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Role of renal DJ-1 in the Pathogenesis of Hypertension Associated with Increased ROS Production

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Abstract

The D_2 dopamine receptor (D_2R) is important in the pathogenesis of essential hypertension. We have already reported that systemic deletion of the D_2R gene in mice results in reactive oxygen species (ROS)-dependent hypertension, suggesting that the D_2R has antioxidant effect. However, the mechanism of this effect is unknown. DJ-1 is a protein which has antioxidant properties. D_2R and DJ-1 are expressed in the mouse kidney and colocalize and co-imunoprecipitate in mouse renal proximal tubule cells. We hypothesized that D_2Rs regulate renal ROS production in the kidney through regulation of DJ-1 expression or function. Heterozygous $D_2+/-$ mice have increased blood pressure, urinary 8-isoprostanes, and renal Nox 4 expression, but decreased renal DJ-1 expression. Silencing D_2R expression in mouse renal proximal tubule cells increases ROS production and decreases the expression of DJ-1. Conversely, treatment of these cells with a D_2R agonist increases DJ-1 expression and decreases Nox 4 expression and NADPH oxidase activity, effects that are partially blocked by a D_2R 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, and renal Nox 4 expression and NADPH oxidase activity. These results suggest that the inhibitory effects of D_2R 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.

Keywords

DJ-1; oxidative stress; dopamine D2 receptor; kidney; hypertension

Introduction

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 two families of G protein-couple receptors D1-like receptors (D_1R and D_5R) and D2–like receptors (D_2R, D_3R, and D_4R). Several lines of evidence show that an intact dopaminergic

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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 D_2R gene have reactive oxygen species (ROS)-dependent hypertension (4). Moreover, dopamine and D_2R agonists have been shown to have antioxidant activity (5, 6). D_2R agonists have free radical scavenging and antioxidant activities both *in vitro* and *in vivo* (7,8). *In vitro* and *in vivo* studies have also shown that the protective effects of the D_2R are abolished in the presence of D_2R antagonists, indicating receptor specificity (9, 10). However the mechanisms involved in the antioxidant effects of the D_2Rs are not known.

DJ-1 (also known as Park 7) is a protein originally described as an oncogene (11). It is present in most rodent and human tissues, such as the brain, heart, kidney, liver, pancreas, and skeletal muscle (11). DJ-1 was also identified as an autosomal recessive gene of Parkinson's disease. DJ-1 is a multifunctional oxidative stress response protein that defends cells against ROS and mitochondrial damage (12). Its protective role against oxidative stress has been demonstrated in several pathological disease models both *in vitro* and *in vivo* (13-16). 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 D_2R. In this study, we found that D_2R physically interacts with DJ-1 and regulates the expression of the DJ-1 in the kidney. Our *in vitro* and *in vivo* studies show that the inhibitory effect of D_2R 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 to oxidative stress.

Materials and methods

D_2R-deficient Mice ($D_2+/-$)

The original F2 hybrid strain (129/SvXC57BL/6J, Oregon Health Sciences University) that contained the mutated *Drd2* allele ($D_2+/-$) 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 (CRI), Children's National Medical Center (CNMC). $D_2+/-$ mice and wild-type littermates ($D_2+/+$) were studied at 6 to 8 months of age; $D_2+/-$ mice were used because similar to $D_2-/-$ mice they have high blood pressure (17) and increased oxidative stress but do not have increased aldosterone production as do $D_2-/-$mice (4). We wanted to study the role of DJ-1 and D_2R 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 CRI/CNMC. Mice were housed in metabolic cages the day before blood pressure measurement for collection of 24-h urine samples. Systolic blood pressures were measured (Cardiomax II, Instruments) from the aorta, via the femoral artery, under pentobarbital anesthesia (50 mg/kg IP). Blood pressures were recorded 1 h 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-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 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 *DJ-1*-specific siRNA (delivery rate 3 μg/day) or non-silencing siRNA as control. The siRNAs were dissolved in an *in vivo* 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.

Urinary isoprostane

Urinary 8-isoprostane, an index of oxidative stress, was determined by enzyme immunoassay (Cayman Chemical Company). Values were corrected for urinary creatinine.

Determination of NADPH oxidase activity

NADPH oxidase activity was determined by measuring NADPH-induced chemiluminescence in the presence of lucigenin (5 μmol/L, Invitrogen) and NADPH (100 mol/L, ICN Biomedicals) (18). The specificity of the NADPH-dependent superoxide anion production was verified by treatment with diphenylene iodinium (DPI, Sigma).

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), isolated from mouse embryo kidneys, following the procedure described by Woost et al. (19). Differentiated cells were cultured to 60-70% confluence and transfected using Hyperfect (Qiagen,) with non-silencing siRNA (30 nmol/L, Qiagen) or *Drd2* siRNA (30 nmol/l, Qiagen) and studied after 72 h. In additional experiments, cells cultured to 90-95 % confluence were serum-starved for 2 h and treated for 24 h with 1 μmol/L quinpirole (D_2R/D_3R agonist, Sigma-Aldrich), or 1 μmol/L quinpirole plus 1 μmol/L L-741,262 (selective D_2R antagonist, Sigma-Aldrich)(20,21).

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), followed by Alexa Fluor 488-goat anti-mouse IgG antibody (Molecular Probes). D_2R was visualized using a polyclonal rabbit anti-D_2R antibody (Millipore), 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 D_2R 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 (4, 17, 18). The primary antibodies used were polyclonal rabbit anti-DJ-1 (NOVUS, #NB300-270), polyclonal rabbit anti-D_2R (Millipore, #AB5084P), polyclonal rabbit anti-Nox4 (Epitomics, #3187-1) and monoclonal mouse anti-GAPDH (Millipore,#MAB374). 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 (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). All assays were performed in duplicate.

Co-immunoprecipitation

Serum-starved mouse renal proximal tubule cells were lyzed using RIPA lysis buffer. Equal amounts of cell lysates (500 μg of protein) were mixed with polyclonal rabbit anti-D_2R antibody (Millipore Laboratories), or normal rabbit IgG (Santa Cruz Biotechnology) 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 two groups used the Student's t test. One-way ANOVA was followed by post-hoc analysis using the Holm-Sidak multiple comparison test to assess significant differences among three or more groups. $P<0.05$ was considered statistically significant.

Results

DJ-1 is expressed in the mouse kidney and physically interacts with D_2R

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 D_2R (Figure 1A). Furthermore, DJ-1 and D_2R 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-D_2R antibody and immunoblotting with an anti-DJ-1 antibody (Figure 1B).

The expression of DJ-1 is increased in mice with decreased D_2R expression

To determine the role of DJ-1 in the antioxidant effect of D_2R we studied mice with heterozygous deletion of D_2R allele ($D_2+/-$). As mentioned above $D_2+/-$ mice were used to avoid the confounding effect of increased aldosterone since they have high blood pressure and increased oxidative stress but urinary aldosterone excretion in $D_2+/-$ mice is similar to those in wild-type littermates (4.3±1.5 vs 5.2±1.3 ng/day, respectively) and lower than those in $D_2-/-$ mice (4), indicating that increased oxidative stress in $D_2+/-$ mice is independent of aldosterone. The expression of D_2R in the renal cortex of $D_2+/-$ mice is decreased about 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 (22) Both systolic and diastolic blood pressures, measured under anesthesia, were increased in $D_2+/-$ mice compared to their wild-type littermates (systolic, 126±6 vs. 92±4 mmHg; diastolic, 97±5 vs. 60±3 mmHg) (Figure 2), in agreement with our previous report (17) and similar to homozygous $D_2-/-$ mice (4). Also in agreement with our previous results in $D_2-/-$ mice (4), 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 $D_2+/-$ mice. In contrast the DJ-1 expression in the renal cortex was decreased by 30% in $D_2+/-$ mice, suggesting that D_2R may regulate DJ-1 expression.

Silencing the D_2R in mouse renal proximal tubule cells decreases DJ-1 expression and increases NADPH oxidase activity

To confirm the results obtained in D_2+/− mice, we silenced D_2R expression in mouse renal proximal tubule cells using D_2R siRNA. D_2R expression was decreased by 70% while DJ-1 expression was decreased by 60%. This was associated with an increase (180%) in ROS production (Figure 3A).

D_2R 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 D_2R agonist quinpirole increased DJ-1 expression by about 40%. This effect was partially blocked by a selective D_2R antagonist. The treatment also modestly decreased NADPH oxidase activity (15%) and Nox4 expression (21%). These data suggest that D_2R may have a role in regulating DJ-1 expression that may affect Nox4 expression and NADPH oxidase activity (Figure 3B). The greater effect of D_2R silencing than D_2R 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 D_2R 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 D_2R silencing (180%) could be taken to suggest that the antioxidant effect of D_2R 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 d) siRNA treatment had a greater effect in decreasing DJ-1 expression and increasing Nox4 expression and NADPH oxidase activity than with acute (24 h) treatment. The infusion also resulted in a 20% increase in systolic blood pressure measured under anesthesia (Figure 5), suggesting that deficient DJ-1 expression 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 anti-hypertensive effect of D_2R can only be partially explained by its inhibitory effect on ROS production. Indeed, the D_2R can negatively regulate sodium transport (23) 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 D_2R. We have already reported that disruption of D_2R in mice causes hypertension that is associated with increased ROS production and oxidative

stress suggesting that D_2R negatively regulates ROS production (4). We now report that DJ-1 mediates at least, in part, the antioxidant effects of the D_2R in the kidney.

Our results provide new evidence that lack of just one allele of the D_2R 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 D_2R alleles (4). We have also reported that the lack of one or both D_2R alleles increases blood pressure to a similar extent (17).

Stimulation of D_2Rs is associated with increased DJ-1 expression. Although we have used the mixed D_2R-D_3R agonist, quinpirole, to determine the effects of D_2R stimulation on the expression of DJ-1 we have ruled out a significant effect of the D_3R for two reasons: 1) the effects of quinpirole on DJ-1 expression are almost completely blocked by the specific D_2R antagonist L741,626, with minimal D_3R antagonism, and 2) we have evidence that the D_3R is not involved in the regulation of oxidative stress in mice; mice with deletion of the D_3R do not have increased oxidative stress in the kidney, increased excretion of 8-isoprostane or production of ROS (unpublished observation)

The mechanisms by which D_2R regulates the expression of DJ-1 are unknown. The D_2R may directly increase DJ-1 expression by activation of the MAP kinase pathway through activation of ERK1/2 (24), a pathway that has been shown to upregulate DJ-1 expression both *in vivo* and *in vitro* (25). Nevertheless, the negative regulation of ROS by D_2R may be related to its positive regulation of DJ-1.

DJ-1 belongs to a protein superfamily which includes archetypical bacterial Thij and Pfpl (26) and in vertebrates is expressed in a variety of tissues, including the brain, kidney, liver, pancreas, and skeletal muscle (11). DJ-1 is a ubiquitous redox-responsive cytoprotective protein that has been associated with oncogenesis, control of gene transcription, and regulation of mRNA stability (27), and acts as an antioxidant and antiapoptotic transcriptional modulator (26). 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 (28). However, most of the antioxidant effects of DJ-1 are due to its ability to increase the expression of other antioxidant genes, such as superoxide dismutase and heme oxygenase-1 during oxidative stress (29) and the modulation of Akt activation and ERK1/2 signalling, key signalling pathways in the modulation of the oxidative response (30-32). 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, e.g., Nox4. DJ-1 silencing in renal proximal tubule cells increases ROS production to a lesser extent than does downregulation of D_2R suggesting that mechanisms other than that related to DJ-1 are involved in the antioxidant effect of D_2R in the kidney.

The role of oxidative stress in the pathogenesis of hypertension has been extensively studied (33, 34). 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 anti-hypertensive effect of D_2R can only be partially explained by its inhibitory effect on ROS production.

Several enzymes and signalling pathways are involved in the antioxidant function of DJ-1 in tissues other than the kidney (30, 35-37). 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 since increases in ROS are known to increase Nox4 transcription (38, 39).

In summary our results show that the inhibitory effects of D_2R 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 or not modulation of renal DJ-1 function is a therapeutic approach in hypertension.

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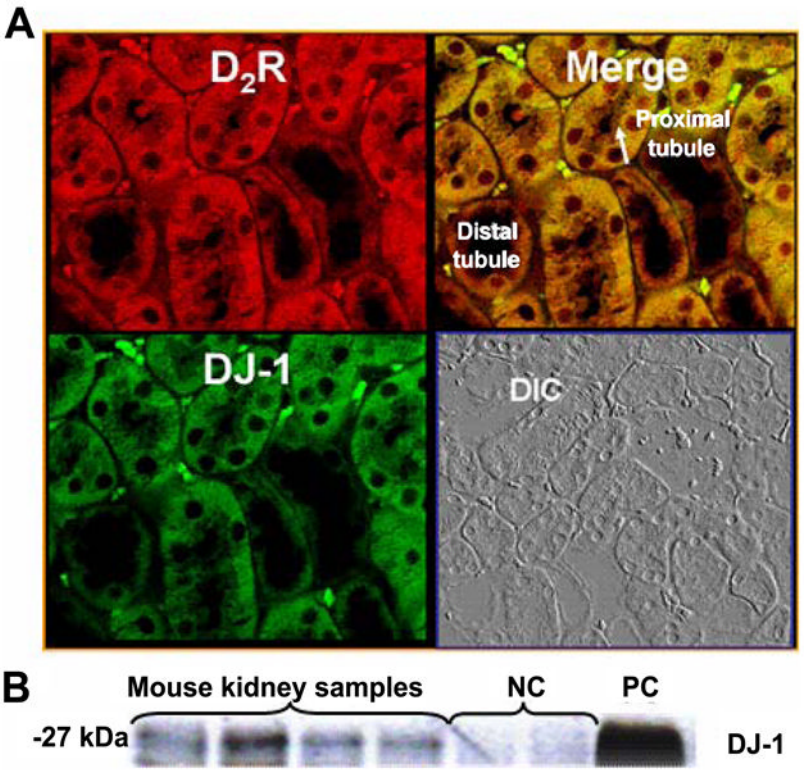

Figure 1. Colocalization and co-inmunoprecipitation of D_2R and DJ-1 in mouse kidney

(A) Formalin-fixed, paraffin-embedded kidney sections of mouse were prepared to determine *in vivo* colocalization of D_2R (pseudocolored *red*) and DJ-1 (pseudocolored *green*) by confocal microscopy. Differential interference contrast (*DIC*) images were also obtained to show cell integrity and boundaries. The arrow indicates the brush border membrane. The colocalization of D_2R and DJ-1 (shown as yellow in merge images) was found in the cytoplasm and brush border of proximal tubules. *Scale bar,* 10 μm, ×600 magnification, $n = 3–5$ independent experiments.

(B) The D_2R and DJ-1 physically interact in mouse kidney. Total mouse kidney lysates were immunoprecipitated with anti-D_2R antibody and immunoblotted with anti-DJ-1 antibody. Negative control (NC), immunoprecipitant is rat IgG and positive control (PC); immunoprecipitant is anti-DJ-1 antibody. The bands that appear in the mouse kidney samples show physical interaction between D_2R and DJ-1.

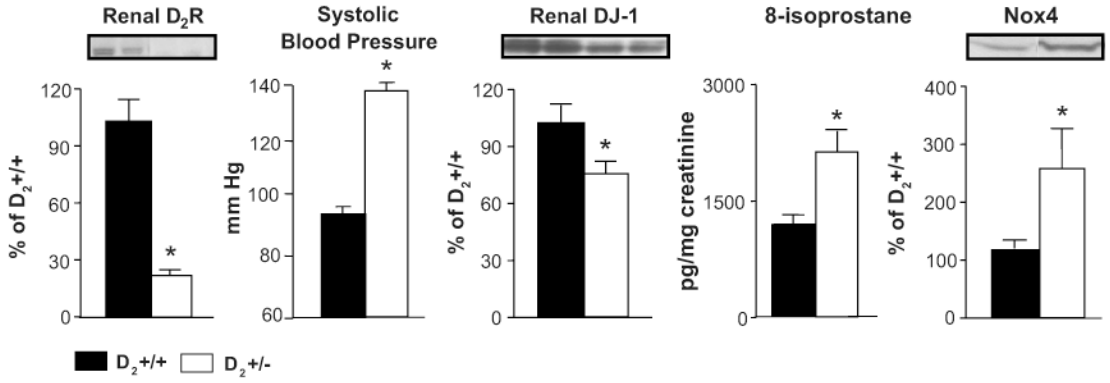

Figure 2. Expression of renal D_2R, systolic blood pressure, renal DJ-1 expression, urinary excretion of 8-isoprostane, and renal Nox4 expression in D_2+/− mice and their wild-type littermates

Renal cortical homogenates were immunoblotted using D_2R, DJ-1, or Nox4 specific antibodies; GAPDH was used for normalization of the data. Urinary 8-isoprostane was determined by enzyme immunoassay and values were corrected for urinary creatinine. Systolic blood pressure was measured from the aorta, via the femoral artery, under pentobarbital anesthesia. Data are expressed as mean ± S.E. n=6 in each group. *, $P< 0.05$ vs. wild-type, t-test,

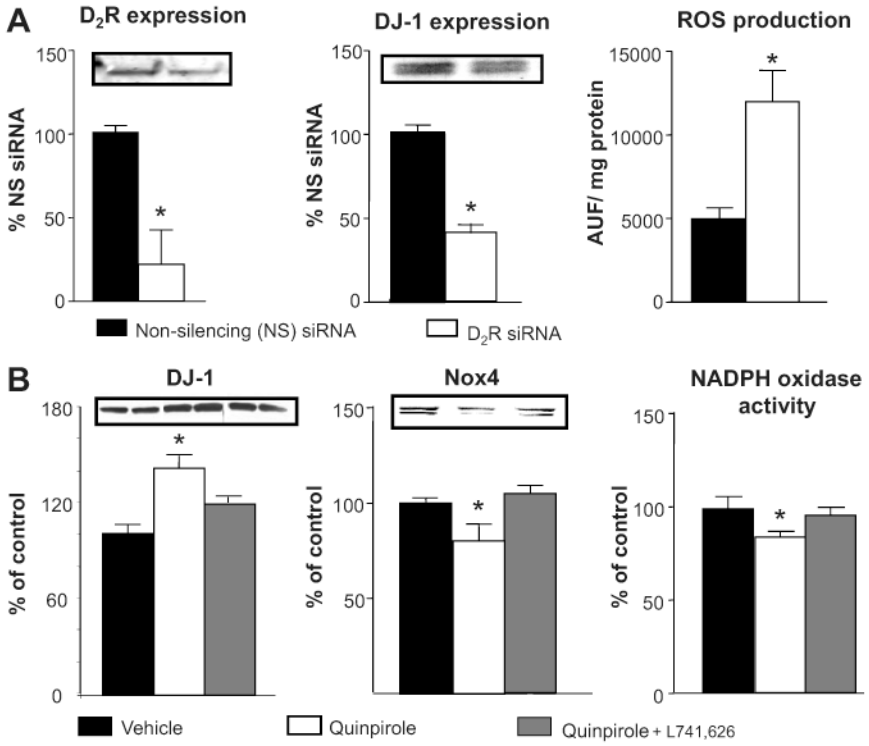

Figure 3. Effect of silencing or stimulation of D_2R on the expression of DJ-1, production of ROS, Nox4 expression, and NADPH oxidase activity in mouse renal proximal tubule cells

(**A**) Mouse renal proximal tubule cells were studied 72 h after transfection with D_2R specific siRNA or non-silencing siRNA.

(**B**). Mouse renal proximal tubule cells were treated with a D_2R agonist, quinpirole (1 μM), in the presence or absence of a D_2R antagonist, L741,626 (1 μM), for 24 h. Total cell lysates were immunoblotted using D_2R, DJ-1, or Nox4 specific antibodies. GAPDH was used for normalization of the immunoblots. ROS production was measured using dichlorofluorescein diacetate (DCFDA). NADPH oxidase activity was quantified by measuring NADPH-induced chemiluminescence in the presence of lucigenin (5 μmol/L) and NADPH (100 μmol/L). Data are expressed as mean ± S.E. *, $P< 0.05$, vs. others, t-test or one-way ANOVA and Holm-Sidak post hoc test, $n = 3$-5 independent experiments

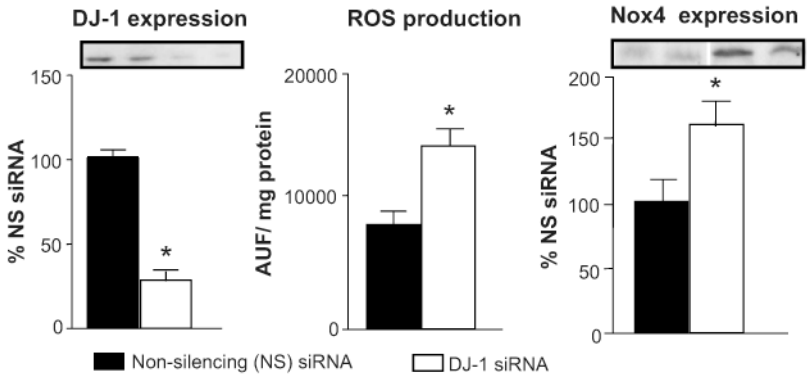

Figure 4. Effect of DJ-1 silencing on ROS production and Nox4 expression in mouse renal proximal tubule cells

Mouse renal proximal tubule cells were transfected with DJ-1-specific siRNA or non-silencing siRNA. Total cell lysates were immunoblotted using DJ-1 or Nox4 specific antibodies. GAPDH was used for normalization of the immunoblotting data. ROS production was measured using dichlorofluorescein diacetate (DCFDA). Data are expressed as mean ± S.E. *, P< 0.05, t –test, n = 3-5 independent experiments.

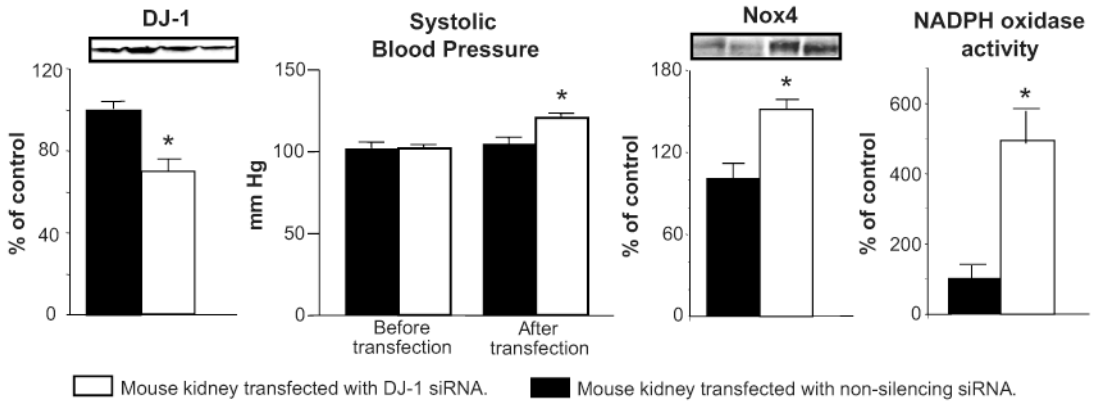

Figure 5. Effect of renal subcapsular infusion of DJ-1 siRNA on DJ-1 and Nox4 expression in the kidney, renal NADPH oxidase activity, and systolic blood pressure

Renal cortical DJ-1 was silenced by a 7-day subcapsular infusion of DJ-1-specific siRNA via an osmotic minipump. Adult male C57BL/6J mice were uninephrectomized one week before implantation of the minipump. Systolic blood pressures were measured from the aorta, via the femoral artery, under anesthesia. Kidney tissue homogenates were immunoblotted using DJ-1 and Nox4 specific antibodies. GAPDH was used for normalization of the immunoblotting data. NADPH oxidase activity was determined by measuring NADPH-induced chemiluminescence, in the presence of lucigenin (5 μmol/L) and NADPH (100 μ mol/L). Data are expressed as mean ± S.E. *P< 0.05, -test or one-way ANOVA and Holm-Sidak post hoc test, n = 3-4 independent experiments