Lipid Rafts Keep NADPH Oxidase in the Inactive State in Human Renal Proximal Tubule Cells

Weixing Han, Hewang Li, Van Anthony M. Villar, Annabelle M. Pascua, Mustafa I. Dajani, Xiaoyang Wang, Aruna Natarajan, Mark T. Quinn, Robin A. Felder, Pedro A. Jose, Peiying Yu

Abstract—Recent studies have indicated the importance of cholesterol-rich membrane lipid rafts (LRs) in oxidative stress-induced signal transduction. Reduced nicotinamide-adenine dinucleotide phosphate (NADPH) oxidases, the major sources of reactive oxygen species, are implicated in cardiovascular diseases, including hypertension. We tested the hypothesis that NADPH oxidase subunits and activity are regulated by LRs in human renal proximal tubule cells. We report that a high proportion of p22phox and the small GTPase Rac1 are expressed in LRs in human renal proximal tubule cells. The D1-like receptor agonist, fenoldopam (1 μmol/L per 20 minutes) dispersed Nox subunits within LRs and non-LRs and decreased oxidase activity (30.7±3.3%). In contrast, cholesterol depletion (2% methyl-β-cyclodextrin [βCD]) translocated NADPH oxidase subunits out of LRs and increased oxidase activity (154.0±10.5% versus control, 103.1±3.4%), which was reversed by cholesterol repletion (118.9±9.9%). Moreover, NADPH oxidase activation by βCD (145.5±9.0%; control: 98.6±1.6%) was also abrogated by the NADPH oxidase inhibitors apocynin (100.4±3.2%) and diphenylene iodonium (9.5±3.3%). Furthermore, βCD-induced reactive oxygen species production was reversed by knocking down either Nox2 (81.0±5.1% versus βCD:162.0±2.0%) or Nox4 (108.0±10.8% versus βCD:152.0±9.8%). We have demonstrated for the first time that disruption of LRs results in NADPH oxidase activation that is abolished by antioxidants and silencing of Nox2 or Nox4. Therefore, in human renal proximal tubule cells, LRs maintain NADPH oxidase in an inactive state. (Hypertension. 2008;51[part 2]:481-487.)

Key Words: NADPH oxidase ■ ROS ■ dopamine receptors ■ lipid rafts

Reduced nicotinamide-adenine dinucleotide phosphate (NADPH) oxidase, originally identified as a key component of human innate host defense,1 and studied extensively in phagocytes,1-3 has been subsequently studied in nonphagocytic cells, including gastrointestinal, neural, pulmonary, renal, and vascular smooth muscle cells.4-17 These studies provided evidence that this enzyme is the major source of reactive oxygen species (ROS) in nonphagocytic cells.5-17 In addition to mediating the intracellular killing of pathogens in leukocytes and macrophages, ROS may serve as signal transducers. However, ROS may also be important mediators of cell injury6-16; oxidative stress is thought to be important in the pathogenesis of hypertension.15-17

The primary redox component of all NADPH oxidases is known as Nox, which has 7 isoforms: Nox1; gp91phox (also termed Nox2); Nox3; Nox4; Nox5; Duox1; and Duox2. Nox 1 is mainly expressed in the colon,2,4 Nox2 was originally found in human neutrophils,1-4,8,18 Nox3 is expressed in fetal liver and the lung, Nox4 is found in the kidney,4,10,11 Nox5 is present in spleen and sperm, and Duox1 and Duox2 are primarily found in the thyroid. However, these Noxs are also expressed in other tissues.4-16 In the kidney, renox/Nox4 (which is homologous to Nox2) is highly expressed in the proximal convoluted tubule,10 whereas Nox4 is highly expressed in distal tubule.11 Activation of Nox2 requires its assembly with a membrane subunit, p22phox; several cytosolic regulatory subunits, p67phox, p47phox, and p40phox; and small GTPase Rac.1-3,18,19

Lipid rafts (LRs) are composed of glycosphosphatidylinositol-linked proteins, glycosphingolipids, and cholesterol that are associated with signaling molecules, eg, receptors such as G proteins.20 There are several types of LRs, but the best described are the caveolae.20-23 These LRs are characterized by the presence of caveolins, which bind to cholesterol. Caveolar and noncaveolar LRs have been implicated in protein trafficking and signal transduction.17,20-23 We and others have reported that there are noncaveolar LRs in human embryonic kidney and rat renal proximal tubule cells because these cells are devoid of measurable caveolin-124,25 and, therefore, could not form caveolae.21,24,25 Nox2 and its subunits are distributed in and their activity regulated by LRs in murine microglial, bovine aortic endothelial, and bovine...
coronary arterial endothelial cells; however, there are no such reports in nonphagocytic human cells.30,31

Dopamine is an important regulator of blood pressure and renal and adrenal function through an independent peripheral dopaminergic system.32,33 Abnormalities in renal dopamine production and receptor function have been described in human essential hypertension and rodent models of genetic hypertension.32,33 D1-like dopamine receptor-mediated inhibition of NADPH oxidase activity has been reported by several groups, including ours. Those studies were performed in vascular smooth muscle cells in rats, renal tubules in mice, and embryonic kidney and peripheral blood mononuclear cells in humans. However, those reports did not describe the membrane microdomain localization of NADPH oxidase subunits and did not provide a cellular biological mechanism for the D1-like receptor-mediated inhibition of NADPH oxidase activity. We tested the hypothesis that NADPH oxidase subunits and activity are regulated by D1-like receptors in LRs of human renal proximal tubule (HRPT) cells. We now report for the first time that the majority of p22phox and Rac1 are distributed in LRs, whereas Nox4 is excluded from LRs. Cholesterol depletion increased NADPH oxidase activity by redistributing NADPH oxidase subunits in non-LRs. In contrast, the D1-like receptor agonist (fenoldopam)-induced inhibition of oxidase activity was accompanied by the dispersal of Nox and subunits in LRs and Nox2 and Nox4 in non-LRs. These studies suggest that, in human nonphagocytic cells, LRs keep NADPH oxidase in the inactive state.

**Methods**

An expanded Methods section is available online at http://hyper.ahajournals.org.

**Measurement of Nox Activity**

Cells treated with the indicated drugs and whole cell membranes were prepared (see the supplemental data). NADPH oxidase activity was determined by measuring superoxide generation in whole cell membranes in the presence of lucigenin (10 μmol/L) and NADPH (100 μmol/L).35,36 The dynamic tracings of NADPH-dependent activity were recorded for 180 seconds (AutoLumat Plus luminometer, LB953, EG&G Berthold). The activities were expressed as arbitrary light units (ALUs) corrected for the protein concentration and duration of the experiment (ALU/second per milligram of protein). To compare the control and drug treatment groups, the percentage difference of the absolute value of each drug treatment from the absolute mean of the controls was calculated. All of the assays were performed in duplicate. All of the protein concentrations were assayed via Bio-Rad kit.

In some experiments, ROS were measured in living cells. Cells (10^6 cells per well), seeded in 6-well plates and treated with drugs, were washed twice with the assay buffer and scraped into an assay tube. The assay was started by the addition of lucigenin and NADPH, and the dynamic tracing was recorded as described above. Activity is expressed as ALUs per well in dynamic tracing graph and converted to the percentage of change from control.

**LR Labeling and Confocal Microscopy**

LRs were labeled using the Vybrant LR labeling kit. Cells grown on coverslips were washed once with serum-free medium and incubated with cholera toxin subunit B conjugated with Alexa Fluor 400 (CTB-488) at 1.0 μg/mL for 10 minutes at 4°C. The cells were washed and incubated with polyclonal anti-CTB antibody (1:200 dilution) for 15 minutes at 4°C. The cells were then stained with monoclonal anti-Nox2 or anti-p22phox antibodies for 30 minutes.24

Fluorescence images were obtained using laser confocal scanning microscopy (Olympus Fluoview FV600) at excitation and emission wavelengths of 488 nm and 505 nm, respectively.

**Statistical Analysis**

The data are expressed as means±SEMs: Significant differences between the 2 groups were determined by Student’s t test. Significance among several groups was determined by 1-way factorial ANOVA followed by the Newman-Keuls test. P<0.05 was considered significant.

**Results**

**Analysis of NADPH Oxidase Subunit mRNA in hRPT Cells**

The expression of mRNA for Nox1, Nox2, Nox4, p22phox, p67phox, p47phox, p40phox, and Rac1 in hRPT cells, shown previously to express D1-like receptors,8 is shown in Figure S1.

**Analysis of Nox and Subunit Proteins in Membranes From Sucrose Gradients by Immunoblotting**

Less than 10% of the membrane proteins were recovered in LRs (fractions 2 to 6; Figure 1A); the LR marker protein, caveolin-1, was mainly found in LRs; the other LR marker, flotillin-1, was found in both LRs and non-LRs (Figure 1A). Dopamine D1 and D5 receptors also cofractionated with the LR markers but were found in non-LR membrane fractions as well (Figure 1A).

The basal distributions of Nox2, Nox4, and NADPH oxidase subunits in LR and non-LR fractions are shown in Figure 1B and 1C and Table S2. The percentage of LR protein expressions, relative to total LR and non-LR were as follows: Nox2, 40.3%; p22phox, 65.7%; and Rac1, 57.7% in LRs; Nox4 was almost exclusively expressed in non-LRs. p67phox tended to be expressed more in LRs than in non-LRs; however, significance was not attained. p40phox and p47phox were mainly localized in non-LRs (Figure 1C).

Fenoldopam shifted the Nox2 bands from fractions 4 and 5 to fraction 3; shifted p22phox bands from fractions 4 and 5 to 3 and 4 in LRs and to fractions 10 to 12 in non-LRs; minimally shifted p67phox band from fractions 5 to 3 and 4; and decreased Rac1 bands in fractions 5 and 4 and redistributed them to fractions 10 and 11 in non-LR fractions (Figure 1B and 1C). Fenoldopam also decreased the protein expression of p22phox and Rac1 in LRs and Nox2 and Nox4 in non-LRs (Figure 1D and Table S3). Moreover, fenoldopam altered the localization of some of the Nox2 and p22phox from membranes into small intracellular vesicles (Figure 1E). Fenoldopam did not affect the distribution of p47phox and minimally altered the distribution of p40phox (Figure 1C).

**Effect of D1-Like Receptor Agonist on NADPH Oxidase Activity in hRPT Cells**

The fenoldopam-induced alterations in NADPH oxidase subunit localization were associated with a decrease in total cell membrane oxidase activity. Thus, fenoldopam decreased NADPH oxidase activity (69.3±3.3% versus control: 100±2.8%) that was blocked by the D1-like receptor antagonist, Sch23390 (101.1±3.0%), which by itself had no effect (98.2±6.8%; Figure 2). These results indicate that the inhib-
itory effect of fenoldopam on NADPH oxidase activity in hRPT cells was specifically mediated by D₁-like receptors (D₁R or D₅R). The inhibition of oxidase activity through the activation of D₁-like receptors at a low concentration of the agonist is in agreement with previous reports.34–36

**Effect of Methyl-β-Cyclodextrin on ROS Production and Membrane NADPH Oxidase Activity in hRPT Cells**

To investigate the functional role of LRs in the regulation of NADPH oxidase, methyl-β-Cyclodextrin (βCD), a well-established cholesterol depleting reagent, was used to disrupt LRs.21,23,24 The effects of βCD on ROS production are shown in Figure 3 and Table S4. βCD dose-dependently increased ROS production, with the maximum effect noted at 15 mmol/L. This effect also increased with time, peaking at 60 minutes. A higher concentration (20 mmol/L) and a longer period of incubation (90 minutes) resulted in a decrease in ROS production relative to the previous dose or time period (Figure 3). Treatment of hRPT cells with βCD (2% per 1 hour at 37°C) decreased membrane cholesterol by 54.9±4.6%, (Figure S4), in agreement with previous reports.21,23 βCD treatment also resulted in a redistribution of Nox2 and other subunits toward the non-LR fractions.7 to 12 (Figure 1B and 1C).

To determine whether the effect of βCD on Nox activity depended on caveolae or dopamine receptors, HEK-293 cells transfected with vector or with D₁ receptors (HEK-hD₁ cells) were studied in addition to hRPT cells, which express both D₁ and D₅ receptors. hRPT cells contain caveolae because...
caveolin-1 is expressed (Figure 1A). In contrast, there are no caveolae in HEK-293 cells because they lack caveolin-1.24 Nox2 and Nox4 are endogenously expressed in human kidney and HEK-293 cells.4 D1-like receptors are endogenously expressed in hRPT cells38 but not in HEK-293 cells.39 Similar to the studies in hRPT cells, βCD increased Nox activity, which was completely reversed by cholesterol repletion (Figure 4A and Table S5). Of note was the greater increase in NADPH oxidase activity caused by βCD in HEK-293 cells, which do not express either the D1 or D5 receptor.39 This could be taken to indicate that NADPH oxidase activity may be negatively regulated by D1-like receptors, because HEK-293 cells that do not express D1-like receptors have higher Nox activity than cells that express them (Figure 4A).

To confirm that the stimulatory effect of βCD on NADPH oxidase activity in human kidney cells was also exerted in other human cells, human coronary artery smooth muscle cells (hCASMs), which also express caveolin-1,40 were studied. In hCASMs, βCD also increased ROS production (8.5±1.0-fold), an effect that was partially reversed by cholesterol repletion (Figure 4B).

Effect of NADPH Oxidase Inhibitors on βCD-Mediated Stimulation of Oxidase Activity in Human Kidney Cells

To investigate the mechanisms responsible for the effect of βCD on NADPH oxidase activity in human kidney cells (hRPT and HEK-hD1), oxidase inhibitors apocynin and di-phenylene iodonium chloride (DPI) were used. The increased NADPH oxidase activity induced by βCD treatment was completely reversed by the addition of apocynin and DPI (Figure 5 and Table S6) in both hRPT and HEK-hD1 cells. Apocynin, by itself, had a modest inhibitory effect, whereas...
Figure 5. Effect of apocynin or DPI on the βCD-induced increase in NADPH oxidase activity in hRPT and HEK-hD1 cells. The cells were treated with vehicle or the indicated drugs, and the cell membranes were assayed for NADPH oxidase activity. Dynamic tracings of oxidase activities (top) and the percentage changes in activity from control (bottom) are shown. Values are means ± SEMs. n = 4 to 6, ANOVA (Newman-Keuls test), *P < 0.001, βCD vs others, **P < 0.001, Apo vs others, †P < 0.001, A/βCD vs others except Con, $ and # P < 0.001, DPI and D/βCD vs others, respectively, for hRPT or HEK-HD1. Vehicle, Con; Apo indicates apocynin; A/βCD, Apo + βCD; D/βCD, DPI + βCD.

Figure 6. Effect of knockdown of Nox2 or Nox4 on the βCD-induced increase in ROS production in hRPT cells. The cells were transfected with siRNA for Nox2 or Nox4 and assayed for ROS activity. Dynamic tracings of ROS activities (top) and the percentage changes of ROS activity from control (bottom) are shown. Values are means ± SEMs. n = 4 to 6, ANOVA (Newman-Keuls), *P < 0.001, #P < 0.01, βCD vs others except Con, siRNA alone, or siRNA + βCD.

Discussion

NADPH oxidases are now recognized to have specific subcellular localizations in caveolae LRs, endosomes, and nuclei.17–21,26–31 There are only few studies that have reported the distribution and regulation of Nox proteins and oxidase subunits in LRs, and most of the studies were performed in nonhuman cells.26–29 except those in human neutrophils.30,31 The novel findings are as follows: (1) NADPH oxidase subunits (p22phox and Rac1) are mainly distributed in LRs rather than in non-LRs; (2) D1-like receptors (D1R and D5R) inhibit membrane NADPH oxidase activity that is accompanied by movement of Nox2 and subunits into more buoyant LR fractions or non-LR fractions and into intracellular vesicles; (3) cholesterol depletion with βCD increases membrane oxidase activity/ROS production; and (4) the stimulatory effect of cholesterol depletion is prevented by direct inhibition of NADPH oxidase or by knockdown of either Nox2 or Nox4 protein expression.

The current studies show that endogenously expressed Nox isoforms and oxidase subunits are regulated in caveolae-LRs and non-LRs. Of the NADPH oxidase subunits, p22phox plays a key role in NADPH oxidase function.4,12 Proper functioning of Nox2 and Nox4 requires association with p22phox,2–4,12 Activation of Nox2 also requires assembly of Rac1 and p67phox with Nox2-p22phox.12,18,19 Under basal conditions, a high proportion of p22phox and Rac1 is located in LRs, whereas a large amount of Nox4, Nox2, p47phox, and p40phox is located in non-LRs. Disruption of LRs with βCD decreases ROS production in hRPT cells. However, knockdown of either Nox2 or Nox4 completely prevented the stimulatory effect of βCD on ROS production, indicating that both Nox2 and Nox4 contributed to the enhanced ROS production when LRs were disrupted.
increased superoxide production (unpublished data). Transfection of rat p22PHOX in HEK-293 cells heterologously expressing rat Nox4 caused a 3-fold increase in ROS production. In contrast, transfection of human p22PHOX in HEK-293 cells heterologously expressing human NADPH oxidase isoforms, Nox1 to 4, did not further increase ROS production. These studies suggest inherent differences between human and rodent NADPH oxidase subunit activity, but the cause of such inherent differences remains to be discovered.

There are caveolae in rodent and bovine vascular smooth muscle and endothelial cells, similar to human RPT cells (current study) and human coronary arterial smooth muscle cells. In contrast, there are no caveolae in human neutrophils, rat renal proximal tubules, and mouse microglial cells. The effect of cholesterol-depleting drugs, eg, βCD, is because of cholesterol depletion and is not caused by nonspecific drug effects, because the drug-induced changes were reversed by cholesterol repletion. These studies support the notion that cholesterol, an important component of LRs, rather than caveolae mediates the effect of cholesterol depletion on basal oxidase activity and ROS production, be it stimulatory or inhibitory. The notion that the activation of NADPH oxidase with cholesterol depletion is secondary to translocation of NADPH oxidase subunits, resulting in its assembly and activation, is supported by the apocynin and DPI experiments. Apocynin and DPI, 2 structurally unrelated NADPH oxidase inhibitors, have been widely used in functional studies of NADPH oxidase. In the current report, both apocynin, which, by itself had a modest inhibitory effect on basal oxidase activity, and DPI, which by itself had a marked inhibitory effect, reversed the βCD-mediated increase in ROS production in hRPT and HEK-293 cells, indicating that NADPH oxidase is a major source of ROS production in these cells. In the basolateral state, cells produce low levels of ROS for self-defense, which requires only a minimal activation of Nox. Apocynin exerts its inhibitory effect by reacting with thiol groups and by inhibiting the translocation of cytosolic factors and their assembly with Nox2-p22PHOX in the cell membrane. DPI inhibits flavoprotein activity by direct binding and inhibition of mitochondrial electron transfer and NO synthase activity.

The pharmacological studies using apocynin and DPI do not directly prove the importance of redistribution of NADPH oxidase subunits in non-LRs in the stimulatory effect of cholesterol depletion on NADPH oxidase activity, and, thus, other mechanisms may be involved. However, the Nox2 and Nox4 knockout studies minimize the need for such alternative explanations. Nox2 and Nox4 are expressed in renal tubules, with Nox4 as the major isoform in the kidney. Inhibition of Nox2 or Nox4 protein expression in hRPT cells by Nox2 or Nox4 siRNA completely reversed the stimulatory effect of βCD on ROS production, suggesting that both Nox2 and Nox4 are critical in this phenomenon. Kawahara et al have reported that p22PHOX serves to stabilize the catalytic oxidase subunits and that Nox1 protein was decreased by silencing of p22PHOX. It is possible that knockdown of either Nox2 or Nox4 could have also reduced the expression of other Nox subunits, such as p22PHOX. Therefore, knocking down 1 oxidase subunit could influence the expression and function of the other subunits.

D1-like receptors decrease NADPH oxidase activity and ROS production, mediated via protein kinase A–dependent and –independent mechanisms and the direct inhibition of phospholipase D and NADPH oxidase activities. Our studies provide the first direct evidence for D1-like dopamine receptor–dependent signaling in membrane microdomains of hRPT cells. We have also reported previously that D1 receptors are regulated in LRs in HEK-hD1 cells. Several studies have shown that caveolar and noncaveolar LRs serve as platforms to store signaling proteins and as vehicles for endocytosis and trafficking during signaling, keeping some proteins in the active or in the inactive state. The current studies provide a cell biological mechanism by which D1-like receptors inhibit NADPH oxidase activity. Thus, NADPH activity can be inhibited by D1-like receptors via 2 mechanisms: redistribution of Nox2 and p22PHOX to more buoyant LR fractions and Rac1 and p22PHOX to non-LR fractions and decreasing the expression of Rac1 and p22PHOX in LRs and Nox2 and Nox4 in non-LRs. The D1-like receptor–mediated decrease in LR expression of NADPH oxidase subunits, such as p22PHOX and Rac1, could be because of translocation to non-LRs. The D1-like receptor–mediated decrease in the expression of Nox2 and Nox4 in non-LRs may have been the result of degradation; fenoldopam induced the movement of Nox2 into small intracellular vesicles (early or late endosomes), possibly en route to their degradation. This segregated compartmentalization of Nox and subunits would impair their assembly, similar to those described for apocynin, resulting in decreased NADPH oxidase activity and ROS production. However, it remains to be determined how D1-like receptors regulate Nox2 and NADPH oxidase subunit degradation and trafficking along with the other receptors in LRs in hRPT cells.

In summary, we show for the first time that D1-like receptors regulate NADPH oxidase activity through the redistribution of Nox2 and subunits (p22PHOX and Rac1) in membrane microdomains and intracellular vesicles. Disruption of LRs enhances NADPH oxidase activity and ROS production, which are abolished by antioxidant reagents and inhibition of Nox2 and Nox4 expression. Therefore, the integrity of LRs plays an important role in maintaining NADPH oxidase in the inactive state in human nonphagocytic cells.

Clinical Perspectives
The integrity of LRs plays an important role in keeping NADPH oxidase inactive in hRPT cells. Marked reductions in total cholesterol have beneficial effects on cardiovascular function. A 50% decrease in cell membrane cholesterol is associated with oxidative stress, and oxidative stress is associated with disease, including hypertension. Thus, it is possible that the marked reduction in serum cholesterol obtained through pharmacological treatment could result in a decrease in cell membrane cholesterol and a potentially deleterious increase in oxidative stress.

Sources of Funding
These studies were supported in part by grants from the National Institutes of Health (HL23081, HL074940, DK39308, and HL68686).
Disclosures

None.

References

Lipid Rafts Keep NADPH Oxidase in the Inactive State in Human Renal Proximal Tubule Cells

Weixing Han, Hewang Li, Van Anthony M. Villar, Annabelle M. Pascua, Mustafa I. Dajani, Xiaoyang Wang, Aruna Natarajan, Mark T. Quinn, Robin A. Felder, Pedro A. Jose and Peiying Yu

_Hypertension._ 2008;51:481-487; originally published online January 14, 2008; doi: 10.1161/HYPERTENSIONAHA.107.103275

_Hypertension_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

Copyright © 2008 American Heart Association, Inc. All rights reserved.

Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:

http://hyper.ahajournals.org/content/51/2/481

Data Supplement (unedited) at:

http://hyper.ahajournals.org/content/suppl/2008/01/24/HYPERTENSIONAHA.107.103275.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in _Hypertension_ can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:

http://www.lww.com/reprints

Subscriptions: Information about subscribing to _Hypertension_ is online at:

http://hyper.ahajournals.org//subscriptions/
ONLINE SUPPLEMENT

Lipid Rafts Keep NADPH Oxidase in the Inactive State in Human Renal Proximal Tubule Cells

1Weixing Han, 1Hewang Li, 1Van Anthony M. Villar, Annabelle M. Pascua, 1Mustafa I. Dajani,
1Xiaoyang Wang, 1Aruna Natarajan, 2Mark T. Quinn, 3Robin A. Felder,
1Pedro A. Jose and 1Peiying Yu

1Department of Pediatrics, Georgetown University School of Medicine, Washington, DC
2Department of Veterinary Molecular Biology, Montana State University, Bozeman, MT
3Department of Pathology, University of Virginia, VA

Address correspondence to
Peiying Yu, MD
Research Assistant Professor
Division of Pediatric Nephrology
Department of Pediatrics
Georgetown University Medical Center
4000 Reservoir Road, NW
Washington, DC 20057
Tel: 202-687-4951
Fax: 202-687-1503
E-mail: yup@georgetown.edu
Materials and Methods

Materials

Previously well-characterized human renal proximal tubule (hRPT) cells were used (1-3). The sources of reagents were: monoclonal antibodies to human NADPH oxidase subunits (Nox2, p22phox, p67phox) (4); anti-Rac1, siRNA for Nox2 and Nox4 and control siRNA (Santa Cruz Biotechnology, Inc., CA); an affinity purified polyclonal anti-Nox4 antibody (5), polyclonal anti-D3R and anti-D1R antibodies generated from our laboratory, specificities of which have been reported (6-8); anti-caveolin-1 and anti-flotillin-1 (BD Biosciences-Transduction Labs., Lexington, KY); secondary antibodies for blotting and immunostaining (Jackson ImmunoResearch Labs. West Grove, PA); ECL western blotting detection reagents (Millipore Corporation, Billerica, MA); BCA protein assay reagent (PIERCE, Rockford, IL); protease inhibitor Cocktail set III, fenoldopam, SCH23390, 2-N-morpholino ethanesulfonic acid (Mes), methyl-β-cyclodextrin (βCD), triethanolamine, lucigenin, NADPH, and cholesterol (SIGMA-Aldrich, St. Louis, MO); cell culture reagents (Life Technologies -GIBCO/BRL, Rockville, MD); Amplex Red cholesterol assay kit and Vybrant LR labeling kit (Molecular Probes Inc., Eugene, OR 97402).

Cell Culture

The hRPT cells from normotensive subjects were maintained in DMEM/F12 supplemented with 10% fetal bovine serum (FBS), epidermal growth factor (10 ng/ml), insulin, transferrin, selenium (5 μg/ml each), hydrocortisone (36 ng/ml) and triiodothyronine (4 pg/ml) at 37°C in humidified 5% CO2/95% air (1-3). Non-transfected human embryonic kidney (HEK-
293) cells and those heterologously expressing the human D₁ receptor (HEK-hD₁ cells) (6-20 passages) were cultured in MEM with 10% FBS, as previously reported (9).

**Cell Treatments**

Our laboratory has reported that the fenoldopam-mediated inhibition of O₂⁻ production in HEK-293 cells heterologously expressing the human D₃R is dose-dependent (10). The inhibitory effect of fenoldopam on ROS production in hRPT cells is also dose-dependent (Figure S3). Based on these data, a fenoldopam concentration of 1 µmol/L was chosen for all experiments in current report. Higher concentrations of D₁-like receptor agonists may increase superoxide production (11). The hRPT cells were grown in 100 cm dishes. At 90% confluence, cells pre-starved in serum-free DMEM/F12 medium (SFM) for 1 hour were treated with vehicle; the D₁-like receptor agonist, fenoldopam (1.0 µmol/L/20 min); the D₁-like receptor antagonist, SCH 23390 (Sch, 1.0 µmol/l) for 20 min; or Sch (2 min) plus fenoldopam for an additional 20 min at the same concentrations. To disrupt lipid rafts (LRs), the cholesterol depleting reagent, methyl-β-cyclodextrin (βCD) was used (12-14). In all cholesterol depletion and cholesterol repletion experiments, the βCD concentration was 2% and incubated at 37°C for 1 hour, except for the dose- and time-dependent experiments. The cells were washed once with SFM and then incubated with vehicle or βCD in SFM. Cholesterol repletion was performed by incubating the cells in a pre-mixed solution containing cholesterol (stock solution in 100% ethanol at 50 mg/ml) at 100 µg/ml along with βCD. In some cases, the cells were pretreated with apocynin (10 µmol/L) or diphenylene iodonium (DPI, 5.0 µmol/L), alone or in combination with βCD (2%/37°C) for 1 hour. The cells were washed in cold PBS after incubated with the drugs and subjected to experiment.
Cell transfections

Cells were seeded in 6-well plates at density of 2x10⁵/well at day 1 and then transfected with siRNA for Nox2 or Nox4 or control siRNA at day 2, following the manufacturer’s directions. ROS, assayed at day 4, as described in ‘Methods’, were expressed as ALU/well (10⁶ cells/well). The results were compared with controls that were given a value of 100%.

Reverse transcription-polymerase chain reaction (RT-PCR) analysis

RNA was extracted from hRPT cells, using the RNeasay RNA Extraction Kit (Qiagen) following the manufacturer’s directions. Total RNA was quantified by Smartspec™ Plus (Bio-Rad) and 1 µg of RNA was used to semi-quantify the mRNA expression of Nox2 and Nox4. The sets of the primers are listed in Supplement Table S1.

RT-PCR was carried out using Superscript III One-step RT-PCR Kit (Invitrogen) at the following conditions: 55°C for 30 min, 35 cycles at 95°C for 30 sec, 55°C for 30 sec, 68°C for 1 min, and one cycle at 68°C for 3 min. β-actin was used as the housekeeping gene to normalize the data. The products were resolved in 2% agarose gel and visualized using a digital gel documentation system (Alpha Innotech Corporation), as shown in Supplement Figure S1.

Whole cell membrane preparation

Cell membranes were prepared, as described previously (10) with slight modifications. Briefly, hRPT cells were treated with the indicated drugs. The cell pellets were collected after centrifugation (1000g/2min/4°C), washed with cold PBS, and re-suspended in 500 µl of ice-cold hypotonic lysis buffer containing (concentration in mmol/L, shown in square brackets) Tris-HCl
[25], EDTA [1], and EGTA [1], and protease inhibitors, pH 7.4. The cell suspensions were sonicated and centrifuged at 1000 g for 10 min. The supernatants were collected, the pellets were re-suspended in lysis buffer, and the sonication and centrifugation steps were repeated. The supernatants were combined and centrifuged at 425,000 g for 60 min. The pellets containing whole cell membranes were resuspended in assay buffer containing (mmol/L) sodium phosphate [10], potassium phosphate [2], potassium chloride [10], triethanolamine [50], NaCl [150], MgCl₂ [2], EDTA [1.0] and protease inhibitors.

Subcellular fractionation

To prepare LRs and non-LRs, hRPT cells were subjected to sucrose density gradient centrifugation using a detergent-free protocol according to Song (15), with slight modifications (9). Briefly, the cell pellets were lysed in 1.5 ml of 500 mmol/L sodium carbonate, pH 11, and homogenized in clear ultracentrifuge tubes. Equal amounts of homogenates (for each group of samples) were diluted 1:2 with 80% sucrose and overlaid with 5-35% sucrose as a discontinuous sucrose gradient and subjected to centrifugation at 160,000 g (Beckman SW40 rotor) at 4°C for 16 hours. After centrifugation, twelve 1-ml fractions were collected, and a light-scattering band confined to the 5-35% sucrose interface were in fractions 2-6 and represent lipid rafts (LRs) while fractions 7-12 represent non-LRs (9, 15, 16). Caveolin-1 and flotillin-1, which are strongly associated with LRs, and considered as LRs marker proteins (12), co-fractionated with these LRs fractions (9, 12-15). All sucrose solutions were prepared in MBS (mmol/L) Mes [25], pH 6.7, NaCl [150]. The fractionated proteins were mixed with Laemmli buffer, boiled, and subjected to immunoblotting (9).
**Immunoblotting**

To assess the quantity of proteins in the sucrose gradient fractions, 30 µl from each of the twelve fractions were subjected to SDS-polyacrylamide gradient (8-16%) gel electrophoresis and transferred onto nitrocellulose membranes. The membranes were probed with the indicated antibodies, and the immunoreactive bands were quantified as reported (9, 16, 17). Proteins of interest were normalized to control groups, with the control given a value of 100%. All protein preparations were carried out at 4°C in the presence of protease inhibitors (Cocktail set III), as described above. All protein concentrations were assayed with a Bio-Rad kit.

**Measurement of cholesterol content**

Cholesterol content was measured using the Amplex Red cholesterol assay kit following the manufacturer’s directions (18). hRPT cells were grown in 6-well plates to 90% confluence. Cells were washed with PBS buffer and incubated with vehicle (control) or βCD (15 mmol/L≈2%) in SFM at the indicated time points at 37°C. The cells were washed 3 times with PBS, and the cell pellets lysed in lysis buffer containing (mmol/L) 20 potassium phosphate, pH 7.4, 100 NaCl, 25 cholic acid, and 0.5% Triton X-100. The reactions were initiated by adding 50µl of Amplex Red reagent to each microplate well containing 50 µl of the diluted sample (1/5 dilution) and incubated for 30 min at 37°C, protected from light. Serially diluted cholesterol samples (0-10 µg) served as standard. The fluorescence was read at excitation and emission wave lengths of 530-560 and 590 nm, respectively, using Vector (Bio-Rad) reader. The cholesterol content was calculated and expressed as % change from control.
References:


Figure legends:

Figure S1. Expression of mRNA for Nox2 and Nox4 and cytosolic subunits in hRPTs

The expressions of Nox2, Nox4, and cytosolic subunit mRNA were evaluated in non-stimulated hRPT cells by RT-PCR using β-actin as control.

Figure S2. Sucrose gradient analysis of p47phox and p40phox in hRPT cells

Fractions from sucrose gradients were probed with monoclonal anti-p47phox and anti-p40phox. Low density fractions (fractions 2-6) contain LRs, while the high density fractions (fractions 7-12) contain non-LRs. The blots are one of three separate experiments.

Figure S3. Dose-dependent effect of the D1-like receptor agonist, fenoldopam, on ROS production in hRPT cells

The cells were treated with vehicle (Con) or varying concentrations of fenoldopam (10^-8, 10^-7, 10^-6 and 10^-5 μmol/L) in serum-free medium for 20 min at 37°C. The cell membranes were subjected to ROS assay, as described in ‘Methods”. The ROS activities are expressed as percentage change from control (left panel). The numerical values of the percentage changes are listed in the right panel. Values are mean ± SEM., n=4, ANOVA (Newman-Keuls test), *P<0.01, Con vs. all doses, 10^-8 vs. 10^-6 and 10^-5 μmol/L, †10^-8 vs. Con, 10^-6 and 10^-5 μmol/L, #P<0.05, 10^-7 vs. 10^-5 and 10^-6 μmol/L.

Figure S4. Effect of βCD on cholesterol content in hRPT cells

Cholesterol content was measured using the Amplex Red cholesterol assay kit, as described in ‘Methods’. The reactions were initiated by adding 50 μl of Amplex Red reagent to each microplate well containing 50 μl of the diluted sample (1:5 dilution) and incubated for
1 hour at 37°C. The assay was performed in triplicate. Values are mean±SEM from 4 independent experiments, t-test, *P<0.001, control vs βCD.

**Figure S5. Effect of siRNA for Nox2 and Nox4 on Nox protein expression in hRPT cells**

Cells were transfected with siRNA for Nox2 or Nox4 or their respective control-siRNAs, as described in the ‘Methods’. The cell lysates were probed with polyclonal anti-Nox4 and monoclonal anti-Nox2 and anti-β-actin antibodies. The immunoreactive bands (75 kDa) were quantified, as described previously (6-9, 10, 16, 17). Values are mean±SEM., n=4, ANOVA (Newman-Keuls), *P<0.01, siRNA vs control (vehicle) and control-siRNA.
Table S1. List of Primers for RT-PCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nox1</td>
<td>TTAACAGCACGCTGATCCTG (sense)</td>
</tr>
<tr>
<td></td>
<td>CTGGATGGGGATTTAGCCAAAG (anti-sense)</td>
</tr>
<tr>
<td>Nox2</td>
<td>GAATTGTACGTGGGCAGACC (sense)</td>
</tr>
<tr>
<td></td>
<td>CACTCCAGCTTGGGACACCTT (anti-sense)</td>
</tr>
<tr>
<td>Nox4</td>
<td>CGGCTGCATCAGTCTTAAACC (sense)</td>
</tr>
<tr>
<td></td>
<td>TTGACCATTGGGATTTCAT (anti-sense)</td>
</tr>
<tr>
<td>p22phox</td>
<td>CGCTTCACCCAGTGCTACTT (sense)</td>
</tr>
<tr>
<td></td>
<td>GTAGATGCCGCTCGCAAT (antisense)</td>
</tr>
<tr>
<td>p67phox</td>
<td>CTTCAACATTGGCTGATGT (sense)</td>
</tr>
<tr>
<td></td>
<td>GTCAGACTTGCATTGCTCGTGT (antisense)</td>
</tr>
<tr>
<td>p47phox</td>
<td>ACCAAGATCTCCCGCTGTC (sense)</td>
</tr>
<tr>
<td></td>
<td>CGCTCTCGCTCTTCTCTACG (antisense)</td>
</tr>
<tr>
<td>p40phox</td>
<td>TCCTCCTCAGTCGGATCAAC (sense)</td>
</tr>
<tr>
<td></td>
<td>ATGAGCGCTACGTCTCTCATC (antisense)</td>
</tr>
<tr>
<td>Rac1</td>
<td>CCCTATCCTATCCGCAAAACA (sense)</td>
</tr>
<tr>
<td></td>
<td>TCGCTTCTCAAAACACTGTC (antisense)</td>
</tr>
<tr>
<td>β-actin</td>
<td>AGAAATCTGGCCACCACACC (sense)</td>
</tr>
<tr>
<td></td>
<td>CTCCTTAATGTCACGCACGA (anti-sense)</td>
</tr>
</tbody>
</table>
Table S2. Distribution of Nox2 and oxidase subunits in lipid rafts

<table>
<thead>
<tr>
<th>Subunits</th>
<th>LRs</th>
<th>Non-LRs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nox2</td>
<td>40.3±3.7*</td>
<td>59.7±3.7</td>
</tr>
<tr>
<td>Nox4</td>
<td>0*</td>
<td>99.0±4.4</td>
</tr>
<tr>
<td>p22phox</td>
<td>65.7±3.3*</td>
<td>34.3±3.3</td>
</tr>
<tr>
<td>Rac1</td>
<td>57.7±3.1*</td>
<td>42.3±3.1</td>
</tr>
<tr>
<td>p67phox</td>
<td>56.5±6.9</td>
<td>43.5±6.9</td>
</tr>
</tbody>
</table>

Proteins from the 12 fractions were probed with the antibodies, as indicated, and the immunoreactive bands were quantified, as described in ‘Methods’. Fractions 2-6 represent lipid rafts (LRs), and fractions 7-12 represent non-lipid rafts (non-LRs). Results are expressed as % change (LRs + non-LRs = 100%). The values are mean ± SEM from 3-4 experiments, t-test, *P<0.01, LRs vs. non-LRs.
Table S3. Quantification of Nox subunits

<table>
<thead>
<tr>
<th>% Change</th>
<th>LRs</th>
<th>non-LRs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Fenoldopam</td>
</tr>
<tr>
<td>Nox2</td>
<td>100.0±6.0</td>
<td>90.0±8.0</td>
</tr>
<tr>
<td>Nox4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>p22phox</td>
<td>100.8±2.6</td>
<td>71.1±3.1*</td>
</tr>
<tr>
<td>Rac1</td>
<td>100.0±5.8</td>
<td>58.0±3.7*</td>
</tr>
</tbody>
</table>

The values are mean ± SEM from 3-4 experiments, paired t-test,*P<0.01, Control vs.
Fenoldopam, n=3-4, LRs = lipid rafts, non-LRs = non-lipid rafts.
Table S4. Dose- and time-dependent effect of βCD on ROS production in hRPT cells

<table>
<thead>
<tr>
<th>Dose (mmol/L)</th>
<th>0</th>
<th>5</th>
<th>10</th>
<th>15</th>
<th>20</th>
</tr>
</thead>
<tbody>
<tr>
<td>ROS (fold increase)</td>
<td>1.1±0.1*</td>
<td>5.3±0.2</td>
<td>8.4±0.6</td>
<td>13.2±0.8 †</td>
<td>10.3±0.7*</td>
</tr>
<tr>
<td>Time (min)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>1.0±0.1*</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>4.8±0.73</td>
<td>6.9±0.75</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>60</td>
<td>9.5±1.0*</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>90</td>
<td>7.8±1.1 ‡</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Cells were treated with βCD at the indicated doses for 1h or with βCD (2%=13 mmol/L) for the indicated time points at 37°C. Cells were assayed for ROS production, as described in ‘Methods’. Values are mean ± SEM from six separate experiments, ANOVA (Newman-Keuls test), *P<0.001, 0 vs. all doses, †P≤0.003, 15 vs 0, 5, 10 and 20 mmol/L, *P<0.001, 20 vs. 0, 5, 15 mmol/L; *P<0.001, 0 vs. all time points, *P<0.001, 60 min vs. 0 and 15 min, ‡P<0.05, 90 min vs. 0 and 15 min.
Table S5. Effect of βCD on NADPH oxidase activity in human kidney cell lines

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>Control</th>
<th>βCD</th>
<th>CCH</th>
</tr>
</thead>
<tbody>
<tr>
<td>hRPT</td>
<td>100.0±8.7</td>
<td>154.0±4.0*</td>
<td>104.5±6.8</td>
</tr>
<tr>
<td>HEK-293</td>
<td>118.6±12</td>
<td>196.6±9.3* †</td>
<td>121.0±11.6</td>
</tr>
<tr>
<td>HEK-hD1</td>
<td>103.1±3.4</td>
<td>164.6±10.5*</td>
<td>118.9±10</td>
</tr>
</tbody>
</table>

Cells were treated with βCD (2%/1h/37°C), or cholesterol plus βCD (CCH), and the membranes were prepared and assayed, as described in ‘Methods’. The data are expressed as activities in %, with control hRPT cells set at 100%. Values are mean ± SEM from 4-6 experiments, ANOVA (Newman-Keuls test), *P<0.001, βCD vs. control and CCH, †P<0.05, βCD in HEK-293 vs. βCD in hRPT and HEK-hD1 cells.
Table S6. Effect of βCD, apocynin, and/or DPI on NADPH oxidase activity in human kidney cell lines

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>Vehicle</th>
<th>βCD</th>
<th>Apo</th>
<th>A/βCD</th>
<th>DPI</th>
<th>D/βCD</th>
</tr>
</thead>
<tbody>
<tr>
<td>hPRT</td>
<td>100 ±1.0</td>
<td>146±9.0*</td>
<td>74.7±6.2 †</td>
<td>100±3.2 ‡</td>
<td>11±2.4*</td>
<td>9.5±3.3*</td>
</tr>
<tr>
<td>HEK-hD₁</td>
<td>100±4.5</td>
<td>153±3.6*</td>
<td>77.2±3.0 †</td>
<td>117±3.1 ‡</td>
<td>10±4.6*</td>
<td>7.9±2.2*</td>
</tr>
</tbody>
</table>

Cells were treated with vehicle, βCD (2%/1h/37°C), apocynin (Apo, 10 µmol/L), βCD plus Apo (A/βCD), DPI (5 µmol/L), or DPI plus βCD (D/βCD). Cell membranes were prepared and assayed, as described in ‘Methods’. Values represent the mean ± SEM from 4-6 experiments, ANOVA (Newman-Keuls test), *P<0.001, βCD vs. others, †Apo vs. others, ‡A/βCD vs. βCD, Apo, DPI and D/βCD, and *DPI and D/βCD vs. others respectively.
Table S7. Effect of Nox2 and Nox4 siRNA on βCD-induced increase in ROS production in hRPT cells

<table>
<thead>
<tr>
<th>Isoform</th>
<th>Control</th>
<th>βCD</th>
<th>siRNA</th>
<th>siRNA+βCD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nox2</td>
<td>100±1.0</td>
<td>162.3±2.0*</td>
<td>83.0±7.1</td>
<td>81.3±5.1</td>
</tr>
<tr>
<td>Nox4</td>
<td>100±1.0</td>
<td>151.7±9.8*</td>
<td>112.8±12.1</td>
<td>108.0±10.8</td>
</tr>
</tbody>
</table>

Cells were transfected with siRNA for Nox2 or Nox4 for 48 h. Cells were then treated with vehicle or βCD and assayed for ROS production, as described in ‘Methods’. The activity in vehicle-treated (Control) cells was set at 100%. Values are the mean ± SEM from 3-6 experiments, ANOVA (Newman-Keuls test), *P<0.001, βCD vs. control, siRNA and siRNA+βCD.
Figure S1
Figure S2

<table>
<thead>
<tr>
<th>Fraction No.</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
</tr>
</thead>
<tbody>
<tr>
<td>Con</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fen</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Con p47phox</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fen p40phox</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure S3

Dose-dependent fenoldopam-mediated inhibition of ROS production in hRPT cells

<table>
<thead>
<tr>
<th>Log (fenoldopam) (M)</th>
<th>% change of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Con</td>
<td>100±5.7%*</td>
</tr>
<tr>
<td>10^{-8}</td>
<td>85.8±4.2%†</td>
</tr>
<tr>
<td>10^{-7}</td>
<td>79.6±5.0%#</td>
</tr>
<tr>
<td>10^{-6}</td>
<td>64.6±6.3%</td>
</tr>
<tr>
<td>10^{-5}</td>
<td>53.9±7.0%</td>
</tr>
</tbody>
</table>
Figure S4

Fluorescence (% changes)

Con βCD

*
Figure S5

The effect of siRNA for Nox2 and Nox4 on protein expression

<table>
<thead>
<tr>
<th>% change</th>
<th>Control</th>
<th>siRNA</th>
<th>Control-siRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nox2</td>
<td>100.2±6.2</td>
<td>39.1±6.1*</td>
<td>121.2 ±6.2</td>
</tr>
<tr>
<td>Nox4</td>
<td>104.5±5.4</td>
<td>53.1±4.8*</td>
<td>105.6±8.1</td>
</tr>
</tbody>
</table>

Values are mean ±SEM., n=4. ANOVA (Student-Newman-Keuls), *P<0.01, siRNA vs. control and control-siRNA.