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Paraoxonase 2 decreases renal reactive oxygen species production, lowers blood pressure, and mediates dopamine D₂ receptor-induced inhibition of NADPH oxidase

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Abstract

The dopamine D₂ receptor (D₂R) regulates renal reactive oxygen species (ROS) production and impaired D₂R function results in ROS-dependent hypertension. Paraoxonase 2 (PON2), which belongs to the paraoxonase gene family, is expressed in various tissues, acting to protect against cellular oxidative stress. We hypothesized that PON2 may be involved in preventing excessive renal ROS production and thus may contribute to maintenance of normal blood pressure. Moreover, the D₂R may decrease ROS production, in part, through regulation of PON2.

D₂R co-localized with PON2 in the brush border of mouse renal proximal tubules. Renal PON2 protein was decreased (-33%±6%) in D₂^{-/-} relative to D₂^{+/+} mice. The renal subcapsular infusion of PON2 siRNA decreased PON2 protein expression (-55%), increased renal oxidative stress (2.2-fold), associated with increased renal NADPH oxidase expression (Nox1: 1.9-fold; Nox2: 2.9-fold; and Nox4: 1.6-fold) and activity (1.9-fold), and elevated arterial blood pressure (systolic: 134±5 vs. 93±6 mmHg; diastolic: 97±4 vs. 65±7 mmHg; mean: 113±4 vs. 75±7 mmHg). To determine the relevance of the PON2 and D₂R interaction in humans, we studied human renal proximal tubule cells. Both D₂R and PON2 were found in non-lipid and lipid rafts and physically interacted with each other. Treatment of these cells with the D₂R/D₃R agonist quinpirole (1μM, 24h) decreased ROS production (-35%±6%), associated with decreased NADPH oxidase activity (-32%±3%) and expression of Nox2 (-41%±7%) and Nox4 (-47%±8%) protein, and increased expression of PON2 mRNA (2.1-fold) and protein (1.6-fold) at 24h. Silencing PON2 (siRNA, 10nM, 48 h) not only partially prevented the quinpirole-induced decrease in ROS production by 36%, but also increased basal ROS production (1.3-fold) which was associated with an increase in NADPH oxidase activity (1.4-fold) and expression of Nox2 (2.1-fold) and Nox4 (1.8-fold).

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protein. Inhibition of NADPH oxidase with diphenylene iodonium (10 μ M/30 min) inhibited the increase in ROS production caused by PON2 silencing.

Our results suggest that renal PON2 is involved in the inhibition of renal NADPH oxidase activity and ROS production and contributes to the maintenance of normal blood pressure. PON2 is positively regulated by D₂R and may, in part, mediate the inhibitory effect of renal D₂R on NADPH oxidase activity and ROS production.

Keywords

Paraoxonase 2; Dopamine D₂ receptor; Reactive oxygen species; NADPH oxidase; Hypertension

Introduction

Dopamine is important in the regulation of renal function, sodium balance, and systemic blood pressure through an independent peripheral dopaminergic system [1-4]. Dopamine exerts its actions via two families of cell surface receptors that belong to the superfamily of G protein-coupled receptors. These include D₁-like (D₁R and D₅R) and D₂-like receptors (D₂R, D₃R, and D₄R) in mammals [1-3]. There is abundant evidence 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 [1-3]. The disruption of any of the dopamine receptor genes in mice produces dopamine receptor subtype-specific hypertension [3, 5-7]. Disruption of the dopamine D₂ receptor (D₂R) gene increases systolic and diastolic blood pressure in mice that is associated with salt sensitivity, depending on the genetic background [6, 7]. D₂R polymorphisms are associated with human essential hypertension and elevated blood pressure [8, 9].

Reactive oxygen species (ROS) are ubiquitous highly diffusible reactive molecules produced by reduction of molecular oxygen, and include a series of oxygen intermediates such as the superoxide anion, hydrogen peroxide, hydroxyl radical, and hypochlorous acid. ROS have also been shown to be important mediators of multiple cellular functions such as growth and differentiation, proliferation, apoptosis and gene expression [10-12]. There are several intracellular sources contributing to ROS generation, including NADPH oxidase, mitochondrial respiration, cyclooxygenases, and lipoxygenases. A major source of ROS in renal and vascular tissues is NADPH oxidase, an enzymatic complex that consists of six subunits including membrane subunits p22^{phox} and gp91^{phox}, cytosolic components p40^{phox}, p47^{phox} and p67^{phox}, and a low-molecular weight G protein Rac1 or Rac2. Several homologs of gp91^{phox} in nonphagocytic tissues, organs, and cell lines have been cloned, including the Nox (NADPH oxidase) subgroup (Nox 1, Nox 2, Nox 3, Nox 4 and Nox 5) and the Duox subgroup (Duox 1 and Duox 2) [13-17].

The paraoxonase (PON) family consists of 3 genes: PON1, PON2 and PON3. These genes are located in a gene cluster on chromosome 7q21.3-22.1 and share a high degree of sequence identity [18, 19]. Although the physiological substrates of PONs are still uncertain, several studies have shown that PONs are lipo-lactonases [20, 21]. PON1 and PON3 proteins are present in plasma and reside in the high-density lipoprotein fraction and protect against oxidative stress by hydrolyzing certain oxidized lipids in lipoproteins, macrophages, and atherosclerotic lesions [22, 23]. In contrast to PON1 and PON3, PON2 is cell-associated and is not found in plasma. It is widely expressed in a variety of tissues, including the kidney, and protects against cellular oxidative stress [24, 25]. Overexpression of PON2 reduces oxidative status, prevents apoptosis in vascular endothelial cells, and inhibits cell-mediated low density lipoprotein oxidation [26, 27]. Mouse peritoneal macrophages harvested from PON2^{-/-} mice are more susceptible to urokinase plasminogen activator-

induced cellular oxidative stress than wild-type macrophages [28]. PON2 also inhibits the development of atherosclerosis in mice, via mechanisms involving the reduction of oxidative stress [29, 30].

D₂R agonists have been reported to have antioxidant effects by both receptor-dependent and receptor-independent mechanisms in the central nervous system. Administration of the D₂R agonist ropirinole increases the activity of catalase and superoxide dismutase in the striatum, resulting in neuroprotection [31]. Moreover, D₂^{-/-} mice have increased oxidative stress in striatal synaptosomes and substantia nigra [32]. In the kidney, increased generation of ROS has been shown to be important in the pathogenesis of hypertension in several animal models of hypertension [33-35]. We have reported that disruption of the D₂R gene is associated with increased ROS production and oxidative stress within the kidney and results in ROS-dependent hypertension [35]. Oxidative stress in D₂^{-/-} mice is associated with increased renal activity of NADPH oxidase and expression of Nox-1, Nox-2 and Nox-4 [35]. However, the mechanisms by which the D₂R regulates NADPH oxidase activity and expression are unclear. We hypothesized that renal PON2 may be involved in counter-regulating renal ROS production and thus may contribute to the maintenance of normal blood pressure. We hypothesize further that PON2 mediates, in part, the inhibitory effect of D₂R on NADPH oxidase expression, activity and ROS production.

Material and Methods

D₂R deficient mice

The original F2 hybrid mouse strain (129/SvXC57BL/6J, Oregon Health Sciences University) that contained the mutated D₂R allele (D₂^{-/-}) was backcrossed to wild-type C57BL/6J for >5 generations and genotyped [6, 35]. Wild-type littermates (D₂^{+/+}) were used as controls. Mice 6 to 8 months old were used in these experiments. Mice were sacrificed (pentobarbital 100 mg/kg) prior to harvesting the kidneys, and tissues were frozen in isopentane at -30°C on dry ice, and stored at -80°C for later study.

Renal silencing of D₂R and PON2 by the chronic subcapsular infusion of D₂R siRNA and PON2 siRNA, and measurement of blood pressure

Renal cortical D₂R and PON2 were silenced by the chronic subcapsular infusion of D₂R and PON2 siRNA via an osmotic minipump which was implanted in the remnant kidney of uninephrectomized mice. One week following the uninephrectomy, the mice were anesthetized with pentobarbital (50 mg/kg body weight, intraperitoneally), tracheotomized, and placed on a heated board to maintain rectal temperature at 37°C. The abdomen was opened under sterile conditions and a small portion of the kidney capsule was gently lifted and separated from the kidney to create a subcapsular space. An osmotic pump (ALZET® Osmotic Pump, model1007D), filled with previously prepared D₂R or PON2 siRNA or non-silencing control siRNA, was fitted with polyethylene delivery (Alzet #0007701) tubing. Using a tuberculin syringe fitted with a 33 gauge needle, the renal capsule was punctured and the tip of the tubing (approximately 1-3 mm) was inserted within the subcapsular space. Surgical glue (Vet-Bond, 3M, Minnesota) was used to seal in the infusion and keep the tubing in place. The body of the minipump was placed in the area previously occupied by the kidney that was removed; stabilization was achieved by suturing (4-0 ethilon) the minipump to the lateral abdominal musculature close to it. Before and after seven days of infusion (3 µg/d), systolic and diastolic blood pressures were measured (Cardiomax II, Columbus Instruments, Columbus OH) from the aorta, via the femoral artery under pentobarbital anesthesia (50 mg/kg). Blood pressures were recorded 1 hour after the induction of anesthesia and when blood pressures were stable. The mice were euthanatized (pentobarbital 100 mg/kg) at the conclusion of the study. The studies were conducted in

accordance with NIH guidelines for the ethical treatment and handling of animals in research and approved by the Institutional Animal Care and Use Committee at Children's Research Institute, Children's National Medical Center.

Cell culture and treatment of immortalized human renal proximal tubule (RPT) cells

Immortalized human RPT cells were maintained at 37°C in an atmosphere containing 5% CO₂ and cultivated in DMEM/F-12 supplemented with 10% FBS (Invitrogen) and 1% penicillin-streptomycin (Invitrogen). Human RPT cells were pretreated for 2 hours with serum-free medium and then treated with 1 μ M quinpirole for 0-24 hours.

RNA interference (RNAi)

Cells were plated in 6-wells plates at 2×10^5 cells per well one day before treatment. Predesigned FlexiTube siRNA (Qiagen) targeting human PON2 mRNA was transfected (10 nM) into human RPT cells using HiperFect (Qiagen) according to the manufacturer's instructions. AllStars siRNA (Qiagen) with a scrambled sequence (10 nM, 48 h) but with a similar G/C-content as PON2 siRNA served as negative control (non-silencing siRNA).

RNA extraction and quantitative RT-PCR (qRT-PCR)

Cells were collected and total RNA was extracted using an RNeasy Mini kit (Qiagen). To prepare cDNA, 500 ng of total RNA were mixed with the SuperScript RT System. cDNA was quantified using the SYBR Green PCR Master Mix (Applied Biosystems) to determine the mRNA expression of PON2. Real-time PCR reactions were carried out in a total volume of 25 μ l using pre-designed QuantiTect Primers for PON2 and GAPDH (Qiagen). All measurements were performed in triplicate to ensure reproducibility. The ratio of mean $\pm$ SEM of expression of each gene to GAPDH was calculated for sample-to-sample comparison.

Immunoblotting

Cells lysates were subjected to immunoblotting, as reported previously [35]. The primary antibodies used were polyclonal anti-D₂R antibody (Millipore), polyclonal anti-PON2 (Abcam), polyclonal anti-Nox-1 antibody (Abcam), monoclonal anti-Nox-2 (a kind gift of Dr. M.T. Quinn, Department of Veterinary Molecular Biology, Montana State University, Bozeman, MT), polyclonal anti-Nox4 (BD bioscience), monoclonal anti-GAPDH (Millipore), and monoclonal anti- β -actin (Sigma). The primary antibodies were detected using goat anti-rabbit or goat anti-mouse horseradish peroxidase-conjugate secondary antibodies (1:5000), and membranes were exposed for chemiluminescence. Quantification was performed using ImageJ software.

Immunofluorescence and confocal microscopy

Thin sections (3 μ m) of formalin-fixed, paraffin-embedded mouse kidneys were deparaffinized in xylene and rehydrated with step-down concentrations of ethanol. PON2 was visualized using a polyclonal rabbit anti-PON2 antibody (Abcam). The polyclonal rabbit anti-PON2 antibody was directly labeled using the Mix-n-Stain™ CFTM555A labeling kit (Biotium). D₂Rs were visualized using a polyclonal rabbit anti-D₂R antibody (Millipore) followed by Alexa Fluor 488-goat anti-rabbit IgG antibody (Molecular Probes). For a negative control, the primary antibodies were replaced with normal rabbit serum at an appropriate dilution. Colocalization of the D₂R receptor and PON2 was identified by the development of a yellow color in the merged images.

Sucrose gradient fractions

Lipid and non-lipid raft fractions were prepared by sucrose gradient ultracentrifugation using a detergent-free protocol, as previously described [36]. Human RPT cells grown to 95% confluence were harvested and lysed in 500 mmol/L sodium carbonate and homogenized by sonication. One ml of the homogenate was diluted with 2 ml 80% sucrose in MBS (25 mM MES, pH 6.7; 150 mM NaCl) and overlaid with 6 ml 35% sucrose and 3 ml 5% sucrose, and spun at 160,000g in a Beckman SW40 rotor at 4°C for 18 hours. After centrifugation, twelve 1-ml fractions were collected and labeled 1 to 12 from top to bottom. Aliquots of each fraction were mixed with Laemmli buffer, boiled, and immunoblotted.

Co-immunoprecipitation

Cell lysates were prepared using RIPA lysis buffer. Equal amounts of cell lysates (500 µg protein) were mixed with polyclonal anti-D₂R (Millipore), normal rabbit IgG (Sigma), as negative control, or polyclonal anti-PON2 (Abcam), as positive control. Protein G agarose beads (30 µL) (Roche, Indianapolis, IN) were added to each sample with rocking for 2 hours. The immune complexes were pelleted out, and the bound proteins were eluted using 30 µl of Laemmli buffer. The samples were immunoblotted with polyclonal anti-PON2 antibody.

Detection of reactive oxygen species (ROS)

The oxidation of 2', 7'-dichlorofluorescein diacetate (DCFDA) was used to measure ROS in human RPT cells and renal homogenates. Briefly, cells or renal homogenates were incubated with fresh DCFDA (10 µM/30 min) at 37 °C. For the effect of cellular NADPH oxidase inhibition on ROS production, human RPT cells were pretreated with an inhibitor of NADPH oxidase, diphenylene iodonium (DPI, 10 µM/30 min) (Sigma), before incubation with DCFDA. DCFDA fluorescence was measured using a microplate reader in 96-well plates at an excitation wavelength of 485 nm and an emission wavelength of 530 nm. ROS production was expressed in arbitrary units corrected for the protein concentration (AU/mg protein). All assays were performed in duplicate.

Determination of NADPH oxidase activity by lucigenin chemiluminescence

Whole cell membranes were prepared as described [37]. NADPH oxidase activity was determined by measuring superoxide generation in whole cell membranes in the presence of lucigenin (5 µM) and NADPH (100 µmol/L, ICN Biomedicals), using a microplate luminometer in 96-well plates (Centro LB 960, Berthold Technologies), as previously reported [37]. The specificity of the NADPH-dependent superoxide anion production was verified by treatment with DPI (10 µM/30 min) (Sigma). Equal amounts of cell membranes were incubated with lucigenin for 10 minutes at 37°C in a final volume of 200 µl of assay buffer. NADPH oxidase activity was expressed as arbitrary units corrected for the protein concentration (AU/mg protein). All assays were performed in duplicate.

Statistical Analysis

The data are expressed as mean ± SEM. Unpaired Student's *t* test was used for a 2-group comparison and factorial ANOVA, followed by the Newman-Keuls test for multigroup comparison. *P*<0.05 was considered significant.

Results

I. *In vivo* studies

Renal PON2 protein expression is regulated by D₂R—We have previously reported that disruption of the Dopamine D₂ receptor (D₂R) gene (D₂^{-/-}) increases systolic and

diastolic blood pressures associated with increased ROS production and oxidative stress in the kidney [35]. To determine whether or not PON2 is involved in the increased blood pressure and oxidative stress caused by disruption of the *D2R* gene, we measured the renal protein expression of PON2 in *D2*^{-/-} and *D2*^{+/+} mice by immunoblotting and immunofluorescence methods. As shown in Figure 1A, two bands (39kDa and 40kDa) were visualized, which corresponded to two isoforms of PON2, previously reported [26]. The expression of PON2 was decreased by 33% in kidneys of *D2*^{-/-} mice as compared with their wild-type littermates (*D2*^{+/+}: 0.452 ± 0.043 ; *D2*^{-/-}: 0.301 ± 0.026 , $P < 0.05$). In addition, renal immunofluorescent staining was weaker in *D2*^{-/-} than *D2*^{+/+} mice (Figure 1B). In order to avoid the confounding effect of an intact contralateral kidney, uninephrectomized mice were used to study the effect of selective renal *D2R* depletion by subcapsular siRNA infusion. Subcapsularly infused siRNA was observed only in proximal tubules and other cortical tubules in the cortex (unpublished data), although we cannot rule out the down regulation of *D2R* in other parts of the nephron. Similarly, renal PON2 protein expression was decreased by 55% in mice after 7 days of continuous renal subcapsular infusion of *D2R* siRNA relative to non-silencing siRNA-infused littermates (non-silencing siRNA: 0.42 ± 0.03 ; *D2R* siRNA: 0.19 ± 0.02 , $P < 0.01$) (Figure 1C).

***D2R* co-localizes with PON2 in the brush border of proximal tubules of mouse kidney**

The co-localization of *D2R* and PON2 was evaluated in formalin-fixed, paraffinembedded mouse kidney sections using laser confocal microscopy. *D2R* was strongly expressed in proximal tubules and was expressed in thick ascending limbs and distal tubules with weak intensity. There was no expression of *D2R* in glomeruli and collecting tubules/ducts. PON2 was mainly expressed in proximal tubules and is poorly expressed in nephron segments beyond the proximal straight tubule. *D2R* (green) and PON2 (red) were strongly coexpressed in the brush border of proximal tubules. The strong co-localization signal (yellow color) was clearly evident in the brush borders of proximal tubules (Figure 1D).

Blood pressure is increased by the chronic renal subcapsular injection of PON2 siRNA

To determine the consequence of silencing PON2 on blood pressure, we measured systolic, diastolic and mean arterial blood pressure before and after 7 days of continuous renal subcapsular infusion of PON2 siRNA or non-silencing siRNA in the remnant kidney of uninephrectomized mice. As shown in Figure 2A, PON2 protein expression (39kDa and 40kDa) [26] was decreased by 55% in PON2 siRNA-infused mice relative to non-silencing siRNA-infused littermates. Anesthetized PON2 siRNA-infused mice had higher systolic (134 ± 5 vs. 93 ± 6 mmHg, $P < 0.001$), diastolic (97 ± 4 vs. 65 ± 7 mmHg, $P = 0.007$) and mean arterial blood pressures (113 ± 4 vs. 75 ± 7 mmHg, $P = 0.003$, one way ANOVA, Newman-Keuls test) than non-silencing siRNA-infused littermates (Figure 2B). The blood pressures in mice were similar before renal subcapsular injection of nonsilencing siRNA or PON2 siRNA. *D2R* protein expression was not altered by PON2 siRNA infusion (Supplemental Figure S1A).

Renal NADPH oxidase expression and activity are up-regulated with chronic renal subcapsular injection of PON2 siRNA

We also measured renal NADPH oxidase activity and protein expression of Nox1, Nox2, and Nox4 in mice infused subcapsularly with PON2 siRNA. Silencing renal PON2 significantly increased the expression of Nox1, Nox2, and Nox4 by 1.9-fold, 2.9-fold, and 1.6-fold, respectively (Figure 2C). Renal NADPH oxidase activity was increased by 1.9-fold (non-silencing siRNA: 1578 ± 335 ; PON2 siRNA: 3066 ± 370 , AU/mg protein, $P < 0.01$) after subcapsular injection of PON2 siRNA relative to non-silencing siRNA (Figure 2D).

Renal oxidative stress is increased in mice with chronic renal subcapsular infusion of PON2 siRNA

—To investigate the effect of renal PON2 silencing on oxidative stress *in vivo*, we measured oxidative stress in mice subcapsularly infused with PON2 siRNA. Renal ROS production, as determined by DCFDA oxidation, showed a 2.2-fold increase (nonsilencing siRNA: 3254 ± 363 ; PON2 siRNA: 7217 ± 546 , AU/mg protein, $P < 0.01$) in mice subcapsularly infused with PON2 siRNA relative to non-silencing siRNA-infused littermates (Figure 2E).

II. *In vitro* studies

PON2 expression is regulated by D₂R in human renal proximal tubule (RPT) cells

—To determine the relevance of PON2 and D₂R interaction in humans, we investigated the role of D₂R on the expression of PON2 in human RPT cells. Quinpirole (1 μ M) increased PON2 protein expression in a time-dependent manner in human RPT cells (Figure 3A). Although quinpirole is a D₂R/D₃R specific agonist, our unpublished data showed that renal D₃R is not involved in the regulation of oxidative stress. We next asked if the increased protein expression of PON2 resulted from increased gene transcription. Therefore, we quantified PON2 gene transcription using real-time RT-PCR. We found that PON2 mRNA expression was increased by 2.1-fold in human RPT cells treated with quinpirole (1 μ M) for 24 hours (quinpirole: $210 \pm 21\%$; vehicle: $100 \pm 14\%$, $P < 0.01$) (Figure 3B).

Distribution of D₂R and PON2 in membrane microdomains—In the plasma membrane, distinct islands of lipids and proteins collectively known as lipid rafts are present and spatially concentrate functionally related sets of proteins to facilitate and augment their interaction, thereby enhancing the efficiency and specificity of their signal transduction [38, 39]. The subfractionation of endogenous D₂R and PON2 in lipid rafts was performed by sucrose gradient ultracentrifugation. Both D₂R and PON2 were found both in lipid (fractions 1-6) and non-lipid raft fractions (fractions 7-12). Forty seven percent of D₂R and 45% of PON2 were present in lipid raft fractions, and 53% of D₂R and 55% of PON2 were found in non-lipid raft fractions (Figure 3C).

D₂R directly interacts with PON2—In order to investigate the physical interaction between D₂R and PON2 proteins, total human RPT cell lysates were immunoprecipitated using a polyclonal anti-D₂R antibody, and the eluates were subsequently immunoblotted with PON2 antibody. As shown in Figure 3D, two bands were visualized, which corresponded to PON2, as similar bands were obtained when whole cell lysate was immunoprecipitated with anti-PON2 antibody. No co-immunoprecipitation was observed when normal rabbit IgG was used as the immunoprecipitant.

Stimulation of D₂R decreases cellular oxidative stress through up-regulation of PON2

—We have previously shown that D₂R decreases cellular oxidative stress in the mouse kidney [35]. We, therefore, measured the anti-oxidant effect of D₂R in human RPT cells. As shown in Figure 4A, cellular oxidative stress as determined by DCFDA oxidation was decreased by 35% (Vehicle: $100 \pm 7\%$; quinpirole: $65 \pm 5\%$, $P < 0.01$) in human RPT cells treated with the D₂R/D₃R agonist quinpirole for 24 hours. To determine whether or not PON2 is involved in the anti-oxidant effect of D₂R, we treated human RPT cells with quinpirole in the presence of siRNA (10 nM, 48 h) targeting human PON2 mRNA (PON2 siRNA). As expected, PON2 mRNA and protein expression were significantly decreased (63% and 60% respectively) in human RPT cells treated with PON2 siRNA (Figures 4B and 4C). Similar to the mouse kidney result (Supplemental Figure S1A), D₂R protein expression was not altered by PON2 siRNA treatment (Supplemental Figure S1B). In contrast, quinpirole treatment decreased ROS production in human RPT cells transfected with non-

silencing siRNA by 39% (vehicle: $100 \pm 4\%$; quinpirole: $61 \pm 5\%$, $P < 0.01$). PON2 siRNA treatment alone increased ROS production by 1.3-fold (PON2 siRNA: $134 \pm 7\%$, $P < 0.01$) and partially prevented the quinpirole-induced decrease in ROS production by 36% (quinpirole + PON2 siRNA: $100 \pm 5\%$, $P < 0.01$) (Figure 4D), indicating that D₂R decreases oxidative stress, in part, by increasing PON2 expression.

NADPH oxidase is involved in the increased cellular oxidative stress caused by depletion of PON2—The NADPH oxidase family is one of the major sources of ROS production [40] and therefore, the increase in NADPH oxidase activity with the silencing of PON2 should be associated with an increase in ROS production. This is indeed the case. As shown in Figure 5A, the increase in ROS production due to silencing of PON2 siRNA is related to NADPH oxidase activity because pretreatment of human RPT cells with the NADPH oxidase inhibitor, DPI ($10 \mu\text{M}/30 \text{ min}$), completely inhibited the PON2 siRNA-induced increase in ROS production (PON2 siRNA+vehicle: $157 \pm 10\%$; PON2 siRNA+DPI: $104 \pm 8\%$, $P < 0.01$). DPI pretreatment alone did not change the ROS production.

PON2 decreases the expression of NADPH oxidase and partially mediates the inhibitory effect of D₂R on NADPH oxidase expression and activity—We have recently shown that NADPH oxidase activity and Nox expression in the renal cortex of D₂^{-/-} mice are increased relative to wild-type littermates [35]. We, therefore, studied the effect of D₂R stimulation on NADPH oxidase system in human RPT cells. Treatment with quinpirole ($1 \mu\text{M}$, 24 h) decreased NADPH oxidase activity ($-32 \pm 3\%$) (Figure 5B) and the protein expressions of Nox2 ($-41 \pm 7\%$) and Nox4 ($-47 \pm 8\%$) (Figure 5C) in human RPT cells.

We next investigated whether or not PON2 plays a role in the inhibition of NADPH oxidase enzyme by D₂R. Silencing PON2 alone significantly increased the expression of Nox2 and Nox4, by 2.1-fold and 1.8-fold, respectively, and completely abolished the inhibitory effect of quinpirole on Nox2 and Nox4 expression (Figure 5D). Silencing of PON2 also increased NADPH oxidase activity by 1.5-fold (Figure 5E), in agreement with the increase in Nox2 and Nox4 protein expression (Figure 5D). In addition, the inhibitory effect of D₂R on NADPH oxidase activity was greater in nonsilencing siRNA-treated cells ($34 \pm 2\%$) than in PON2 siRNA-treated cells ($16 \pm 4\%$, $P < 0.01$); all comparisons are relative to vehicle+non-silencing siRNA (Figure 5E). These results suggest that the inhibitory effect of quinpirole on Nox2 and Nox4 expression is mediated solely by PON2. However, the inhibitory effect of quinpirole on NADPH oxidase activity is only partially due to PON2, indicating that quinpirole may inhibit Nox proteins other than Nox2 and Nox4, the identity of such Nox protein isoforms remains to be determined. As shown in Figure 4D, the inhibitory effect of D₂R on ROS production is also partly mediated by PON2, indicating that D₂R inhibits ROS production by mechanisms other than Nox2, Nox4, and PON2 pathway. Indeed, we have reported that the D₂R also decreases ROS production, in part, via DJ-1⁴¹. The PON2 pathway is distal to D₂R because silencing PON2 does not decrease D₂R expression (Supplemental Figure S1A and S1B). DJ-1 pathway is also distal to D₂R because silencing DJ-1 does not decrease D₂R expression (unpublished data).

Discussion

PON2 serves as a cellular antioxidant enzyme and is widely expressed in many tissues [24-30]. The increase in renal ROS production caused by the selective renal silencing of PON2 through the renal subcapsular injection of PON2 siRNA provides concrete evidence of the importance of PON2 in the regulation of renal ROS *in vivo*. We have reported that D₂^{-/-} mice had increased systemic oxidative stress and D₂R negatively regulates renal ROS production [35]. Therefore, we determined if the antioxidant action of D₂R [35] is mediated by PON2. We demonstrate, for the first time, that PON2 expression is positively regulated

by D₂R *in vivo*. D₂-/- mice, relative to wild-type littermates, have decreased renal PON2 protein expression. These studies in mice are relevant to humans because stimulation of D₂R in human RPT cells increases PON2 expression and decreases ROS production. Silencing the PON2 gene in human RPT cells increases ROS production and impairs the ability of D₂R stimulation to decrease ROS production, indicating that the antioxidant action of D₂R is mediated, in part, via PON2.

Our results show that PON2 mRNA and protein expressions are increased by D₂R, suggesting that the D₂R can positively regulate renal PON2 expression at the transcriptional or post-transcriptional level. We further show that D₂R and PON2 colocalize in the brush borders of proximal tubules of the mouse kidney and lipid rafts of human RPT cells. Lipid rafts are cholesterol and sphingolipid enriched microdomains in the outer leaflet of the lipid bilayer of plasma membranes. These specialized membrane microdomains function as unique signal transduction platforms and serve as compartments for the recruitment of cell signaling components, resulting in increased efficiency of signal transduction [36-40]. Our results show that D₂R and PON2 are found in the same microdomains and physically interact with each other, as evidenced by the co-immunoprecipitation experiments. These results raise the possibility that lipid rafts provide a favorable environment for D₂R and PON2 cross-talk and allow these molecules to achieve effective concentrations for direct protein-protein interaction. However, further investigation is needed to study the exact interaction between D₂R and PON2 which results in the regulation of PON2.

We and others have reported that renal D₂R contributes to the regulation of salt and water balance blood pressure [3, 6, 7]. While increased ROS production can cause hypertension [34], the increased oxidative stress in D₂-/- mice is due in part to increased expression and activity of NADPH oxidase [35]. Indeed, apocynin, an NADPH oxidase inhibitor, decreased the urinary excretion of 8-isoprostanes and normalized Nox1 but not Nox2 or Nox4 expression D₂-/- mice [35]. In this study, using human RPT cells, the D₂R-mediated decrease in Nox2 and Nox4 expression is completely abolished by PON2 silencing, suggesting that D₂R regulation of Nox2 and Nox4 expression is solely mediated by PON2. However, when the *PON2* gene is silenced, D₂R stimulation can still decrease NADPH oxidase activity, in the face of complete prevention of the D₂R-mediated decrease in Nox2 and Nox4 expression. The ability of D₂R to decrease NADPH oxidase activity during complete prevention of the D₂R-mediated decrease in Nox2 and Nox4 expression indicates that D₂R can negatively regulate Nox isoforms other than Nox2 and Nox4, but their identities remain to be determined. The increase in ROS production in human RPT cells, when *PON2* gene is silenced, can be decreased by D₂R stimulation. This implies the existence of other mechanisms by which D₂R regulates ROS independent of Nox2 and Nox4 and PON2. This suggestion is consistent with our previous report, which showed that impaired D₂R function may increase ROS caused by increased aldosterone production and decreased heme-oxygenase 2 expression [35], as well as by decreased DJ-1 expression⁴¹. Another explanation is that because PON2 expression was not completely eliminated by siRNA, D₂R may still decrease ROS production via the cellular PON2.

The mechanism by which PON2 modulates ROS production is still unclear. Our observations in human RPT cells, of the involvement NADPH oxidase isoforms, e.g., Nox2 and Nox4, are corroborated by a report that the increases in macrophage triglycerides accumulation and biosynthesis in PON2-deficient mice are mediated by NADPH oxidase-derived increases in oxidative stress [42]. It has also been shown that the antioxidant effects of PON2 are, in part, mediated by its ability to regulate mitochondrial function associated with respiratory complex III [43]. Lactones are suggested to be the natural substrates of PON2 and its lactonase activity has been shown to correlate with this enzyme's biological antioxidant properties. PON2 protein has two free cysteines at positions 284 and 311 and it

has been suggested that loss of its enzymatic activity is probably the result of cysteine oxidation, leading to disulfide bond formation between cysteine residues in different PON2 molecules [44]. However, it was recently shown that the PON2 lactonase activity and antioxidant functions are separate. Mutations that abolish lactonase activity do not alter the antioxidant action of PON2 [45]. The fact that DPI completely prevented the increase in ROS production with PON2 silencing indicates that the antioxidant activity of PON2 is solely mediated by inhibition of NADPH oxidase activity, probably via Nox2 and Nox4.

We only studied renal proximal tubule cells in our experiments because both D₂R and PON2 are expressed mainly in proximal tubules of mouse kidney. Both D₂R [46] and PON2 (unpublished data) are poorly expressed in the medullary collecting duct. ROS are apparently predominantly formed in the renal cortex, whereas the medulla which is susceptible to hypoxia may normally produce less amounts of ROS production relative to the renal cortex [47]. One limitation of this study is that we do not know the contribution of other cells of the kidney in the production of ROS. The increase in renal ROS production, associated with activation of the intrarenal renin-angiotensin system (RAS), alteration of apoptosis-inducing enzymes and a decreased in transcription of hypoxiasensitive genes, can cause sodium and fluid retention and ultimately hypertension [47]. The negative regulation of ROS production by D₂R is important to keep blood pressure in the normal range [35]. This may be due, in part, to the positive regulatory effect of D₂R on PON2 expression and function. PON2 expression is decreased in D₂^{-/-} mice which are hypertensive [35]. Selective renal silencing of D₂R decreased PON2 expression, and increased oxidative stress and blood pressure [35, 48]. Similarly, selective renal silencing of PON2 increases oxidative stress and blood pressure, shown in the current study. D₂R expression is not affected by the silencing of PON2 in the kidney *in vivo* and human RPT cells *in vitro*. In contrast, selective renal D₂R silencing decreases PON2 expression. These studies suggest that D₂R is upstream of the PON2 pathway.

In conclusion, we suggest that renal PON2 is positively regulated by D₂R and mediates, in part, the inhibitory effect of renal D₂R on NADPH oxidase activity and ROS production. D₂R and PON2 are found in the brush border of the proximal tubule of the kidney and physically interact with each other. D₂R and PON2 are involved in counter-regulating renal NADPH oxidase activity and ROS production which contributes to the maintenance of normal blood pressure.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

D₂R	dopamine D ₂ receptor
ROS	reactive oxygen species
PON2	paraonase 2
Nox 1	NADPH oxidase 1
Nox 2	NADPH oxidase 2
Nox4	NADPH oxidase 4
RPT	Renal proximal tubule
DCFDA	2', 7'-dichlorofluorescein diacetate
DPI	diphenylene iodonium

Highlights

- D₂R and PON2 are found in the brush border of the proximal tubule of the kidney.
- PON2 is physically interacts with D₂R and is positively regulated by D₂R.
- PON2 mediates the inhibitory effect of D₂R on NADPH oxidase and ROS production.
- D₂R and PON2 contribute to maintenance of normal blood pressure.

Figure 1.

(A) Immunoblot analysis of PON2 from kidneys of $D_2^{-/-}$ and $D_2^{+/+}$ mice. Results were normalized to the expression of GAPDH and shown as mean $\pm$ SEM; n=3/group, *P<0.05; t-test. The inset shows representative immunoblots of PON2 and GAPDH. (B) Renal PON2 protein expression in $D_2^{-/-}$ mice and $D_2^{+/+}$ mice. Formalin-fixed paraffin-embedded $D_2^{-/-}$ and $D_2^{+/+}$ mouse kidney sections were stained using PON2 antibody (red). PON2 was expressed in the brush border (BB) of proximal tubules (PT) (original magnification, 400 $\times$, 300 DPI). The nuclei are blue. G = glomerulus. (C) Immunoblot analysis of PON2 in mouse kidneys which were continuously injected subcapsularly with D_2R siRNA or non-silencing siRNA (3 μ g/d) for 7 days. Results are normalized by expression of β -actin and shown as mean $\pm$ SEM, n=3/group, *P<0.01, t-test. The inset shows representative immunoblots of PON2 and β -actin. (D) D_2R and PON2 colocalization in mouse kidney. D_2R (green) and PON2 (red) are expressed in the brush border (BB) (original magnification, 630 $\times$, 300 DPI). The immunofluorescence images were acquired using a Zeiss 510 confocal laser scanning microscope. Colocalization of D_2R and PON2 is indicated by the yellow color in the merged image.

Figure 2.

(A) Immunoblot analysis of PON2 in mouse kidneys which were continuously infused subcapsularly with PON2 siRNA or non-silencing siRNA (3 μ g/d) for 7 days. Results are normalized by expression of β -actin and shown as mean $\pm$ SEM, n=3/group, *P<0.05, t-test. The inset shows representative immunoblots of PON2 and β -actin. (B) Systolic, diastolic, and mean arterial blood pressures in mice before and after the kidneys were continuously injected subcapsularly with PON2 siRNA or non-silencing siRNA (3 μ g/d) for 7 days. Values are shown as mean $\pm$ SEM, n=3/group, *P<0.001, systolic blood pressure, *P=0.007, diastolic blood pressure, *P=0.003, mean arterial blood pressure, one-way ANOVA, Newman-Keuls test. (C) Immunoblot analysis of Nox1, Nox2, and Nox4 expression in mouse kidneys which were continuously infused subcapsularly with PON2 siRNA or non-silencing siRNA (3 μ g/d) for 7 days. Results are normalized by β -actin and expressed as percentage of change compared with group treated with non-silencing siRNA. Results are shown as mean $\pm$ SEM, n=3/group, *P<0.01, t-test. The inset shows representative immunoblots of Nox1, Nox2, Nox4, and β -actin. (D) NADPH oxidase activity in mouse kidneys which were continuously infused subcapsularly with PON2 siRNA or non-silencing siRNA (3 μ g/d) for 7 days. NADPH oxidase activity was measured using the lucigenin chemiluminescence method. Values were normalized by protein concentration and calculated as AU/mg protein. Results are shown as mean $\pm$ SEM, n=3/group, *P < 0.01, t-test. (E) ROS production, measured by the DCFDA method, in mouse kidneys which were continuously infused subcapsularly with PON2 siRNA or non-silencing siRNA (3 μ g/d) for 7 days. Values were normalized by protein concentration and calculated as AU/mg protein. Results are shown as mean $\pm$ SEM, n=3/group, *P < 0.01, t-test.

Figure 3.

(A) Time-dependent effect of quinpirole (1 μ M) on PON2 protein expression in human renal proximal tubule (RPT) cells. Results are normalized by the expression of β -actin and shown as mean $\pm$ SEM, $n=3$ /group, * $P < 0.05$ vs. control (0h), ANOVA, Newman-Keuls test. The inset shows representative immunoblots of PON2 and β -actin. (B) PON2 mRNA expression in human RPT cells treated with quinpirole (1 μ M) for 24 hours. Total RNA of human RPT cells treated with quinpirole or vehicle was reverse-transcribed and the cDNA was subjected to quantitative PCR. GAPDH was used as an internal standard to normalize PON2 expression. $n=9$ /group, * $P < 0.01$, t-test. (C) Distribution of endogenous D₂R and PON2 in membrane microdomains of human RPT cells. Lipid and non-lipid membrane fractions of human RPT cells were prepared by sucrose gradient centrifugation to determine the basal membrane distribution of both D₂R and PON2. Twelve fractions were obtained (fractions 1-6 correspond to lipid rafts and fractions 7-12 to nonlipid rafts) and immunoblotted for D₂R and PON2. (D) D₂R and PON2 co-immunoprecipitation in human RPT cells. Lysates of cells were immunoprecipitated (IP) using a polyclonal anti-D₂R antibody, normal rabbit IgG, as a negative control, or polyclonal anti-PON2 antibody, as positive control and the eluates were immunoblotted with PON2 antibody. To determine the specificity of the bands, immunoblots of whole cell lysate (WCL) with PON2 antibody were used.

Figure 4.

(A) Human RPT cells were treated with quinpirole (1 μ M/24 h) or vehicle, and ROS production measured using the DCFDA method. Values were normalized by protein concentration and expressed as percentage of group treated with vehicle (control). Results are shown as mean $\pm$ SEM, n=9/group, *P < 0.01, t-test. (B) Real-time PCR analysis of PON2 mRNA in human RPT cells transfected with PON2 siRNA (10nM) for 48 hours. mRNA results are normalized by the GAPDH gene and expressed as the percentage of non-silencing siRNA group (control). All results are shown as mean $\pm$ SEM, n=9/group, *P < 0.01, t-test. (C) Immunoblot analysis of PON2 protein expression in human RPT cells transfected with PON2 siRNA (10 nM) for 48 hours. Immunoblot results are normalized by β -actin and expressed as percentage non-silencing siRNA group (control). All results are shown as mean $\pm$ SEM, n=9/group, *P < 0.01, t-test. The inset shows representative immunoblots of PON2 and β -actin. (D) ROS production in human RPT cells treated with quinpirole and PON2 siRNA. Cells were transfected with PON2 siRNA (10nM) or non-silencing siRNA (10nM) for 24 hours and then treated with quinpirole 1 μ M or vehicle for 24 hours. ROS production was measured using the DCFDA method. Values were normalized by protein concentration and expressed as percentage vehicle+non-silencing siRNA group (control). Results are shown as mean $\pm$ SEM, n=9/group, *P < 0.01, vs. vehicle + non-silencing siRNA; # P < 0.01, vs. others; ** P < 0.01, vs. quinpirole + non-silencing siRNA. ANOVA, Newman-Keuls test.

Figure 5.

(A) Human RPT cells were transfected with PON2 siRNA (10 nM) for 48 hours and then incubated with DPI (10 μ M) for 30 minutes. ROS production was measured using the DCFDA method. Values were normalized by protein concentration and expressed as percentage of vehicle+non-silencing siRNA group (control). Results are shown as mean $\pm$ SEM, n=9/group, *P < 0.01, vs. vehicle+non-silencing siRNA; ** P < 0.01, vs. PON2 siRNA +DPI. ANOVA, Newman-Keuls test. (B) After human RPT cells were treated with quinpirole (1 μ M) for 24 hours, NADPH oxidase activity was measured using the lucigenin chemiluminescence method. Values were normalized by protein concentration and expressed as percentage of group treated with vehicle (control). Results are shown as mean $\pm$ SEM, n=9/group, *P < 0.01, t-test. (C) Immunoblot analysis of Nox2 and Nox4 expression in human RPT cells treated with quinpirole (1 μ M) for 24 hours. Results were normalized by β -actin and expressed as percentage of group treated with vehicle (control). The results are shown as mean $\pm$ SEM, n=9/group, *P<0.01, t-test. The inset shows representative immunoblots of Nox2, Nox4, and β -actin. (D) Immunoblot analysis of Nox2 and Nox4 expression in human RPT cells treated with quinpirole and PON2 siRNA. Cells were transfected with PON2 siRNA (10 nM) or non-silencing siRNA for 24 hours and then treated with quinpirole (1 μ M) or vehicle for 24 hours. Results were normalized by β -actin and expressed as percentage of vehicle+nonsilencing siRNA group (control). All results are shown as mean $\pm$ SEM, n=9/group, *P < 0.01, vs. vehicle+non-silencing siRNA; #P < 0.01, vs. vehicle+non-silencing siRNA; ** P < 0.01, vs. quinpirole+non-silencing siRNA.

ANOVA, Newman-Keuls test. **(E)** Human RPT cells were transfected with PON2 siRNA (10 nM) or non-silencing siRNA for 24 hours and treated with quinpirole (1 μ M) or vehicle for 24 hours. NADPH oxidase activity was measured using the lucigenin chemiluminescence method. Values were normalized by protein concentration and expressed as percentage of vehicle+nonsilencing siRNA group (control). The specificity of the NADPH-dependent superoxide anion production was verified by treatment with diphenylene iodonium (DPI). Results are shown as mean $\pm$ SEM, n=9/group, *P < 0.01, vs. vehicle+non-silencing siRNA; #P < 0.01, vs. vehicle+non-silencing siRNA; **P < 0.01, vs. quinpirole + non-silencing siRNA; *** P < 0.01, vs. any group treated with DPI. ANOVA, Newman-Keuls test.