Sestrin2 Decreases Renal Oxidative Stress, Lowers Blood Pressure, and Mediates Dopamine D\textsubscript{2} Receptor–Induced Inhibition of Reactive Oxygen Species Production

Yu Yang, Santiago Cuevas, Sufei Yang, Van Anthony Villar, Crisanto Escano, Laureano Asico, Peiyng Yu, Xiaoliang Jiang, Edward J. Weinman, Ines Armando, Pedro A. Jose

Abstract—The dopamine D\textsubscript{2} receptor (D\textsubscript{2}R) decreases renal reactive oxygen species (ROS) production and regulates blood pressure, in part, via positive regulation of paraoxonase 2. Sestrin2, a highly conserved antioxidant protein, regulates intracellular ROS level by regenerating hyperoxidized peroxiredoxins. We hypothesized that sestrin2 may be involved in preventing excessive renal ROS production and thus contribute to the maintenance of normal blood pressure. Moreover, the D\textsubscript{2}R may decrease ROS production, in part, through the regulation of sestrin2. Renal sestrin2 expression was lower (−62±13\%) in D\textsubscript{2}R\textsuperscript{−/−} than in D\textsubscript{2}R\textsuperscript{+/+} mice. Silencing D\textsubscript{2}R in human renal proximal tubule cells decreased sestrin2 expression (−53±3\%) and increased hyperoxidized peroxiredoxins (2.9-fold). Stimulation of D\textsubscript{2}R in renal proximal tubule cells increased sestrin2 expression (1.6-fold), decreased hyperoxidized peroxiredoxins (−61±3\%), and reduced ROS production (−31±4\%). Silencing sestrin2 in renal proximal tubule cells increased hyperoxidized peroxiredoxins (2.1-fold) and ROS production (1.3-fold). Silencing sestrin2 also abolished D\textsubscript{2}R-induced decrease in peroxiredoxin hyperoxidation and partially prevented the inhibitory effect of D\textsubscript{2}R stimulation on ROS production. Silencing paraoxonase 2 increased sestrin2 ubiquitinylation (2.8-fold), decreased sestrin2 expression (−30±3\%), and increased ROS production (1.3-fold), peroxiredoxin hyperoxidation (2.9-fold), and lipid peroxidation (2.3-fold), and blocked the increase in sestrin2 that occurs with D\textsubscript{2}R stimulation. In vivo renal selective silencing of sestrin2 by the renal subcapsular infusion of sestrin2 small interfering RNA (3 \(\mu\)g/day; 7 days) in mice increased renal oxidative stress (1.3-fold) and blood pressure. These results suggest that the D\textsubscript{2}R, via paraoxonase 2 and sestrin2, keeps normal renal redox balance, which contributes to the maintenance of normal blood pressure. (Hypertension. 2014;64:825-832.) • Online Data Supplement

Key Words: aryl dialkylphosphatase ■ hypertension ■ peroxiredoxins ■ reactive oxygen species ■ receptors, dopamine D\textsubscript{2}

Dopamine is important in the regulation of renal function, sodium balance, and systemic blood pressure through an independent peripheral dopaminergic system. 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. The disruption of any of the dopamine receptor genes—D\textsubscript{1}-like (D\textsubscript{1}R and D\textsubscript{3}R) and D\textsubscript{2}-like receptors (D\textsubscript{2}R, D\textsubscript{3}R, and D\textsubscript{4}R)—in mice produces dopamine receptor subtype–specific hypertension. Disruption of the dopamine D\textsubscript{2} receptor (D\textsubscript{2}R) gene increases blood pressure in mice that is associated with salt sensitivity, depending on the genetic background. D\textsubscript{2}R polymorphisms are associated with human essential hypertension and elevated blood pressure. Increased generation of reactive oxygen species (ROS) in the kidney has been shown to be important in the pathogenesis of hypertension in several animal models. Disruption of Drd2 (D\textsubscript{2}R\textsuperscript{−/−}) increases ROS production and oxidative stress in the kidney and results in ROS-dependent hypertension. The renal activity of NADPH oxidase and the expression of Nox-1, Nox-2, and Nox-4 are increased in D\textsubscript{2}R\textsuperscript{−/−}. We have recently shown that renal paraoxonase 2 (PON2), a protein that possesses antioxidant properties and is positively regulated by D\textsubscript{2}R, mediates, in part, the inhibitory effect of renal D\textsubscript{2}R on NADPH oxidase activity and ROS production and contributes to the maintenance of normal blood pressure. 

ROS production is limited not only by decreased oxidant activity but also by increased antioxidant defense. Peroxiredoxins are thiol-based antioxidant enzymes detoxifying ROS by oxidation of their 2 cysteine groups to cysteine sulfenic acid (Cys-SO\textsubscript{2}H) or cysteine sulfonic acid (Cys-SO\textsubscript{3}H), causing inactivation of peroxidase activity. Sestrin2, also...
known as Hi95 and initially identified as one of the hypoxia-inducible genes, is a member of a family of antioxidant proteins.\textsuperscript{20-22} The expression of sestrin2 is responsive to hypoxia, oxidative stress, and DNA damage. Overexpression of sestrin2 protects cells against hydrogen peroxide or ischemia.\textsuperscript{18,21,22} Sestrin2 negatively regulates intracellular ROS levels by catalyzing the reduction of hyperoxidized peroxiredoxins (Prx-SO\textsubscript{2}\textsuperscript{−}\textsubscript{−}H) that cannot be reduced by typical cellular reductants, such as thioredoxin or glutathione.\textsuperscript{18,23,24}

\textit{D}_{2}\textit{R} agonists have been reported to have antioxidant effects by both receptor-dependent and receptor-independent mechanisms in the central nervous system.\textsuperscript{25} The \textit{D}_{2}\textit{R} agonist ropinirole increases the activity of catalase and superoxide dismutase in the striatum and protects striatal neurons against oxidative stress.\textsuperscript{25} In the current study, we tested the hypothesis that sestrin2 inhibits renal ROS production and may contribute to the maintenance of normal blood pressure. Moreover, the \textit{D}_{2}\textit{R} inhibits ROS production not only by inhibiting NADPH oxidase expression/activity but also by increasing sestrin2, via PON2.

\section*{Methods}

\subsection*{D\textsubscript{2}R-Deficient Mice}

The original F\textsubscript{2} hybrid mouse strain (129/Sv/c57bl/6j; Oregon Health Sciences University) that contained the mutated \textit{Drd2} allele (\textit{D},\textsubscript{2R}\textsuperscript{−/−}) was backcrossed to wild-type c57bl/6j for \textit{≥}5 generations and genotyped.\textsuperscript{5,12} Wild-type littermates (\textit{D},\textsubscript{2R}+/+) were used as controls. Mice were euthanized (pentobarbital 100 mg/kg) before harvesting the kidneys.

\subsection*{Renal Selective Silencing of Sestrin2 and Measurement of Blood Pressure}

The method of renal cortical silencing of the gene of interest was developed in our laboratory.\textsuperscript{13,26-28} C57BL/6 mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Renal cortical sestrin2 was silenced by the chronic renal subcapsular infusion (3 \textmu g/day; 7 days) of sestrin2 small interfering RNA (siRNA) via an osmotic minipump, which was implanted into the space previously occupied by the removed kidney in right uninephrectomized mice. Blood pressure was recorded 1 hour after the induction of anesthesia when blood pressure was stable. The studies were conducted in accordance with National Institutes of Health guidelines and approved by the Institutional Animal Care and Use Committee at University of Maryland. For detailed methods, see the online-only Data Supplement.

A detailed description of all other methods, including cell culture, RNA interference, RNA extraction and quantitative reverse transcriptase polymerase chain reaction, immunoblotting, coimmunoprecipitation, detection of ROS, and measurement of lipid peroxidation (thiobarbituric acid reactive substances assay), is presented in the online-only Data Supplement.

\section*{Statistical Analysis}

The data are expressed as mean±SEM. Unpaired Student \textit{t} test was used for a 2-group comparison and factorial ANOVA, followed by the Newman–Keuls test for multigroup (≥3) comparison. \textit{P}<0.05 was considered significant.

\section*{Results}

\subsection*{Sestrin2 Expression Is Upregulated by \textit{D}_{2}\textit{R}}

Previous studies from our laboratory have shown that disruption of \textit{Drd2} (\textit{D},\textsubscript{2R}\textsuperscript{−/−}) increases systolic and diastolic blood pressures that are associated with increased ROS production and oxidative stress in the kidney.\textsuperscript{12,15,26-27} To determine if sestrin2 is involved in these alterations, we first measured the renal protein expression of sestrin2 in \textit{D},\textsubscript{2R}\textsuperscript{−/−} and \textit{D},\textsubscript{2R}+/+ mice. The expression of sestrin2 was lower (−62±13%) in kidneys of \textit{D},\textsubscript{2R}−/− mice compared with their wild-type littermates (Figure 1A). To determine the relevance of sestrin2 and \textit{D},\textsubscript{2R} interaction in humans, we investigated the role of \textit{D},\textsubscript{2R} on the expression of sestrin2 in human renal proximal tubule cells (RPTCs). Consistent with the observation in \textit{D},\textsubscript{2R}−/− mice, silencing \textit{D},\textsubscript{2R} resulted in decreased sestrin2 expression by 53±3% relative to nonsilencing siRNA-treated human RPTCs (Figure 1B). Conversely, treatment with the \textit{D},\textsubscript{2R} agonist quinpirole increased sestrin2 expression (1.6-fold) in human RPTCs. This effect was completely blocked by a selective \textit{D},\textsubscript{2R} antagonist, L741, 262 (Figure 1C), which by itself had no effect. Stimulation or silencing of the \textit{D},\textsubscript{2R} had no effect on the expression of other proteins (sestrin1 and sestrin3) belonging to the same sestrin family (Figure S1A–S1D in the online-only Data Supplement).

\textit{Stimulation of \textit{D},\textsubscript{2R} Decreases Cellular Oxidative Stress, in Part, via Upregulation of Sestrin2}

The \textit{D},\textsubscript{2R} decreases cellular oxidative stress in human RPTCs.\textsuperscript{33} To determine whether or not sestrin2 is involved

![Image](http://hyper.ahajournals.org/)

\begin{figure}[h]
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  \caption{Sestrin2 expression is upregulated by dopamine \textit{D}, receptor (\textit{D},\textit{R}). A, Sestrin2 expression in renal homogenates from \textit{D},\textsubscript{2R}−/− mice and wild-type (\textit{D},\textsubscript{2R}+/+) littermates (\textit{n}=4 per group). B, Sestrin2 in human renal proximal tubule cells (RPTCs) transfected with \textit{D},\textsubscript{2R} small interfering RNA (siRNA) or nonsilencing siRNA (15 \textmu mol/L; 48 h). Protein expression of sestrin2 was analyzed by immunoblotting. \textbf{Insets}: Representative immunoblots of sestrin2 and \textit{β}-actin. C, Sestrin2 in human RPTCs pretreated with vehicle or \textit{D},\textsubscript{2R} antagonist, L741, 262 (1 \textmu mol/L; 1 h) and then treated with vehicle or \textit{D},\textsubscript{2R} agonist quinpirole (1 \textmu mol/L; 24 h). Results are mean±SE from 3 independent experiments; \textsuperscript{*}\textit{P}<0.01; \textit{t} test (B) or 1-way ANOVA Newman–Keuls test (C).}
\end{figure}
in the antioxidant effect of D₉R, we treated human RPTCs with the D₉R agonist quinpirole in the presence of siRNA targeting human sestrin2. Sestrin2 mRNA and protein expressions were significantly decreased (59% and 73%, respectively) in human RPTCs treated with sestrin2 siRNA (Figure 2A and 2B). Quinpirole treatment decreased ROS production by 31±4% in human RPTCs transfected with nonsilencing siRNA. However, in human RPTCs treated with quinpirole and sestrin2 siRNA, ROS production was decreased by only 17%±4% (Figure 2C), suggesting that D₉R decreases oxidative stress, in part, by increasing sestrin2 expression.

**Sestrin2 Regulates Peroxiredoxin Hyperoxidation and Mediates D₉R-Induced Reduction of Hyperoxidized Peroxiredoxins**

Sestrin2 can protect peroxiredoxins from hyperoxidation. To investigate whether or not sestrin2 in human RPTCs can regulate the redox state of peroxiredoxins, we determined the ratio of Cys-SO₂⁻/³ peroxiredoxins to total 2-Cys peroxiredoxins. Only 1 major band was detected using a total 2-Cys peroxiredoxins antibody in human RPTCs. Sestrin2 siRNA treatment alone increased hyperoxidized peroxiredoxins (2.1-fold) (Figure 3A), ROS production (1.3-fold; Figure 3B), and lipid peroxidation (1.7-fold; Figure 3C), compared with nonsilencing siRNA treatment. Silencing D₉R also increased hyperoxidized peroxiredoxins (2.9-fold; Figure 3D). Conversely, quinpirole treatment of human RPTCs significantly reduced peroxiredoxin hyperoxidation (−61±3%), but this protective effect of quinpirole on peroxiredoxins was completely abolished by pretreatment with sestrin2 siRNA (Figure 3E).

Sulfiredoxin and thioredoxin–peroxiredoxin system repair hyperoxidized peroxiredoxins by catalyzing or facilitating the reduction of its hyperoxidized forms. The expression of sulfiredoxin was not altered by treatment with quinpirole or D₉R siRNA in human RPTCs (Figure S2A and S2B). Similarly, the expression of thioredoxin-interacting protein that binds thioredoxin and inhibits its activity was also not altered by D₉R stimulation or silencing (Figures S2C and S2D).

**D₉R-Induced Sestrin2 Activation Is Dependent on PON2, and Depletion of PON2 Increases Peroxiredoxin Hyperoxidation and Sestrin2 Degradation**

PON2, which directly interacts with and is positively regulated by D₉R, mediates, in part, the inhibitory effect of renal D₉R on ROS production. We next investigated if PON2 has a role in the activation of sestrin2 by D₉R. Treatment of human RPTCs with PON2 siRNA decreased the expression of sestrin2 by 31±5% and completely abolished its upregulation by quinpirole (Figure 4A), indicating that the stimulatory effect of quinpirole on sestrin2 is mediated solely by PON2. Sestrin2 is downstream of PON2 because silencing sestrin2 does not decrease PON2 expression (Figure S3A). Silencing PON2 increased peroxiredoxins hyperoxidation by 2.9-fold (Figure 4B), ROS production by 1.3-fold (Figure 4C), and lipid peroxidation by 2.3-fold (Figure 4D). The increase in ROS production induced by PON2 silencing is mediated, in part, by an increase in NADPH oxidase expression and activity. However, silencing sestrin2 did not alter NADPH oxidase activity (Figure S3B), indicating that the involvement of sestrin2 in the increased oxidative stress caused by PON2 silencing is independent of NADPH oxidase. PON2 depletion did not alter the transcription of sestrin2 (Figure S3C); PON2 and sestrin2 did not communoprecipitate, suggesting they may not physically interact (Figure S3D). Because silencing PON2 was associated with decreased sestrin2 expression without decreased transcription, post-translational modification may be involved, for example, protein degradation. Sestrin2 ubiquitylation was determined as a parameter of protein degradation. PON2 downregulation was associated with a 30% reduction in sestrin2 expression (Figure 4E) and a 2.8-fold increase in ubiquitylated sestrin2, suggesting that PON2 silencing decreases sestrin2 expression.

**Figure 2.** Stimulation of dopamine D₂ receptor decreases cellular oxidative stress, in part, via upregulation of sestrin2. Sestrin2 mRNA expression, analyzed by real-time reverse transcriptase polymerase chain reaction (A) and protein expression, analyzed by immunoblotting (B) in human renal proximal tubule cells (RPTCs) transfected with sestrin2 small interfering RNA (siRNA) or nonsilencing siRNA (15 nmol/L; 48 h). Inset: Representative immunoblots of sestrin2 and β-actin. Results are shown as mean±SE from 4 independent experiments; *P<0.01; t test. C. Reactive oxygen species (ROS) production measured using the 2', 7'-dichlorofluorescein diacetate method in human RPTCs transfected with sestrin2 siRNA or nonsilencing siRNA (15 nmol/L; 24 h) and then treated with quinpirole (1 μmol/L) or vehicle for 24 h. Inhibitory effect of quinpirole on ROS production is expressed as percentage decrease in ROS production compared with vehicle. Results are showed as mean±SE from 4 independent experiments; *P<0.05; t test.
by increasing its ubiquitinylation and presumably resulting in its degradation (Figure 4E).

**Selective Renal Silencing of Sestrin2 in Mice Increases Arterial Blood Pressure and ROS Production**

Renal subcapsular infusion of sestrin2 siRNA decreased renal sestrin2 expression by 52% (Figure 5A). However, sestrin2 siRNA treatment did not alter the expression of hyperoxidized peroxiredoxins (Figure S4A). Because hyperoxidized form of peroxiredoxins could be repaired by sulfiredoxin, we measured the renal sulfiredoxin expression after renal subcapsular sestrin2 siRNA infusion. The sulfiredoxin protein expression was increased (1.5-fold) in mice kidneys subcapsularly infused with sestrin2 siRNA compared with nonsilencing siRNA infusion (Figure S4B). Anesthetized sestrin2 siRNA–infused mice had higher systolic (116±3 versus 94±2 mm Hg) and diastolic (84±2 versus 66±3 mm Hg) blood pressures compared with nonsilencing siRNA–infused littermates (Figure 5B). The blood pressures in mice were similar in the 2 groups before renal subcapsular infusion of nonsilencing siRNA or sestrin2 siRNA (data not shown). Renal ROS production was increased (1.3-fold) in mice kidneys subcapsularly infused with sestrin2 siRNA relative to nonsilencing siRNA–infused littermates (Figure 5C).

**Discussion**

Our results demonstrate for the first time that the antioxidant action of D2R is mediated, in part, by positive regulation of sestrin2 expression. D2R deletion in mice or D2R silencing in human RPTCs decreases, whereas D2R stimulation increases sestrin2 expression. Sestrin2 decreases ROS production and may prevent cellular damage from oxidative stress by catalyzing the reduction of hyperoxidized peroxiredoxins.18,23,24 We have shown that PON2 mediates the inhibitory effect of renal D2R on ROS production.15 Our current results show that D2R-induced sestrin2 upregulation is solely mediated by PON2; PON2 is upstream of sestrin2 because silencing of sestrin2 does not affect PON2 or D2R expression. This work...
also demonstrates that endogenous renal sestrin2 protects against oxidative stress and is involved in the maintenance of normal blood pressure, because selective renal silencing of sestrin2 increases both renal ROS production and blood pressure.

The protective effect of D2R stimulation on peroxiredoxins was completely abolished by sestrin2 downregulation. These results are in accordance with previous reports showing that NO preincubation prevented peroxiredoxin hyperoxidation in macrophages via sestrin2 upregulation23; FOXO3 activated a ROS rescue pathway by regenerating hyperoxidized peroxiredoxins through the elevation of the expression of sestrin331; and the histone deacetylase inhibitor trichostatin A increased sestrin2 expression and inhibited the formation of hyperoxidized peroxiredoxins caused by H2O2 in neurons.21

The mechanism by which PON2 regulates sestrin2 expression is still unclear and it seems to be independent of protein–protein interaction, because PON2 and sestrin2 do not coimmunoprecipitate. However, weak or transient interactions between PON2 and sestrin2 could not be excluded because it is often not detectable by coimmunoprecipitation. Our studies suggest that it may involve posttranslational modifications because PON2 silencing increases ubiquitinylation of sestrin2, which may increase its degradation.22 We have previously shown that D2R and PON2 decrease renal...
oxidative stress, in part, by decreasing NADPH oxidase activity. Our current study indicates that the decreased oxidative stress attributable to D2R and PON2 is, in part, the consequence of the upregulation of sestrin2 expression. However, the increased NADPH oxidase activity caused by PON2 silencing was not altered after sestrin2 siRNA treatment, suggesting that sestrin2 is not involved in the negative regulation of NADPH oxidase by PON2.

The negative regulation of ROS production by D2R is, in part, because of positive regulation of sestrin2 expression and function that is important to keep blood pressure in the normal range. Our results clearly show that renal sestrin2 is decreased in D2R−/− mice, which are hypertensive. The crucial role of the antioxidant effect of sestrin2 is proved by the increase in renal oxidative stress and arterial blood pressure with renal selective silencing of sestrin2. This is the first report that demonstrates the role of sestrin2 in the regulation of blood pressure. The increase in renal oxidative stress and arteriolar blood pressure with renal selective silencing of sestrin2. This is the first report that demonstrates the role of sestrin2 in the regulation of blood pressure. The increase in renal ROS production, associated with the activation of adrenergic nervous system and intrarenal renin–angiotensin system, may affect renal sodium transport causing sodium and fluid retention and ultimately hypertension.

Interestingly, we found that sestrin2 silencing increased hyperoxidized peroxiredoxins in human PRTCs. However, sestrin2 silencing in vivo in mice using renal subcapsular sestrin2 siRNA treatment did not increase hyperoxidized peroxiredoxins, although it increased renal ROS production and blood pressure. Our in vivo results are agreement to some extent with those of Woo et al., which showed that sulfiredoxin 2-Cys peroxiredoxin reduction was similar in sestrin2-knockout and wild-type mice. It is possible that the in vivo silencing of renal sestrin2 induces a mechanism that prevents an increase in hyperoxidized peroxiredoxins in an effort to mitigate the increases in ROS production. It has been reported that sulfiredoxin promotes the recovery of hyperoxidized peroxiredoxins. Our study showing increased sulfiredoxin expression after renal subcapsular sestrin2 siRNA treatment suggests that the lack of an increase in hyperoxidized peroxiredoxins in this model may be related to increased sulfiredoxin activity. However, sulfiredoxin expression is not increased in human PRTCs when sestrin 2 is downregulated; this may be explained by different temporal dynamics of the 2 models (7 days in vivo versus 2 days in vitro) or in vivo versus in vitro conditions. It is also possible that additional mechanisms are involved in the renal sestrin2 silencing–induced increase in ROS production and blood pressure. It has been reported that sestrin silencing in Drosophila resulted in chronic activation of mammalian target of rapamycin signaling, leading to ROS accumulation and development of a variety of age-related pathologies.

Sestrin2 was reported to inhibit p70S6K and S6 phosphorylation independent of peroxiredoxin 1. Further studies are needed to determine the exact mechanism by which sestrin2 decreases renal oxidative stress in vivo. Based on our results, we propose a hypothetical model for the D2R-induced activation of PON2 and sestrin2, which negatively regulates renal oxidative stress and helps maintain a normal blood pressure (Figure 6).

Perspective

Our study shows that D2R increases sestrin2 expression via PON2 and catalyzes the reduction of hyperoxidized peroxiredoxins, which in turn decreases renal oxidative stress. Endogenous renal sestrin2 expression protects against oxidative stress and is involved in the maintenance of normal blood pressure. This study identifies a mechanism by which D2R decreases renal ROS-induced high blood pressure, sheds light into the understanding of the pathogenesis of human essential hypertension, and gives insights into

Figure 5. Sestrin2 knockdown by the chronic renal subcapsular infusion of sestrin2 small interfering RNA (siRNA) decreased renal sestrin2 expression and increased blood pressure and renal oxidative stress. Mice kidneys were continuously infused underneath the renal capsule with sestrin2 siRNA or nonsilencing siRNA (3 μg/d) for 7 d. A, Immunoblot analysis of sestrin2 expression. Inset: Immunoblots of sestrin2 and β-actin. B, Diastolic and systolic blood pressures. C, Reactive oxygen species production, measured by the 2′, 7′-dichlorofluorescein diacetate method, in mouse kidneys. Results are mean±SE; n=5 per group; *P<0.05; t test.
Institutes of Health, HL068686, HL023081, HL074940, HL092196. This work was supported, in part, by grants from the National Institutes of Health, HL068686, HL023081, HL074940, HL092196.

Hypertension, leading to normal blood pressure. Peroxiredoxins (Prx), with both pathways decreasing renal sestrin2 expression, catalyzing the reduction of hyperoxidized peroxiredoxin (Prx), with both pathways decreasing renal oxidative stress, leading to normal blood pressure.

The modulation of renal sestrin2 function as a therapeutic approach in hypertension.

Sources of Funding
This work was supported, in part, by grants from the National Institutes of Health, HL068686, HL023081, HL074940, HL092196, DK090918, and DK039308.

Disclosures
None.

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**Novelty and Significance**

**What Is New?**

- Antioxidant action of dopamine D2 receptor (D2R) is mediated in part by positive regulation of sestrin2 expression.
- Sestrin2 counter-regulates renal reactive oxygen species (ROS) production and may contribute to the maintenance of normal blood pressure.

**What Is Relevant?**

- This study identifies a mechanism by which D2R decreases renal ROS-induced high blood pressure and sheds light into the understanding of the pathogenesis of human essential hypertension.
- Activation of sestrin2 may serve as a potential therapeutic agent in the treatment of ROS-induced hypertension. Our study gives insights into the design of personalized treatment for patients with hypertension.

**Summary**

We have demonstrated that sestrin2 expression is positively regulated by D2R, which in turn reduces peroxiredoxin hyperoxidation and decreases renal oxidative stress. Silencing sestrin2 increases peroxiredoxin hyperoxidation and impairs the ability of D2R to decrease oxidative stress. Silencing of paraoxonase 2 decreases sestrin2 expression and completely blocks the ability of D2R to upregulate sestrin2 expression. Selective renal silencing of sestrin2 increases renal ROS production and blood pressure. Our study suggests that sestrin2 counter-regulates renal ROS production and may contribute to the maintenance of normal blood pressure. Moreover, D2R inhibits ROS production by increasing sestrin2 via paraoxonase 2.
Sestrin2 Decreases Renal Oxidative Stress, Lowers Blood Pressure, and Mediates Dopamine D2 Receptor–Induced Inhibition of Reactive Oxygen Species Production

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Hypertension. 2014;64:825-832; originally published online July 14, 2014;
doi: 10.1161/HYPERTENSIONAHA.114.03840

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

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Sestrin2 decreases renal oxidative stress, lowers blood pressure, and mediates dopamine D2 receptor-induced inhibition of ROS production

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Material and Methods:

Renal selective silencing of sestrin2 and measurement of blood pressure

C57BL/6 mice were purchased from Jackson Laboratory (Bar Harbor, ME). Right uninephrectomy was first performed under pentobarbital anesthesia. One week following right 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 remaining left kidney capsule was gently lifted and separated from the kidney to create a subcapsular space. An osmotic minipump (ALZET® Osmotic Pump, model1007D), filled with sestrin2 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 the puncture site to prevent spillage and keep the tubing in place. The body of the minipump was then placed into the space previously occupied by the kidney that was removed; stabilization was achieved by suturing (4-0 ethilon) the minipump to the adjacent lateral abdominal musculature. An additional injection of the analgesic buprenorphine was given in the days after the surgery if and when needed. Before and after seven days of infusion of the siRNAs (3 µg/d), systolic and diastolic blood pressures were measured (Cardiomax II, Columbus Instruments, Columbus OH) from the aorta, via a catheter inserted into 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 surgeon was blinded to the experimental intervention. The studies were conducted in accordance with NIH
guidelines and approved by the Institutional Animal Care and Use Committee, University of Maryland.

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

Immortalized human RPTCs 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). RPTCs were serum-starved for 2 hours then treated with 1 μmol/L quinpirole (D₂R agonist) and/or pretreated with 1 μmol/L L741,262 (D₂R antagonist) (1 hour before quinpirole) for 24 hours.

**RNA interference**

RPTCs were plated in 6-wells plates at 2 x 10⁵ cells per well one day before treatment. Pre-designed FlexiTube siRNA of D₂R (15nmol/L, 48h), PON2 (10nmol/L, 48h), sestrin2 (15nmol/L, 48h) (Qiagen) were transfected into RPTCs using HiperFect (Qiagen), according to the manufacturer’s instructions. AllStars siRNA (Qiagen) with a scrambled sequence 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 sestrin2. Real-time PCR reactions were carried out in a total volume of 25 μl using pre-designed QuantiTect Primers for sestrin2 and GAPDH (Qiagen). All measurements
were performed in triplicate to ensure reproducibility. The ratio of mean ± 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 [10]. The primary antibodies used were polyclonal anti-D$_2$R antibody (Millipore), polyclonal anti-PON2 (Abcam), polyclonal anti-sestrin2 antibody (Proteintech), peroxiredoxin-Cys and SO$_3$ (Abcam), sulfiredoxin (Santa Cruz), Txnip (MBL International Corporation), and 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.

**Co-immunoprecipitation**

Cell lysates were prepared using lysis buffer (Roche, Indianapolis, IN). Equal amounts of cell lysates (500 μg protein) were mixed with polyclonal anti-sestrin2 (Proteintech), or normal rabbit IgG (Sigma) which served as negative control. Protein G agarose beads (30 μL) (Roche, Indianapolis, IN) were added to each sample with rocking, overnight. The immune complexes were pelleted out, and the bound proteins were eluted using 30 μl of Laemmlsi buffer. The samples were immunoblotted with polyclonal anti-ubiquitin (Cell Signaling) or anti-sestrin2 antibody.

**Detection of reactive oxygen species (ROS)**
The oxidation of 2’, 7’-dichlorofluorescin diacetate (DCFDA) was used to measure ROS in human RPTCs and whole kidney homogenates. Briefly, cells or whole kidney homogenates were incubated with fresh DCFDA (10 μmol/L/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 an emission wavelength of 530 nm. ROS production was expressed in arbitrary units corrected for protein concentration (AU/mg protein). All assays were performed in duplicate.

**Measurement of lipid peroxidation (TBARS assay)**

Lipid peroxidation (LPO) in human RPTCs was measured by the formation of thiobarbituric acid reactive substances (TBARS). 100μl cell lysates were added into test tubes followed by the addition of thiobarbituric acid (TBA), acetic acid, and sodium hydroxide. The mixture was boiled for 60 min and then cooled in an ice bath for 10 min followed by centrifugation at 1600g for 10 min. The absorbance of the samples was measured at 535 nm against TBA as blank. A standard curve was prepared correlating the concentration of MDA in the solution. The concentration of MDA was read from standard calibration curve.

**Determination of NADPH oxidase activity by lucigenin chemiluminescence**

NADPH oxidase activity was determined by measuring superoxide generation in whole cell homogenates in the presence of lucigenin (5 μmol/L) and NADPH (100 μmol/L, ICN Biomedicals), using a microplate luminometer in 96-well plates (Centro LB 960, Berthold Technologies), as previously reported [15]. The specificity of the NADPH-dependent superoxide anion production was verified by treatment with DPI (10 μmol/L/30 min) (Sigma). Equal amounts of cell lysates were incubated with lucigenin for 10 min 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.

**Results:**

*Figure S1: Sestrin1 and sestrin3 protein expressions were analyzed by immunoblotting in human RPTCs treated with quinpirole (1 µmol/L) or vehicle for 24 hours (A, C) or transfected with D2R siRNA or non-silencing siRNA (15 nmol/L, 48 h) (B, D). Insets: representative immunoblots of sestrin1, sestrin3 and β-actin. Results are mean±SE from three independent experiments. There are no significant differences between vehicle vs. quinpirole or non-silencing siRNA vs. D2R siRNA, *t*- test.*
Figure S2: Sulfiredoxin and Txnip protein expressions were analyzed by immunoblotting in human RPTCs treated with quinpirole (1 µmol/L) or vehicle for 24 hours (A, C) or transfected with D2R siRNA or non-silencing siRNA (15 nmol/L, 48h) (B, D). Insets: representative immunoblots of sulfiredoxin, Txnip, and β-actin. Results are mean ± SE from three independent experiments. There are no significant differences between vehicle vs. quinpirole or non-silencing siRNA vs. D2R siRNA, t-test.
Figure S3: 

(A) PON2 protein expression, analyzed by immunoblotting in human RPTCs transfected with sestrin2 siRNA or non-silencing siRNA (15 nmol/L, 48 h). Inset: representative immunoblots of PON2 and β-actin. 

(B) NADPH oxidase activity, measured by the lucigenin chemiluminescence method in human RPTCs transfected with PON2 siRNA (10 nmol/L, 48 h) with or without sestrin2 siRNA (15 nmol/L, 48 h). 

(C) Sestrin2 mRNA expression, analyzed by real-time qRT-PCR in human RPTCs transfected with PON2 siRNA (10 nmol/L, 48 h) or non-silencing siRNA (10 nmol/L, 48 h). 

(D) Co-immunoprecipitation between sestrin2 and PON2 in human RPTCs. Inset: representative immunoblots of PON2. All results are mean ± SE (A, B, C) from three independent experiments. There are no significant differences between groups, t-test.
Figure S4: Mouse kidneys were continuously infused underneath the renal capsule with sestrin2 siRNA or non-silencing siRNA (3 µg/d) for 7 days. Hyper-oxidized peroxiredoxin (Prx) (A) and sulfiredoxin protein expressions (B) were measured by immunoblotting. Inset: representative immunoblots of Prx-SO$_{2/3}$ and Prx-2Cys (A), and sulfiredoxin and β-actin (B). Results are mean ± SE, n=5/group. *P<0.05 vs. non-silencing siRNA, t-test.