Cholesterol affects flow-stimulated cyclooxygenase-2 expression and prostanoid secretion in the cortical collecting duct

Yu Liu,¹ ²* Daniel Flores,¹ ²* Rolando Carrisoza-Gaytán,³ and Rajeev Rohatgi¹ ² ³

¹Department of Medicine, Icahn School of Medicine at Mount Sinai, New York, New York; ²Department of Medicine, The James J. Peters Veterans Affairs Medical Center, New York, New York; ³Department of Pediatrics, Icahn School of Medicine at Mount Sinai, New York, New York

Submitted 26 November 2014; accepted in final form 9 March 2015

Cholesterol affects flow-stimulated cyclooxygenase-2 expression and prostanoid secretion in the cortical collecting duct. Am J Physiol Renal Physiol 308: F1229–F1237, 2015. First published March 11, 2015; doi:10.1152/ajprenal.00635.2014.—Essential hypertension (eHTN) is associated with hypercholesterolemia, but how cholesterol contributes to eHTN is unknown. Recent evidence demonstrates that short-term dietary cholesterol ingestion induces epithelial Na channel (ENaC)-dependent Na absorption with a subsequent rise in blood pressure (BP), implicating cholesterol in salt-sensitive HTN. Prostaglandin E₂ (PGE₂), an autocrine/paracrine molecule, is induced by flow in endothelia to vasodilate the vasculature and inhibit ENaC-dependent Na absorption in the renal collecting duct (CD), which reduce BP. We hypothesize that cholesterol suppresses flow-mediated cyclooxygenase-2 (COX-2) expression and PGE₂ release in the CD, which, in turn, affects Na absorption. Cortical CDs (CCs) were microperfused at 0, 1, and 5 nl·min⁻¹·mm⁻¹, and PGE₂ release was measured. Secretrion of PGE₂ was similar between no- and low-flow (151 ± 28 vs. 121 ± 48 pg·ml⁻¹·mm⁻¹) CCs, but PGE₂ was greater from high-flow (578 ± 146 pg·ml⁻¹·mm⁻¹; P < 0.05) CCs. Next, mice were fed either a 0 or 1% cholesterol diet, injected with saline to generate high urine flow rates, and CCs were microdissected for PGE₂ secretion. CCs isolated from cholesterol-fed mice secreted less PGE₂ and had a lower PGE₂-generating capacity than CCs isolated from control mice, implying cholesterol repressed flow-induced PGE₂ synthesis. Next, cholesterol extraction in a CD cell line induced COX-2 expression and PGE₂ release while cholesterol incorporation, conversely, suppressed their expression. Moreover, fluid shear stress (FSS) and cholesterol extraction induced COX-2 protein abundance via p38-dependent activation. Thus cellular cholesterol composition affects biomechanical signaling, which, in turn, affects FSS-mediated COX-2 expression and PGE₂ release via a p38-dependent mechanism.

cholersterol; collecting duct; shear

Essential hypertension (eHTN) affects millions of adults in the United States and worldwide and contributes to end-organ damage, including kidney failure. The epidemic of eHTN is, in part, related to environmental changes, and in particular, the high-sodium (Na) and -cholesterol diets consumed in the United States. The link between Na consumption and eHTN is established; however, the relationship between cholesterol and eHTN is less well understood.

Hypercholesterolemia enhances biochemical incorporation of cholesterol into the plasma membrane (PM), and this raises PM viscosity (53). Recent data demonstrate that cholesterol accumulation in tissues contributes to organ dysfunction; in particular, it can induce diabetes (by suppressing insulin secretion) and diabetic nephropathy (31). In fact, cholesterol chelation by cyclodextrin improved insulin secretion and diabetic nephropathy (less proteinuria) while treatment with statins had no effect, implying tissue cholesterol contributes to organ dysfunction (31). In hypertensive rodents, blood pressure correlates with elevated erythrocyte PM viscosity, which is observed in human eHTN and is reflected in changes in renal epithelial PM, but how this change in the PM affects ion transport is limited (21, 23, 33, 37, 46). Short-term cholesterol ingestion in rodents (without vasculopathy), in the absence of hyperinsulinemia or hyperaldosteronism, induced a marked activation of epithelial Na channels (ENaCs) and antinatriuresis that preceded a rise in blood pressure (2). In Dahl salt-sensitive rats, PM viscosity is increased, renal intracellular calcium concentration ([Ca²⁺]) is decreased, and prostaglandin E₂ (PGE₂) release is reduced, findings that stimulate renal Na avidity (5, 21). However, the mechanisms by which tissue cholesterol leads to functional alterations in renal cation transport, Na retention, and eHTN remain elusive (2).

Tubular flow through biomechanical forces, fluid shear stress (FSS) and circumferential stretch (CS) (4), regulate Na and potassium (K) transport in the collecting duct (CD) (26, 34). In addition, our laboratory and others have shown that tubular flow and FSS induce paracrine factors, like PGE₂ (12), endothelin-1 (29), and nucleotides (18, 47), that secondarily regulate calcium cation transport in the CD. The mechanism by which mechanical forces are transduced into an intracellular signal that stimulates autocrine/paracrine secretion of molecules is an area of active investigation.

The biomechanical sensors which induce intracellular signaling have principally been studied in endothelial cells, leading to a rich literature suggesting cilia, caveolae, the glycocalyx, and other proteins/organelles can induce mechanically sensitive signaling (25, 45, 52). Caveolae are particularly interesting to consider since evidence points to flow stimulation of MAPKs through this structure (6), and evidence from our laboratory and others demonstrates that MAPKs regulate COX expression (27, 43). Caveolae and/or lipid rafts (LRs; a more general term) are cholesterol- and sphingomyelin-enriched regions of the PM that express high concentrations of proteins. The cholesterol content of LR can alter the interaction of proteins localized within the LR, which, in turn, can affect downstream signaling (41). Thus this led us to hypothesize that cholesterol modulates flow-stimulated PGE₂ secretion and PGE₂-generating capacity by altering mechanical signaling of the CD to FSS. We speculate the changes in cholesterol-modulated, flow-mediated COX-2 and PGE₂ generation may, in turn, affect ENaC-dependent Na absorption. To
test the effect of cholesterol on flow-induced PGE2 release and COX-2 stimulation, we utilized a combination of in vitro cell culture and in vivo murine studies in which we manipulated the cellular cholesterol content of the a CD cell line or native CD.

**MATERIALS AND METHODS**

**Cell Culture**

Murine immortalized inner medullary CD3 (IMCD3) cells were grown in DMEM/F12 (with 10% FBS) on 25 × 75-mm slides and studied when they reached confluence (between 4 and 7 days). Cells were serum starved for at least 16 h before experimentation. We only used cells up to passage 10 due to the risk of genetic drift.

**Induction of FSS**

Cells grown on slides were placed in a laminar flow chamber (Glycotech) maintained at 37°C and subjected to FSS of 0.4 dynes/cm² without exposure to FSS. One milliliter of serum- and phenol red-free DMEM/F12 was incubated with either static or sheared cells for 1 h without exposure to FSS. One milliliter of serum- and phenol red-free DMEM/F12 was exposed to the same solution and duration as sheared cells, but was not recirculated into the perfusion chamber. Static control cells were exposed to the same solution and duration as sheared cells, but without exposure to FSS. One milliliter of serum- and phenol red-free DMEM/F12 was incubated with either static or sheared cells for 1 h for measurement of PGE2 secretion (12, 30). Cells from the Glycotech chamber were then collected for total protein.

**Western Blotting**

Western blot analysis was performed as previously described (10). Protein lysates were generated from IMCD3 cells by incubating and homogenizing cells in lysis buffer [10 mM Tris, pH 7.2, 1 mM EGTA, 1 mM EDTA, 150 mM NaCl, 0.5% NP-40, 1.0% Triton X-100, and protease inhibitors (1 mM PMSF, 10 μg/ml leupeptin, 5 μM pepstatin A, 1 mM benzamidine, 30 mM sodium fluoride, 2 mM sodium orthovanadate, and 1 μg/ml aprotinin)] on ice. The supernatant was collected and assayed for protein content using the BCA protein assay (Thermo Scientific). Thirty to one hundred micrograms (depending on the abundance of the signal) of protein lysate were resolved electrophoretically and transferred to Immobilon filters (Millipore, Billerica, MA). Filters were blocked in 5% non-fat dry milk and 0.05% Tween and immunoblotted with a primary antibody (see Reagents). After washing, blots were incubated with a horseradish peroxidase-conjugated secondary antibody (Sigma, St. Louis, MO), and bands were visualized with a West Pico enhanced chemiluminescence kit (Pierce, Rockford, IL). After stripping and blocking of the membrane, the blot was incubated with an anti-β-actin-specific antibody and visualized using the same methods as for the primary antibody.

**Rabbits.** Adult female New Zealand White (NZW) rabbits were obtained from Covance (Denver, PA) and housed at the Icahn School of Medicine for 6 h, the mice were euthanized by compressed CO2, kidneys were extracted, and cortical CD (CCDs) were identified, microdissected, and isolated under a dissecting microscope by hand (9) to measure PGE2 release. All protocols were approved by the Institutional Animal Care and Use Committee of the Icahn School of Medicine at Mount Sinai. Animals were euthanized in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

**Tests for FSS**

Rabbit kidneys were removed via a midline incision, and CCDs were dissected in cold (4°C) Ringer solution, affixed to poly-L-lysine-coated 0.5 × 0.2-cm coverslips, and micropunched in vitro as previously described (9). From each animal, three CCDs were microdissected and perfused at 0, 1, or 5 nL·min⁻¹·mm⁻³ for 1 h, respectively. Briefly, isolated tubules were immediately transferred to a temperature- and O2/CO2-controlled specimen chamber, and those tubules that are exposed to flow were mounted on concentric glass pipettes and perfused and bathed at 37°C with Burg's perfusate containing (in mM) 120 NaCl, 25 NaHCO3, 2.5 K2HPO4, 2.0 CaCl2, 1.2 MgSO4, 4.0 Na lactate, 1.0 Na citrate, 6.0 L-alanine, and 5.5 D-glucose, pH 7.4, 290 ± 2 mosmol/kgH2O (9). During the 45-min equilibration period and thereafter, the perfusion chamber was continuously suffused with a gas mixture of 95% O2:5% CO2 to maintain the pH of the Burg’s solution at 7.4 at 37°C. The CCD which was not exposed to flow was incubated for the same duration and in Burg’s solution as the flow-exposed CCDs, except the tubule was not mounted on concentric glass pipettes. The bathing solution was continuously exchanged at a rate of 10 ml/h with a syringe pump (Razel, Stamford, CT). After the CCD was exposed to the flow or no-flow condition, the coverslip-affixed CCD was carefully removed by manipulating the coverslip. CCDs were then transferred to cold Ringer lactate (RL) for 30 min at 37°C, and PGE2 was measured in the conditioned media.

**PGE2 Secretion by Microdissected CCDs and PGE2-Generating Capacity**

Approximately 1–3 mm of CCDs (1–4 tubules/sample) were microdissected by hand (27) in RL from cholesterol- and control-fed mice after injection with saline. The CCDs were affixed to poly-L-lysine-coated 0.5 × 0.2-cm coverslips, and transferred to RL and incubated at 37°C for 30 min. The conditioned media was collected and stored at −80°C for measurement of PGE2 (17). To compute total PGE2-synthesizing activity in individual CCDs, the coverslips were incubated in RL containing 100 μM arachidonic acid (the substrate for COXs) for 30 min at 37°C, and PGE2 was measured in the supernatant (17, 48).

**PGE2 was measured in supernatants using a PGE2 Enzyme Immunoassay (EIA) kit (Cayman Chemical) (12).**

**Altering Cellular Cholesterol Content**

To effectuate a reduction in cellular cholesterol, IMCD3 cells were treated with 10 mM methyl-β-cyclodextrin (MβC), a cholesterol-chelating agent, for 1 h (50). To raise cholesterol, cells were incubated for 36 h with a cholesterol/MβC mixture. Because the solubility of cholesterol is poor, 10 mM MβC was incubated with 1 mM cholesterol to enhance the solubility of the compound. Next, this mixture was added at a 1:10 dilution to the media bathing the IMCD3 cells (54), generating a 100 μM cholesterol solution. To confirm the effect of these treatments, cholesterol content was measured by an Ampex Red cholesterol assay (Life Technologies, Grand Island, NY).
Reagents

The following reagents were used: stimulator: arachidonic acid (AA), 100 μM (27) (Cayman Chemical); inhibitors: SB203580, 10 μM (12) (Cayman Chemical); antibodies: rabbit anti-COX-2 (1:1,000; Cayman Chemical), rabbit anti-COX-1 (1:1,000; Cayman Chemical), mouse anti-β-actin (1:1,000; Cell Signaling) antibody, and goat anti-rabbit conjugated to horseradish peroxidase or goat anti-mouse conjugated to horseradish peroxidase (1:5,000; Sigma).

Statistics

Data are given as means ± SE (n = number of slides or number of animals). Statistical analyses were performed using unpaired t-tests (SigmaStat version 2.03; SPSS, San Rafael, CA) for cell culture and CCD experiments.

RESULTS

Tubular Flow Rate Affects PGE2 Secretion in CCDs

Prior studies of CCDs isolated from volume-expanded animals suggested that high tubular flow rates induce PGE2 synthesis in vivo. To confirm this, rabbit CCDs were microdissected and microperfused ex vivo at flow rates of 0, 1, and 5 nl·min⁻¹·mm⁻¹, removed from the rig, and incubated in RL solution, and secreted PGE2 was measured in the conditioned media. No difference in PGE2 secretion was found in CCDs exposed to no and low flow (Fig. 1); however, PGE2 secretion increased in the high-flow CCDs (Fig. 1) (5 nl·min⁻¹·mm⁻¹; 578 ± 146 pg·ml⁻¹·mm⁻¹; *P < 0.05 compared with no or low flow), confirming that fast tubular flow rates in the CCD induce PGE2 synthesis and that a threshold flow rate needs to be achieved to generate this response.

Biochemical Alteration of Cholesterol and Its Effect on FSS-Induced PGE2 Release

Cholesterol was extracted with MβC or augmented with 100 μM cholesterol (cholesterol:MβC mixture) in IMCD3 cells which were exposed to FSS (0.4 dynes/cm²), and PGE2 was measured in the media. MβC-treated static, untreated sheared, and MβC-treated sheared cells secreted significantly more PGE2 than static untreated cells (Fig. 2A; *P < 0.05). PGE2 concentration was similar between static MβC-treated and shear untreated cells; however, PGE2 was greatest in cells pretreated with MβC and sheared (Fig. 2A; *P < 0.05). Conversely, cholesterol incorporation into cells significantly suppressed FSS-induced PGE2 release (Fig. 2B, *P < 0.05 compared with untreated sheared cells). Cellular cholesterol was measured and confirmed that MβC reduced and cholesterol:MβC mixture enhanced cellular cholesterol content (Fig. 2C; *P < 0.05 compared with untreated control).

Short-Term Dietary Cholesterol Affects PGE2 Synthesis by CCD

Next, we tested whether short-term cholesterol ingestion alters this CCD flow response. Mice were fed either a control (0% cholesterol) or 1% cholesterol diet and then injected with isotonic saline. Serum cholesterol was greater in cholesterol (17.4 ± 0.9 μM)- vs. control (11.0 ± 0.8 μM; n = 13, *P < 0.05)-fed mice. The urine volume [2.2 ± 0.2 vs. 2.5 ± 0.2 ml; P = not significant (NS)] and urinary PGE2 concentration (448 ± 68 vs. 360 ± 40 pg/ml; P = NS) did not differ between control (n = 15)- vs. cholesterol (n = 15)-fed mice after saline injection. Immunoblotting of medullary protein lysates with an anti-COX-2 antibody did not demonstrate any difference in expression between control- and cholesterol-fed mice (data not shown). However, the PGE2 concentration, normalized to the length of the CCD, was significantly less in the CCDs isolated from cholesterol (124 ± 13 pg·ml⁻¹·mm⁻¹)- vs. control (269 ± 39 pg·ml⁻¹·mm⁻¹)-fed mice (Fig. 3A; *P < 0.05), a finding similar to that observed in vitro (Fig. 2B). Less PGE2 secretion by the CCD is expected to enhance transepithelial Na transport in the CCD (12).

Next, we tested whether total PGE2-generating capacity (the maximum PGE2 a tubule can generate) was affected by cholesterol ingestion. In a recent report by our laboratory, CCDs isolated from saline-injected mice secrete more PGE2 (607 ± 108 pg·ml⁻¹·mm⁻¹) after incubation in 100 μM AA than CCDs isolated from un.injected sham controls (328 ± 16 pg·ml⁻¹·mm⁻¹) (27). Control- and cholesterol-fed mice were both injected with saline, permitted to diuresis for 6 h, euthanized, and then CCDs were isolated from the kidneys. CCDs were incubated with 100 μM AA, and PGE2 was measured in the conditioned media. As expected, the CCDs isolated from control animals secreted 668 ± 58 pg·ml⁻¹·mm⁻¹; however, CCDs isolated from cholesterol-fed mice secreted significantly less PGE2 (Fig. 3B; 313 ± 36 pg·ml⁻¹·mm⁻¹; *P < 0.05) after exposure to AA, implying a reduction in PGE2-generating potential and, possibly, total cyclooxygenase activity (27). To determine whether dietary cholesterol affected tubular cholesterol composition, CCDs were isolated from each group, cholesterol content was measured, and normalized to CCD length (in mm). The cholesterol content was greater in CCDs of cholesterol (Fig. 3C; *P < 0.05)- vs. control-fed mice, implying that dietary cholesterol affects tubular cholesterol content, which, in turn, affects flow-mediated PGE2 release.
Cellular Cholesterol Affects COX-2 Expression

Since cholesterol ingestion and incorporation into the CCD suppressed total PGE2-generating capacity in murine microdissected CCDs, we speculated that the inducible COX-2 was affected by altering cellular cholesterol content. Utilizing MβC, cholesterol was extracted from static IMCD3 cells and COX-2 steady-state abundance was evaluated by immunoblotting. COX-2 abundance in cholesterol-extracted cells was significantly greater (Fig. 4, A and B; *P < 0.05) than in control cells, while cholesterol enrichment of static IMCD3 cells did not alter COX-2 expression compared with control cells (Fig. 4, A and B). We hypothesized this difference in response to extraction vs. enrichment of cholesterol on COX-2 expression as related to the fact that cholesterol extraction fluidizes the PM (22), similar to FSS, to activate intracellular signaling while cholesterol addition simply incorporates into the PM without activating signaling. In fact, we suspect cholesterol incorporation does, indeed, suppress COX-2 expression, however, but only under conditions of FSS. To test this, control (n = 3) and cholesterol (n = 3)-enriched IMCD3 cells were exposed to FSS (0.4 dynes/cm²) for 4 h and COX-2 protein expression was measured by immunoblotting (Fig. 5A). The FSS-exposed, cholesterol-enriched cells expressed significantly less COX-2 (Fig. 5B; *P < 0.05) compared with shear-exposed control cells. On the other hand, FSS-induced COX-1 protein abundance was unaffected by alteration in cellular cholesterol content (Fig. 5, C and D).

FSS and Cholesterol Extraction, Processes Which Increase PM Fluidity, Induce COX-2 Through p38-Dependent Pathways

FSS (16) and cholesterol extraction (22) both enhance apical membrane fluidity, which would permit translocation out of LRs and, hence, derepression of these signaling molecules (20) participating in PGE2 and COX-2 regulation. To this end, IMCD3 cells were exposed to either FSS or MβC, a p38 inhibitor (SB203580), and then assayed by immunoblotting for COX-2 protein expression. Inhibition of the p38 pathway was selected because prior studies in our laboratory suggested a role for p38 in flow-induced PGE2 release (12). Immunoblotting of COX-2 was performed on protein lysates of IMCD3 cells exposed to FSS of 0.4 dynes/cm² for 4 h in the absence or presence of SB203580 (10 μM). In a representative Western blot, p38 inhibition suppressed FSS-induced COX-2 protein expression (Fig. 6A), while densitometric analysis of several experiments confirmed COX-2 expression was significantly less in p38 inhibitor-treated vs. untreated controls (Fig. 6B).

To test whether cholesterol extraction also induces COX-2 through a p38 mechanism, static IMCD3 cells were untreated,
treated with MβC ($n = 6$), or treated with MβC and a p38 inhibitor (SB203580) ($n = 6$), and then COX-2 protein expression was evaluated (Fig. 7A). Immunoblotting demonstrates a significant reduction of COX-2 expression by treating with a p38 inhibitor (Fig. 7B; *$P < 0.05$).

**DISCUSSION**

The stimulation of COX-2 and PGE2 in the distal nephron is critical to enhance Na excretion and maintain Na balance in a diverse array of situations, including, but not limited to, high dietary Na ingestion (12), chronic kidney disease (15, 35), and physiological renal compensation postnephrectomy (32). Each of these conditions is attended by high urine and tubular flow rates that are able to induce COX-2 abundance and PGE2 secretion in CDs, which, in turn, affect Na reabsorption (12). Inhibition of the COX-2/PGE2 pathway, as occurs with non-steroidal anti-inflammatory drugs, enhances Na retention, which contributes to hypertension. In this study, we wanted to test whether cellular cholesterol, an important mediator of FSS-induced signaling in endothelia, can alter flow-induced COX-2 production and, in turn, PGE2 release in the CD. To this end, we utilized in vivo and in vitro preparations to identify that 1) tubular flow rate directly influences the quantity of PGE2 released by the native CCD; 2) cholesterol content of CDs from control fed mice is less than that of cholesterol fed mice, while the cholesterol content of CCDs isolated from cholesterol-fed mice is also greater than that of control-fed mice. A: CCDs were isolated from control- and cholesterol-fed mice 6 h after injection with normal saline to complete the diuresis. CCDs were placed in RL, and PGE2 was measured in the conditioned media. The PGE2 concentration, normalized to the length of CCD, was significantly less in the CCDs isolated from cholesterol- vs. control-fed mice (*$P < 0.05$, 11 mice/group). B: as in the above experiments, CCDs were isolated from control- and cholesterol-fed mice 6 h after injection with normal saline, but to measure total PGE2-generating capacity, the CCDs were incubated in RL containing 100 μM arachidonic acid. The secreted PGE2 concentration was suppressed in CCDs of cholesterol-fed mice by ~50% (*$P < 0.05$) compared with CCDs of control-fed mice. C: the total cholesterol content of CCDs isolated from cholesterol-fed mice was also significantly greater than that of control-fed mice (*$P < 0.05$).

**Fig. 3.** Flow-induced PGE2 release (A) and PGE2-generating capacity (B) in CCDs isolated from cholesterol-fed mice is suppressed compared with control-fed mice, while the cholesterol content (C) of CCDs isolated from cholesterol-fed is also greater than that of control-fed mice. A: CCDs were isolated from control- and cholesterol-fed mice 6 h after injection with normal saline to complete the diuresis. CCDs were placed in RL, and PGE2 was measured in the conditioned media. The PGE2 concentration, normalized to the length of CCD, was significantly less in the CCDs isolated from cholesterol- vs. control-fed mice (*$P < 0.05$, 11 mice/group). B: as in the above experiments, CCDs were isolated from control- and cholesterol-fed mice 6 h after injection with normal saline, but to measure total PGE2-generating capacity, the CCDs were incubated in RL containing 100 μM arachidonic acid. The secreted PGE2 concentration was suppressed in CCDs of cholesterol-fed mice by ~50% (*$P < 0.05$) compared with CCDs of control-fed mice. C: the total cholesterol content of CCDs isolated from cholesterol-fed mice was also significantly greater than that of control-fed mice (*$P < 0.05$).

**Fig. 4.** Cholesterol extraction by MβC induces cyclooxygenase-2 (COX-2) expression in static IMCD3 cells (A and B). Under static conditions, IMCD3 cells were untreated, treated with MβC to extract cholesterol, or treated with cholesterol/MβC to integrate cholesterol; then, the cells were lysed, and protein was extracted. A: immunoblotting performed on the cell lysates utilizing an anti-COX-2 antibody demonstrated an increase in steady-state expression of COX-2 in cells in which cholesterol was extracted, and this was statistically significant (*$P < 0.05$) over several samples. B: treatment with cholesterol/MβC to integrate cholesterol into static IMCD3 did not affect COX-2 expression compared with control.
the CCD alters flow-induced PGE₂ release and PGE₂-generating capacity, partly by regulating COX-2 protein abundance; and 3) FSS and cholesterol extraction both induce COX-2 expression in CD cells by p38 activity.

The regulation of renal Na transport is modulated by several overlapping, biomechanically mediated autocrine/paracrine factors including molecules such as nucleotides (18, 47), endothelin-1 (29), epoxyeicosatrienoic acid (40, 44), nitric oxide (1), and prostanoids (12). The overlapping mechanisms support the maintenance of Na balance, especially, if one or more pathways are inhibited; then, other pathways are activated to compensate and ensure Na balance. Under normal dietary Na intake, inhibition or knockout of a single autocrine/paracrine pathway will not affect net Na balance/homeostasis. However, in the setting of high dietary Na intake and inhibition of an autocrine/paracrine pathway, the ability to excrete the entire Na load is incomplete, which can lead to Na retention and hypertension. For example, PGE₂ secretion and COX-2 activity increased in the IMCD of endothelin-1 knockout mice compared with control mice under either control or high Na diets (14), a finding believed to be a compensatory response. A CD-specific knockout of the endothelin-B receptor (13) and a global knockout of microsomal prostaglandin E synthase-1 (19) raised their blood pressure in response to Na loading compared with diet-matched wild-type mice, implying Na retention. In short, suppression of a single intrarenal autocrine/paracrine pathway regulating Na transport may not induce hypertension; however, when exposed to a higher filtered load of Na, the kidney’s ability to maximally excrete Na is impaired.

Cholesterol was the focus of this manuscript for two main reasons: 1) cellular, and specifically PM cholesterol, plays a critical role in shear-induced signaling in endothelial cells (3, 39); and 2) recent evidence demonstrates that tissue cholesterol content induces organ dysfunction in nonvascular tissues (2, 31, 42). This manuscript extends the evidence that cholesterol incorporation into nonvascular tissue, in this case the renal CCD, can alter its physiological responses to stimuli and

Fig. 5. Cholesterol integration suppress FSS-induced COX-2 expression (A and B) but does not affect COX-1 protein expression (C and D): A: control and cholesterol-enriched IMCD3 cells were exposed to 4 h of FSS, and COX-2 protein expression was assessed by immunoblotting. B: densitometric analysis of COX-2 expression was significantly less (*P < 0.05) in cholesterol-enriched cells exposed to FSS than controls cells exposed to FSS. On the other hand, COX-1 expression (C and D) was unaffected by cholesterol enrichment.

Fig. 6. FSS (A and B) induces p38-dependent expression of COX-2. A: to test whether FSS-induced COX-2 expression is regulated by p38, IMCD3 cells were preincubated with SB203580 (a p38 inhibitor), exposed to FSS, and then immunoblotting was performed for COX-2. The white space indicates the deletion of an unrelated pilot experiment from the original blot (editors have viewed the original blot). B: the p38 inhibitor suppressed FSS-induced COX-2 expression (*P < 0.05) in cells.
Fig. 7. Cholesterol extraction (A and B) induces p38-dependent expression of COX-2. A: to test whether cholesterol extraction similarly induced COX-2 expression through a p38-dependent mechanism, IMCD cells were preincubated with SB203580 (a p38 inhibitor), exposed to MβC to extract cholesterol, and then immunoblotting was performed for COX-2. B: the p38 inhibitor suppressed COX-2 expression (*P < 0.05) cells compared with cells not treated by the p38 inhibitor.

Specifically to tubular flow and/or FSS, our data derived from mice and CD cells demonstrate that cholesterol incorporation into cells of the CCD suppresses flow/shear-induced PGE2 release and COX-2 expression, which is expected to enhance Na absorption. We speculate that Na avidity at the CCD would enhance the chance of developing salt-sensitive high blood pressure, as in Liddle’s syndrome. However, it is of interest to note that we did not observe differences in urinary PGE2 excretion or medullary COX-2 protein expression between the control- and cholesterol-fed mice. This difference may reflect the differing effects of cholesterol integration within a heterogeneous population of nephron segments which comprise the kidney. Therefore, even though renal COX-2 expression and gene expression through a p38-dependent mechanism, IMCD3 cells were preincubated with SB203580 (a p38 inhibitor), exposed to MβC to extract cholesterol, and then immunoblotting was performed for COX-2. B: the p38 inhibitor p38-dependent expression of COX-2. A: to test whether cholesterol extraction similarly induced COX-2 expression through a p38-dependent mechanism, IMCD cells were preincubated with SB203580 (a p38 inhibitor), exposed to MβC to extract cholesterol, and then immunoblotting was performed for COX-2. B: the p38 inhibitor suppressed COX-2 expression (*P < 0.05) cells compared with cells not treated by the p38 inhibitor.

Environmental factors may be driving the high prevalence of eHTN, including high dietary Na, fat, and cholesterol intake, sedentary life style, and obesity. While the underlying mechanisms by which diet influences the development of eHTN are, in some cases, revealed, the role of cholesterol is unknown. eHTN is associated with dyslipidemia, specifically increased levels of LDL and very low-density lipoproteins (VLDL) (53), and elevated PM viscosity (36). In a prospective observational study of normotensive American Indians, a reduction in HDL and increase in LDL were highly correlated with the development of eHTN (8). Dyslipidemia and eHTN are also associated with alterations in PM biochemical composition (specifically, cholesterol incorporation) that led to a rise in the viscosity (decrease in fluidity) of the PM (53). These changes in PM biochemical and biophysical characteristics are known to alter integral membrane function, such as ion channels (24). HMG CoA reductase inhibitors (statins) do not reduce the cholesterol integrated into tissues and, thus, limit their ability to reverse the deleterious effects of cholesterol on tissue function (31). Agents such as cyclodextrins, which extract cholesterol from membranes, may be a novel treatment to address the elevated cholesterol content of tissues, like the CCDs, which may be contributing to eHTN by producing less renal PGE2, which enhances renal Na avidity and high blood pressure.

ACKNOWLEDGMENTS

We gratefully acknowledge the assistance of Lisa M. Satlin. The data were presented, in part, as a poster presentation at the 2014 American Society of Nephrology Meeting in Philadelphia, PA.

GRANTS

This work was supported by Department of Veterans Affairs Merit Review 1101BX00388 (R. Rohatgi), a Pilot and Feasibility Grant from the Hepato/Renal Fibrocystic Diseases Core Center (DK074038 to R. Rohatgi), and the Bronx Veterans Research Foundation. This work was also supported by...
National Institute of Diabetes and Digestive and Kidney Diseases Grant P30 DK079307 (Pittsburgh Center for Kidney Research, Core B).

DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS
Author contributions: Y.L., D.F., and R.C.-G. performed experiments; Y.L. and R.R. analyzed data; R.R. provided conception and design of research; R.R. interpreted results of experiments; R.R. prepared figures; R.R. drafted manuscript; R.R. edited and revised manuscript; R.R. approved final version of manuscript.

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