression of Nox 4 by 61%, supporting a role of DJ-1 in the regulation of renal Nox4 expression and ROS production (Figure 4). The lesser increase in ROS production with DJ-1 silencing (60%) relative to D2R silencing (180%) could be taken to suggest that the antioxidant effect of D2R can only be partially explained by DJ-1.

Selective Renal Silencing of DJ-1 Expression in Mice Increases Renal Nox4 Expression, Renal NADPH Oxidase Activity, and Systolic Blood Pressure
To determine the renal effects of DJ-1 on oxidative stress and systemic blood pressure, we selectively silenced DJ-1 expression in the mouse kidney by the subcapsular infusion of DJ-1 siRNA for 7 days. The infusion decreased DJ-1 expression by 30% and increased Nox4 expression by 50%, which was associated with a 380% increase in NADPH oxidase activity. It should be noted that prolonged (7 days) siRNA treatment had a greater effect in decreasing DJ-1 expression and increasing Nox4 expression and NADPH oxidase activity than acute (24 hours) treatment. The infusion also resulted in a 20% increase in systolic blood pressure measured under anesthesia (Figure 5), suggesting that deficient DJ-1 expres-
sion or function may result in increased renal ROS production and subsequently in increased blood pressure. The percent increase in renal NADPH oxidase activity was greater than the percent increase in blood pressure. This could be taken to suggest that the antihypertensive effect of D2R can only be partially explained by its inhibitory effect on ROS production. The D2R can negatively regulate sodium transport21 that may or may not be related to antioxidant mechanisms.

Discussion

The present results provide evidence that DJ-1 is expressed in the kidney, mainly in proximal tubules, and is regulated by D2R. We have already reported that disruption of D2R in mice causes hypertension that is associated with increased ROS production and oxidative stress, suggesting that D2R negatively regulates ROS production.4 We now report that DJ-1 mediates at least, in part, the antioxidant effects of the D2R in the kidney.

Our results provide new evidence that lack of just 1 allele of the D2R results in an increase in the expression of the NADPH oxidase isoform Nox4 and excretion of 8-isoprostane, similar to what we have already reported in mice lacking both D2R alleles.4 We have also reported that the lack of 1 or both D2R alleles increases blood pressure to a similar extent.15

Stimulation of D2Rs is associated with increased DJ-1 expression. Although we have used the mixed D2R-D3R agonist, quinpirole, to determine the effects of D2R stimulation on the expression of DJ-1, we have ruled out a significant effect of the D3R for 2 reasons: (1) the effects of quinpirole on DJ-1 expression are almost completely blocked by the specific D3R antagonist L741,626, with minimal D2R antagonism and (2) we have evidence that the D3R is not involved in the regulation of oxidative stress in mice. Mice with deletion of the D3R do not have increased oxidative stress in the kidney, increased excretion of 8-isoprostane, or production of ROS (unpublished observation).

The mechanisms by which D2R regulates the expression of DJ-1 are unknown. The D2R may directly increase DJ-1 expression by activation of the MAP kinase pathway through activation of ERK1/2,22 a pathway that has been shown to upregulate DJ-1 expression both in vivo and in vitro.23 Nevertheless, the negative regulation of ROS by D2R may be related to its positive regulation of DJ-1.

DJ-1 belongs to a protein superfamily that includes archetypical bacterial ThiJ and PfpI24 and in vertebrates is expressed in a variety of tissues, including the brain, kidney, liver, pancreas, and skeletal muscle.9 DJ-1 is a ubiquitous redox-responsive cytoprotective protein that has been associated with oncogenesis, control of gene transcription, and regulation of mRNA stability,25 and acts as an antioxidant and antiapoptotic transcriptional modulator.24 Downregulation of DJ-1 expression in renal proximal tubule cells is associated with increased Nox4 expression and ROS production, suggesting that in these cells DJ-1 also has antioxidant effects. DJ-1 exerts its antioxidant effects at several levels, although it may have intrinsic activity as an atypical peroxiredoxin-like peroxidase that plays a role in scavenging mitochondrial ROS.26 However, most of the antioxidant effects of DJ-1 are attributable to its ability to increase the expression of other antioxidant genes, such as superoxide dismutase and heme oxygenase-1 during oxidative stress27 and the modulation of Akt activation and ERK1/2 signaling, key signaling pathways in the modulation of the oxidative response.28–30 Therefore, the increase in ROS production in renal proximal tubule cells when DJ-1 is silenced may be related to decreased activity of antioxidant enzymes and also increased expression/activity of prooxidant enzymes, eg, Nox4. DJ-1 silencing in renal proximal tubule cells increases ROS production to a lesser extent than does downregulation of D2R, suggesting that mechanisms other than that related to DJ-1 are involved in the antioxidant effect of D2R in the kidney.
The role of oxidative stress in the pathogenesis of hypertension has been extensively studied.\textsuperscript{31,32} The present study shows that renal selective downregulation of DJ-1 expression in mice is associated with increased Nox4 expression, NADPH oxidase activity, and a 20% increase in blood pressure. The greater percent increase in renal NADPH oxidase activity than the percent increase in blood pressure could be taken to suggest that the antihypertensive effect of D2R can only be partially explained by its inhibitory effect on ROS production.

Several enzymes and signaling pathways are involved in the antioxidant function of DJ-1 in tissues other than the kidney.\textsuperscript{28,33–35} However, there are no reports on the effect of DJ-1 on NADPH oxidase expression or function. Our results suggest that DJ-1 may directly or indirectly act as an inhibitor of Nox 4 transcription/translation; the mechanisms by which these occur remain to be determined. It is possible that changes in the redox state of the cells could indirectly be responsible for changes in Nox4 expression because increases in ROS are known to increase Nox4 transcription.\textsuperscript{36,37}

In summary, our results show that the inhibitory effects of D2R on renal ROS production are at least, in part, mediated by positive regulation of DJ-1 expression or function, which in turn is involved in decreasing NADPH oxidase expression/activity. This is the first report providing evidence of a role of renal DJ-1 in the regulation of oxidative stress and ROS-dependent hypertension.

**Perspectives**

The results of the present study show that DJ-1 may have an important role in the regulation of the antioxidant activity in the kidney. DJ-1 appears to have protective effects in the kidney by dampening oxidative stress that can cause hypertension and kidney disease. Further studies are needed to establish whether modulation of renal DJ-1 function is a therapeutic approach in hypertension.

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**Disclosures**

None.

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