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Unique role of NADPH oxidase 5 in oxidative stress in human renal proximal tubule cells

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ABSTRACT

NADPH oxidases are the major sources of reactive oxygen species in cardiovascular, neural, and kidney cells. The NADPH oxidase 5 (NOX5) gene is present in humans but not rodents. Because Nox isoforms in renal proximal tubules (RPTs) are involved in the pathogenesis of hypertension, we tested the hypothesis that NOX5 is differentially expressed in RPT cells from normotensive (NT) and hypertensive subjects (HT). We found that NOX5 mRNA, total NOX5 protein, and apical membrane NOX5 protein were 4.2 ± 0.7 -fold, 5.2 ± 0.7 -fold, and 2.8 ± 0.5 -fold greater in HT than NT. Basal total NADPH oxidase activity was 4.5 ± 0.2 -fold and basal NOX5 activity in NOX5 immunoprecipitates was 6.2 ± 0.2 -fold greater in HT than NT ($P < 0.001$, $n = 6-14$ /group). Ionomycin increased total NOX and NOX5 activities in RPT cells from HT ($P < 0.01$, $n = 4$, ANOVA), effects that were abrogated by pre-treatment of the RPT cells with diphenyleneiodonium or superoxide dismutase. Silencing NOX5 using NOX5-siRNA decreased NADPH oxidase activity ($-45.1 \pm 3.2\%$ vs. mock-siRNA, $n = 6-8$) in HT. D₁-like receptor stimulation decreased NADPH oxidase activity to a greater extent in NT ($-32.5 \pm 1.8\%$) than HT (-14.8 ± 1.8). In contrast to the marked increase in expression and activity of NOX5 in HT, NOX1 mRNA and protein were minimally increased in HT, relative to NT; total NOX2 and NOX4 proteins were not different between HT and NT, while the increase in apical RPT cell membrane NOX1, NOX2, and NOX4 proteins in HT, relative to NT, was much less than those observed with NOX5. Thus, we demonstrate, for the first time, that NOX5 is expressed in human RPT cells and to greater extent than the other Nox isoforms in HT than NT. We suggest that the increased expression of NOX5, which may be responsible for the increased oxidative stress in RPT cells in human essential hypertension, is caused, in part, by a defective renal dopaminergic system.

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Introduction

The reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidases have been studied extensively in phagocytic and non-phagocytic cells, including those from cardiovascular, neural, pulmonary, and renal tissues [1–3]. The NADPH oxidase family, which has seven members, is classified into three groups:

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group 1, comprised of NOX1, NOX2, NOX3, NOX4; group 2, comprised of NOX5; and group 3, comprised of DOUX1 and DOUX2 [1]. NADPH oxidase-dependent enzymes are the major sources of reactive oxygen species (ROS) in non-phagocytic cells, including cardiovascular, neural, and renal cells [2,4–9]. ROS are important in intracellular signaling. However, excessive ROS production can lead to cell injury and death [2,9,10]. Indeed, oxidative stress is thought to be important in the pathogenesis of many diseases, such as cardiovascular and renal diseases and hypertension [2,4,5,9–11].

Nox1, Nox2, and Nox4 are predominantly expressed in cardiovascular and renal tissues in humans and rodents [2,4–8]. The NOX5 gene, which is present in humans but not rodents [12,13]

was initially reported to be expressed in human testis, spleen, and lymph node [14]. More recently, NOX5 has been reported in human vascular endothelial and smooth muscle cells [9,15–17]. Five splice variants of human NOX5, namely NOX5 α , NOX5 β , NOX5 γ , NOX5 δ , and short NOX5, have been identified [14,15]. NOX5 differs from the other NADPH oxidase isoforms in that it contains an N-terminal calmodulin-like domain with four binding sites for Ca²⁺ (EF hands) and does not require p22^{phox} or other subunits for its activation [1,3]. NOX5 can be activated by thrombin, platelet-derived growth factor, angiotensin II, endothelin 1, and protein kinase C [15–22]. NOX5 can also be directly regulated by intracellular Ca²⁺, the binding of which induces a conformational change, leading to enhanced ROS generation [18–23].

Dopamine is an important regulator of sodium balance and blood pressure through an independent peripheral dopaminergic system [11,24,25]. Knockout of any of the dopamine receptor subtypes (D₁R, D₂R, D₃R, D₄R, and D₅R) results in hypertension, the pathogenesis of which is receptor subtype-specific [24]. D₁-like receptor (comprised of D₁R and D₅R) regulation of renal proximal tubule (RPT) sodium transport is defective in genetically hypertensive rats and patients with essential hypertension [11,24–32]. More specifically, D₁R function is impaired in RPT cells from humans with essential hypertension [33]. Both decreased dopamine receptor function and increased ROS production contribute to the pathogenesis of essential hypertension [2,11,24,25]. D₁-like receptors inhibit oxidative stress in vascular smooth muscle and RPT cells [11,28–31]. However, oxidative stress can also cause dysfunction of D₁R in rat RPT cells [32] and ROS production is increased in RPT cells from spontaneously hypertensive rats (SHRs) [34]. NADPH oxidase activity and protein levels of Nox2 and Nox4 are greater in kidneys and cerebral arteries of SHRs than those from their normotensive controls (Sprague-Dawley and Wistar-Kyoto [WKY]) rats [29–31,35]. We now report for the first time that NOX5 is the predominant NADPH oxidase isoform expressed in human RPT cells, the expression of which is greater in RPT cells from hypertensive (HT) than normotensive subjects (NT). We suggest that increased NOX5 expression and activity may contribute to the increased oxidative stress in human essential hypertension.

To read materials and some methods, please see the online supplement.

Methods

Cell sources and cell culture

RPT cells, from histologically normal sections of human kidneys freshly obtained from adult normotensive (NT: NT9, NT16, and NT22) and hypertensive subjects (HT: HT2, HT14, and HT19) who had unilateral nephrectomy due to renal carcinoma or trauma, were grown in culture [33]. These cells have been characterized for D₁-like receptor expression and function (i.e., response to dopamine and dopamine receptor agonists and antagonists) [33,36].

The human RPT cells were maintained in phenol-free Dulbecco's modified Eagle's medium/F12 medium supplemented with 10% fetal bovine serum (FBS), epidermal growth factor (10 ng/ml), ITSTM premix (insulin, transferrin, and selenium), hydrocortisone (36 ng/ml), triiodothyronine (4 pg/ml), and primocin (500 μ g/ml) at 37 °C in humidified 5% CO₂/95% air [31,33,36].

Measurement of NADPH oxidase activity

NADPH oxidase activity was measured in triplicate, using whole cell membranes in the presence of lucigenin (10 μ mol/L) and NADPH (100 μ mol/L), as described previously [8,29,31]. The

dynamic tracings of NADPH oxidase-dependent activity were recorded for 180 s (AutoLumat Plus luminometer, LB953, EG&G Berthold, Germany) and the results expressed as arbitrary light units (ALU), corrected for protein concentration. We also measured ROS production in whole cell homogenates, in the presence of lucigenin (10 μ mol/L) and NADPH (100 μ mol/L), using a microplate luminometer (MicroWin 2000). NADPH oxidase activity was expressed as relative luminescence units (RLU), corrected for protein concentration. Protein concentrations were determined by a bicinchoninic acid kit. To compare vehicle and drug treatment groups, the percentage of the absolute value of each drug treatment relative to the absolute mean of the vehicle-treated controls was calculated.

In some experiments, calcium-dependent NADPH oxidase activity in RPT cells was measured [9,18,19,23]. Whole RPT cell membranes were prepared as described above and membrane oxidase activity was measured in the absence or presence of CaCl₂ (1.0 mmol/L) [9,19,23]. The difference between NADPH oxidase activity in the absence calcium (total NADPH oxidase activity) and NADPH oxidase activity in the presence of Ca²⁺ (NOX5 activity) represented Ca²⁺-independent oxidase activity [9]. The NADPH oxidase activities were expressed as RLU/50 μ g protein and converted to % change of NT as described previously [8,29,31].

Measurement of NOX5 activity in NOX5-immunoprecipitates

Because there is no specific test for NOX5 activity, we first isolated NOX5 from the other NADPH oxidase isoforms by immunoprecipitation, as described under “Materials and Methods”, [Supplementary information](#). The immunoprecipitates (IPs) were washed twice with ice-cold PBS and then finally washed once with assay buffer. NOX5 assay buffer was reconstituted as described [9,19,23]. NOX5 activity was measured in duplicate, by incubating the NOX5 immunoprecipitates with assay buffer (200 μ l) containing 1 \times PBS buffer, pH 7.4, (mmol/L) 100 KCl, 2 MgCl₂, and 1 EGTA, 100 μ M FAD (Sigma-Aldrich), in the presence of 1.0 mmol/L CaCl₂ and 50 μ mol/L lucigenin. The reaction was allowed to proceed for 20 min at 37 °C in a water bath. The supernatants, in duplicate, were immediately transferred into a 96-well microplate and read on a luminometer (MicroWin 2000) at 25 °C following the injection (20 μ l) of NADPH (200 μ mol/L, final concentration) and monitored for 30 min. Normal rabbit IgG IPs served as negative controls. NOX5 activity was expressed as relative luminescence units (RLU) and converted to % of control values [8,29,31]. In some cases RPT cell membrane NADPH oxidase activity was measured using the same protocol.

Measurement of extracellular ROS production

Extracellular ROS production was measured in triplicate by the superoxide dismutase (SOD)-inhibitable reduction of cytochrome c (absorbance at 550 nm). Cells (5 \times 10⁶ cells/well) cultured on 12-well plates were incubated in 0.5 ml phenol-free DMEM/F12 medium containing reduced cytochrome c (200 μ mol/L) with or without SOD (200 μ mol/L) for 15 min at 37 °C. The incubation was continued for another 30 min in the presence of vehicle or the D₁-like receptor agonist, fenoldopam (1 μ mol/L), and the absorbance of reduced cytochrome c was measured at 550 nm optical density (OD), using a Victor spectrophotometer (PerkinElmer Life, Shelton, CT). SOD-inhibitable reduction of cytochrome c was calculated by the formula [nmol = 159 \times (OD – SOD – OD + SOD)] [37,38]. The results were expressed as nmol/5 \times 10⁶ cells. To compare vehicle and drug treatment groups, the percentage of the absolute value of each drug treatment relative to the absolute mean of the vehicle-treated controls was calculated.

Measurement of ROS production in live RPT cells by a fluorogenic probe

Cells were initially grown on coverslips in DMEM/F12 complete medium. The medium was then changed to phenol-free DMEM/F12 medium containing 1% dialyzed FBS and the fluorogenic probe CellROX Deep Red reagent (5 $\mu\text{mol/L}$, final concentration) and incubated for 30 min at 37 °C. The cells were washed with PBS buffer three times, fixed with 4% paraformaldehyde in PBS buffer for 10 min, and mounted on coverslips using ProLong[®] Gold reagent. ROS fluorescence images were obtained using laser confocal scanning microscopy (Zeiss LSM 510) at excitation/emission wavelengths of 644/655 nm at 630x magnification. Using ZEN 2012 software NOX5 was colored green, ROS was colored red, and the merged image was yellow. In some cases 5-(and-6)-carboxy-2',7'-dichlorofluorescein diacetate (carboxy-DCFDA) was also used to measure ROS production ("Materials and Methods", Supplementary information).

Real-time quantitative PCR

Total RNA was extracted from RPT cells with the RNeasy Mini Kit (Qiagen Inc., Valencia, CA) and reverse transcription (RT) was performed using M-MLV reverse transcriptase (Invitrogen). Two micro liters of the RT product was used for real-time PCR (SYBR Green qPCR Supermix UDG Kit, Invitrogen): 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 63 °C for 60 s. The human NOX5 primers were 5'-AACTTCTG-GAAGTGGCTGCT-3' (sense, nt #1251-71), and 5'-GAGGAGATGAGT-GACCTTGGGA-3' (antisense, nt # 1376-86). Human GAPDH primers were 5'-GTCGTGGAGTCTACTGGCGTCTT-3' (sense), and 5'-CAGTCTTCTGAGTGGCAGTGATGG-3' (antisense). Expression of the target gene was normalized against the expression of GAPDH (ABI Prism 7700 sequence detector system, Applied Biosystems, SDS (Ver. 1.91, Foster City, CA).

Statistical analysis

Numerical data were expressed as mean \pm standard error of the mean (SEM). Significant difference between two groups was determined by the Student's *t*-test. Significant differences among groups (>2) were determined by one-way factorial ANOVA followed by the Newman-Keuls test. $P < 0.05$ was considered significant.

Results

Expression of NOX5 mRNA

Previous studies have shown that mRNA and protein levels of Nox isoforms (Nox2, and Nox4) are greater in RPT cells from SHR than WKY rats [31,39,40]; Nox1, Nox2, and Nox4 expressions are also increased in vascular smooth muscles cells of SHR [4]. In the current report, we determined which NADPH oxidase isoform contributed to the enhanced NADPH oxidase activity in RPT cells from HT, relative to NT. In contrast to the studies in rats, we found that in human RPT cells, NOX2 and NOX4 mRNAs were similar in NT and HT. In agreement with the studies in vascular smooth muscle cells of SHR [4], NOX1 mRNA was greater in HT than NT (Supplementary Fig. S1). The mRNA expression of NOX5, which is found in humans but not rodents, was also greater in HT (3.5 \pm 0.1 fold) than NT (1.0 \pm 0.2) (Fig. 1A). The increased basal NOX5 mRNA in HT relative to NT was verified by real-time qPCR; NOX5 mRNA was 4.2 \pm 0.7-fold greater in HT than NT ($P < 0.001$, vs. NT, $n = 4$, *t*-test) (Fig. 1B).

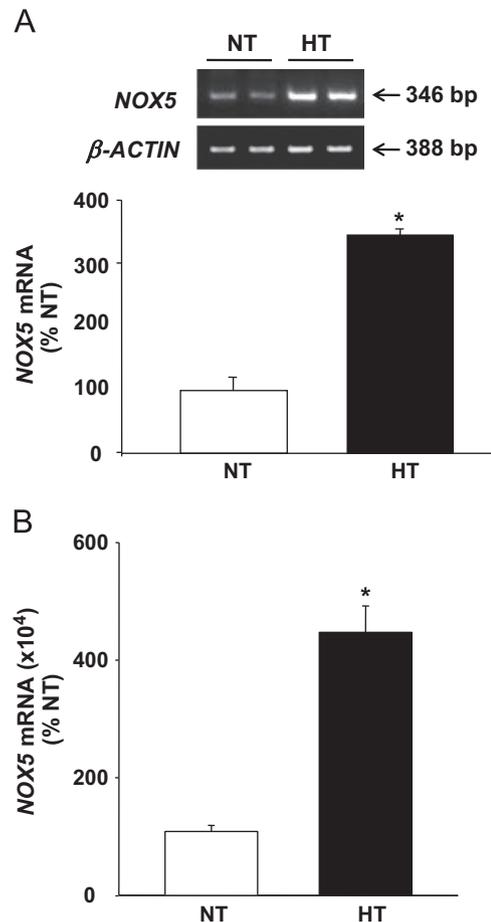


Fig. 1. Expression of NOX5 mRNA in human RPT cells. (A). RT-PCR analysis of human NOX5 mRNA in RPT cells from normotensive (NT) and hypertensive subjects (HT). The results were normalized with β -ACTIN mRNA, converted to % of NT, and shown as mean \pm SEM. * $P < 0.01$ vs. NT, $n = 4$, *t*-test. Two of four experiments are shown above the bar graphs. (B). Real-time quantitative PCR analysis of human NOX5 mRNA in human RPT cells. Reverse transcription (RT) utilized M-MLV reverse transcriptase and real-time quantitative PCR used the SYBR Green qPCR Supermix system. Results were normalized with GAPDH mRNA, converted to % of NT and shown as mean \pm SEM. * $P < 0.01$ vs. NT, $n = 4$, *t*-test.

Protein profile of NADPH oxidase isoforms in Human RPT cells

Except for NOX3 and DUOX, the five other NOX isoforms are ubiquitously expressed in tissues and organs of humans. NOX1, NOX2, and NOX4 proteins, which are present in RPT cells from humans [8] and rats, are expressed to a greater extent in SHR than WKY rats [4,31,39,40]. We, therefore, determined whether or not NADPH oxidase isoform protein expression is also greater in RPT cells from hypertensive than normotensive humans (i.e., HT vs. NT). In agreement with the mRNA data, total cellular NOX2 and NOX4 proteins were similar in NT and HT (Fig. 2A and B), while total cellular NOX1 protein was slightly greater in HT (1.2 \pm 0.-fold) than NT ($P = 0.016$) (Fig. 2A and B). By contrast, total cellular NOX5 protein was markedly greater in HT (5.2 \pm 0.7-fold) than NT (Fig. 2A and B). Similar results were obtained using four other RPT cell lines from HT ($n = 2$) and NT ($n = 2$) (Fig. S2, supplementary file). NOX3 protein was not found in human RPT cells, in agreement with a previous report [13]. We also studied the effect of the number of cell passages on NOX5 mRNA expression, NOX5 protein, and ROS production (6 vs. 20 passages). NOX5 expression and ROS production were not different after 6 or 20 passages of HT cells (Fig. S3 supplementary file). In addition, 3 pairs of human RPT primary cells (4–10 passages) were studied for NOX5 protein expression and activity. In these lower passaged RPT cells, NOX5

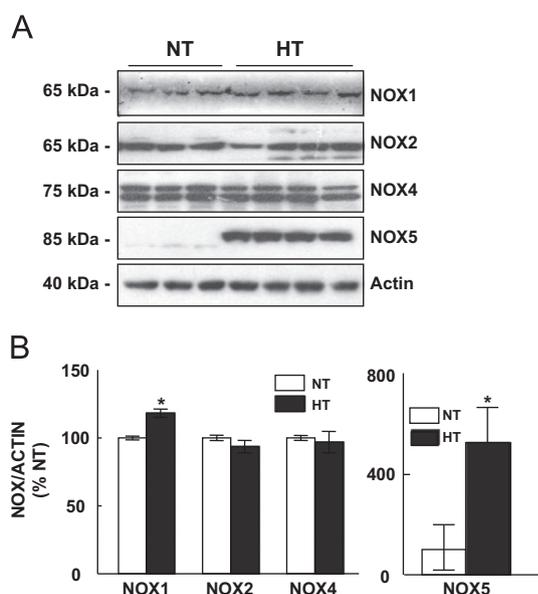


Fig. 2. Total cellular NOX isoform proteins in human RPT cells. (A). Immunoblotting analysis of **total** cellular NOX isoform proteins in RPT cells from normotensive (NT) and hypertensive subjects (HT). Human RPT cells were lysed in 2% SDS buffer and the cell lysates were immunoblotted with specific antibodies against NADPH oxidase isoforms, as indicated. The specificity of the antibody against NOX5 (Santa Cruz) was determined by immunizing peptide blocking study (Fig. S2A, supplementary file). (B). Quantification of **total** cellular NOX isoform proteins in human RPT cells. The immunoreactive bands from Fig. 2A were quantified by densitometry, as described under "Section 2"; the NOX isoform bands were normalized with the ACTIN bands, converted to % of NT and shown as mean \pm SEM of 4/groups, * $P < 0.05$, vs. NT, t -test.

protein was also greater in HT than NT (Fig. S4A, supplementary file); ROS production was also greater in HT ($349 \pm 91\%$ of NT) than NT cells (100 ± 3.0) (Fig. S4B, supplementary file).

Apical and basolateral membrane localization of NADPH oxidase isoform proteins

NOX5 was found in the perinuclear region and plasma membrane in human endothelial cells [15] and NOX5-transfected human embryonic kidney (HEK) 293 cells [20,21]. In RPT cells from NT, endogenous NOX5 was localized to a greater extent at the perinuclear area and to a lesser extent at the cell membrane (Fig. 3A, upper panel). In contrast, in RPT cells from HT, NOX5 was predominantly localized at the cell membrane but was present also in the cytosol and nucleus and co-localized with ROS (Fig. 3A, lower panel, and Fig. S5 supplementary file). ROS production was abrogated by pretreatment of the HT cells with the NADPH oxidase inhibitor, diphenyleneiodonium (DPI), and superoxide dismutase (SOD) which dismutates superoxide to hydrogen peroxide (Fig. 3B). The presence of NOX5 protein at the RPT cell membrane in HT was confirmed by biotinylation studies (Fig. S6, supplementary file). We quantified the protein levels of NOX isoforms (NOX1, 2, 4, and 5) at the apical membrane by cell surface biotinylation, immunoprecipitation, and immunoblotting with specific anti-NOX isoform antibodies. NOX1, NOX2, and NOX4 proteins were expressed to a greater extent in apical than basolateral membranes of RPT cells from both NT and HT (Fig. 3C and D). Furthermore, NOX1, NOX2, NOX4, and NOX5 protein levels were greater in the apical membranes of RPT cells from HT than NT; NOX5 protein, in particular, was 2.8 ± 0.5 -fold greater in HT than NT (Fig. 3C and D, and Table S1, supplementary file). Thus, while total cellular NOX1 and NOX5 proteins but not NOX2 and NOX4 were greater in RPT cells from HT than NT, apical membrane expressions of NOX1, NOX2, NOX4, and NOX5 were all increased in HT relative to NT.

However, of these NOX isoforms, the total cellular and apical membrane expression of NOX5 was increased to a greater extent than the other NOX isoforms in HT, relative to NT.

NADPH oxidase activity and ROS production

We used four different methods to measure NADPH oxidase activity and/or ROS production in RPT cells. First, total NADPH oxidase activity was measured in the presence of lucigenin and NADPH using whole cell membranes (Fig. 4A), as reported previously [8,31]. We have previously reported that basal NADPH oxidase activity was greater in RPT cells from SHR than from WKY rats [31]. In the current study, basal total NADPH oxidase activity was also greater in RPT cells from HT (4.5 ± 0.2 -fold) than NT (Fig. 4A). In contrast, the D_1 -like receptor agonist fenoldopam, using a concentration that is less than the V_{max} [29], decreased NADPH oxidase activity to a greater extent in RPT cells from NT ($-32.5 \pm 1.8\%$) than HT ($-14.8 \pm 1.8\%$) ($P < 0.01$, t -test) (Fig. 4B). The effect of fenoldopam was specific to the D_1 -like receptor because the inhibitory effect of fenoldopam on NADPH oxidase activity was reversed by the addition of the D_1 -like receptor antagonist Sch23390 in both NT and HT (Fig. 4B).

Calcium-dependent NADPH oxidase activity in total cell membranes was measured in the presence of $1.0 \text{ mmol/L CaCl}_2$ since NOX5 is a calcium-dependent enzyme [9,14,18,19,23]. NADPH oxidase activity expressed % control (no ionomycin) or RLU is shown in Fig. S7A and B in the supplementary file. As shown in Fig. 4C, Left figure, calcium-independent NADPH oxidase activity was 2.3 ± 0.3 -fold greater in HT ($230 \pm 33\%$) than NT cells ($100 \pm 1.6\%$) ($P = 0.003$). However, calcium-dependent NADPH oxidase activity was even greater (3.6 ± 0.7 -fold) in HT ($362 \pm 35\%$) than NT ($100 \pm 7.6\%$) ($P = 0.001$) (Fig. 4C, right figure).

NOX5 is a calcium-dependent enzyme and ionomycin, which raises the intracellular level of Ca^{2+} , stimulates NOX5 activity [9,14,18,19]. Therefore, we studied the effect of ionomycin on NOX5 activity using whole cell membranes from HT cells only because NOX5 protein expression was minimally expressed in NT cells (Figs. 1 and 2). Ionomycin stimulated NADPH oxidase activity only in the presence of calcium (Fig. S7A, supplementary file) [18]. Ionomycin increased membrane NADPH oxidase activity ($+127.3 \pm 6.0\%$ vs. $100.6 \pm 1.8\%$, vehicle control) ($P = 0.008$, vs. control, ANOVA) (Fig. 4D). The stimulatory effect of ionomycin on NADPH oxidase activity was decreased below control values by SOD, or DPI.

Second, NOX5-specific oxidase activity was measured in immunoprecipitated NOX5 in the presence of CaCl_2 . In HT and NT cells (4–18 passages) NOX5 activity in NOX5 immunoprecipitates was $2791.9 \pm 200.7 \text{ RLU}$ in HT vs. $432.3 \pm 128.5 \text{ RLU}$ in NT ($P = 0.003$, Table S2, supplementary file). Fig. 5A, left figure, shows that basal NOX5 activity (measured immediately after the injection of NADPH, in the presence of calcium) was 6.2-fold greater in HT ($624 \pm 25\%$ of NT) than NT ($100 \pm 4\%$) ($P < 0.001$, t -test, $n = 6-14$). The amount of NOX5 pulled down (Fig. S8 supplementary file) was also greater in HT ($244 \pm 5\%$) than NT ($102 \pm 2\%$) ($P = 0.03$). Fig. 5A, right figure shows the NOX5 activity monitored over a period of 30 min. Following the injection of NADPH, NOX5 activity increased with the time, peaked at 10 min and started to decline at 15 min. Fig. 5B shows that in HT cells, ionomycin increased NOX5 activity by $25 \pm 6.3\%$ compared with control (vehicle-treatment) ($P < 0.01$ vs. control, one-way ANOVA). The effect of ionomycin on NOX5 activity was abrogated by pre-treatment of HT cells with either DPI or SOD, both of which decreased NOX5 activity below control values, similar to those observed for total NADPH oxidase activity (Fig. 4D).

Third, extracellular ROS production was measured by SOD-inhibitable reduction of cytochrome c in live RPT cells [19–21,37,38].

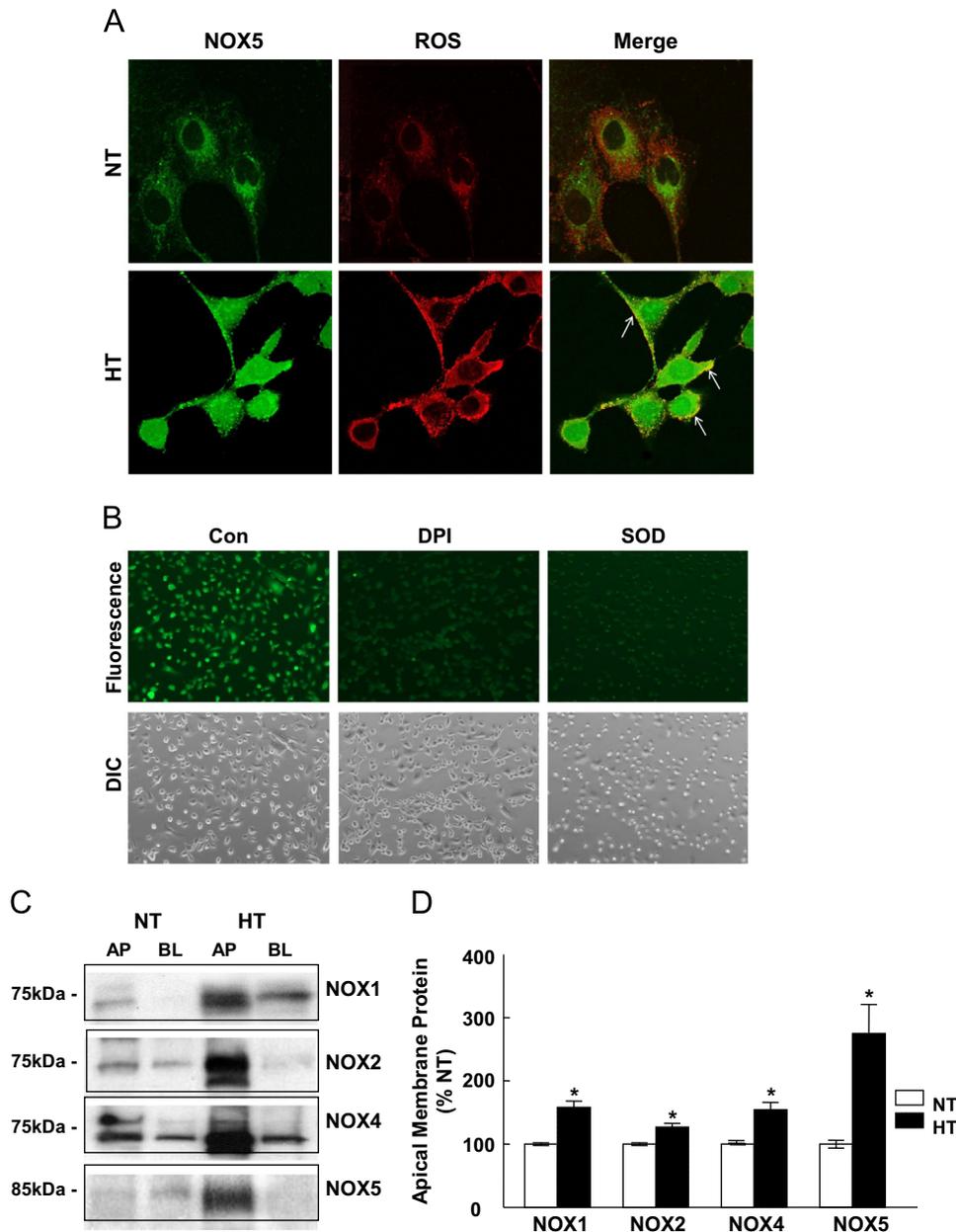


Fig. 3. Co-localization of cellular NOX5 protein and reactive oxygen species (ROS) and localization of NOX5 in apical membranes of human RPT cells. (A). Cellular NOX5 protein and ROS co-localization determined by confocal microscopy. ROS were labeled in RPT cells from normotensive (NT) and hypertensive subjects (HT) using the fluorogenic probe, CellROX Deep Red reagent (5 $\mu\text{mol/L}$, final concentration) as described under "Section 2". At the end of the incubation period, the coverslips were washed and fixed with 4% paraformaldehyde for 10 min at room temperature. The cells were stained with anti-NOX5 antibody. The fluorescence images were obtained using laser confocal scanning microscopy (Zeiss LSM 510) at excitation and emission wavelengths of 644/655 nm and 488/505 nm, respectively at 630x magnification. NOX5 is green, ROS is red, and the merged image is yellow. Arrows in HT, merge lower panel, indicate colocalization of NOX5 and ROS at the plasma membrane. (B). Effect of diphenyleneiodonium (DPI) and superoxide dismutase (SOD) on ROS production in RPT cells from HT. Cells were seeded to coverslips in 12-well plates on day one. The cells were pre-treated on day two with vehicle, DPI (30 $\mu\text{mol/L}$), or SOD (400 units/ml) for 30 min and then the cells were labeled with DCFDA at a final concentration of 5 $\mu\text{mol/L}$ for 10 min/37 $^{\circ}\text{C}$. The cells were washed 3 times with PBS buffer and the coverslips were mounted. The fluorescence images were obtained using laser confocal scanning microscopy (Zeiss LSM 510) at excitation and emission wavelengths of 485 and 535 nm, respectively. The upper panel shows fluorescence image and the lower panel shows differential interference contrast (DIC). (C). Expression of NOX isoform proteins in **apical** (AP) and **basolateral** (BL) membranes of human RPT cells. RPT cells from HT and NT, grown on Transwells, were separately biotinylated with a cell impermeable biotinylation reagent (EZ-link sulfo-NHS-LC-LC-biotin, 500 $\mu\text{g/ml}$) in the upper chamber (AP) or lower chamber (BL) of the Transwells, as described under "Methods", [Supplementary information](#). The cells were washed, lysed in MBST lysis buffer, and the cell lysates were immunoprecipitated with antibodies against NADPH oxidase isoforms. The proteins eluted from the immunoprecipitates were probed with horseradish peroxidase-conjugated streptavidin, and the biotinylated protein bands were visualized by ECL reagents. One of 4–5 separate experiments is shown. (D). Quantification of NOX isoform proteins in **apical** membranes of human RPT cells. The immunoreactive bands of apical membranes (AP) shown in Fig. 3C and those in the other 4–5 experiments were quantified by densitometry, as described under "Section 2". The results were expressed as % of NT and shown as mean \pm SEM of 4–5/groups, * $P < 0.05$, vs. NT, *t*-test.

To evaluate the relationship between the localization of the NOX isoforms at the apical membranes and extracellular ROS production, we measured extracellular ROS production using the SOD-inhibitable reduction of cytochrome *c*. We found that extracellular ROS production was 2.6-fold greater in HT ($45.9 \pm 2.0 \text{ nmol/5} \times 10^6$

cells) than NT ($18.4 \pm 3.5 \text{ nmol/5} \times 10^6$ cells) (Fig. 5C) ($P < 0.001$, one-way ANOVA, Newman–Keuls test, $n = 5/\text{group}$). D₁-like receptor function, assessed by production of cAMP or inhibition of sodium transporter or pump activity, is impaired in kidney tissues and cells from humans with essential hypertension and SHRS

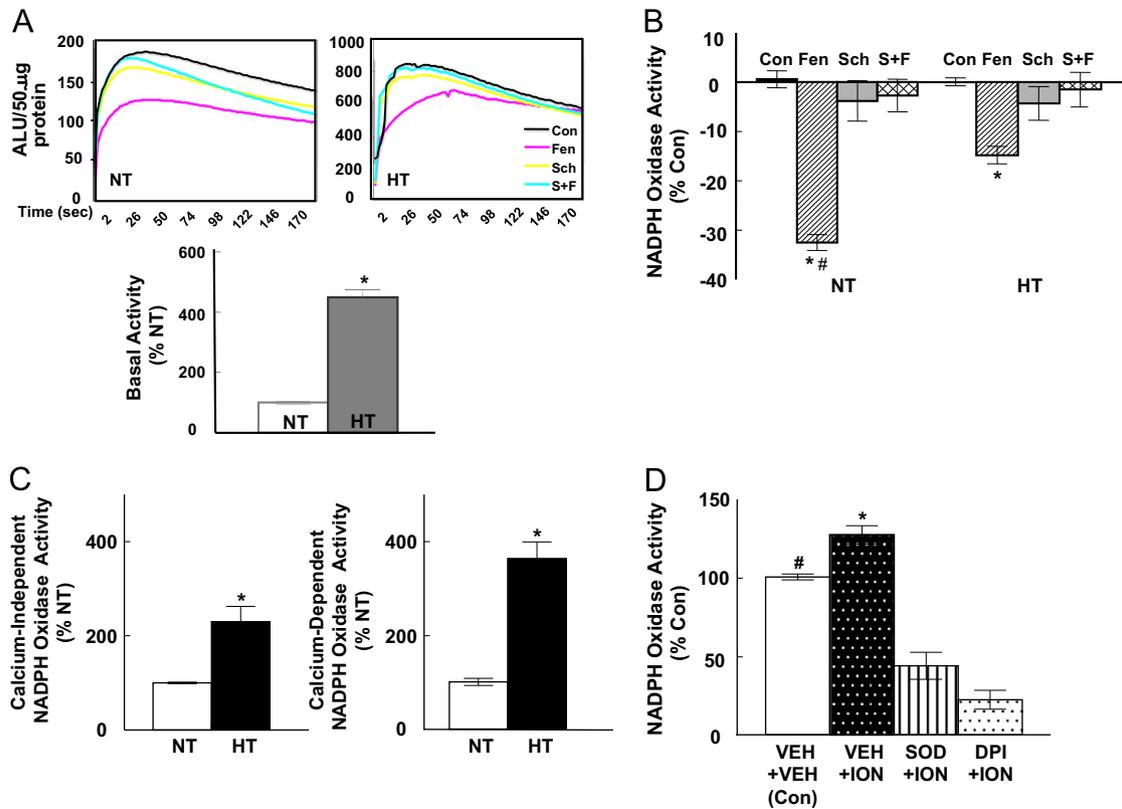


Fig. 4. Measurement of NADPH oxidase activity in human RPT cells. (A). Measurement of NADPH oxidase activity in whole cell membranes of human RPT cells. RPT cells, from normotensive (NT) and hypertensive subjects (HT), grown to 90% confluence and pre-starved in serum-free DMEM/F12 medium (SFM) for 1 h, were treated for 20 min at 37 °C with vehicle (Con), the D₁-like receptor agonist fenoldopam (Fen, 1.0 µmol/L), the D₁-like receptor antagonist Sch23390 (Sch, 5.0 µmol/L) alone, or a combination of both antagonist and agonist (S+F); Sch was added to the cells 5 min prior to the addition of fenoldopam. Whole cell membranes were prepared and NADPH oxidase activity was measured in the presence of 10 µmol/L lucigenin and 100 µmol/L NADPH as described under “Section 2”. NADPH oxidase activity is expressed as arbitrary light units (ALU) per 50 µg protein. The upper figures show the dynamic tracings of NADPH oxidase-dependent chemiluminescence recorded over a period of 180 s. The lower figure shows basal NADPH oxidase activity expressed as % of NT and shown as mean ± SEM ($n=4-7$ /group). $P < 0.001$, vs. NT, t -test. (B). Summary of NADPH oxidase activity from the studies shown in Fig. 4A. NADPH oxidase activity was calculated and converted to % of control (Con). Values are mean ± SEM of 4–7 separate experiments per group. $*P < 0.001$, vs. others, # $P < 0.05$ vs. Fen-HT, one-way ANOVA, Newman–Keuls test. (C). Measurement of calcium-independent and calcium-dependent NADPH oxidase activity in RPT cells. Whole cell membranes were prepared as described under “Section 2”. NADPH oxidase activity was measured in the absence or presence of CaCl₂ (1.0 mmol/L). The calcium-independent (left figure) and -dependent (right figure) NADPH oxidase activities were expressed as RLU/50 µg protein (Fig. S7B) and expressed as % of NT (Fig. 4C). Values are mean ± SEM ($n=8-12$ /group), $*P=0.003$ for left panel, and $*P=0.001$ for right panel, vs. NT, t -test. (D). Effect of ionomycin on NADPH oxidase activity in RPT cells from HT. RPT cells from HT were pre-treated with vehicle (VEH), superoxide dismutase (SOD, 400 units/ml), or diphenyleiodonium (DPI, 30 µmol/L) for 45 min. The cells were then treated with vehicle (VEH+VEH, control [Con]) or ionomycin (ION, 1 µmol/L) (VEH+ION, SOD+ION, DPI+ION) for an additional 15 min in the presence of 0.65 mmol/L CaCl₂ (equivalent to 47 µmol/L of free Ca²⁺) (18). The cell pellets were collected and whole cell membranes were prepared as in Fig. 4A. NADPH oxidase activity was measured using a microplate luminometer as described under “Section 2”. NADPH oxidase activity was expressed as % of control and shown as mean ± SEM ($n=4$ /group). $*P=0.008$, vs. all others, # $P < 0.001$, vs. SOD+ION and DPI+ION, one-way ANOVA, Newman–Keuls test.

[31–33, 36, 40]. These previous reports were corroborated by the current studies in that the D₁-like receptor agonist fenoldopam decreased ROS production to a greater extent in NT (-39.1 ± 5.8) than HT (-17.1 ± 2.6) (Fig. 5C), in agreement with its decreased ability to inhibit NADPH oxidase activity in HT (Fig. 4B).

Finally, we used a fluorescence kit to label live RPT cells and visualized ROS production by confocal microscopy. The fluorescence intensity, measured under the same microscope setting, was much stronger in RPT cells from HT than NT, indicating that basal ROS production was greater in HT than NT (Fig. 5D), corroborating the results using the other methods to measure ROS production (Figs. 4A, C, and 5C). The finding that NOX5 colocalized with ROS in RPT cells from HT (Fig. 3A and Fig. S5) indicated that intracellular ROS abundance was related to NOX5 protein abundance.

Effect of knockdown of NOX5 expression on NADPH oxidase activity

To determine if the increased NOX5 activity in RPT cells from HT was causal of the increased ROS production, we measured

NADPH oxidase activity following the silencing of NOX5, using NOX5-siRNA. Because NOX5 protein in NT cells was barely detectable (Fig. 3A, Figs. S2, S4 and S5), only RPT cells from HT were used in the silencing experiments. To determine whether or not loss of NOX5 expression altered cellular behavior, cell number was measured following the silencing of NOX5 with NOX5-siRNA in HT cells. Silencing NOX5 decreased NOX5 protein by $56 \pm 9\%$ (Fig. 6A) but did not alter cell number (Figs. S9A and B, supplementary file). In another set of studies, NOX5 protein expression was decreased by $-40 \pm 6.2\%$ (data not shown) and NADPH oxidase activity was decreased by $-45 \pm 3.2\%$ (Fig. 6B) in NOX5-siRNA treated HT RPT cells, relative to mock-siRNA-treated HT RPT cells. NOX5 activity was also measured with or without ionomycin following the silencing of NOX5 gene. Ionomycin increased NOX5 activity to a greater extent in mock-silenced (mock-siRNA) HT cells ($+31.3 \pm 2.8\%$) than in NOX5-silenced (NOX5-siRNA) HT cells ($+14.6 \pm 3.2\%$) ($P < 0.02$, ANOVA) ($n=6$) (Fig. 6C); SOD or DPI decreased NADPH oxidase activity in both mock-siRNA and NOX5-siRNA-treated RPT cells, to levels below control values, similar to HT cells not treated with NOX5-siRNA (Fig. 5B).

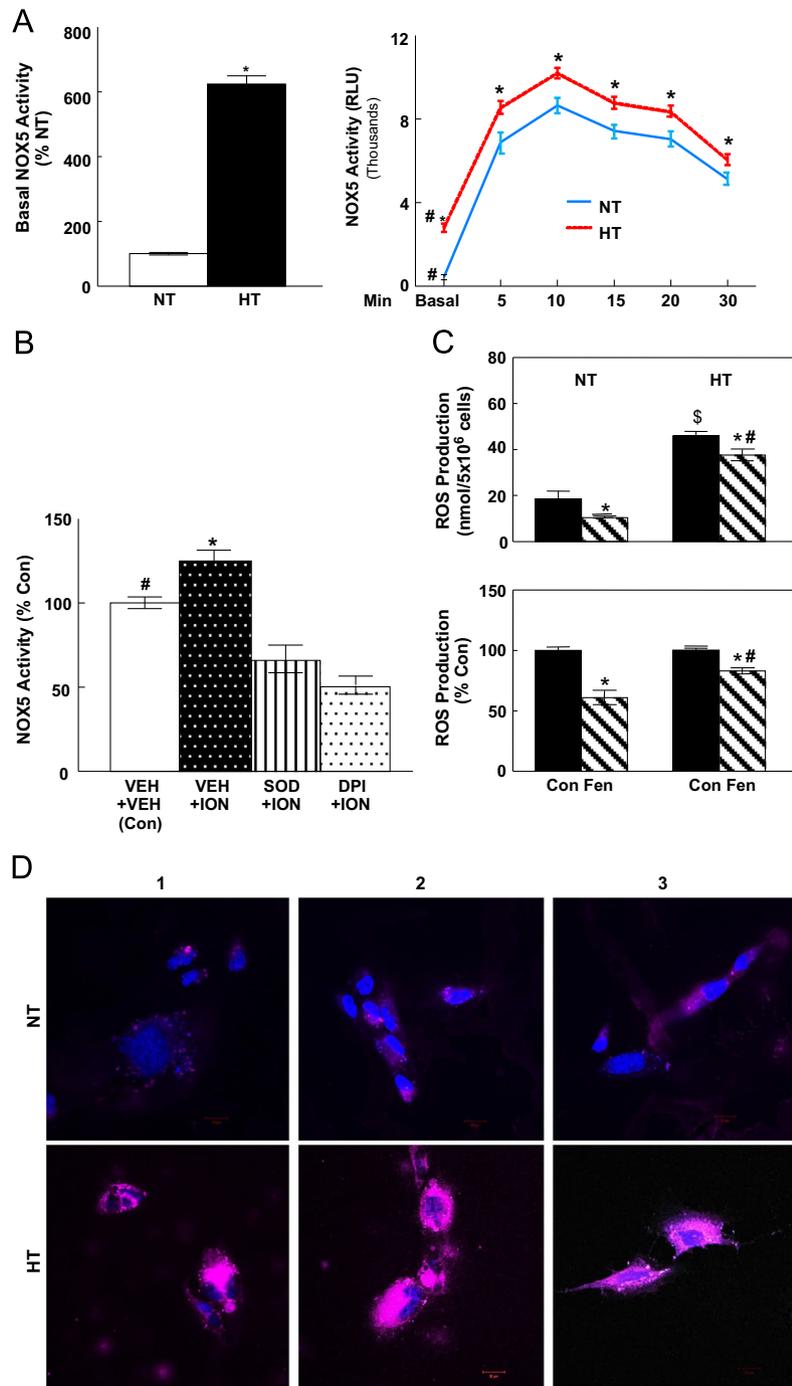


Fig. 5. Measurement of NOX5 activity and extracellular ROS production in RPT cells. (A). Measurement of NOX5 activity in RPT cells. **Left figure** shows basal NOX5 activity in NOX5 immunoprecipitates of RPT cells from normotensive (NT) and hypertensive subjects (HT). Human RPT cells were lysed as described under “Section 2”. The cell lysates were immunoprecipitated with polyclonal anti-NOX5 antibody. NOX5-dependent oxidase activity was measured by incubating the NOX5-immunoprecipitates with assay buffer in the presence of CaCl_2 (1.0 mmol/L), luciferin (50 $\mu\text{mol/L}$) and NADPH (200 $\mu\text{mol/L}$) using a microplate luminometer, as described under “Section 2”. The first readings, which were obtained immediately following the injection of NADPH (final concentration = 200 $\mu\text{mol/L}$), represented **basal** NOX5 activity (**left figure**). NOX5 activity was calculated and expressed as RLU and converted to % of NT. Values are mean \pm SEM of 6–14/groups, $*P < 0.001$, vs. NT, *t*-test. **Right figure** shows the NOX5 activity monitored for a period of 30 min following the injection of NADPH, in the presence of calcium. Values are mean \pm SEM of 6–14/groups, $*P < 0.001$, vs. NT, # $P < 0.001$, vs. all other time points, one way ANOVA, Newman–Keuls test. (B). Effect of ionomycin on NOX5 activity in HT cells. Cells were pre-treated with vehicle (VEH), ION (ionomycin), SOD (superoxide dismutase), or DPI (diphenyleneiodonium) as described in Fig. 4D. The cell lysates were subjected to immunoprecipitation and NOX5 activity was measured as in Fig. 5A. Values are mean \pm SEM of 4/groups, $*P < 0.01$, vs. other groups, # $P < 0.001$, vs. SOD + ION and DPI + ION, one-way ANOVA, Newman–Keuls test. (C). Measurement of extracellular ROS production in RPT cells. Extracellular ROS production was measured by SOD-inhibitable reduction of cytochrome c in live RPT cells. Cells, cultured on 12-well plates (5×10^6 /well), were incubated in 0.5 ml phenol-free DMEM/F12 medium containing reduced cytochrome c (200 $\mu\text{mol/L}$) with or without SOD (200 $\mu\text{mol/L}$) for 15 min at 37 °C. The incubation was continued for another 30 min in the presence of vehicle (control, Con) or fenoldopam (Fen, 1 $\mu\text{mol/L}$). The absorbance of reduced cytochrome c was measured at 550 nm, and SOD-inhibitable reduction of cytochrome c was calculated (37). The upper figure shows ROS production expressed as nmol/ 5×10^6 cells and the lower figure shows values expressed as % of Con. Values are mean \pm SEM of 5/group. Upper panel: $*P < 0.05$, vs. Con (NT or HT), $^{\$}P < 0.01$, vs. NT Con, # $P < 0.01$, vs. NT Fen, $n = 5$, one-way ANOVA, Newman–Keuls test. Lower panel: $*P < 0.01$, vs. Con (NT or HT), # $P < 0.05$, vs. NT-Fen, $n = 5$, one-way ANOVA, Newman–Keuls test. (D). Visualization of ROS in live cells. ROS were labeled in live cells with CellROX Deep Red fluorogenic probe and visualized by confocal microscopy. Cells, grown on coverslips, were incubated in phenol-free DMEM/F12, containing 1% dialyzed FBS with fluorogenic probe at a final concentration 5 $\mu\text{mol/L}$ for 30 min at 37 °C. The coverslips were washed, fixed, and mounted as described under “Section 2”. NT cells served as control for HT cells. ROS (triplicate: 1, 2, 3) were detected by confocal microscopy (excitation and emission wave lengths at 640 and 655 nm, respectively) at 630 × magnification.

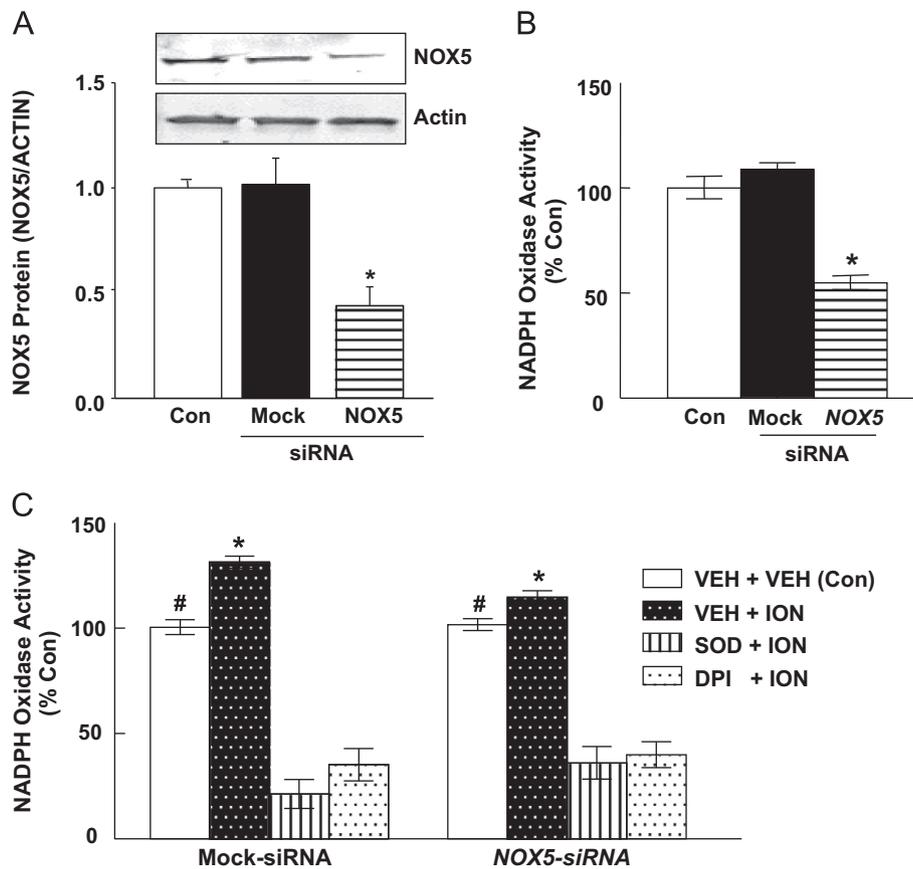


Fig. 6. Effect of silencing of *NOX5* gene on *NOX5* protein and NADPH oxidase activity in RPT cells from HT. (A). Effect of silencing of *NOX5* gene on *NOX5* protein expression. RPT cells from hypertensive subjects (HT) were transfected with vehicle (Con) that contained only transfection reagent, mock siRNA (aka control-siRNA) that consisted of scrambled RNA and served as a negative control, or *NOX5*-siRNA for 48 h described under "Section 2". Cell lysates were immunoblotted with polyclonal anti-*NOX5* antibody. The immunoreactive bands were semi-quantified and expressed as density units. Values are mean \pm SEM ($n=6$ /group). * $P < 0.05$, vs. others, one-way ANOVA, Newman-Keuls test. One set of studies is shown on top of the bar graphs. (B). Effect of silencing *NOX5* gene on basal NADPH oxidase activity. RPT cells were transfected with vehicle (Con), mock-siRNA, or *NOX5*-siRNA for 48 h, as in the Fig. 6A. NADPH oxidase activity was measured in whole cell homogenates in the presence of lucigenin (10 μ mol/L) and NADPH (200 μ mol/L) using a microplate luminometer, as described under "Section 2". The activity, expressed as RLU/mg protein, was converted to % of Con. Values are mean \pm SEM of 6/groups, * $P < 0.001$, vs. others, one-way ANOVA, Newman-Keuls test. (C). Effect of ionomycin on NADPH oxidase activity in *NOX5*-siRNA-treated HT cells. Cells were transfected with mock-siRNA or *NOX5*-siRNA for 48 h as in Fig. 6A and B. The cells were treated with vehicle (VEH, Con), ionomycin (ION), superoxide dismutase (SOD), or diphenyleneiodonium (DPI), as described in Figs. 4D and 5B. NADPH oxidase activity was measured as in Fig. 6B. Values are mean \pm SEM of 6/groups, * $P < 0.001$, vs. others, # $P < 0.001$, vs. DPI and SOD, one one-way ANOVA, Newman-Keuls test.

Discussion

The role of NOX isoforms in physiological and pathological states is well documented. However, studies of *NOX5* are relatively rare because this gene is not found in rodents [12,13]. By contrast, the *NOX5* gene is present in humans and its protein is expressed in fetal [13] and non-fetal organs and tissues, including the lung, testis, spleen [14], and vascular endothelial, smooth muscle [3,7,9,10,15–17,41], and gastrointestinal [42] cells. We now show for the first time that *NOX5* mRNA and protein are expressed in human RPT cells.

NADPH oxidase enzymes are the major sources of ROS production in renal and cardiovascular tissues [2,4–9]. The increased generation of ROS contributes to human [10,11] and animal hypertension [2,4,5,10,29–32,43]. NADPH oxidase activity and superoxide formation are increased in endothelial [2,10,41,43], neural [5,43], renal [2,29–32,43,44], and vascular smooth muscle cells [4,28,41,43] in genetic and acquired hypertension. Of the Nox isoforms that are present in rodents (Nox1, Nox2, and Nox4) [2,7–9,29–32,39], Nox4 has been claimed to be the major NADPH oxidase in renal [6,43], vascular smooth muscle [4,7,41,43], and endothelial cells [41,43], and its expression is markedly increased in hypertensive rodents [2,31,35,43]. It has also been reported that injection of siRNA against-*NOX2* or *NOX4* into the hypothalamic

paraventricular nucleus attenuates the development of aldosterone/salt-induced hypertension [5]. Therefore, Nox4 may play vital role in the pathogenesis of animal hypertension. By contrast, human microvascular endothelial cells possess functionally active *NOX5* which generates ROS and is regulated by angiotensin II and endothelin 1 [14–16,41]. In patients with coronary artery disease, *NOX5* protein and mRNA levels and NADPH oxidase-dependent production of ROS in coronary arterial membranes are increased [9]. We now report for the first time that NADPH oxidase activity and mRNA and total cellular protein expressions of *NOX5* and *NOX1*, but not *NOX2* and *NOX4* are increased in RPT cells from HT relative to NT. We also report that the apical membrane expressions of *NOX1*, *NOX2*, *NOX4*, and *NOX5* are increased in RPT cells from HT relative to NT. However, the increase in *NOX5* expression in HT is greater than the other NOX isoforms. Moreover, the increase in *NOX5* expression and activity in HT is associated with increased production of ROS. Therefore, we speculate that increased *NOX5* expression may be important in the pathogenesis of human essential hypertension.

The increase in NOX proteins, especially *NOX5* at the apical and plasma membranes in RPT cells from HT, is associated with increased extracellular ROS production. This may be related to polybasic domains within the N-terminus of *NOX5*, that can bind phosphatidylinositol 4,5- bisphosphate at the plasma membrane,

and promote the trafficking and cell surface expression of NOX5 [20,21]. NOX5 in the endoplasmic reticulum and nucleus may also contribute to intracellular ROS production [20,21,23]. The colocalization of NOX5 with ROS in RPT cells from HT could be taken to indicate that the increased intracellular ROS in HT is related to NOX5 protein abundance.

NOX5 protein is expressed predominantly in the plasma membrane and to a lesser extent in the cytosol and nucleus. NOX1–5 proteins have been reported to be expressed in many subcellular areas, including the nucleus in human endocardial and vascular endothelial, vascular smooth muscle, esophageal adenocarcinoma, prostate cancer, and leukemia cells [18,41,42,44,45]. In agreement with these reports, confocal imaging shows that NOX5 protein and ROS colocalize in the plasma membrane and nucleus of RPT cells from HT. Therefore, nuclear NOX5-dependent ROS production may also contribute to total cellular oxidative stress. Whether or not nuclear NOX5 is involved in redox responsive gene expression remains to be determined [44].

Ionomycin, an ionophore that increases intracellular Ca^{2+} and NADPH oxidase activity [14,18,19], also stimulates NOX5 activity in HT cells. The stimulatory effect of ionomycin on ROS production is abrogated by DPI, an NADPH oxidase inhibitor, and SOD which catalyzes the dismutation of superoxide to H_2O_2 . These data support the notion that activation of NOX5 is responsible for the increase in ROS production, specifically superoxide. There are at least five splice variants of NOX5 (α , β , δ , γ , and ϵ) [15,23]. Ionomycin stimulates NOX5 α and β but not the other NOX5 isoforms to produce superoxide [23]. It remains to be determined whether these NOX5 isoforms are responsible for the increased ROS production in RPT cells from HT or NOX5 polymorphisms, if they exist, are associated with the hypertensive phenotype. Nevertheless, increased NOX5 expression and activity are likely to be important in the increased ROS production in RPT cells from HT because silencing of NOX5 with siRNA decreases basal NADPH oxidase activity in RPT cells from HT.

We and others have previously reported that ROS production is negatively regulated by D_1 -like receptors in RPT cells from humans [8] and RPT and vascular smooth muscle cells from rats [28–32]. The inhibitory effect of fenoldopam, a D_1 -like receptor agonist, on NADPH oxidase activity is probably exerted at the D_1 -like receptor because a D_1 -like receptor antagonist, SCH23390, blocks the fenoldopam effect. The decreased ability of fenoldopam to inhibit NADPH oxidase activity in HT relative to NT is in agreement with our previous report that fenoldopam inhibits NADPH oxidase activity to a lesser extent in RPT cells from SHR than those from normotensive WKY rats [31]. A defective D_1 -like receptor may have caused the impaired ability of fenoldopam to inhibit NADPH oxidase activity in the RPT cells from HT. We and others have reported a decreased ability of D_1 -like receptors to inhibit RPT sodium reabsorption [27], and stimulate cAMP production and inhibit sodium transport in RPT cells from hypertensive humans [33], three animal models of hypertension [11,24,32], and obese hypertensive rats [46]. The impairment in renal proximal tubular D_1 -like receptor function in hypertension is caused by constitutive phosphorylation of D_1R , as a consequence of increased activity of type 2 and type 4 G protein-coupled receptor kinases [24,33,46,47].

In conclusion, we report for the first time that NOX5 is the predominant NOX isoform expressed in human RPT cells and its expression and activity in RPT cells are increased in humans with essential hypertension. Although the latter conclusion is limited by the small number of subjects (three normotensive and three hypertensive subjects), NOX5 expression in RPT cells was always greater in HT than in NT. Thus, it is likely that increased NOX5 expression and activity may be responsible for the oxidative stress that contributes to the pathogenesis of human essential hypertension.

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Appendix A. Supplementary information

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.redox.2014.01.020>.

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