AMERICAN JOURNAL OF PHYSIOLOGY
RENAL PHYSIOLOGY

Am J Physiol Renal Physiol 323: F633–F641, 2022.
First published October 6, 2022; doi:10.1152/ajprenal.00107.2022

RESEARCH ARTICLE

NOXA1-dependent NADPH oxidase 1 signaling mediates angiotensin II activation of the epithelial sodium channel

Elena Mironova,[1] Crystal R. Archer,[1] Aleksandr E. Vendrov,[2] Marschall S. Runge,[2] Nageswara R. Madamanchi,[2] William J. Arendshorst,[3] James D. Stockand,[1] and Tarek Mohamed Abd El-Aziz[1,4]

[1]*Department of Cellular and Integrative Physiology, University of Texas Health Science Center at San Antonio, San Antonio, Texas;* [2]*Department of Medicine, University of Michigan, Ann Arbor, Michigan;* [3]*Department of Cell Biology and Physiology, University of North Carolina at Chapel Hill School of Medicine, Chapel Hill, North Carolina; and* [4]*Department of Zoology, Minia University, El-Minia, Egypt*

Abstract

The activity of the epithelial Na^+ channel (ENaC) in principal cells of the distal nephron fine-tunes renal Na^+ excretion. The renin-angiotensin-aldosterone system modulates ENaC activity to control blood pressure, in part, by influencing Na^+ excretion. NADPH oxidase activator 1-dependent NADPH oxidase 1 (NOXA1/NOX1) signaling may play a key role in angiotensin II (ANG II)-dependent activation of ENaC. The present study aimed to explore the role of NOXA1/NOX1 signaling in ANG II-dependent activation of ENaC in renal principal cells. Patch-clamp electrophysiology and principal cell-specific Noxa1 knockout (PC-*Noxa1* KO) mice were used to determine the role of NOXA1/NOX1 signaling in ANG II-dependent activation of ENaC. The activity of ENaC in the luminal plasma membrane of principal cells was quantified in freshly isolated split-opened tubules using voltage-clamp electrophysiology. ANG II significantly increased ENaC activity. This effect was robust and observed in response to both acute (40 min) and more chronic (48–72 h) ANG II treatment of isolated tubules and mice, respectively. Inhibition of ANG II type 1 receptors with losartan abolished ANG II-dependent stimulation of ENaC. Similarly, treatment with ML171, a specific inhibitor of NOX1, abolished stimulation of ENaC by ANG II. Treatment with ANG II failed to increase ENaC activity in principal cells in tubules isolated from the PC-*Noxa1* KO mouse. Tubules from wild-type littermate controls, though, retained their ability to respond to ANG II with an increase in ENaC activity. These results indicate that NOXA1/NOX1 signaling mediates ANG II stimulation of ENaC in renal principal cells. As such, NOXA1/NOX1 signaling in the distal nephron plays a central role in Na^+ homeostasis and control of blood pressure, particularly as it relates to regulation by the renin-ANG II axis.

NEW & NOTEWORTHY Activity of the epithelial Na^+ channel (ENaC) in the distal nephron fine-tunes renal Na^+ excretion. Angiotensin II (ANG II) has been reported to enhance ENaC activity. Emerging evidence suggests that NADPH oxidase (NOX) signaling plays an important role in the stimulation of ENaC by ANG II in principal cells. The present findings indicate that NOX activator 1/NOX1 signaling mediates ANG II stimulation of ENaC in renal principal cells.

collecting duct; hypertension; reactive oxygen species; renal physiology; sodium excretion

INTRODUCTION

Discretionary Na^+ excretion by the kidneys contributes to the regulation of blood pressure (1–3). Because of its location in the final segments of the nephron, including the late distal convoluted tubule, connecting tubule (CNT), and collecting duct, the epithelial Na^+ channel (ENaC) is the final arbiter of discretionary Na^+ excretion by the kidneys (4–7). Consequently, ENaC activity contributes to the control of blood pressure. This is highlighted by the fact that gain and loss of ENaC function in humans leads to inheritable forms of hypertension and renal salt wasting associated with low blood pressure, respectively (8, 9).

ENaC is a Na^+-selective ion channel that is expressed in the luminal plasma membrane of principal cells in the distal nephron, where it functions as a key end effector of the renin-angiotensin-aldosterone system (RAAS) (10–12). The RAAS signaling system has been shown to be an important regulator of blood pressure through its effects on vascular tone and renal Na^+ transport, including ENaC activity. Aldosterone increases the activity of ENaC, decreasing renal Na^+ excretion, and, consequently, increasing blood pressure (13–15). Existing evidence is also consistent with angiotensin II (ANG II), a hormone upstream of aldosterone in the RAAS, exerting a direct effect on ENaC in principal cells independently of aldosterone actions on the

Correspondence: J. D. Stockand (stockand@uthscsa.edu); T. M. Abd El-Aziz (Mohamedt1@uthscsa.edu).
Submitted 15 April 2022 / Revised 3 October 2022 / Accepted 4 October 2022

channel. The first compelling evidence of this was provided in 2002 by Peti-Peterdi and colleagues, when they demonstrated that ANG II stimulated benzamil-sensitive Na^+ entry in perfused cortical collecting ducts (CCDs) isolated from the rabbit and rat (16). Benzamil, like amiloride, is an ENaC blocker (17). Recent findings have demonstrated directly using patch-clamp electrophysiology that ANG II increases the activity of ENaC in principal cells of isolated tubules from mice and rats in a manner that is parallel to aldosterone and not redundantly (18–20). The current consensus is that ANG II stimulates ENaC in principal cells through ANG II type 1 (AT_1) receptors (16, 18, 19, 21).

However, the signal transduction pathway linking AT_1 receptors to ENaC in principal cells is less understood. Emerging evidence suggests that NADPH oxidase (NOX) signaling plays an important role in the stimulation of ENaC by ANG II in principal cells (18, 19, 22). This is in accordance with the general finding that NOX signaling and reactive oxygen species (ROS) play a crucial role in regulating ENaC activity in the kidneys and lungs (23–25).

The NOX catalytic subunits (NOX1, NOX2, and NOX4) are expressed in vascular, glomerular, and tubular cells of the kidney (26, 27). NOXs are the main producers of ROS in the vasculature in response to ANG II stimulation of AT_1 receptors (28). Activation of membrane-bound NOX1 requires interactions with cytosolic regulatory subunits. NOX activator 1 (NOXA1) is one such regulatory subunit that stimulates NOX1, causing constitutive production of superoxides (29). In vascular smooth muscle cells (VSMCs), NOXA1 is required for NOX1 activation (30). The preponderance of evidence indicates that ANG II predominantly stimulates NOX1 (31, 32). We have recently reported that ANG II-activated renal NOXA1/NOX1-dependent ROS increases tubular ENaC expression and Na^+ reabsorption, leading to increased blood pressure (22).

To further investigate the possible role that NOXA1/NOX1 signaling might play in ANG II-mediated regulation of ENaC activity, we tested the effects of ANG II on ENaC activity using patch-clamp electrophysiology in principal cells in isolated, split-open CNT/CCDs from wild-type littermates and principal cell-specific *Noxa1*$^{-/-}$ [PC-*Noxa1* knockout (KO)] mice. As expected, ANG II significantly increased ENaC activity in principal cells of wild-type tubules. Losartan, an AT_1 inhibitor, abrogated this effect of ANG II. Similarly, the NOX1 inhibitor ML171 (33) inhibited activation of ENaC by ANG II in isolated wild-type tubules. Moreover, ANG II had no effect on ENaC activity in tubules from PC-*Noxa1* KO mice. These results demonstrate that NOXA1/NOX1 signaling is necessary for ENaC stimulation by ANG II in renal principal cells. As such, NOXA1/NOX1 signaling in the distal nephron likely plays an important role in Na^+ homeostasis and blood pressure control, particularly as it pertains to control by ANG II.

METHODS

Animal Care and Use

All animal use and welfare adhered to the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* following protocols reviewed and approved by the Institutional Animal Care and Use Committee of the University of Texas Health Science Center at San Antonio. Mice were housed and cared for by Laboratory Animal Resources at the University of Texas Health Science Center at San Antonio, which is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care and licensed by the US Department of Agriculture.

We used young healthy adults (2–3 mo old) male and female of wild-type (C57BL/6J) and PC-*Noxa1* KO mice as a source of the CNT/CCD for patch-clamp analysis of ENaC activity in principal cells in these experiments. The assignment to the treatment or vehicle group within a genotype was random. Mice were maintained on a normal 12:12-h light-dark cycle at room temperature with free access to water and standard chow (TD.7012 and/or TD.8656, Envigo, Indianapolis, IN) and were housed socially with littermates and peers. The present study was not designed to test for sexually dimorphic responses and, thus, we make no claim about any sex-dependent effects of ANG II or Nox1 signaling on ENaC in this study.

Creation and Validation of the PC-*Noxa1* KO Mouse

Creation and validation of the PC-*Noxa1* KO mouse was done following previously described procedures (22, 34). In brief, PC-*Noxa1* KO mice were homozygous for the floxed *Noxa1* transgene and heterozygous for the aquaporin 2 (*Aqp2*)-*Cre* transgene. Floxed *Noxa1* mice [B6(Cg)-(*Noxa1*^{tm1Brg}/Brgj), Strain No. 014601] were purchased from The Jackson Laboratory (Bar Harbor, ME) and have been previously described (35). The B6.Cg-Tg (Aqp2-cre)1Dek/J (Strain No. 006881) mouse was also from The Jackson Laboratory and has been previously described (36–38). In this model, Cre-dependent recombination deletes the coding region of the *Noxa1* gene from exon 3 to exon 6. Littermate controls lacked the *Aqp2-Cre* transgene. The *Aqp2-Cre* transgene was transmitted only through the female line. Mouse genotype was confirmed by PCR, as shown in Fig. 1, in

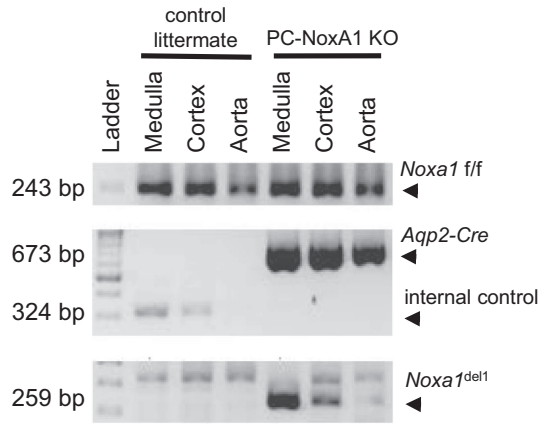

Figure 1. Genotype of the principal cell-specific NADPH oxidase activator 1 knockout (PC-*Noxa1* KO) mouse. Shown is an inverted image of representative gels showing typical PCR products from genotyping reactions with genomic DNA from littermate controls and PC-*Noxa1* KO mice. Arrowheads indicate expected products of aquaporin 2 (*Aqp2*)-Cre and floxed *Noxa1* transgenes as well as the *Noxa1* allele that has undergone recombination (*Noxa1*del1). Genomic DNA was extracted from the renal medulla and cortex as well as the aorta. To maximize clarity, the gray scale of this image was inverted for presentation purposes.

accordance with Jackson Laboratory protocols. In brief, genomic DNA was extracted from tissues using a Quick-DNA Miniprep Plus Kit (D4069, Zymo Research, Irvine, CA), and PCR was performed using MyTaq HS Red Mix (BIO-25047, Meridian Bioscience, Memphis, TN).

Immunofluorescence

Mice were euthanized with an inhaled isoflurane overdose, and the kidneys and aortas were dissected and embedded in OCT compound (Sakura Finetek, Torrance, CA). Serial coronal sections (10 μm) were fixed in acetone, permeabilized in 0.1% Triton X-100, and stained using an antibody against NOXA1 (1:100 dilution, Ab199, gifted by Dr. Ralf Brandes, Institut für Kardiovaskuläre Physiologie, Goethe-Universität, Frankfurt am Main, Germany) (39) followed by a secondary antibody conjugated to Alexa Fluor 594 (1:200 dilution, A11072, ThermoFisher, Waltham, MA) or Alexa Fluor 488-conjugated anti-Aqp2 antibody (bs-4611R-A488, Bioss, Woburn, MA). Sections were mounted with Vectashield mounting medium for fluorescence with DAPI (H-1200, Vector Laboratories, Burlingame, CA). Fluorescence images were acquired with a Nikon Microphot-FX microscope using the same exposure, gain, and offset values. Images were analyzed using ImageJ 1.53 (National Institutes of Health, Bethesda, MD).

Split-Open Tubule Preparation and Single-Channel Patch-Clamp Electrophysiology

Renal tubules were isolated, split open, and prepared for patch-clamp electrophysiology as previous described (40–42). In brief, kidneys were sectioned transversely, and segments of the CNT and CCD were manually microdissected with forceps and adhered to a glass coverchip coated with 0.01% poly-L-lysine. Next, the chips were placed under an inverted microscope, and the top layer of the CNT/CCD was split open using sharpened pipettes. Gap-free, single-channel voltage-clamp experiments in the cell-attached configuration were then performed using standard procedures on the luminal plasma membranes of principal cells in split-open tubules (40–42). For these experiments, the bath solution contained (in mM) 150 NaCl, 5 KCl, 1 $CaCl_2$, 2 $MgCl_2$, 5 glucose, and 10 HEPES (pH 7.4). The pipette solution contained (in mM) 140 LiCl, 2 $MgCl_2$, and 10 HEPES (pH 7.4). Cell-attached patches were held at a negative pipette potential of 60 mV. Channel activity [NP_o; open probability (P_o) multiplied by channel number (N)] was calculated as previously described (40–42). For experiments testing the chronic (48–72 h) effects of ANG II on ENaC, mice were treated with 500 ng/kg/min ANG II using implanted Alzet microosmotic minipumps (model 1007 D, Durect, Cupertino, CA).

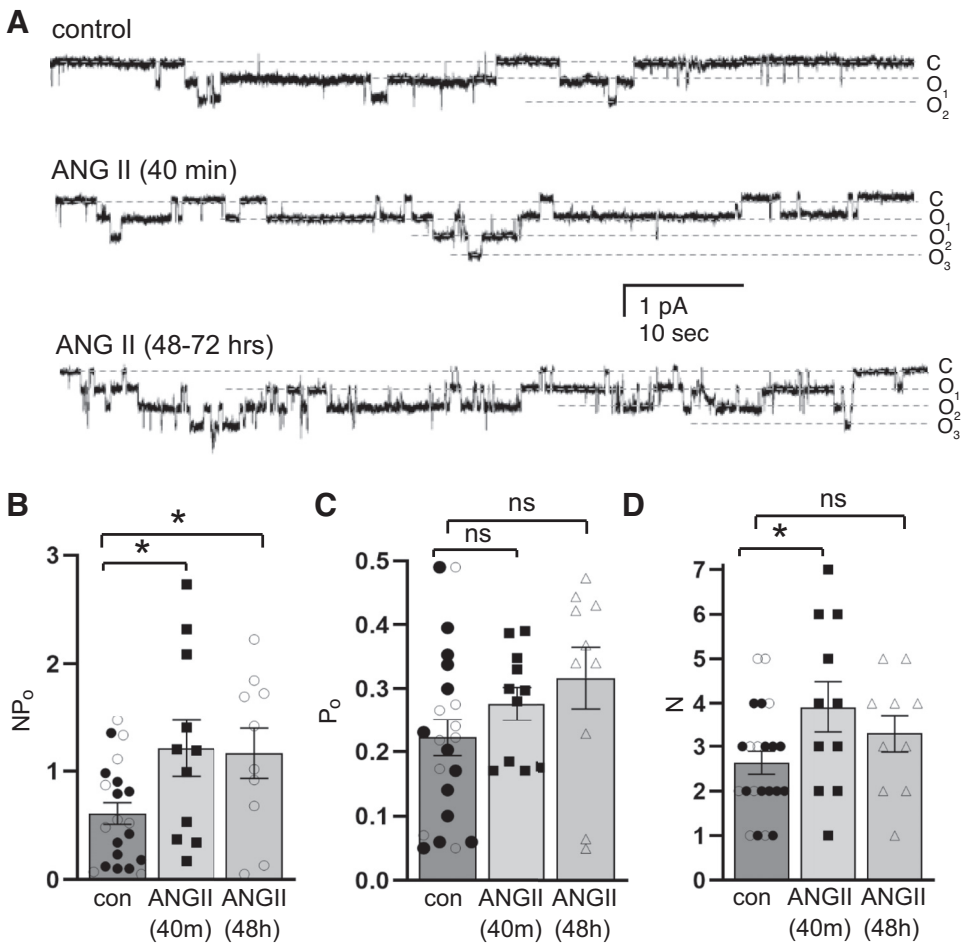

Figure 2. Angiotensin II (ANG II) increases epithelial Na^+ channel (ENaC) activity in principal cells of the connecting tubule (CNT)/cortical collecting duct (CCD). *A*: representative current traces of ENaC in cell-attached patches on apical plasma membranes of principal cells in the split-open CNT/CCD from wild-type C57BL/6J mice. Tubules were treated with vehicle (*top*) or 500 nM ANG II for 40 min (*middle*) or were from mice treated with 500 ng/kg/min ANG II (osmotic minipump) for 48-72 h (*bottom*). Summary graphs show means ± SE (bars) and individual data points (circles, boxes, and triangles; solid symbols indicate males and open symbols indicate females) for ENaC activity (NP_o; *B*), open probability (P_o; *C*), and number of active channels per patch (N; *D*) in tubules from the vehicle control (con; n = 21 cells from 3 male and 3 female mice), 40-min ANG II treatment (n = 11 cells from 3 male mice), and 48- to 72-h ANG II treatment (n = 10 cells from 2 females) groups. *Significantly different compared with the control by one-way ANOVA with a Tukey highly significant difference post hoc test. ns, not significant. C and O indicate closed and open channels, respectively.

Statistics

Data were analyzed, compared, and plotted using GraphPad Prism 9 (GraphPad Software, San Diego, CA). Summarized data are reported as means ± SE. Unpaired summarized data were compared with one-way ANOVA using a post hoc Tukey highly significant difference test. $P \leq 0.05$ was considered significant.

RESULTS

ANG II Increases ENaC Activity in Principal Cells

ANG II has been previously reported to enhance EnaC activity (18–20, 43). To confirm these earlier observations, we examined whether ANG II is capable of increasing EnaC activity in principal cells in the isolated, split-open CNT/CCD from C57BL/6J wild-type mice. ANG II increased the activity of EnaC in native principal cells in intact tubules, as demonstrated by the results shown in Fig. 2. The representative current traces shown in Fig. 2*A* demonstrate that treatment with 500 nM ANG II rapidly within 40 min increased ENaC activity. A similar finding was observed when mice were chronically (48–72 h) exposed to 500 ng/kg/min ANG II. ENaC activity was markedly increased. The summary data shown in Fig. 2, *B–D*, demonstrate that ANG II significantly increased ENaC NP_o within 40 min primarily by increasing N expressed in the luminal plasma membrane with a lesser effect on P_o of these channels. NP_o, P_o, and N for vehicle-treated control tubules were 0.50 ± 0.11, 0.22 ± 0.04, and 2.33 ± 0.26 (n = 12), respectively. Treatment with ANG II for 40 min and 2–3 days increased these values to 1.21 ± 0.26, 0.28 ± 0.03, and 3.91 ± 0.58 (n = 11) and 1.17 ± 0.23, 0.32 ± 0.05, and 3.30 ± 0.42 (n = 10), respectively. Thus, ANG II stimulates ENaC by increasing channel activity in principal cells of the CNT and CCD.

ANG II Increases ENaC Activity by Stimulating AT_1 Receptors

To affirm that ANG II signals to ENaC through the AT_1 receptor in principal cells (18–20), we measured ENaC activity in response to ANG II treatment in the presence of the AT_1 receptor inhibitor losartan. As shown in Fig. 3, as opposed to treatment of isolated tubules with 500 nM ANG II alone, treatment of isolated tubules with 500 nM ANG II in the presence of 500 nM losartan did not change ENaC activity. In fact, ENaC NP_o, P_o, and N were 0.22 ± 0.08, 0.10 ± 0.03, and 1.60 ± 0.27 (n = 10), respectively, in the presence of ANG II plus losartan. There was a significant decrease in all three values from those in the presence of ANG II alone to levels similar to those of the vehicle treatment. This is consistent with ANG II stimulating ENaC via AT_1 receptors in principal cells of the intact tubule.

ANG II-Induced Activation of EnaC Is Abolished by Inhibition of NOX1

Several lines of evidence suggest that NOXA1/NOX1 signaling plays a central role in ANG II-dependent activation of ENaC in principal cells. To directly test this possibility, we measured ENaC activity in response to treatment with ANG II in the presence of the NOX1 inhibitor ML171. As shown in Fig. 4, treatment with ANG II for 40 min in the presence of

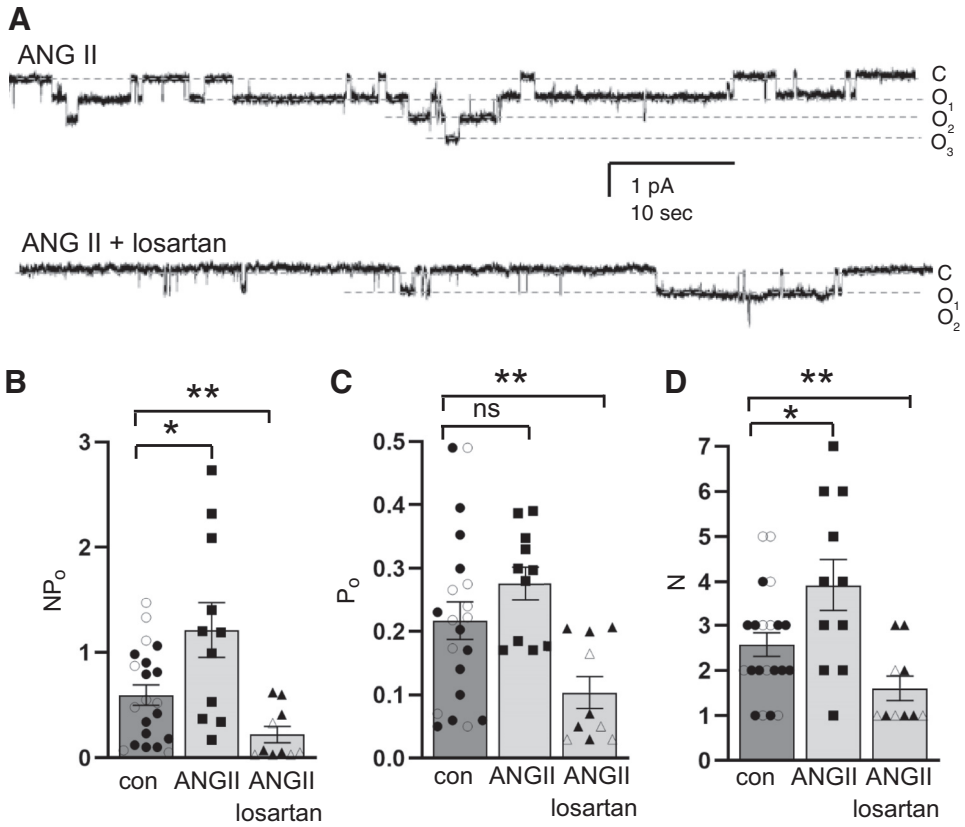

Figure 3. Angiotensin II (ANG II) type 1 receptor blockade abolishes ANG II effects on the epithelial Na^+ channel (ENaC). *A*: representative current traces of ENaC in cell-attached patches on apical plasma membranes of principal cells in the split-open connecting tubule/cortical collecting duct from wild type-C57BL/6J mice treated with 500 nM ANG II (*top*) or 500 nM ANG II plus 500 nM losartan for 40 min (*bottom*). The ANG II trace is identical to that shown in Fig. 2*A* and is reshown here for presentation purposes. Summary graphs showing means ± SE (bars) and individual data points (circles, boxes, and triangles; solid symbols indicate males and open symbols indicate females) for ENaC activity (NP_o; *B*), open probability (P_o; *C*), and number of active channels per patch (N; *D*) in tubules from the vehicle control (con; n = 21 cells from 3 male and female mice), 40-min ANG II treatment (n = 11 cells from 3 male mice), and 40-min ANG II plus losartan treatment (n = 10 cells from 2 male and 1 female mice) groups. The vehicle and ANG II treatment groups are identical those shown in Fig. 2, *B–D*, and are reshown here for presentation purposes. Significantly different compared with *control and **ANG II treatment groups by one-way ANOVA with a Tukey highly significant difference post hoc test. ns, not significant. C and O indicate closed and open channels, respectively.

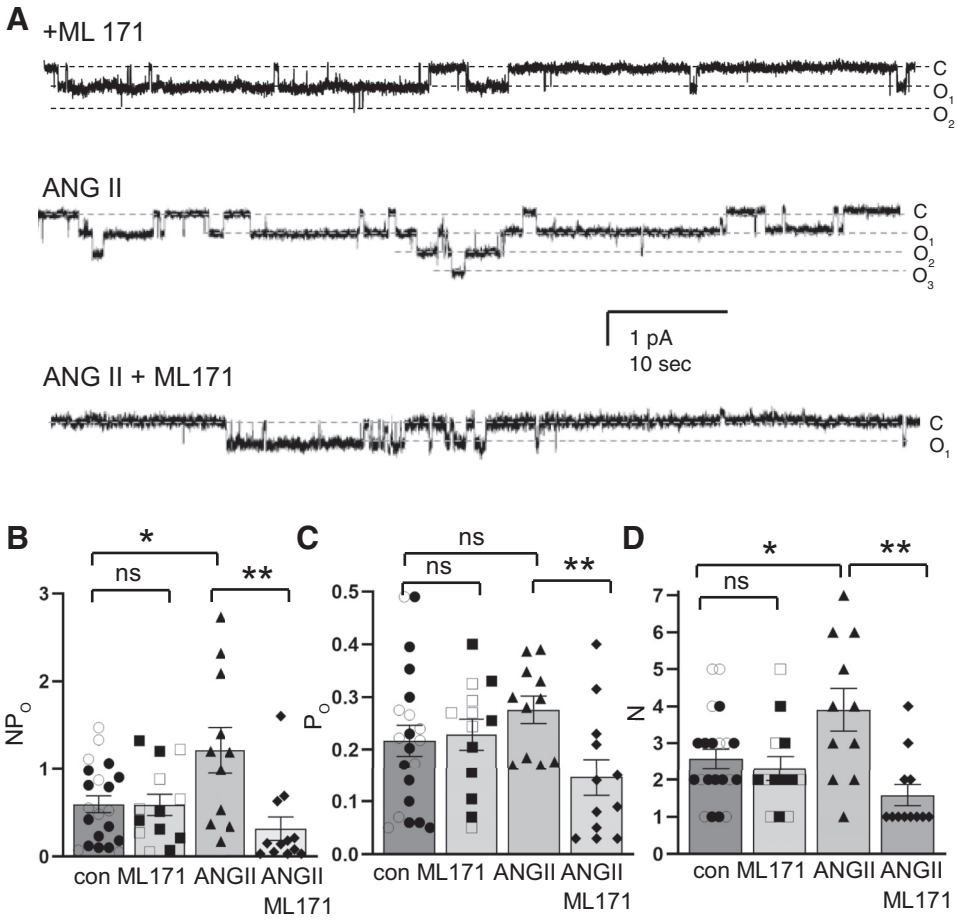

Figure 4. Inhibition of NADPH oxidase 1 (NOX1) prevents epithelial Na^+ channel (ENaC) activation by angiotensin (ANG II). *A*: representative current traces of ENaC after treatment with 500 nM ANG II for 40 min in the absence (*top*) and presence of 500 nM ML171 (*bottom*). The ANG II trace is identical to that shown in Fig. 2 and is reshown here for presentation purposes. Summary graphs showing means ± SE (bars) and individual data points (circles, boxes, and triangles; solid symbols indicate males and open symbols indicate females) for ENaC activity (NP_o; *B*), open probability (P_o; *C*), and number of active channels per patch (*N*; *D*) in tubules from the vehicle control (con; n = 21 cells from 3 male and 3 female mice), 40-min ML171 treatment (n = 13 cells from 2 male and 2 female mice), 40-min ANG II treatment (n = 11 cells from 3 male mice), and 40-min ANG II plus ML171 treatment (n = 12 from 3 male mice) groups. The vehicle and ANG II treatment groups are identical to those shown in Fig. 2. Significantly different compared with the *control and **ANG II treatment groups by one-way ANOVA with a Tukey highly significant difference post hoc test. ns, not significant. C and O indicate closed and open channels, respectively.

500 nM ML171 failed to increase the activity of ENaC. This is in contrast to the effects of ANG II alone (see Fig. 2). In the presence of ANG II plus ML171, ENaC NP_o, P_o, and N were 0.32 ± 0.13, 0.15 ± 0.03, and 1.58 ± 0.29 (n = 12), respectively. This represents a significant decrease in all three values from those in the presence of ANG II alone, to levels that were not different from treatment with vehicle. Such findings are consistent with NOX1 signaling being necessary for ANG II-dependent stimulation of ENaC in principal cells in the intact tubule.

Noxa1 Is Deleted in Principal Cells of the PC-*Noxa1* KO Mouse

As demonstrated by the results shown in Fig. 5, NOXA1 was deleted specifically in renal principal cells but not from aortic smooth muscle cells in the PC-*Noxa1* KO mouse. NOXA1 expression was not affected in renal principal cells and aortic smooth muscle cells of littermate controls, as expected. Immunofluorescence micrographs of fixed sections from the renal cortex and medulla as well as the aorta from PC-*Noxa1* KO mice and their littermate controls probed with anti-NOXA1 (red) and anti-Aqp2 (green) antibodies, as well as DAPI (blue), are shown. NOXA1 was robustly expressed in cells in each of these tissues in the littermate controls but was primarily in VSMCs only in the aorta as opposed to principal cells of the medulla in the PC-*Noxa1* KO mouse. In addition, NOXA1 and Aqp2 signals did not overlap in cells of the renal cortex of the PC-*Noxa1* KO mouse. These observations are consistent with *Noxa1* being deleted only in renal principal cells in the PC-*Noxa1* KO mouse.

NOXA1/NOX1 Signaling Is Necessary for ANG II Activation of ENaC in Principal Cells

Comparing PC-*Noxa1* KO mice with their littermate controls, we examined the role of NOXA1/NOX1 signaling in ANG II-dependent activation of ENaC. ENaC activity was assessed in voltage-clamped principal cells in split-opened tubules. The effects of 500 nM ANG II for 40 min (*bottom* traces) compared with vehicle (*top* traces) were quantified in tubules isolated from littermate controls (Fig. 6*A*) and PC-*Noxa1* KO mice (Fig. 6*B*). As shown in Fig. 6, *C*–E, ENaC NP_o, P_o, and N were 0.57 ± 0.20, 0.19 ± 0.04, and 2.39 ± 0.51 (n = 13) and 1.42 ± 0.23, 0.35 ± 0.03, and 3.73 ± 0.40 (n = 15) in the presence of vehicle and 500 nM ANG II, respectively, in principal cells of tubules isolated from littermate controls. Similar to control wild-type C57BL/6J mice (see Fig. 2), ANG II significantly increased ENaC activity in these intact tubules from littermates, having a marked effect on both P_o and N. In contrast, ANG II had no effect on ENaC activity in principal cells in tubules isolated from PC-*Noxa1* mice. NP_o, P_o, and N were 0.70 ± 0.25, 0.17 ± 0.04, and 2.69 ± 0.49 (n = 13) and 0.65 ± 0.16, 0.21 ± 0.04, and 2.69 ± 0.38 (n = 13) in principal cells of vehicle- and ANG II-treated tubules, respectively, isolated from the PC-*Noxa1* KO mouse. For tubules isolated

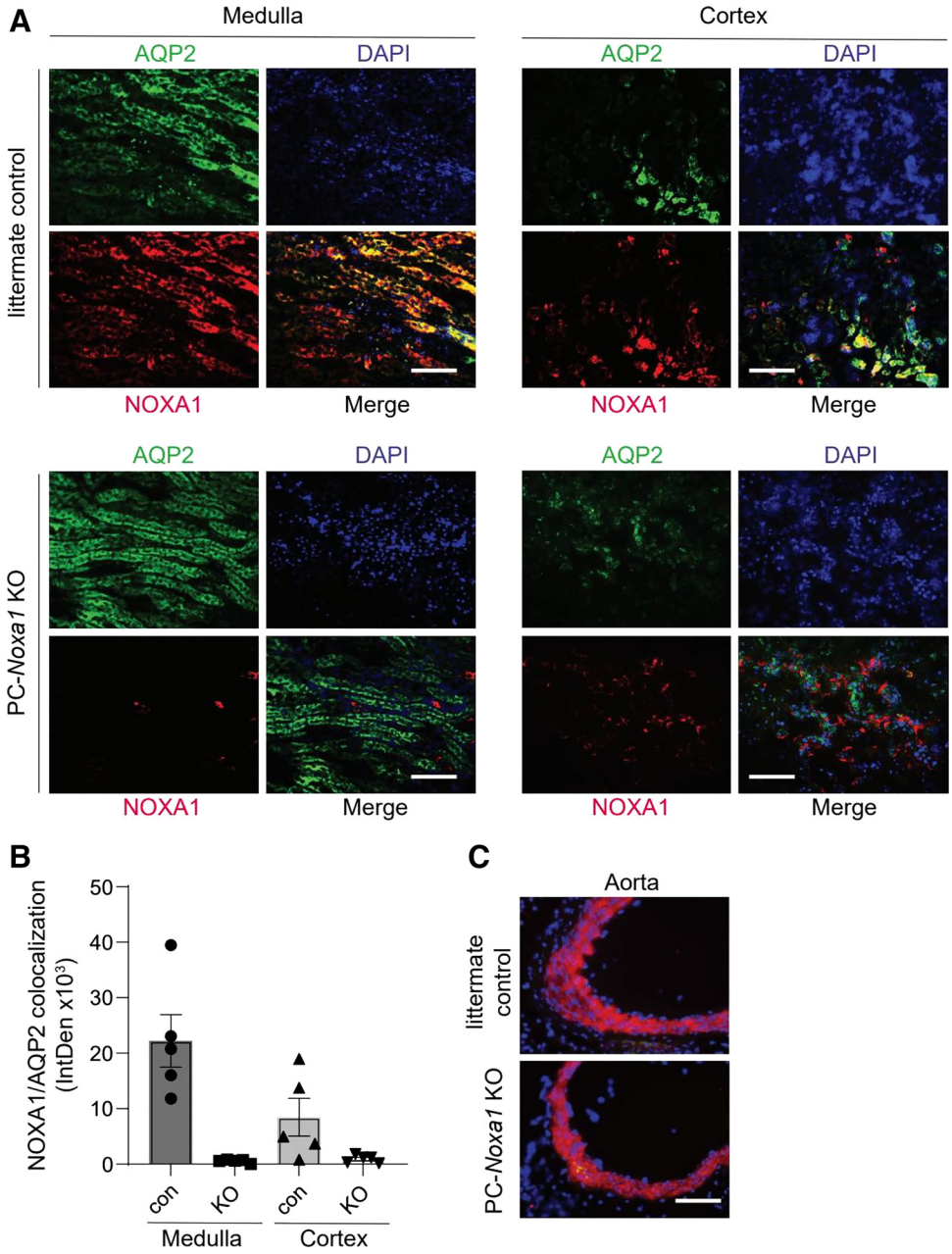

Figure 5. NADPH oxidase activator 1 (*Noxa1*) is specifically deleted in renal principal cells of the principal cell-specific *Noxa1* knockout (PC-*Noxa1* KO) mouse. *A*: representative fluorescence microscopy images of frozen renal medulla and cortex sections from littermate controls (*top*) and PC-*Noxa1* KO (*bottom*) mice stained for immunoreactive NOXA1 (red), aquaporin 2 (Aqp2; green), and DAPI (blue). Scale bars = 100 μm. *B*: quantification of NOXA1 colocalization with AQP2 in the renal medulla and cortex. Data are fluorescence integrated density of merged fluorescence images (means ± SE, n = 5). *C*: representative fluorescence microscopy images of the frozen aorta from littermate controls (*top*) and PC-Noxa1 KO (*bottom*) mice stained for immunoreactive NOXA1 (red) and DAPI (blue). Scale bar = 100 μm.

from the PC-*Noxa1* mouse, these values for ANG II treatment did not differ from those for vehicle treatment. In fact, NP_o, P_o, and N in ANG II- and vehicle-treated tubules from the PC-*Noxa1* KO mouse did not differ from those for vehicle treatment. These results support the notion that NOXA1/NOX1 signaling in principal cells is necessary for ANG II-induced activation of ENaC in these cells.

DISCUSSION

Our findings demonstrate that ANG II activates ENaC activity via AT_1 receptors in renal principal cells. This ANG II effect on ENaC requires functional NOX1. Similarly, NOXA1 expression is required for ANG II stimulation of ENaC activity in principal cells. These findings demonstrate the central role of NOXA1/NOX1 signaling in ANG II-dependent activation of ENaC in principal cells. Therefore, NOXA1/NOX1 signaling in the distal nephron likely plays a central role in Na^+ homeostasis and blood pressure control.

The results shown in Fig. 2, demonstrating that ANG II increases ENaC activity in renal principal cells rapidly and robustly, were not unexpected and are in accordance with previously published results (16, 18–20). Treatment of isolated tubules with ANG II for 40 min increased ENaC activity as effectively as giving ANG II systemically over 2–3 days. Although ANG II increased both ENaC P_o and N, its primary effect was on N. These results are consistent with our recent findings that ANG II increases the expression of ENaC in principal cells (22). As ANG II stimulates the secretion of aldosterone from the adrenal gland, and since aldosterone can

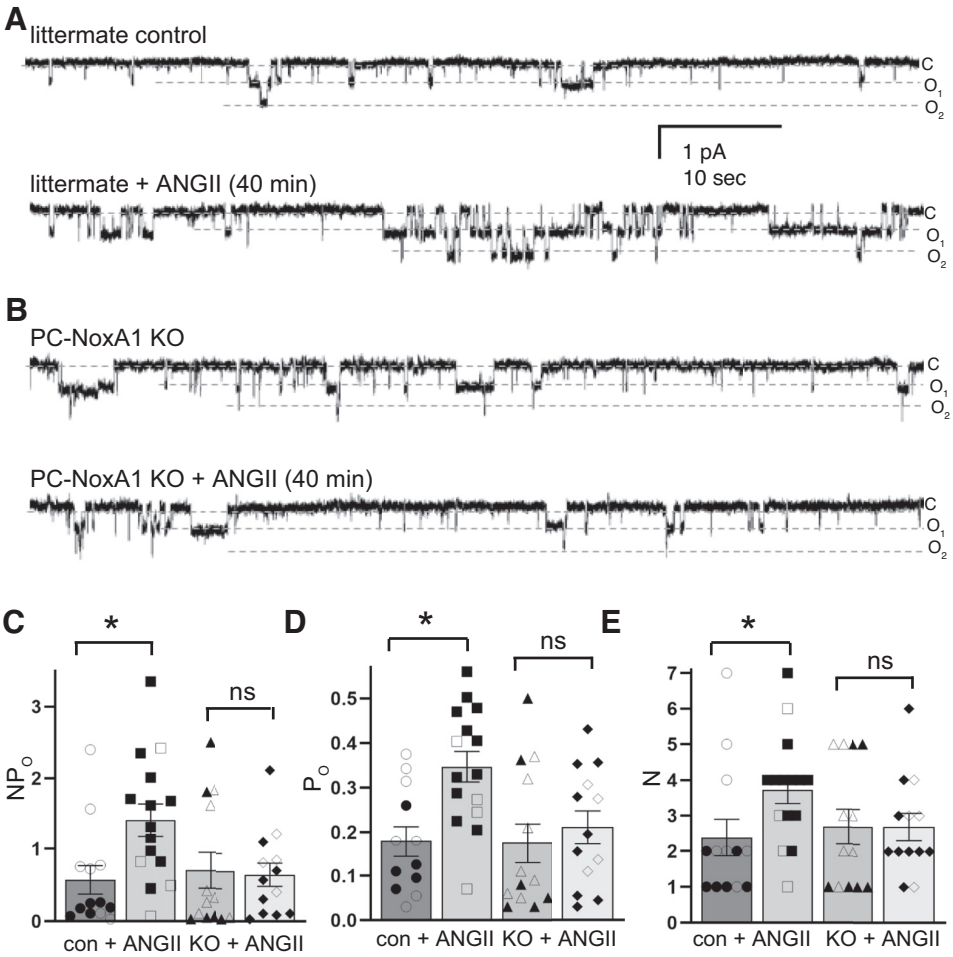

Figure 6. Angiotensin II (ANG II) had no effect on epithelial Na^+ channel (ENaC) activity in principal cells of the principal cell-specific *Noxa1* knockout (PC-*Noxa1* KO) mouse. Representative current traces of ENaC in cell-attached patches on the apical plasma membranes of principal cells in the split-open connecting tubule/cortical collecting duct from littermate controls (*A*) and PC-*Noxa1* KO mice (*B*) treated with vehicle (*top*) or 500 nM ANG II for 40 min (*bottom*). Summary graphs of means ± SE (bars) and individual data points for ENaC activity (NP_o; *C*), open probability (P_o; *D*), and number of channels (*N*; *E*) in principal cells from littermate controls (con; n = 13 cells from 2 male and 1 female mice, circles) without and with ANG II treatment (n = 15 cells from 2 male and 1 female mice, squares) and from PC-*Noxa1* KO mice (n = 13 cells from 1 male and 3 female mice, triangles) without and with ANG II (n = 13 cells from 2 male and 2 female mice, diamonds). These experiments included mice of both sexes at approximately equal proportions. *Significantly different by one-way ANOVA with a Tukey highly significant difference post hoc test. ns, not significant. C and O indicate closed and open channels, respectively.

affect ENaC activity independently of ANG II, we were surprised to find that chronic treatment with ANG II had no additional effect on ENaC activity than exposing isolated tubules to ANG II for 40 min. However, in hindsight, this observation likely reflects the complex interaction between ANG II and aldosterone during stimulation of ENaC (20, 21). Although blockade of mineralocorticoid receptors alone often fails to reduce the inappropriate Na^+ excretion and hypertension associated with high ANG II levels (44, 45), inhibition of ENaC with amiloride is effective at lowering blood pressure in the ANG II-infused rat (46). In line with this and the present findings, ENaC activity is elevated well above the physiological range and not responsive to inhibition of the aldosterone-mineralocorticoid receptor axis when ANG II is elevated or when ANG II-dependent hypertension is present (20).

Figure 3 shows that ANG II activates ENaC in principal cells by stimulating AT_1 receptors. This is in line with previous reports (18, 19, 21). These earlier studies implicated that ROS and NOX signaling may be downstream of AT_1 in the ANG II to ENaC transduction pathway in principal cells (18, 19). This inhibitory effect most likely reflects low-level, tonic ANG II-dependent ENaC activity under control conditions, which is fully inhibited in the presence of losartan. Accordingly, the findings shown in Fig. 4 demonstrate that functional NOX1 is necessary for ENaC activity to increase in response ANG II. The broad-spectrum NOX inhibitors apocynin and diphenyleneiodonium block ANG II effects on ENaC (18, 19), and ROS, like ANG II, increase ENaC activity in principal cells (19, 23).

It is unclear which form of NOX is most important for ENaC stimulation by ANG II. The present results, as shown in Fig. 4, are most consistent with NOX1 being the primary mediator. NOX4, though, has been implicated in the control of ENaC in the distal nephron of salt-sensitive Dahl hypertensive rats and in A6 and mpkCCD principal cell models (23, 47, 48). Rac1-mediated NOX2 generation of superoxide, on the other hand, stimulates ENaC activity in alveolar cells (24).

ANG II is unable to elevate ENaC activity in tubules isolated from PC-*Noxa1* KO mice, as shown in Figs. 5 and 6, supporting the notion that NOXA1 is critical for ANG II actions on ENaC. This is further evidence that NOXA1/NOX1 signaling is paramount for ANG II effects on ENaC in the mammalian principal cell. This is in agreement with findings that NOXA1 stimulation of NOX1 production of ROS in principal cells in response to ANG II results in elevated ENaC expression, Na^+ and water retention, and ultimately increased blood pressure during the developmental stage of hypertension (22). To date, the emerging evidence seems most consistent with NOXA1/NOX1 signaling being obligatory in principal cells for ANG II stimulation of

ENaC and subsequent changes in renal Na^+ excretion that favor the development of hypertension.

GRANTS

This work was supported by National Heart, Lung, and Blood Institute Grant R01HL139841 (to W.J.A. and N.R.M.).

DISCLOSURES

M.S.R. is a member of the Board of Directors at Eli Lilly & Company. None of the other authors has any conflicts of interest, financial or otherwise, to disclose.

AUTHOR CONTRIBUTIONS

E.M., A.E.V., N.R.M., W.J.A., and J.D.S. conceived and designed research; E.M., C.R.A., A.E.V., N.R.M., J.D.S., and T.M.A. performed experiments; E.M., C.R.A., A.E.V., N.R.M., W.J.A., J.D.S., and T.M.A. analyzed data; E.M., C.R.A., A.E.V., M.S.R., N.R.M., W.J.A., J.D.S., and T.M.A. interpreted results of experiments; C.R.A., A.E.V., N.R.M., W.J.A., J.D.S., and T.M.A. prepared figures; E.M., C.R.A., A.E.V., N.R.M., J.D.S., and T.M.A. drafted manuscript; E.M., C.R.A., A.E.V., M.S.R., N.R.M., W.J.A., J.D.S., and T.M.A. edited and revised manuscript; E.M., C.R.A., M.S.R., N.R.M., W.J.A., J.D.S., and T.M.A. approved final version of manuscript.

REFERENCES

1. **Lifton RP**, **Wilson FH**, **Choate KA**, **Geller DS.** Salt and blood pressure: new insight from human genetic studies. *Cold Spring Harb Symp Quant Biol* 67: 445–450, 2002. doi:10.1101/sqb.2002.67.445.
2. **Pitzer AL**, **Van Beusecum JP**, **Kleyman TR**, **Kirabo A.** ENaC in salt-sensitive hypertension: kidney and beyond. *Curr Hypertens Rep* 22: 69, 2020. doi:10.1007/s11906-020-01067-9.
3. **Guyton AC.** Blood pressure control–special role of the kidneys and body fluids. *Science* 252: 1813–1816, 1991. doi:10.1126/science.2063193.
4. **Garty H**, **Palmer LG.** Epithelial sodium channels: function, structure, and regulation. *Physiol Rev* 77: 359–396, 1997. doi:10.1152/physrev.1997.77.2.359.
5. **Rotin D**, **Schild L.** ENaC and its regulatory proteins as drug targets for blood pressure control. *Curr Drug Targets* 9: 709–716, 2008. doi:10.2174/138945008785132367.
6. **Booth RE**, **Johnson JP**, **Stockand JD.** Aldosterone. *Adv Physiol Educ* 26: 8–20, 2002. doi:10.1152/advan.00051.2001.
7. **Toney GM**, **Vallon V**, **Stockand JD.** Intrinsic control of sodium excretion in the distal nephron by inhibitory purinergic regulation of the epithelial Na^+ channel. *Curr Opin Nephrol Hypertens* 21: 52–60, 2012. doi:10.1097/MNH.0b013e32834db4a0.
8. **Rossier BC**, **Pradervand S**, **Schild L**, **Hummler E.** Epithelial sodium channel and the control of sodium balance: interaction between genetic and environmental factors. *Annu Rev Physiol* 64: 877–897, 2002. doi:10.1146/annurev.physiol.64.082101.143243.
9. **Bonny O**, **Hummler E.** Dysfunction of epithelial sodium transport: from human to mouse. *Kidney Int* 57: 1313–1318, 2000. doi:10.1046/j.1523-1755.2000.00968.x.
10. **Canessa CM**, **Horisberger JD**, **Rossier BC.** Epithelial sodium channel related to proteins involved in neurodegeneration. *Nature* 361: 467–470, 1993. doi:10.1038/361467a0.
11. **Canessa CM**, **Schild L**, **Buell G**, **Thorens B**, **Gautschi I**, **Horisberger JD**, **Rossier BC.** Amiloride-sensitive epithelial Na^+ channel is made of three homologous subunits. *Nature* 367: 463–467, 1994. doi:10.1038/367463a0.
12. **Hamilton KL**, **Eaton DC.** Single-channel recordings from two types of amiloride-sensitive epithelial Na^+ channels. *Membr Biochem* 6: 149–171, 1986. doi:10.3109/09687688609065447.
13. **Kemendy AE**, **Kleyman TR**, **Eaton DC.** Aldosterone alters the open probability of amiloride-blockable sodium channels in A6 epithelia. *Am J Physiol Cell Physiol* 263: C825–C837, 1992. doi:10.1152/ajpcell.1992.263.4.C825.
14. **Lifton RP.** Genetic determinants of human hypertension. *Proc Natl Acad Sci USA* 92: 8545–8551, 1995. doi:10.1073/pnas.92.19.8545.
15. **Lifton RP.** Genetic factors in hypertension. *Curr Opin Nephrol Hypertens* 2: 258–264, 1993. doi:10.1097/00041552-199303000-00013.
16. **Peti-Peterdi J**, **Warnock DG**, **Bell PD.** Angiotensin II directly stimulates ENaC activity in the cortical collecting duct via AT_1 receptors. *J Am Soc Nephrol* 13: 1131–1135, 2002. doi:10.1097/01.asn.0000013292.78621.fd.
17. **Hamilton KL**, **Eaton DC.** Single-channel recordings from amiloride-sensitive epithelial sodium channel. *Am J Physiol Cell Physiol* 249: C200–C207, 1985. doi:10.1152/ajpcell.1985.249.3.C200.
18. **Mamenko M**, **Zaika O**, **Ilatovskaya DV**, **Staruschenko A**, **Pochynyuk O.** Angiotensin II increases activity of the epithelial Na^+ channel (ENaC) in distal nephron additively to aldosterone. *J Biol Chem* 287: 660–671, 2012. doi:10.1074/jbc.M111.298919.
19. **Sun P**, **Yue P**, **Wang W-H.** Angiotensin II stimulates epithelial sodium channels in the cortical collecting duct of the rat kidney. *Am J Physiol Renal Physiol* 302: F679–F687, 2012. doi:10.1152/ajprenal.00368.2011.
20. **Mamenko M**, **Zaika O**, **Prieto MC**, **Jensen VB**, **Doris PA**, **Navar LG**, **Pochynyuk O.** Chronic angiotensin II infusion drives extensive aldosterone-independent epithelial Na^+ channel activation. *Hypertension* 62: 1111–1122, 2013. doi:10.1161/HYPERTENSIONAHA.113.01797.
21. **Mamenko M**, **Zaika O**, **Tomilin V**, **Jensen VB**, **Pochynyuk O.** Compromised regulation of the collecting duct ENaC activity in mice lacking AT_{1a} receptor. *J Cell Physiol* 233: 7217–7225, 2018. doi:10.1002/jcp.26552.
22. **Vendrov A**, **Stevenson MD**, **Lozhkin A**, **Hayami T**, **Holland NA**, **Yang X**, **Moss N**, **Pan H**, **Wickline SA**, **Stockand JD**, **Runge MS**, **Madamanchi NR**, **Arendshorst WJ.** Renal NOXA1/NOX1 signaling regulates epithelial sodium channel and sodium retention in angiotensin II-induced hypertension. *Antioxid Redox Signal* 36: 550–566, 2022. doi:10.1089/ars.2021.0047.
23. **Trac D**, **Liu B**, **Pao AC**, **Thomas SV**, **Park M**, **Downs CA**, **Ma H-P**, **Helms MN.** Fulvene-5 inhibition of Nadph oxidases attenuates activation of epithelial sodium channels in A6 distal nephron cells. *Am J Physiol Renal Physiol* 305: F995–F1005, 2013. doi:10.1152/ajprenal.00098.2013.
24. **Takemura Y**, **Goodson P**, **Bao HF**, **Jain L**, **Helms MN.** Rac1-mediated NADPH oxidase release of O_2^- regulates epithelial sodium channel activity in the alveolar epithelium. *Am J Physiol Lung Cell Mol Physiol* 298: L509–L520, 2010. doi:10.1152/ajplung.00230.2009.
25. **Downs CA**, **Trac DQ**, **Kreiner LH**, **Eaton AF**, **Johnson NM**, **Brown LA**, **Helms MN.** Ethanol alters alveolar fluid balance via Nadph oxidase (NOX) signaling to epithelial sodium channels (ENaC) in the lung. *PLoS One* 8: e54750, 2013. doi:10.1371/journal.pone.0054750.
26. **Gill PS**, **Wilcox CS.** NADPH oxidases in the kidney. *Antioxid Redox Signal* 8: 1597–1607, 2006. doi:10.1089/ars.2006.8.1597.
27. **Lassegue B**, **San Martin A**, **Griendling KK.** Biochemistry, physiology, and pathophysiology of NADPH oxidases in the cardiovascular system. *Circ Res* 110: 1364–1390, 2012. doi:10.1161/CIRCRESAHA.111.243972.
28. **Nguyen Dinh Cat A**, **Montezano AC**, **Burger D**, **Touyz RM.** Angiotensin II, NADPH oxidase, and redox signaling in the vasculature. *Antioxid Redox Signal* 19: 1110–1120, 2013. doi:10.1089/ars.2012.4641.
29. **Takeya R**, **Ueno N**, **Kami K**, **Taura M**, **Kohjima M**, **Izaki T**, **Nunoi H**, **Sumimoto H.** Novel human homologues of $p47^{phox}$ and $p67^{phox}$ participate in activation of superoxide-producing NADPH oxidases. *J Biol Chem* 278: 25234–25246, 2003 [Erratum in *J Biol Chem* 290: 6003, 2015]. doi:10.1074/jbc.M212856200.
30. **Ambasta RK**, **Schreiber JG**, **Janiszewski M**, **Busse R**, **Brandes RP.** Noxa1 is a central component of the smooth muscle NADPH oxidase in mice. *Free Radic Biol Med* 41: 193–201, 2006. doi:10.1016/j.freeradbiomed.2005.12.035.
31. **Mollnau H**, **Wendt M**, **Szöcs K**, **Lassègue B**, **Schulz E**, **Oelze M**, **Li H**, **Bodenschatz M**, **August M**, **Kleschyov AL**, **Tsilimingas N**, **Walter U**, **Förstermann U**, **Meinertz T**, **Griendling K**, **Münzel T.** Effects of angiotensin II infusion on the expression and function of NAD(P)H oxidase and components of nitric oxide/cGMP signaling. *Circ Res* 90: E58–E65, 2002. doi:10.1161/01.res.0000012569.55432.02.

32. **Rajagopalan S**, **Kurz S**, **Münzel T**, **Tarpey M**, **Freeman BA**, **Griendling KK**, **Harrison DG.** Angiotensin II-mediated hypertension in the rat increases vascular superoxide production via membrane NADH/NADPH oxidase activation. Contribution to alterations of vasomotor tone. *J Clin Invest* 97: 1916–1923, 1996. doi:10.1172/JCI118623.
33. **Chiou J-T**, **Huang N-C**, **Huang C-H**, **Wang L-J**, **Lee Y-C**, **Shi Y-J**, **Chang L-S.** NOXA-mediated degradation of MCL1 and BCL2L1 causes apoptosis of daunorubicin-treated human acute myeloid leukemia cells. *J Cell Physiol* 236: 7356–7375, 2021. doi:10.1002/jcp.30407.
34. **Vendrov AE**, **Sumida A**, **Canugovi C**, **Lozhkin A**, **Hayami T**, **Madamanchi NR**, **Runge MS.** NOXA1-dependent NADPH oxidase regulates redox signaling and phenotype of vascular smooth muscle cell during atherogenesis. *Redox Biol* 21: 101063, 2019. doi:10.1016/j.redox.2018.11.021.
35. **Flaherty JP**, **Spruce CA**, **Fairfield HE**, **Bergstrom DE.** Generation of a conditional null allele of NADPH oxidase activator 1 (NOXA1). *Genesis* 48: 568–575, 2010 [Erratum in *Genesis* 49: 103, 2011]. doi:10.1002/dvg.20655.
36. **Ge Y**, **Ahn D**, **Stricklett PK**, **Hughes AK**, **Yanagisawa M**, **Verbalis JG**, **Kohan DE.** Collecting duct-specific knockout of endothelin-1 alters vasopressin regulation of urine osmolality. *Am J Physiol Renal Physiol* 288: F912–F920, 2005. doi:10.1152/ajprenal.00432.2004.
37. **Ge Y**, **Stricklett PK**, **Hughes AK**, **Yanagisawa M**, **Kohan DE.** Collecting duct-specific knockout of the endothelin A receptor alters renal vasopressin responsiveness, but not sodium excretion or blood pressure. *Am J Physiol Renal Physiol* 289: F692–F698, 2005. doi:10.1152/ajprenal.00100.2005.
38. **Zhang H**, **Zhang A**, **Kohan DE**, **Nelson RD**, **Gonzalez FJ**, **Yang T.** Collecting duct-specific deletion of peroxisome proliferator-activated receptor gamma blocks thiazolidinedione-induced fluid retention. *Proc Natl Acad Sci USA* 102: 9406–9411, 2005. doi:10.1073/pnas.0501744102.
39. **Niu X-L**, **Madamanchi NR**, **Vendrov AE**, **Tchivilev I**, **Rojas M**, **Madamanchi C**, **Brandes RP**, **Krause K-H**, **Humphries J**, **Smith A**, **Burnand KG**, **Runge MS.** Nox activator 1: a potential target for modulation of vascular reactive oxygen species in atherosclerotic arteries. *Circulation* 121: 549–559, 2010. doi:10.1161/CIRCULATIONAHA.109.908319.
40. **Soares AG**, **Mironova E**, **Archer CR**, **Contreras J**, **Stockand JD**, **Abd El-Aziz TM.** Cisplatin decreases ENaC activity contributing to renal salt wasting syndrome. *Cancers (Basel)* 12: 2140, 2020. doi:10.3390/cancers12082140.
41. **Mironova E**, **Suliman F**, **Stockand JD.** Renal Na^+ excretion consequent to pharmacogenetic activation of G_q-DREADD in principal cells. *Am J Physiol Renal Physiol* 316: F758–F767, 2019. doi:10.1152/ajprenal.00612.2018.
42. **Berman JM**, **Mironova E**, **Stockand JD.** Physiological regulation of the epithelial Na^+ channel by casein kinase II. *Am J Physiol Renal Physiol* 314: F367–F372, 2018. doi:10.1152/ajprenal.00469.2017.
43. **Zaika O**, **Mamenko M**, **Staruschenko A**, **Pochynyuk O.** Direct activation of ENaC by angiotensin II: recent advances and new insights. *Curr Hypertens Rep* 15: 17–24, 2013. doi:10.1007/s11906-012-0316-1.
44. **Ortiz RM**, **Graciano ML**, **Seth D**, **Awayda MS**, **Navar LG.** Aldosterone receptor antagonism exacerbates intrarenal angiotensin II augmentation in ANG II-dependent hypertension. *Am J Physiol Renal Physiol* 293: F139–F147, 2007. doi:10.1152/ajprenal.00504.2006.
45. **Zhao D**, **Seth DM**, **Navar LG.** Enhanced distal nephron sodium reabsorption in chronic angiotensin II-infused mice. *Hypertension* 54: 120–126, 2009. doi:10.1161/HYPERTENSIONAHA.109.133785.
46. **Gonzalez AA**, **Liu L**, **Lara LS**, **Seth DM**, **Navar LG**, **Prieto MC.** Angiotensin II stimulates renin in inner medullary collecting duct cells via protein kinase C and independent of epithelial sodium channel and mineralocorticoid receptor activity. *Hypertension* 57: 594–599, 2011. doi:10.1161/HYPERTENSIONAHA.110.165902.
47. **Pavlov TS**, **Palygin O**, **Isaeva E**, **Levchenko V**, **Khedr S**, **Blass G**, **Ilatovskaya DV**, **Cowley AW Jr**, **Staruschenko A.** NOX4-dependent regulation of ENaC in hypertension and diabetic kidney disease. *FASEB J* 34: 13396–13408, 2020. doi:10.1096/fj.202000966RR.
48. **Lu X**, **Wang F**, **Liu M**, **Yang KT**, **Nau A**, **Kohan DE**, **Reese V**, **Richardson RS**, **Yang T.** Activation of ENaC in collecting duct cells by prorenin and its receptor PRR: involvement of Nox4-derived hydrogen peroxide. *Am J Physiol Renal Physiol* 310: F1243–F1250, 2016. doi:10.1152/ajprenal.00492.2015.