Introduction

There are more than 40 million people in the US alone with hypertension, and of these, the majority have salt-sensitive hypertension. In addition, at least a quarter of normotensive individuals also show salt sensitivity (1). Although the etiology of salt-sensitive hypertension is undoubtedly multifactorial, there is experimental and epidemiologic evidence linking abnormalities in the cyclooxygenase/prostaglandin (COX/PG) system to its pathogenesis. COX is the rate-limiting enzyme in metabolizing arachidonic acid to PGG2 and subsequently to PGH2, which serves as the precursor for subsequent metabolism by PG and thromboxane synthases. Prostanoid cellular responses are mediated by specific membrane–associated G-protein–coupled receptors. Receptor affinity for the prostanoids is in the nanomolar range, and prostanoids act locally associated G-protein–coupled receptors. Receptor affinity for the prostanoids is in the nanomolar range, and prostanoids act locally.

Two isoforms of COX exist in mammals, “constitutive” COX-1 and “inducible” COX-2. Both nonselective COX inhibitors (NSAIDs) and selective COX-2 inhibitors (coxibs) can elevate blood pressure (BP) and antagonize the BP-lowering effect of antihypertensive medication in many users (9). NSAIDs and COX-2 inhibitors can also induce peripheral edema (10, 11). A COX-2 polymorphism that reduces enzymatic activity has been associated with increased risk of stroke in African-Americans (12). Selective inhibition of COX-2 has also been implicated in increased cardiovascular mortality, which appears to be multifactorial and may involve increases in BP and salt and water retention in addition to accelerated thrombogenesis (13, 14).

The mechanism by which COX-2 inhibition leads to development or exacerbation of hypertension has been attributed to inhibition of intrinsic renal COX-2 activity, which leads to increased sodium retention by the kidney (9). However, recent studies have indicated an important role for immune cells in mediation and exacerbation of hypertension (15–17), with increased infiltration of both macrophages and lymphocytes in target organs (vasculature and kidney). In addition, studies by Titze and coworkers have shown that the skin is an important reservoir in the body for sodium, which is thought to interact with the negatively charged glycosaminoglycan extracellular matrix (18). Skin macrophages appear to play an important role in preventing skin sodium accumulation, at least in part by promoting skin lymphangiogenesis, and macrophage depletion can predispose to development of salt-sensitive hypertension (19, 20). Macrophages express COX-2 and are a rich source of PGs, and macrophage-dependent COX-2 expression has been shown to be important for tumor- or inflammation-associated lymphangiogenesis (21). Therefore, in the current studies, we determined the role of COX-2–derived PG expression and activity in BM-derived cells in mediation of salt-sensitive hypertension.
Results

**Global deletion of Cox2 led to salt-sensitive hypertension.** Initial studies were performed in 129/SvJ mice, which are relatively resistant to the development of hypertension. However, these mice developed salt-sensitive hypertension when administered a high-salt diet plus the COX-2 inhibitor SC58236 (Figure 1A) and had increased renal macrophage infiltration (Supplemental Figure 1A; supplemental material available online with this article; doi:10.1172/JCI81550DS1). In C57BL/6 WT mice, BP was similar when the animals were fed a low-salt diet, a normal-salt diet, or a high-salt diet, consistent with the notion that C57BL/6 mice are resistant to development of salt-sensitive hypertension. In contrast, although C57BL/6 Cox2–/– mice had BPs similar to those of WT mice in response to a low-salt diet, they exhibited increased BP in response to a normal-salt diet (~20 mmHg systolic BP [SBP]) and had further increases in BP in response to a high-salt diet (~40 mmHg SBP) (Figure 1B). Cox2–/– mice also had increased renal macrophage infiltration (Supplemental Figure 1B). The increased salt sensitivity in the global Cox2–/– mice compared with WT mice treated with a COX-2 inhibitor may reflect more complete COX-2 inhibition as well as the well-described defects in postnatal nephrogenesis seen in global Cox2–/– mice (22, 23).

**COX-2 expression and activity in BM-derived cells.** Previous studies by us and others demonstrated that COX-2 is highly expressed in renal medullary interstitial cells and its expression is increased in response to high-salt intake. However, cells of monocytic lineage, tissue macrophages and dendritic cells, also reside in the renal interstitium and increase in response to hypertensive stimuli. Macrophages and dendritic cells are known to express high levels of COX-2. Therefore, we isolated renal macrophages/dendritic cells from WT mice on a high-salt diet using mouse CD11b microbeads, as we have previously described (24), and determined that they had increased mRNA levels of Cox2 but not Cox1 compared with mice on a normal-salt diet. There was also increased expression of microsomal PGE synthase–1 (mPGES-1), the enzyme primarily mediating PGE₃ synthesis from COX-2–derived PGH₃ (ref. 25 and Figure 2). In BM-derived cells from WT mice, PGE₃ was the predominant prostanoid produced (PGE₃: 101 ± 12; PGD₂: 60 ± 1; PGF₂α: 14 ± 3; PGI₂: 0.92 ± 0.28; 11δ-TXB₂: 0.26 ± 0.01 ng/mg protein). Of note, in BM-derived cells from Cox2–/– mice, the major decrease in prostanoids resulted from decreased PGE₃ production (PGE₃: 69 ± 3 [P < 0.05]; PGD₂: 62 ± 14; PGF₂α: 13 ± 3; PGI₂: 0.24 ± 0.03 [P < 0.05]; 11δ-TXB₂: 0.26 ± 0.03 ng/mg protein; n = 3 in each group).

**Hematopoietic COX-2 deficiency increased BP in response to chronic high-salt exposure.** In order to investigate the role of immune cells in COX-2–mediated regulation of BP, BM transplantation (BMT) was performed from either WT or Cox2–/– males into syngeneic animals. In initial studies, BM from males was transplanted into females in order to assess effective engraftment. In all subsequent studies, male BM was transplanted into males, and all BP studies were therefore performed on male animals. In animals on a normal-salt diet, there were no differences in serum electrolytes or hematocrit (Supplemental Table 1).
A high-salt diet increased the percentage of monocytes in blood of Cox2–/– WT BMT mice compared with WT-WT BMT mice (Figure 3A). However, peritoneal macrophages isolated from Cox2–/– WT BMT mice had decreased mRNA expression of anti-inflammatory and reparative (“M2”) phenotypic markers (Ym1, arginase-1, mannose receptor [MR, Cd206], Tgfβ) (Figure 3B). Kidneys from Cox2–/– WT BMT mice treated with high salt had increased expression of the pan-T cell marker CD3 and the macrophage marker F4/80, but had decreased expression of a marker of Tregs, forkhead box P3 (FOXP3) (Figure 4A). High-salt–treated Cox2–/– WT BMT kidneys also had decreased expression of the macrophage M2 markers MR and arginase-1 (Figure 4D), but increased expression of the M1 marker iNOS (Figure 4A) and increased mRNA expression of M1/Th1 markers/cytokines (Inos, Ccl3, Tnfα, Il1a, Il1b) (Figure 4E). Treatment with the COX-2 inhibitor SC58236 also resulted in decreased expression of macrophage M2 markers and increased iNOS and Th1 cytokines in kidneys from WT mice on a high-salt diet (Supplemental Figure 2A). Furthermore, freshly isolated peritoneal macrophages incubated with SC58236 for 24 hours had decreased expression of the M2 markers (MR, arginase-1) and increased expression of the proinflammatory (“M1”) marker TNF-α (Supplemental Figure 2B).

When mice (on either a C57BL/6 or a 129/SvJ background) were maintained on a regular diet, there were no BP differences noted between WT-WT BMT mice and Cox2–/– WT BMT mice. However, when placed on a high-salt diet for 4 to 6 weeks, there was a significant increase in BP in Cox2–/– WT BMT mice compared with WT-WT WT mice (10 to 15 mmHg, n = 6–8) (Figure 5A), in association with increased heart hypertrophy (heart weight/weight ratios: 0.00466 ± 0.00013 vs. 0.00386 ± 0.00017; P < 0.01, n = 4) (Supplemental Figure 3A), suggesting that the hematopoietic cell COX-2 plays an important role in maintenance of BP in response to chronic high-salt intake. As validation, we also monitored BP in high-salt–treated WT-WT and Cox2–/– WT mice with radiotelemetry and found significantly increased BP in the mice with Cox2 deletion in their hematopoietic cells (Figure 5B). However, we saw no differences in urinary PGE-M excretion (17.16 ± 1.56 vs. 15.74 ± 2.35 ng/mg creatinine of WT-WT BMT, n = 4).

Salt-sensitive hypertension was also noted in mice with BMT from mice with deletion of mPGES-1 (mPGES-1–/– WT BMT) (Figure 6A). WT-WT BMT mice on a high-salt diet significantly increased BP when treated with SC58236, but there was no further increase with COX-2 inhibitor treatment of Cox2–/– WT BMT mice (Figure 6B). When BM from Cox2–/– mice was transplanted into global Cox2–/– mice, they continued to demonstrate salt-sensitive hypertension, but when BM from WT mice was transplanted into Cox2–/– mice, there was partial amelioration of salt-sensitive hypertension (Figure 6C).

Deletion of EP4 in macrophages increased BP in response to chronic high-salt exposure. The PGE2 type 4 (EP4) receptor is the most highly expressed PGE2 receptor subtype in macrophages, with lower expression levels of other PGE2 receptor subtypes, EP2 and EP3 (26). We confirmed EP4 expression in the mouse macrophage cell line RAW 264.7 (Supplemental Figure 4A) and found that administration of a selective EP4 inhibitor, L-161,982, increased expression of M1 markers (Supplemental Figure 4B), while administration of PGE2 decreased expression of Inos mRNA, which was reversed by simultaneous treatment with L-161,982 (Supplemental Figure 4C). We generated mice with selective monocyte/macrophage/dendritic EP4 deletion by crossing EP4/fl/fl mice with Cd11b-Cre mice, all on a C57BL/6 background, and confirmed effective deletion in peritoneal macrophages from the Cd11b-Cre EP4/fl/fl mice (Supplemental Figure 5). Similarly to the mice with either global or selective hematopoietic deletion of Cox2, Cd11b-
**Figure 4.** Kidneys from mice with hematopoietic cell CDX-2 deficiency had higher levels of Th1 cytokines and exhibited M1 phenotypic macrophages/dendritic cells in response to high-salt intake. (A) Immunoblotting demonstrated that high-salt diet–treated Cox2−/−-WT BMT mouse kidneys had increased protein levels of F4/80 (a marker of macrophages/dendritic cells), CD3 (a marker of T cells), and INOS (a marker of M1 phenotype), but decreased protein levels of a Treg marker, FOXP3. (B-D) High-salt diet–treated Cox2−/−-WT BMT mouse kidneys had decreased mRNA levels (B) and decreased immunoreactivity (C) of MR as well as decreased protein levels of arginase-1, markers of an M2 phenotypic macrophages/dendritic cells (D). ***P < 0.001 vs. high-salt diet–treated WT-WT BMT mice.

**Figure 4 continued.** A Student’s t test was used to determine statistical differences. n = 4 in each group. Original magnification, ×250.

**Figure 5.** Total RNA was isolated from kidney samples of WT and Cox2−/−-WT BMT mice fed a high-salt diet. (A) Cox2−/−-WT BMT mouse kidneys had decreased mRNA levels of M1/Th1 markers/cytokines Il1b, Tnfα, Il1a, and Il1b. *P < 0.05; **P < 0.001 vs. high-salt diet–treated WT-WT BMT mouse kidneys.

**Figure 6.** (A) Immunoblotting demonstrated that high-salt diet–treated Cox2−/−-WT BMT mouse kidneys had increased mRNA levels of M1/Th1 markers/cytokines Il1b, Tnfα, Il1a, and Il1b. *P < 0.05; **P < 0.001 vs. high-salt diet–treated WT-WT BMT mouse kidneys. 

**Figure 7.** Immunohistochemical analysis of WT-WT and Cox2−/−-WT BMT mouse skin treated with high-salt diet for 5 hours. (A) Neutrophils (Ly-6G), M1 macrophages (CD11b+), M2 macrophages (F4/80+), and lymphatic vessels (MR+). (B) Histology and immunohistochemistry staining of WT-WT and Cox2−/−-WT BMT mouse skin treated with high-salt diet for 5 hours. (C) Immunohistochemistry staining of WT-WT and Cox2−/−-WT BMT mouse skin treated with high-salt diet for 5 hours. (D) Immunohistochemistry staining of WT-WT and Cox2−/−-WT BMT mouse skin treated with high-salt diet for 5 hours. (E) Immunohistochemistry staining of WT-WT and Cox2−/−-WT BMT mouse skin treated with high-salt diet for 5 hours.

**Figure 8.** Immunohistochemical analysis of WT-WT and Cox2−/−-WT BMT mouse skin treated with high-salt diet for 5 hours. (A) Neutrophils (Ly-6G), M1 macrophages (CD11b+), M2 macrophages (F4/80+), and lymphatic vessels (MR+). (B) Histology and immunohistochemistry staining of WT-WT and Cox2−/−-WT BMT mouse skin treated with high-salt diet for 5 hours. (C) Immunohistochemistry staining of WT-WT and Cox2−/−-WT BMT mouse skin treated with high-salt diet for 5 hours. (D) Immunohistochemistry staining of WT-WT and Cox2−/−-WT BMT mouse skin treated with high-salt diet for 5 hours. (E) Immunohistochemistry staining of WT-WT and Cox2−/−-WT BMT mouse skin treated with high-salt diet for 5 hours.

**Figure 9.** Kidneys from mice with hematopoietic cell CDX-2 deficiency had higher levels of Th1 cytokines and exhibited M1 phenotypic macrophages/dendritic cells in response to high-salt intake. (A) Immunoblotting demonstrated that high-salt diet–treated Cox2−/−-WT BMT mouse kidneys had increased protein levels of F4/80 (a marker of macrophages/dendritic cells), CD3 (a marker of T cells), and INOS (a marker of M1 phenotype), but decreased protein levels of a Treg marker, FOXP3. (B-D) High-salt diet–treated Cox2−/−-WT BMT mouse kidneys had decreased mRNA levels (B) and decreased immunoreactivity (C) of MR as well as decreased protein levels of arginase-1, markers of an M2 phenotypic macrophages/dendritic cells (D). ***P < 0.001 vs. high-salt diet–treated WT-WT BMT mice. 

**Figure 10.** Total RNA was isolated from kidney samples of WT and Cox2−/−-WT BMT mice fed a high-salt diet. (A) Cox2−/−-WT BMT mouse kidneys had decreased mRNA levels of M1/Th1 markers/cytokines Il1b, Tnfα, Il1a, and Il1b. *P < 0.05; **P < 0.001 vs. high-salt diet–treated WT-WT BMT mouse kidneys. 

**Figure 11.** (A) Cox2−/−-WT BMT mouse kidneys had increased expression of Vegfc mRNA (Figure 10). Increased medium NaCl led to upregulation of Cox2 and Vegfc mRNA expression, with the greatest proportional increase seen in M2 macrophages. Furthermore, the NaCl-treated macrophages all had increased expression of Nfat5 and Vegfc mRNA (Figure 10). Increased medium NaCl led to upregulation of Cox2 and Vegfc mRNA by 2.5 hours, while Vegfc was only increased at 5 hours (Supplemental Figure 7A). PGE2 administration increased mPGES-1 mRNA expression in isolated peritoneal macrophages from WT mice (Supplemental Figure 7B).

In preliminary studies, we determined skin sodium content in ashed skin (19) and found a marked, 20%–25%, increase in the skin from Cox2−/−-WT BMT mice compared with WT-WT BMT mice fed a high-salt diet (Supplemental Figure 8A). We also found increased skin water content (Supplemental Figure 8B). Skin from WT-WT BMT mice had increased Cox2 expression on a high-salt diet compared with normal diet (relative Cox2 mRNA levels: 0.152 ± 0.024 vs. 0.062 ± 0.010; P < 0.05, n = 4). Skin macrophages from high-salt–treated WT-WT BMT mouse expressed COX-2 (Figure 11A). Compared with high-salt–treated WT-WT BMT mice, skin macrophages from high-salt–treated Cox2−/−-WT BMT mice also had markedly decreased expression of VEGF-C (Figure 11B). High-salt–treated Cox2−/−-WT BMT mice had increased macrophages in skin compared with those of WT-WT BMT mice, but the Cox2−/−-WT BMT macrophages had decreased expression of the M2 marker MR (Figure 11C).
There were similar increases and altered polarization of skin macrophages in Cd11b-Cre EP4fl/fl and SC58236-treated EP4fl/fl mice on a high-salt diet (Figure 12A).

The skin of Cox2–/– WT BMT mice on a high-salt diet exhibited abnormal lymphangiogenesis, as indicated by a decreased number of lymphatic ducts detected by the lymphatic markers LYVE and podoplanin (Figure 13, A and B). In addition, the skin lymphatic ducts observed in the Cox2–/– WT BMT mice were markedly dilated compared with those of WT-WT BMT mice (Figure 13C). Similar decreased skin lymphatic ducts and increased ductal dilation were also observed in high-salt-treated Cd11b-Cre EP4fl/fl mice and in high-salt plus SC58236-treated EP4fl/fl mice (Figure 13, D–F).

As a functional measure of lymphatic function, tail lymphatic flow was measured by injecting a large (2,000 kDa) FITC-labeled dextran that is taken up by lymphatics but not capillaries (31, 32). Lymphatic flow was markedly decreased in high-salt–treated Cox2–/– WT BMT mice compared with those of WT-WT BMT mice (Figure 14A). Cd11b-Cre EP4fl/fl mice also had marked decreases in lymphatic flow rates compared with EP4fl/fl mice on a high-salt diet. Furthermore, chronic administration of SC58236 to high-salt–treated EP4fl/fl mice also led to decreased tail lymphatic flow (Figure 14B).

**Discussion**

COX-2 inhibitors, as well as nonselective NSAIDs, can elevate BP and exacerbate existing hypertension to an extent that may potentially increase hypertension-related morbidity (9, 33). NSAIDs and COX-2 inhibitors have also been reported to induce peripheral edema in up to 5% of the general population (10, 11). The mechanism by which COX-2 inhibition leads to development or exacerbation of salt-sensitive hypertension has been generally posited to be due primarily to inhibition of intrinsic renal COX-2 activity, since salt loading upregulates COX-2 expression in renal medulla (34, 35) and COX-2 inhibitors reduce urinary sodium excretion during the initial period of treatment when sodium loaded (36–40). In addition to intrarenal COX-2, the current studies indicated that with chronic salt loading, COX-2–generated PGs from BM-derived cells mediate BP homeostasis such that selective deletion of either COX-2 expression (Cox2–/– WT BMT) or PGE2 production (mPGES-1–/– WT BMT) in these cells predisposed to salt-sensitive hypertension. Furthermore, selective deletion of the PGE2 receptor subtype EP3 in monocytes/macrophages also led to development of salt-sensitive hypertension.

The mechanism by which deletion of PGE2 production or signaling in hematopoietic cells leads to development of hypertension appears to be multifactorial. BM-derived cells are known to be a rich source of PGs, and cells of monocytic origin express high levels of COX-2. Myeloid cell COX-2–derived PGE2 is implicated in colonic tumorigenesis (41), and macrophage-derived PGE2 is essential for promoting the M2/Th2 phenotype seen in infiltrating cells in tumors (42), an effect that is mediated by EP3 activation. EP2 is the predominant PG receptor in macrophages (26), and EP2 activation in macrophages inhibits macrophage cytokine and chemokine release (43). In the current studies, lack of COX2 or inhibition of EP2 signaling altered macrophage/dendritic cell polarization, leading to a proinflammatory or M1-like phenotype rather than an M2-like phenotype; these findings are consistent with previous studies (21, 43). The net effect was a relative increase in macrophages as well as T cells and neutrophils in the kidney, which may then promote a proinflammatory phenotype.

Macrophage infiltration of the kidney is a consistent finding in experimental models of hypertension (44). Decreased macrophage infiltration has been associated with amelioration of hypertension in some (45–47), but not all studies (48, 49). It has been...
suggested that the lack of improvement in the latter studies may be due to global deletion of all macrophages, both proinflammatory (M1) and antiinflammatory (M2) phenotypes (44).

Recent studies have indicated an important role for T cells in development of hypertension (reviewed in refs. 44 and 50) and have also indicated an important role for dendritic cell activation of T cells in this mediation of hypertension (51). COX-2–derived PGs from dendritic cells can suppress T cell activation (52). PGE2 can act directly on T cells, including CD4, CD8, and Th17 subtypes. PGE2 suppresses CD4 Th cell differentiation to a Th1 phenotype (53), and PGE2 reduces production of Th1 cytokines such as IFN-γ and IL-2, blocks cell-surface expression of cytokine receptors, and directly inhibits CD8 T cell proliferation and differentiation (54). In addition, both T cells and B cells can themselves express COX-2 (55, 56).

In the current studies, it was also noteworthy that both NCC expression and phosphorylation were increased in the kidneys of mice with alterations in macrophage/dendritic cell COX-2 expression or activity, since NCC phosphorylation indicates increased activation of the transporter (57). In preliminary studies, we also noted increased expression of epithelial sodium channel (ENaC) mRNA in high-salt diet–treated Cox2–/–WT BMT and mPGES–/–WT BMT mice (Supplemental Figure 9). Previous studies have indicated activation of distal sodium transporters in angiotensin-mediated hypertension, an effect proposed to be due at least in part to T cell activation (58, 59). Of note, previous studies by Jia et al. in global mPGES knockout mice reported relative increases in expression of NCC and ENaC in response to aldosterone-induced hypertension (60). There have not been previous studies indicating a role for PGs in direct regulation of NCC, and in the current studies, we did not determine whether the observed NCC phosphorylation was a direct effect of decreased PGE2 or was mediated by other cytokines or factors released by macrophages or T cells.

To confirm that the effects of hematopoietic COX-2 inhibition did...
not mediate renal responses to an acute salt load, we evaluated sodium and water balance with acute salt loading in Cox2−/−-WT and CD-11b-Cre EP4fl/fl mice and found no difference from control mice on sodium and water homeostasis (Supplemental Figure 10).

In addition to potential alterations of renal function, disruption of hematopoietic COX-2 expression or signaling also led to alterations in skin lymphangiogenesis. Titze and coworkers found that skin macrophages sense alterations in skin interstitial electrolyte accumulation and thereby increase local lymphangiogenesis via increased expression of the transcription factor, TonEBP (NFAT5), which is implicated in increased expression of the lymphangiogenesis promoter VEGF-C (19, 20). Macrophage depletion or inhibition of TonEBP decreased skin VEGF-C levels, inhibited skin lymphangiogenesis, and led to development of salt-sensitive hypertension (19, 20, 61).

Previous studies have demonstrated an important role for M2 macrophages and macrophage COX-2–derived PGs in lymphangiogenesis associated with tumors, inflammation, or secondary lymphedema via increased VEGF-C expression (62–66). The present studies demonstrate that treatment with a COX-2 inhibitor inhibited high-salt–mediated increases in skin VEGF-C expression, and skin macrophages from high-salt–treated Cox2−/−-WT BMT mice had decreased VEGF-C expression. Furthermore, incubating cultured macrophages with 40 mM additional NaCl increased mRNA expression for Cox2 as well as Nfat5 and Vegfc. In this regard, previous studies by Wiig et al. indicated that, with salt accumulation in the skin, there was substantially higher sodium concentration in the skin lymphatic fluid compared with plasma (61). In addition, there was a marked decrease in lymphangiogenesis in the skin of high-salt–treated mice with...
Cox2 deletion in hematopoietic cells or with EP<sub>4</sub> receptor deletion in macrophages as well as pharmacologic COX-2 inhibition in association with decreased lymphatic flow. Further studies will be required to determine the relative importance of alterations in skin versus kidney responses to chronic salt loading when hematopoietic COX-2 is inhibited.

In summary, these studies suggest that COX-2–derived PGE<sub>2</sub> in hematopoietic cells plays an important role in both kidney and skin in maintaining homeostasis in response to chronically increased dietary salt and that inhibiting COX-2 expression or activity in these cells can predispose to salt-sensitive hypertension.

Methods

Animal studies. Cox2–/– mice on a 129/BL6 background were originally generated by Dinchuk et al. (67). Heterozygous breeding pairs were obtained from Jackson Laboratory (stock 002476) and backcrossed onto a C57BL/6 background or a 129/SvJ background for 12 generations. Homozygous C57BL/6 mPGES-1–/– mice as BM donors were also purchased from Jackson Laboratory (stock 009135). EP<sub>4</sub>fl/fl mice were generated in M. Breyer’s laboratory (68), and CD11b-Cre mice with transgene integration in the Y chromosome were generated in J. Vacher’s laboratory (69); both types of mice were on a C57BL/6 background. Mice were crossed to generate EP<sub>4</sub>fl/fl mice (WT control) and Cd11b-Cre EP<sub>4</sub>fl/fl mice (mice with macrophage and dendritic cell EP<sub>4</sub> deletion). For experiments with alterations of dietary salt intake, mice were given a low-salt diet (0.02% to 0.03% NaCl, ICN Biochemicals) for 4 weeks or a normal-salt diet (1% NaCl, LabDiet) or a high-salt diet (8% NaCl, Research Diets) for 4 to 6 weeks. The COX-2 inhibitor SC58236 (a gift from Searle Monsanto) was given at a dose of 2 mg/kg by daily gastric gavage. Unless otherwise indicated, the mice were maintained on a normal chow diet.

Creation of chimeric mice. BMT was performed as previously described (70). Briefly, recipient mice were lethally irradiated with 9 Gy using a cesium γ source. BM cells were harvested from the donor femurs and tibias. Recipient mice received 5 × 10<sup>6</sup> BM cells in 0.2 ml medium through tail-vein injection. Five weeks after transplantation,
blood was sampled to determine chimerism by determination of Cox2 expression with PCR.

**BP measurement.** BP was measured in awake, chronically catheterized mice except for studies depicted in Figure 1, which utilized a tail-cuff microphonic manometer (71). For tail-cuff measurements, mice were trained for 3 consecutive days at room temperature (Monday to Wednesday) before SBP was recorded on the following 2 days (Thursday and Friday) using a tail-cuff monitor (BP-2000 BP Analysis System, Visitech Systems). SBPs recorded over 2 days were averaged and used as SBP from 1 mouse. For catheterization BP measurements, BP was recorded every minute for 1 hour, and the average of all recorded BPs was used as BP from 1 mouse. Our preliminary data indicated that BP measured by tail-cuff microphonic manometer and carotid catheterization was comparable in Cox2–/–-WT and WT-WT mice on a normal-salt diet (Supplemental Figure 11).

BP measurement using carotid catheterization was performed through Vanderbilt Mouse Metabolic Phenotyping Centers. Mice were anesthetized with 80 mg/kg of ketamine (Fort Dodge Laboratories) and 8 mg/kg of inactin (Byk Gulden) by i.p. injection and were placed on a temperature-controlled pad. After tracheostomy, phycoerythrin 10 tubing was inserted into the right carotid artery. The catheter was tunneled under the skin, exteriorized, secured at the back of the neck, filled with heparinized saline, and sealed. The catheterized mouse was housed individually, and 24 hours later, BP was determined with a Blood Pressure Analyzer (Micro-Med). In addition, in a subset of mice, BPs were monitored by radiotelemetry. Mice were anesthetized with nembutal (50 mg/kg, i.p.). Radiotelemetric catheters (PA-C10, Data Sciences International) were inserted into the left common carotid artery with the transmitter implanted s.c. Mice were housed individually. After 14 days, mice had recovered from...
Measurement of tail lymphatic flow. Measurement of lymphatic flow was carried out according to a previous report, with modifications (73). Briefly, mice were anesthetized with s.c. injection of a solution containing xylazine hydrochloride (1 mg/kg, Sigma-Aldrich) and ketamine hydrochloride (10 mg/kg, Butler Animal Health Supply) and kept on a heating pad with the tail fixed. One microliter of 25% FITC-dextran (average molecular weight: 2,000 kDa, catalog 52471, Sigma-Aldrich) was injected intradermally at the tip of the tail using a microsyringe (Hamilton). The caudal-to-rostral lymphatic flow in the tail was monitored using a Nikon AZ100M microscope with fluores-.surgery, and heart rate and BP were recorded for the duration of the study. The data from the telemetric device were collected using the Dataquest ART system, version 4.0 (Data Sciences International) by way of an RPC-1 receiver placed under the mouse cage (72).

Sodium and water balance studies with acute salt loading. Mice were housed individually to acclimate in metabolic cages for 3 days on a normal-salt diet with free access to tap water, and then a single dose of 1 mEq of NaCl was given by gavage. Urine was collected every 12 hours for 72 hours for urine volume and Na excretion. Urinary sodium excretion was determined using a flame photometer.

**Figure 13.** Mice with a deficient hematopoietic cell COX-2 pathway had abnormal skin lymphatic ducts in response to high-salt intake. (A) Ear lymphatic ducts were dilated in high-salt diet–treated Cox2−/−-WT BMT mice, as indicated by immunostaining of LYVE-1 and podoplanin, 2 lymphatic markers. Arrows indicate lymphatic vessels. Original magnification: ×400. (B and C) High-salt–treated Cox2−/−-WT BMT mice had decreased numbers of lymphatic ducts, but more dilated ducts compared with high-salt diet–treated WT-WT BMT mice. ***P < 0.001, n = 4. (D-F) Both high-salt diet–treated Cd11b-Cre EP4fl/fl and high-salt diet plus SC58236–treated EP4fl/fl mice also had reduced numbers, but more dilated lymphatic ducts compared with high-salt diet–treated EP4fl/fl mice. ***P < 0.001. n = 4. All values are shown as mean ± SEM. All P values were calculated by Student’s t test.
cent imaging mode (Vanderbilt Image Core) at ×2 magnification with time lapse for 20 minutes. The lymphatic flow rate was expressed as mm/20 minutes.

**Cell culture.** Murine macrophage RAW 264.7 cells purchased from ATCC were grown in DMEM supplemented with 4,500 mg/l glucose, 2 mM L-glutamine, 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin in 5% CO₂ and 95% air at 37°C. The cells were starved for 16 hours in medium containing 0.5% FBS, then treated for an additional 3 hours with vehicle (DMSO), EP₁, receptor antagonist, L-161,982 (20 μM dissolved in DMSO, Cayman Chemical), 10 μM PGE₂, or PGE₃, plus L-161,982, which was added 30 minutes before PGE₂. The cells were harvested for quantitative PCR (qPCR) measurements.

**Polarization of RAW 264.7 cells.** For ex vivo polarization of RAW 264.7 cells, M0 phenotype was achieved by culturing RAW 264.7 cells in DMEM for 48 hours, M1 phenotype by culturing RAW 264.7 cells in medium containing 1 μg/ml LPS for 24 hours, and M2 phenotype by culturing RAW 264.7 cells, M0 phenotype was achieved by culturing RAW 264.7 cells in medium containing IL-4 and IL-13 (10 μg/ml for each) for 48 hours (24).

**Isolation of kidney and peritoneal monocytes/macrophages/dendritic cells.** CD11b-expressing cells in kidney single-cell suspensions or cells isolated from the peritoneal cavity were enriched using mouse CD11b cells (sc-80170), and mouse anti-SKGl (sc28338) were from Santa Cruz Biotechnology Inc. Sheep anti-p-NCC (at Thr45, Thr50, and Thr55) was obtained from Hillary McAulchan/James Hastie (University of Dundee, Dundee, United Kingdom).

**RNA isolation and qRT-PCR.** Total RNA from tissues and cultured cells was isolated using TRIzol reagents (Invitrogen). Quantitative reverse-transcriptase PCR (qRT-PCR) was performed using TaqMan real-time PCR (7900HT, Applied Biosystems). The Master Mix and all gene probes were also purchased from Applied Biosystems. The probes used in the experiments included mouse S18 (Mm002601778), Cox2 (Mm00478374), Cox1 (Mm00477214), mPGES-1 (Mm00452105), Inos (Mm00440502), Cx3 (Mm00441258), arginase-1 (Mm00475993), MR (Mm01329362), Tgfb (Mm00441726), Ym1 (Mm00657889), Tnfa (Mm99999068), Il1a (Mm00439621), Il1b (Mm00434228), Vegf (Mm00437313), podoplanin (Mm00497257), NFAT5 (Mm00497257), EP1 (Mm00443097), EP2 (Mm00436051), EP3 (Mm01316856), EP4 (Mm00436053), Il6 (Mm00446190), Enaca (Mm00803386), Enacb (Mm00441215), ENACc (Mm00441228), and NCC (Mm00490213).

**Immunofluorescence/immunohistochemistry staining and quantitative image analysis.** The animals were anesthetized with Nembutal (70 mg/kg, i.p.) and given heparin (1,000 units/kg, i.p.) to minimize coagulation. One kidney was removed for immunoblotting and qRT-PCR, and the animal was perfused with FPAS (3.7% formaldehyde, 10 mM sodium m-periodate, 40 mM phosphate buffer, and 1% acetic acid) through the aortic trunk cannulated by means of the left ventricle. The fixed kidney and skin were dehydrated through a graded series of ethanol, embedded in paraffin, sectioned (4 μm), and mounted on glass slides. Immunostaining was carried out as in previous reports (74). For both immunofluorescent and immunohistochemical staining of p-NCC, antigen retrieval was achieved by boiling in citric acid buffer (100 mM, pH 6.0) for 3 × 5 minutes. For F4/80 immunofluorescent staining, antigen retrieval was achieved by incubating in trypsin solu-
tion for 15 minutes (T-7186, Sigma-Aldrich). For double-immunofluorescent staining of F4/80 with COX-2, MR, or VEGF-C, deparaffinized sections were blocked with 10% normal goat serum plus 2% BSA for 1 hour and then incubated with rat anti-F4/80 antibodies overnight at 4°C. After washing with PBS, the section was incubated with anti-rat biotinylated IgG for 1 hour, washed with PBS, and then incubated with FITC-streptavidin. After thorough washing with PBS, the section was incubated with second primary antibodies (rabbit anti–COX-2, anti-VEGF-C, or anti-MR) for 1.5 hours at room temperature, washed with PBS, and then incubated with Cy3 anti-rabbit IgG. VECTASCREEN mounting medium with DAPI was used for nuclear staining (H-1200, Vector Laboratories). Sections were viewed and imaged with a Nikon TE300 fluorescence microscope and SPOT-cam digital camera (Diagnostic Instruments). On the basis of the distinctive density and color of immunostaining in video images, the number, size, and position of stained area were quantified by using the BIOQUANT True-Color Windows System (R & M Biometrics). Four representative fields from each animal were quantified at ×160 magnification, and their average was used as data from 1 animal sample.

**Immunoblotting.** Cultured cells were lysed and kidneys were homogenized with buffer containing 10 mM Tris-HCl (pH 7.4), 50 mM NaCl, 2 mM EGTA, 2 mM EDTA, 0.5% Na2VO4, 100 mM NaF, 0.5% sodium deoxycholate, 10 mM sodium pyrophosphate, 1 mM PMSF, 10 µl/g ml aprotinin, and 10 µg/ml leupeptin. The homogenate was centrifuged at 15,000 g for 20 minutes at 4°C. An aliquot of supernatant was taken for protein measurement. This work was supported by funds from the Department of Veterans Affairs (to R.C. Harris) and NIH grants CA122620, DK38226, DK62794, and DK95785. R.C. Harris and Ming-Zhi Zhang receive NIH–National Institute of Diabetes and Digestive and Kidney Diseases funding.

**Acknowledgments**

We would like to thank Jens Titze and David Harrison for helpful discussions (Vanderbilt University), Jean Vacher for provision of CD11b-Cre mice (Clinical Research Institute of Montreal, Montreal, Quebec, Canada), and the Vanderbilt Clinical/Translational Research Center for help in telemetry measurements.

**Address correspondence to:** Raymond C. Harris, Division of Nephrology, C3121 MCN, Vanderbilt University School of Medicine, and Nashville Veterans Affairs Hospital, Nashville, Tennessee 37232, USA. Phone: 615.322.2150; E-mail: ray.harris@vanderbilt.edu. Or to: Ming-Zhi Zhang, S-3206 MCN, Vanderbilt University Medical Center, Nashville, Tennessee 37232, USA. Phone: 615.343.1548; Fax: 615.343.2675; E-mail: ming-zhi.zhang@vanderbilt.edu.

---


