Salt Restriction Leads to Activation of Adult Renal Mesenchymal Stromal Cell–Like Cells via Prostaglandin E2 and E-Prostanoid Receptor 4

Yanqiang Yang, Jose A. Gomez, Marcela Herrera, Romelia Perez-Marco, Peter Repenning, Zhiping Zhang, Alan Payne, Richard E. Pratt, Beverly Koller, William H. Beierwaltes, Thomas Coffman, Maria Mirotsou, Victor J. Dzau

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Abstract—Despite the importance of juxtaglomerular cell recruitment in the pathophysiology of cardiovascular diseases, the mechanisms that underlie renin production under conditions of chronic stimulation remain elusive. We have previously shown that CD44+ mesenchymal-like cells (CD44+ cells) exist in the adult kidney. Under chronic sodium deprivation, these cells are recruited to the juxtaglomerular area and differentiate to new renin-expressing cells. Given the proximity of macula densa to the juxtaglomerular area and the importance of macula densa released prostanoids in renin synthesis and release, we hypothesized that chronic sodium deprivation induces macula densa release of prostanoids, stimulating renal CD44+ cell activation and differentiation. CD44+ cells were isolated from adult kidneys and cocultured with the macula densa cell line, MMD1, in normal or low-sodium medium. Low sodium stimulated prostaglandin E2 production by MMD1 and induced migration of CD44+ cells. These effects were inhibited by addition of a cyclooxygenase 2 inhibitor (NS398) or an E-prostanoid receptor 4 antagonist (AH23848) to MMD1 or CD44+ cells, respectively. Addition of prostaglandin E2 to CD44+ cells increased cell migration and induced renin expression. In vivo activation of renal CD44+ cells during juxtaglomerular recruitment was attenuated in wild-type mice subjected to salt restriction in the presence of cyclooxygenase 2 inhibitor rofecoxib. Similar results were observed in E-prostanoid receptor 4 knockout mice subjected to salt restriction. These results show that the prostaglandin E2/E-prostanoid receptor 4 pathway plays a key role in the activation of renal CD44+ mesenchymal stromal cell–like cells during conditions of juxtaglomerular recruitment; highlighting the importance of this pathway as a key regulatory mechanism of juxtaglomerular recruitment. (Hypertension. 2015;65:1047-1054. DOI: 10.1161/HYPERTENSIONAHA.114.04611.) • Online Data Supplement

Key Words: cyclooxygenase 2 • mesenchymal stem cell • sodium

The renin–angiotensin aldosterone system plays a key physiological role in the regulation of blood pressure, electrolyte homeostasis, and kidney development. Renin is an aspartyl protease that catalyzes the first and rate-limiting step in the activation of the renin–angiotensin system. Factors regulating renin expression have the potential to significantly impact overall renin–angiotensin system activity and potentially can lead to novel therapeutic targets for renin–angiotensin system and cardiovascular/renal disease.

The primary source of renin in the circulation is the kidney. In the adult kidney, renin expression is restricted to the terminal portion of the afferent arterioles in the juxtaglomerular area and is expressed in specialized cells termed juxtaglomerular cells. Despite this restricted expression of renin in the adult kidney, environmental stimuli, such as chronic ischemia, prolonged adrenergic activation, volume, and sodium depletion, produce an increase of the number of cells expressing renin along the afferent arteriole, in the interstitium and inside the glomerulus, in a pattern partially recapitulating embryonic distribution of renin expression.4,5 This process, known as juxtaglomerular recruitment, involves re-expression of renin in renal smooth muscle cells (vascular smooth muscle cells) along the arteriole and differentiation of pericytes and adult CD44+ mesenchymal-like cell populations to renin-expressing cells.6 Despite the importance of this mechanism in regulating renin, the exact cellular processes, as well as the identities of local mediators and cellular receptors involved, are not fully understood.
The macula densa (MD) is an area of highly specialized cells at the thick ascending limb of the loop of Henle that are located adjacent to the afferent arterioles where they are in a unique position to affect changes within the juxtaglomerular area. It is well established that in response to salt restriction, MD cells secrete molecules that directly regulate renin expression and release. Among them, prostaglandin E2 (PGE2) derived from the cyclooxygenase 2 (Cox-2)/prostaglandin syn-thase cascade plays a central role. PGE2 acts via a class of EP1-4 receptors. From those, E-prostanoid receptor 4 (EP4) is of prime importance for renin regulation.

Several studies have shown that stem cell/progenitor like cells exist in the adult kidney and that these cells are activated under conditions of injury of physiological stress. Our laboratory has recently shown that CD44+ mesenchymal-like cells (CD44+ cells) exist in the adult kidney. Under conditions of salt restriction, these cells are activated, accumulate in the juxtaglomerular area, and differentiate to renin-expressing cells. Here, we tested the hypothesis that MD-derived prostanoids by stimulating the receptors on renal CD44+ cells are crucial for their activation and differentiation to renin-expressing cells under conditions of salt restriction.

Methods

Reagents

PGE2, EP4 receptor antagonist (AH23848), prostaglandin I2 (PGI2), and prostacyclin receptor antagonist (CAY10441) were purchased from Cayman Chemical; Forskolin, 1-methyl-3-isobutylxanthine, N-[2-(cyclhexyloxy)]-4-nitrophenyl]-methanesulfonamide (NS398), and lipopolysaccharide from Sigma. Cell DMEM was purchased from Sigma and the MesenCult Proliferation Kit from Stem cell technology. PGE2, EP4 receptor antagonist (AH23848), prostaglandin I2 (PGI2), and prostacyclin receptor antagonist (CAY10441) were purchased from Cayman Chemical; Forskolin, 1-methyl-3-isobutylxanthine, N-[2-(cyclhexyloxy)]-4-nitrophenyl]-methanesulfonamide (NS398), and lipopolysaccharide from Sigma. Cell DMEM was purchased from Sigma and the MesenCult Proliferation Kit from Stem cell Technology.

MMDD1 Cells

MMDD1, a renal epithelial cell line with properties of MD cells (eg, expression of Cox-2 and NckC2), was kindly supplied by Dr J. Schnermann (National Institutes of Health). The cells were routinely maintained in DMEM/nutrient mixture Ham's F-12, supplemented with 10% fetal bovine serum, penicillin (100 U/mL), and streptomycin (100 mg/mL), incubated at 37°C in the presence of 5% CO2. The medium was changed every 2 days. Where necessary, MMDD1 cells were exposed to a reduced extracellular NaCl concentration. Here, confluent MMDD1 monolayers were exposed to serum-free DMEM in a 1:1 mixture with isotonic saline (control) and 300 mmol/L mannitol to reduce NaCl concentration to one half as described in Yang et al.

Isolation and Culture of Renal Progenitor Cells

Renal CD44+ mesenchymal stromal cells (MSCs) were isolated from mice kidneys as described. Briefly, kidneys were perfused in vivo with saline and then harvested, minced, and digested with 0.1% collagenase type I for 30 minutes at 37°C. The cell suspensions were washed and filtered through 70-μm and 40-μm mesh filters, and residual red blood cells removed by treatment with cold ammonium–chloride–potassium buffer (0.15 mol/L potassium-ammonium chloride). CD44+ cells were isolated by 2 cycles of fluorescence-activated cell sorting via specific gates. Dead cells were excluded with 7-aminoactinomycin D, and doublets were excluded on the basis of 3 hierarchical gates (forward/side scatter area, forward scatter height/width, and side scatter height/width). Renal CD44+ cells collected by fluorescence-activated cell sorter were cultured in growth medium MesenCult Proliferation Kit (stem cell technology) at 37°C in the presence of 5% CO2. Medium was changed every 2 to 3 days. Cells were used for experiments during passages 3 to 5.

RT-PCR and Quantitative RT-PCR

The mRNA levels of all the genes checked in this study were quantified by reverse transcriptase polymerase chain reaction (RT-PCR) and quantitative RT-PCR. Total RNA was isolated from tissues or cells using Trizol reagent according to manufacturer’s recommendations (Invitrogen). First strand cDNA was synthesized from 2 μg of total renal RNA using the Omniscript RT kit (Qiagen) and oligo-dT as the primer. Two microliters per reaction of cDNAs were used as the template for real-time PCR amplification. Quantitative RT-PCR was performed using ABI Prism 7700 Applied Biosystems Sequence Detection System and SYBR Green PCR kit (Qiagen) or TaqMan probe set and TaqMan PCR kit (Applied Biosystems).

In Vitro Cell Differentiation

The differentiation assay was performed as described. Briefly, 8-bromoadenosine 3’, 5’-cyclic monophosphate cAMP (1 mmol/L), 3-isobutyl-1-methylxanthine (0.1 mmol/L), or vehicle control (dimethyl sulfoxide) was added to culture media daily during the treatment period. In differentiated C57BL/6 Ren1c–yellow fluorescent protein (YFP) renal CD44+ cells, the renin expression was determined by fluorescence microscopy, using YFP expression as a surrogate for renin expression.

Immunofluorescence or Immunohistochemical Staining

Immunohistochemistry of kidney sections (5 μm thick) was performed using standard procedures. Kidney tissue sections were fixed in 4% paraformaldehyde and permeabilized with 0.2% Triton X-100. After blocking with 5% serum/PBS for 1 hour, sections were incubated with primary antibodies diluted in 5% serum/PBS overnight at 4°C. Slides subsequently were washed in PBS and incubated with secondary fluorochrome-conjugated antibodies for 45 minutes. The following primary antibodies were used: anti-CD44 (immunohistochemistry: BioLegend, no. 103001, 1/50 dilution, immunofluorescence: Abcam no. ab6124, 1/100 dilution), sheep antirenin (immunohistochemistry: Innovative Res 1206, 1/100 dilution), or rabbit antirenin (immunofluorescence: 1/100/00 dilution, kindly provided by Dr Tedashi Inagami, Vanderbilt University). The following secondary antibodies were used at a 1:500 dilution for 45 minutes to 1 hour at room temperature: Alexa 488 goat anti-rabbit IgG (A-11008), Alexa 594 goat anti-rabbit IgG (A-11012), Alexa 594 goat anti-rat IgG (A-11007), Alexa 633 donkey anti-sheep IgG (A-21100). Secondary antibodies were purchased from Invitrogen. Nuclei were counterstained with 4',6-diamidino-2-phenylindole. Kidneys were embedded into optimal cutting temperature compound, coronal sectioned, and 5 micron slices cut. Confocal images were taken in the cortex and acquired with a LSM 510 Meta DuoScan microscope (Zeiss) and processed using LSM 5 software, version 4.2. Images were acquired and analyzed by a blinded investigator.

Cell Migration Assay

Migration of CD44+ cells was assessed using 24-well plates with Transwell inserts (8.0-μm pore Costar), as described. MDMDD1 cells were set up for migration assays in serum-free normal salt DMEM media supplemented 1× Penicillin-Streptomycin. Renal CD44+ MSCs were pretreated with various inhibitors as indicated.

Immunoblotting

Immunoblotting was performed as described. Protein lysates were prepared with radioimmunoprecipitation assay buffer (50 mmol/L Tris pH 7.8, 150 mmol/L NaCl, 0.5% Na deoxycholate, 1% Triton...
X-100) containing protease inhibitors (Roche). Denatured proteins (20–40 μg) were separated by SDS-PAGE and transferred onto polyvinylidene fluoride. Blocking and antibody incubation were performed with 5% nonfat dry milk in TBS-Tween (62.5 mmol/L Tris pH 7.5, 150 mmol/L NaCl, 0.1% Tween-20). Rabbit anti-murine polyclonal antibody to EP4 was used at a dilution of 1:1000. Visualization of the bands was performed with a goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (Cell Signaling; 1:1000) and ECL-Prime (GE Healthcare).

**Animals Studies**

Male C57BL/6 wild-type mice 6 to 8 weeks of age (Charles River Laboratories), male C57BL/6 Ren1c-YFP mice 6 to 8 weeks of age (kindly provided by Dr Gomez at University of Virginia Medical Center), and 6 to 8 week recombinant inbred background EP4−/− and their iso- genetic controls (kindly provided by Dr Coffman at Duke University and Dr Beverly Keller at University of North Carolina) were used for the mouse studies. The generation and maintenance of EP4−/− mice have been reported previously.16 On a C57BL/6 background, EP4−/− die 62.5 mg/kg and furosemide in drinking water for 10 days. Controls were identified at weaning and cohoused for the duration of the experiment. All the animals were maintained on a 12-hour light/12-hour dark cycle at an ambient temperature of 24°C and 60% humidity. Food and water were provided as indicated in each experiment. For low-salt diet plus Cox-2 inhibitor administration, animals received normal chow (0.4% NaCl). All animal procedures were approved by the Institutional Animal Care and Use Committees at Duke University. Male C57BL/6 wild-type mice 6 to 8 weeks of age (Charles River Laboratories), male C57BL/6 Ren1c-YFP mice 6 to 8 weeks of age (Charles River Laboratories), male C57BL/6 Ren1c-YFP mice 6 to 8 weeks of age (kindly provided by Dr Gomez at University of Virginia Medical Center), and 6 to 8 week recombinant inbred background EP4−/− and their iso- genetic controls (kindly provided by Dr Coffman at Duke University and Dr Beverly Keller at University of North Carolina) were used for the mouse studies. The generation and maintenance of EP4−/− mice have been reported previously.16 On a C57BL/6 background, EP4−/− die postnaturally as a result of patent ductus arteriosus. Therefore, a recombinant inbred strain was generated by 35 generations of successive intercross of EP4−/− mice of mixed genetic background composed of 129P2C57BL/6 and DBA/2 alleles. EP4−/− and their congenic wild-type controls were identified at weaning and cohoused for the duration of the experiment. All the animals were maintained on a 12-hour light/12-hour dark cycle at an ambient temperature of 24°C and 60% humidity. Food and water were provided as indicated in each experiment. For low-salt diet plus furosemide administration (in drinking water, 2.28 mmol/L), animals were placed on a low-salt diet (0.02% NaCl) for 10 days. Control animals received normal chow (0.4% NaCl). All animal procedures were approved by the Institutional Animal Care and Use Committees at Duke University. For low-salt diet plus Cox-2 inhibitor administration, animals were placed on a low-sodium diet (0.02% NaCl) with Cox-2 inhibitor 62.5 mg/kg and furosemide in drinking water for 10 days.

**Results**

**PGE2 Promotes CD44+ MSC Migration and Renin Expression**

To investigate the effects of prostanoids on adult renal mesenchymal-like cells, we treated renal CD44+ mesenchymal-like cells (CD44+ cells) directly with PGE2 or PGI2 and tested effects on cell proliferation, migration, and differentiation to renin-expressing cells. CD44+ cells were isolated from kidneys of adult male C57BL/6 wild-type mice by fluorescence-activated cell sorting, and cells were passaged 3× to 5× before use. To study the effects of PGE2 on migration, we performed transwell migration assays using a Boyden chamber. Renal CD44+ cells were seeded on the upper layer of the cell permeable membrane and treated overnight with different concentrations of PGE2 or PGI2 (1–1000 nmol/L). Twenty-four hours later, the number of cells which had migrated through the membrane to the bottom well was quantified. Treatment with PGE2, but not PGI2, resulted in significantly increased migration of CD44+ cells (Figure 1A). PGE2 did not affect CD44+ cell proliferation at the concentrations tested (Figure 1B). CD44+ cells isolated from Ren1c-YFP were treated with PGE2 for 10 days and then examined under immunofluorescence microscopy for YFP expression. We found that prolonged treatment of CD44+ cells with high doses of PGE2 (10 nmol/L) induced renin expression (Figure 1C). No YFP was detected in vehicle-treated cells. YFP and renin were found to coexpress in the same cells validating the YFP model (Figure S1A in the online-only Data Supplement). Moreover, PGE2 increased renin expression in wild-type CD44+ cells (Figure S1B). Collectively, these data demonstrate that PGE2 enhances renal CD44+ cell migration and differentiation to renin-expressing cells.

**PGE2 Promotes CD44+ MSC Migration and Renin Expression via the EP4 Receptor**

The above results suggested that PGE2, but not PGI2, induces migration and differentiation of CD44+ cells. Because PGE2 has been shown to stimulate renin via activation of E-Prostanoid receptor 2 and 4 (EP2 and EP4),19 we first examined the expression of these genes in renal CD44+ cells. CD44+ cells were isolated from kidneys of adult male C57BL/6 wild-type mice by fluorescence-activated cell sorter and mRNA was isolated. As a control, mRNA was isolated from vehicle or lipopolysaccharide-treated J774 macrophage cell line. CD44+ cells were found to express the EP4 but not the EP2 receptor as determined by RT-PCR (Figure 2A). This result was subsequently validated by quantitative RT-PCR (Figure 2B and 2C), and expression of EP4 protein was confirmed by immunoblotting (Figure 2D). EP2 expression was either very low or not detectable in CD44+ and CD44− cells (Figure 2A–2C).
To determine the importance of EP4 receptor in CD44+ cell biology, we pretreated renal CD44+ cells with an EP4 antagonist before PGE2 treatment. Migration was examined using the transwell Boyden chamber. Similar to the data in Figure 1, PGE2 induced a 2-fold increase in migration while the EP4 antagonist AH23848 significantly attenuated PGE2-induced renal CD44+ cell migration (Figure 3A). Moreover, when renal CD44+ cells were isolated from mice lacking the EP4 receptor, the effect of PGE2 on CD44+ cell migration was abrogated (Figure 3B). Similarly, pretreatment of the CD44+ cells with AH23848 abolished the effects of PGE2 in renin induction of these cells (Figure 3C and 3D). The results show that PGE2 induces renal CD44+ cell migration and differentiation to renin-expressing cells through the EP4 receptor.

**MD-Derived PGE2 Activates Adult Renal CD44+ In Vitro via the EP4 Receptor**

The MD is an important source of PGE2 and plays a major role in the regulation of juxtaglomerular renin expression and
cells with MMDD1 cells exposed to normal salt conditions. Migration assays compared with coculture of renal CD44+ cells to low-salt conditions and increased CD44+ cell migration. The coculture was conducted in normal salt DMEM without serum. CD44+ cells were allowed to migrate through the filters at 37°C overnight. AH23848 (E-prostanoid receptor 4 antagonist) 10 μmol/L were added where appropriate (N=3). Comparisons made to the vehicle control in normal sodium media *P<0.05. ns indicates not significant.

Figure 4. Cyclooxygenase 2 (Cox-2) inhibition abrogates macula densa (MD) cell–induced CD44+ cell migration in vitro. MMDD1 cells (25×10⁴ cells/well) were seeded in a 24-well plate and cultured overnight in normal or low sodium medium (to induce Cox-2 expression) in the presence or absence of Cox-2 inhibitor NS398 10 μmol/L. Low-salt medium was used to induce Cox-2 expression in the MMDD1 cells. Serum-starved renal CD44+ cells (1×10⁴) were plated on the transwell inserts, and inserts were placed in the wells containing the MMDD1 cells. The coculture was conducted in normal salt DMEM without serum. CD44+ cells were allowed to migrate through the filters at 37°C overnight. AH23848 (E-prostanoid receptor 4 antagonist) 10 μmol/L and CAY10441 (prostaglandin I2 receptor antagonist) 10 μmol/L were added where appropriate (N=3). Comparisons made to the vehicle control in normal sodium media *P<0.05. ns indicates not significant.

To study the macular densa–mediated effects on the CD44+ cell in vitro, we used the MMDD1 cell line. To enhance secretion of PGE2, the MMDD1 cells were incubated with DMEM medium with reduced sodium chloride (67 mmol/L) where appropriate. Serum-starved renal CD44+ MSCs were plated on transwell inserts and the inserts placed into the wells containing the MMDD1 cells. The coculture was conducted in normal salt DMEM without serum, and CD44+ cells were allowed to migrate through the filters at 37°C overnight. Overnight coculture of renal CD44+ cells with MMDD1 cells exposed to low-salt conditions and increased CD44+ cell migration by 2-fold as measured using transwell Boyden chamber migration assays compared with coculture of renal CD44+ cells with MMDD1 cells exposed to normal salt conditions (Figure 4). This effect on migration was abrogated by addition of a Cox-2 inhibitor (NS398), which blocks PGE2 production in MMDD1 cells, or by addition of the EP4 receptor antagonist AH23848 to the CD44+ cells (upper chamber; Figure 4). Treatment of the CD44+ cells with a PGI2 receptor antagonist (CAY 10441) had no effect on this migratory response (Figure 4), demonstrating that PGE2 secreted from the MMDD1 cells is the prostanoid affecting the CD44+ cell migration and confirming that these effects are mediated via the EP4 receptor.

Cox-2 Inhibition Blocked Renal CD44+ Cell Activation In Vivo After Low-Salt and Furosemide Treatment

Next, to investigate whether the Cox-2 and PGE2 mechanisms played a role in renal CD44+ renal progenitor cell activation in vivo, wild-type C57BL/6 mice were subjected to salt restriction by low-salt diet and furosemide treatment (2.28 mmol/L, drinking water) for 10 days in the presence or absence of the Cox-2 inhibitor rofecoxib (62.5 mg/kg). Low-salt diet increased plasma renin concentration (Figure S2). This increase in plasma renin concentration was significantly inhibited by rofecoxib (Figure S1). Kidneys were harvested and processed for immunofluorescent microscopy using CD44 and renin-specific antibodies. As anticipated from previous studies, salt restriction resulted in increased renin expression and juxtaglomerular cell number, as well as an increase in the number of CD44+ cells (Figure 5A, with quantification provided in Figure 5B and 5C). However, in rofecoxib-treated mice, both renin expression and the number of CD44+ cells were significantly attenuated (Figure 5A, with quantification provided in Figure 5B and 5C). These results suggest that PGE2 is important for the activation of renal CD44+ cells during salt restriction–induced juxtaglomerular cell recruitment.

Discussion

Several studies have shown that stem cell/progenitor cells exist in the adult kidney and are activated after sustained stress or injury. Our laboratory has recently shown that adult renal CD44+ MSC-like cells act as renin progenitor cells. These CD44+ cells are activated during conditions of sodium constriction and contribute to the increased renin response by accumulating in the juxtaglomerular area and differentiating to renin-producing cells.

In the current study, we extend these findings and illustrate that adult renal CD44+ cells express EP4 receptors and that coculture of MMDD1 cells with adult renal CD44+ cells stimulate migration of CD44+ cells; the effect was attenuated with treatment of MMDD1 cells with Cox-2 inhibitor or treatment of the CD44+ cells with an EP4 antagonist. Direct exposure to PGE2 increased CD44+ cell migration in an EP4-dependent manner. In addition, our results indicated that treatment of CD44+ cells with higher doses of PGE2 induced renin expression in vivo. Finally, both the activation of renal CD44+ and the increase in renin expression in response to salt restriction are diminished in mice lacking the EP4 receptor.
Because CD44+ cells lose CD44 expression concurrently with an increase in renin expression, it is difficult to find CD44/renin double positive cells in vivo; however, previous lineage tracing studies have shown that the CD44+ cells are a precursor to the renin+ cells. Thus, taken together, our data support the novel concept that renal stem/progenitor cells contribute to the process of juxtaglomerular recruitment and suggest that MD, via the secretion of PGE2 and activation of the EP4 receptor on the renal progenitor cells, are involved in this process.

It is well established that MD-derived prostaglandin E2 (PGE2) is one of the principal mediators of local control of renin release in the kidney. According to the classic paradigm, MD cells sense salt and other biochemical signals that induce the production of PGE2. PGE2 acts on EP2 or EP4 receptors in juxtaglomerular cells and stimulates renin release; although more recent evidence supports the notion
that EP4 is the receptor responsible in mediating the PGE2-induced renin release under conditions of salt restriction in vivo.13 Our data suggest that the MD/PGE2/EP4 axis plays a broader role in renin regulation by promoting renin expression in CD44+ cells in the adult kidney. PGE2 has recently gained much attention as a mediator of in vitro and in vivo migration and differentiation of hematopoietic stem cells, endothelial progenitor cells, and mesenchymal stem cells.21–23 Our studies provide additional insights into the role of PGE2 in stem cell biology and, to our knowledge, provide the first such evidence for PGE2 affecting the fate of renal stem cells. Future experiments to discern how a PGE2 dose gradient affects renin expression during salt restriction in adult or during renal development, as well as elucidating the downstream pathways involved, would be of high interest. For example, Len Zon and colleagues have demonstrated that PGE2 affects hematopoietic stem cell biology by the cAMP-dependent activation of the Wnt signaling pathway.23 Moreover, cAMP is a known regulator of transcription, and an examination of the transcription factors and transcripts regulated by cAMP in CD44+ cells after exposure to PGE2 may identify targets for future study.

Although our studies highlight the role of MD/PGE2/EP4 axis, we cannot exclude that other molecules or receptors such as PGI2, nitric oxide, or prostacyclin receptors play a role in vivo10 and might be involved in the CD44+ cell response during salt restriction. Although PGE2 treatment did not affect CD44+ cell proliferation in vitro, Cox-2 inhibition was sufficient to abrogate the CD44+ cell expansion after low-sodium furosemide treatment in vivo. Similar results were obtained from with EP4 receptor knockout mice. Similarly, we cannot exclude that the MD/PGE2/EP4 mechanisms affect other cellular processes involved in juxtaglomerular recruitment. Juxtaglomerular recruitment involves several mechanisms such as proliferation of the existing renin-expressing juxtaglomerular cells, induction of renin expression in vascular smooth muscle cells of the afferent arteriole,24 and transdifferentiation of pericytes.1 Studying the effects of MD/PGE2/EP4 on these pathways would be of great interest.

**Perspectives**

Our data show the activation of renal stem/progenitor cells in response to salt restriction requires PGE2/EP4. These studies provide new insights about role of renal stem cells in renal physiology and diseases, as well as advancing our understanding regarding juxtaglomerular recruitment and regulation of the renin–angiotensin system.

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**Disclosures**

None.

**References**


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### Novelty and Significance

**What Is New?**

- Prostaglandin E2 (PGE2) released by a macula densa cell line under conditions of low sodium induced CD44+ cell migration, an effect inhibited by an E-prostanoid receptor 4 (EP4) antagonist.

- In vivo, genetic ablation of EP4 or inhibition of cyclooxygenase 2 by rofecoxib attenuated the ability of low sodium to induce both renal CD44+ cell activation during juxtaglomerular recruitment and the production of renin.

**What Is Relevant?**

- Juxtaglomerular cell recruitment is important in the pathophysiology of hypertension; however, mechanisms that underlie renin production under conditions of chronic stimulation are relatively unknown.

**Summary**

The PGE2/EP4 pathway is a key regulatory mechanism of juxtaglomerular recruitment.

- We have previously shown that CD44+ mesenchymal-like cells exist in the adult kidney. Under chronic sodium deprivation, these cells are recruited to the juxtaglomerular area where they differentiate into new renin-expressing cells.
Salt Restriction Leads to Activation of Adult Renal Mesenchymal Stromal Cell–Like Cells via Prostaglandin E2 and E-Prostanoid Receptor 4

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**Supplementary Methods and Figures**

**SALT RESTRICTION LEADS TO ACTIVATION OF ADULT RENAL MSC-LIKE CELLS VIA PGE2 AND EP4 RECEPTOR**

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**Supplementary Methods**

**Plasma renin concentration.** To determine plasma renin concentration, blood samples were obtained from mice using EGTA as an anticoagulant and assayed for Angiotensinogen I generation as previously described ¹. From each sample, 2 µl of plasma was added to an incubation cocktail containing 158 µl of modified phosphate incubation buffer (0.1 M phosphate buffer, 0.06 M disodium EDTA, 0.2% Gelatin, pH 6.5), 2 µl of PMSF (phenyl methyl sulfonyl fluoride), and 138 µl substrate (containing 250 ng angiotensinogen, obtained from nephrectomized rats). Substrate consumption had to be less than 15% of the total added to insure first-order kinetics of the enzymatic reaction. This sample was incubated at 37°C for 90 minutes, boiled for 10 min to stop the reaction, centrifuged for 10 min at 2500g, and the supernatant removed and frozen for later analysis (typically in less than 48 hours). Angiotensin I generation was analyzed by radioimmunoassay using a Gamma Coat PRA kit (DiaSorin, Stillwater, MN) according to the manufacturer’s instructions. The assay was controlled using buffer blanks and internal quality controls.
Supplementary References


Supplementary Figures
Figure S1. PGE2 promotes renin expression in CD44+ cells.

(A) Renal CD44+ cells isolated from Ren1c-YFP were treated with PGE2 (10 μM) for 10 days, and then examined under immunofluorescence microscopy for YFP and renin expression. Scale bar 100 microns.

(B) Renal CD44+ cells isolated from wild-type mice were treated with vehicle or PGE2 (10 μM) for 10 days, and then examined under immunofluorescence microscopy for renin expression. Scale bar 100 microns.
Figure S2. Low salt induces renin response.

Wild Type C57BL/6 mice were administered a normal salt diet with vehicle or low salt diet accompanied by furosemide treatment for 10 days with or without the Cox-2 inhibitor Rofecoxib. Plasma renin concentration was determined as previously described \(^1\). Angiotensin I generation was determined by radioimmunoassay. N=3. Comparisons made to the normal salt vehicle control, ***P<0.001.