Deletion of Microsomal Prostaglandin E Synthase-1 Increases Sensitivity to Salt Loading and Angiotensin II Infusion

Zhanjun Jia, Aihua Zhang, Hui Zhang, Zheng Dong, Tianxin Yang

Abstract—Microsomal prostaglandin E synthase-1 (mPGES-1), a membrane-associated protein, is critically involved in the inflammatory response and may also be involved in physiological processes as well. The present study examined the role of mPGES-1 in regulation of sodium balance and blood pressure in the settings of salt loading and angiotensin II infusion. mPGES-1 −/− mice developed severe and progressive hypertension associated with an inappropriate increase in sodium balance when fed a high-salt diet. These mice exhibited a significantly impaired ability to excrete an acute enteral load of NaCl. Under these 2 settings of salt loading, urinary excretion of prostaglandin E2 and nitrate/nitrite were remarkably increased in wild-type animals but not in mPGES-1 −/− mice. The changes of urinary cGMP paralleled that of urinary nitrate/nitrite. mPGES-1 −/− mice exhibited a remarkable inhibition of high salt–induced increase in gene expression of all 3 NO synthase isoforms, whereas these mice had upregulated expression of NO synthase III but not NO synthase I and NO synthase II at basal state. Chronic salt loading remarkably induced mPGES-1 protein expression exclusively in the distal nephron. In primary cultures of CD cells, mPGES-1 expression was significantly increased following exposure to hypertonic NaCl, in parallel with increased prostaglandin E2 release. These findings have revealed a mPGES-1/prostaglandin E2/NO/cGMP pathway that appears to be critically important in salt adaptation. In addition, we provide evidence that mPGES-1 deficiency sensitizes the hypertensive effect of angiotensin II. Overall, this study has characterized the natriuretic and antihypertensive role of mPGES-1 that likely contributes to blood pressure homeostasis. (Circ Res. 2006;99:1243-1251.)

Key Words: mPGES-1 ■ mean arterial pressure ■ prostaglandin E2 ■ nitric oxide ■ angiotensin II ■ collecting duct

Dietary salt is an important environmental factor involved in the pathogenesis of hypertension. A large amount of epidemiologic evidence has established a link between sodium intake and blood pressure (Bp). Genetic studies of rare Mendelian forms of hypertension have identified mutations or polymorphisms of genes, almost all of which are related to renal sodium handling. It is most likely that abnormal renal sodium handling underlies the pathogenesis of essential hypertension. Prostaglandins (PGs) are the products of cyclooxygenase-mediated metabolism of arachidonic acid and are known to be involved in the control of renal sodium reabsorption and Bp, especially under physiological stress. The physiological function of PGs is highlighted by the deleterious side effects of nonsteroidal antiinflammatory drugs, and recently by the selective cyclooxygenase (COX)-2 inhibitors (coxibs). Further investigation of this subject may provide knowledge essential for development of safer antiinflammatory drugs and also may lead to a better understanding of PG-dependent regulation of Bp.

Among the PGs, PGE2 exists in a wide variety of cells and tissues and plays important roles in various physiological functions. PGE2 is an important autocrine/paracrine factor that contributes to sodium balance and Bp control through mechanisms that primarily involve the regulation of renal excretory function and vasculature tone. In isolated perfused thick ascending limbs and collecting ducts, PGE2 can inhibit NaCl transport directly, suggesting natriuretic properties of this PG. In the vasculature, PGE2 is considered to be involved in buffering the vasoconstrictive action of angiotensin II (Ang II).

Prostaglandin E2 synthase (PGES) terminally converts COX-derived PGH2 into PGE2. Five mammalian PGES have been cloned and are referred to as microsomal PGES (mPGES-1), mPGES-2, GSTM2-2, GSTM3-3, cytosolic PGES (cPGES). Among these isoforms, mPGES-1 has emerged as a novel PG synthase critically important for inflammation and is considered to act downstream of COX-2. mPGES-1 has been found in distinct renal cell types,
including the macula densa, collecting duct (CD), and renal medullary interstitial cells, and also has been found in the vascular cells, suggesting a potential role of mPGES-1 in regulation of renal and vascular functions. Therefore, the major aims of the present study were to: (1) characterize the phenotype of mPGES-1 knockout (KO) mice with respect to alteration of salt sensitivity and BP; (2) study the regulatory phenotype of mPGES-1 knockout (KO) mice with respect to major aims of the present study were to: (1) characterize the Na, PGE2, and nitrate/nitrite.

every 12 hours for 72 hours for determination of concentrations of hypertonic solution was administered by gavage. Urine was collected for 24 hours, and 24-hour BP measurements were performed. Measurements of daily food intake and 24-hour urine collections were performed to determine sodium balance.

Telemetric Recordings of BP and Chronic High-Salt Loading
The telemetric device was implanted into male mice (3 to 4 months of age) through catheterization of the carotid artery. Briefly, under general anesthesia, a catheter was placed in the carotid artery, and the transmitter body that connected to the catheter was placed under the skin in the ventral abdominal area, according to the instructions of the manufacturer (model no. TA11PA-C20; DSI, St Paul, Minn.). Animals were allowed to recover from surgery and to habituate to metabolic cages and gelled diet for 1 week. After the acclimatization, the telemetry probe was turned on and animals were placed on a normal-salt diet for 2 days, followed by a high-salt diet (3.2% Na) plus saline as drinking fluid for 1 week. The animals moved freely, and 24-hour BP measurements were performed. Measurements of daily food intake and 24-hour urine collections were performed to determine sodium balance.

Acute Salt-Loading Experiments
Male mPGES-1 +/+ and mPGES-1 −/− mice (3 to 4 months of age) were allowed to habituate to metabolic cages for 3 days. Animals were fed a normal-salt diet with free access to tap water for the entire experimental period. A single dose of 1 mEq of Na+ in the hypertonic solution was administered by gavage. Urine was collected every 12 hours for 72 hours for determination of concentrations of Na, PGE2, and nitrate/nitrite.

Immunohistochemistry
Kidneys from the normal salt– and high-salt–treated mice were fixed in 4% paraformaldehyde and embedded in paraffin. Kidneys from the normal salt– and high salt–treated mice were fixed with 3% paraformaldehyde and embedded in paraffin. Kidney sections (4-μm thickness) were incubated in 3% H2O2 for 10 minutes at room temperature to block endogenous peroxidase activity. The slides were boiled in antigen retrieval solution (1 mmol/L Tris-HCl, pH 8.0) for 15 minutes at high power in a microwave oven. The sections were incubated overnight at 4°C with primary antibodies at appropriate dilutions (anti–mPGES-1, Cayman; anti–aquaporin-2, gift from Mark A. Knepper, NIH/National Heart, Lung, and Blood Institute). After washing with PBS, the secondary antibody was applied and the signals visualized using the ABC kit (Santa Cruz Biotechnology).

Real-Time RT-PCR
Total RNA isolation and reverse transcription were performed as previously described. Oligonucleotides were designed using Primer3 software (available at http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). The sequences of the oligonucleotide primers in the public sequence are as follows: NO synthase I, 5′-CTGGTGTCGCCTGGTCTTTA-3′ (sense) and 5′-TGTTGAGGACCA-3′ (antisense) (GenBank accession no. D14552); NO synthase II, 5′-ACTGTGGTGGCCTGGAGTT-3′ (sense), and 5′-TCTTGCTATCCTGGTCTTG-3′ (antisense) (GenBank accession no. NM_010927); NOS III, 5′-GAAGGCAGGCTGTTGTTG-3′ (sense) and 5′-TGATGGCTAAGGAGATTG-3′ (antisense) (GenBank accession no. NM_00713); mPGES-1, 5′-AGCA CACTGCGTGATCATCA-3′ (sense) and 5′-CTCACTCATGTGCTCAC-3′ (antisense) (GenBank accession no. BC024960); β-actin, 5′-GCCCTGCTTCTGGACCAT-3′ (sense) and 5′-GCCACCGTACTCAAGAGTG-3′ (antisense) (GenBank accession no. NM_007939). Real-time PCR amplification was performed using the SYBR Green Master Mix (Applied Biosystems) and the Prism 7500 Real-Time PCR Detection System (Applied Biosystems). Cycling conditions were 95°C for 10 minutes, followed by 40 repeats of 95°C for 15 s and 60°C for 1 minute.

Enzyme Immunoassay
Urine samples were centrifuged for 5 minutes at 10,000 rpm and diluted 1:1 with enzyme immunoassay buffer. Concentrations of PGE2, thromboxane B2, PGD2, 6-keto PGF1α, PGF2α, and cGMP were determined by enzyme immunoassay (Cayman Chemicals).

Primary Cultures of CD Cells
Primary cultures of kidney IM cells were prepared as previously described. In brief, under anesthesia, kidneys were harvested and the renal inner medulla was dissected, minced, and digested for 45 minutes with 0.2% collagenase and 0.2% hyaluronidase (wt/vol) at 37°C. After incubation, 20 mL of distilled water was added to lyse cells other than collecting duct cells by osmotic shock (100 mosmol/kg). Cells were incubated in a 6-well plate for at least 10 days until confluence was reached.

Ang II Infusion Experiments
The telemetric device was implanted into male mPGES-1 +/+ and mPGES-1 −/− mice (3 to 4 months of age) as described above. One week after surgery, microosmotic pumps were implanted subcutaneously in the midscapular region and telemetry was turned on. The pumps delivered Ang II at a rate of 0.7 mg/kg per day.

Statistical Analysis
All values are presented as mean±SE. For the time-course studies of BP, sodium balance, urinary excretion of nitrate/nitrite, and cGMP in mPGES-1 +/+ and mPGES-1 −/− mice, repeated-measures ANOVA was used to detect an interaction (time×strain). The unpaired Student’s t test was used to identify differences between the 2 strains of mice at a single time point. For the end-point studies of urinary PGE2 excretion, gene expression and PGE2 release from cultured cells, the unpaired Student’s t test was used for comparisons between mPGES-1 +/+ and mPGES-1 −/− mice and the paired Student’s t test for comparisons within mPGES-1 +/+ and mPGES-1 −/− mice. Differences were considered to be significant when the probability value was less than 0.05.

Results
Chronic Salt-Loading Experiments
At basal state, mPGES-1 −/− mice had a lower BP level as compared with mPGES-1 +/+ mice (P<0.05, unpaired Student’s t test). A 1-week high-salt diet resulted in increased mean arterial pressure (MAP) from 105.0±1.3 to 126±2.3 mm Hg in mPGES-1 +/+ mice and from 98.3±0.8 to 144±8.6 mm Hg in mPGES-1 −/− mice (Figure 1A). An interaction (time×strain) was detected by repeated measures ANOVA (P=0.045). The slope of the BP response over the 7 days of high-salt loading was also significantly higher in mPGES-1 −/− mice than in mPGES-1 +/+ mice (7.3±1.3 versus 3.0±0.4 mm Hg/d; P=0.014, unpaired Student’s t test).
Metabolic studies revealed that following initiation of the high-salt diet, sodium intake was increased immediately, which paralleled with increased urinary sodium excretion in both animal groups. Sodium intake was not significantly different between the 2 groups, although sodium excretion in mPGES-1/−/− (KO) mice was less than that in mPGES-1+/+/+ (WT) mice on day 2 (Figure 1). Sodium balance, expressed as intake subtracted by excretion, was remarkably increased in mPGES-1/−/− mice on days 1 and 2 and returned to normal levels thereafter.

A 1-week high-salt loading induced a marked increase in urinary PGE\(_2\) excretion in mPGES-1+/+/+ mice (Figure 2A).

In contrast, this increase was significantly inhibited in mPGES-1/−/− mice. Following chronic high-salt loading, urinary PGD\(_2\) excretion was significantly increased in mPGES-1+/+/+ mice and this increase was much greater in mPGES-1/−/− mice (Figure 2B). Urinary excretion of 6-keto PGF\(_1\alpha\), the metabolite of PGH\(_2\), was affected neither by dietary salt nor mPGES-1 deficiency (Figure 2C). mPGES-1−/− mice had increased basal levels of urinary thromboxane B\(_2\) (Figure 2D) that was suppressed by chronic high-salt loading, and these mice had a tendency of reduced urinary excretion of PGF\(_2\alpha\) regardless of dietary salt (Figure 2E).

**Acute Salt-Loading Experiments**

To examine whether mPGES-1 deficiency would cause an impaired ability to eliminate acute sodium load, we compared
natriuretic response between the 2 mouse strains after enteral loading of 1 meq of Na via gavage. Twelve-hour urine collections were performed before and 72 hours after the acute sodium load. mPGES-1+/+ mice were able to eliminate the entire sodium load within 24 hours, whereas mPGES-1−/− mice required 48 hours (Figure 3A).

In mPGES-1+/+ mice, enteral salt loading induced a transient and significant increase in urinary PGE2 excretion at 12 hours, paralleling with the changes in urinary sodium excretion. In contrast, this treatment did not significantly affect urinary PGE2 excretion in mPGES-1−/− mice at any time point (Figure 3B).

Alteration of NO Production
Renal medullary PGE2 and NO share regulatory and functional characteristics during high salt intake, suggesting a potential interaction between the 2 pathways. Therefore, we examined whether NO production was altered in mPGES-1−/− mice in the settings of chronic and acute sodium loading. As expected, chronic high-salt loading gradually and significantly increased urinary nitrate/nitrite excretion in mPGES-1+/+ mice with a maximal increase of 15-fold on day 7. In a sharp contrast, this treatment induced a gradual and remarkable reduction of urinary nitrate/nitrite excretion in mPGES-1−/− mice. Strikingly, the value decreased to undetectable levels in mPGES-1−/− mice after 3 days of high-salt loading (Figure 4A). Urinary cGMP followed the same pattern of changes as for urinary nitrate/nitrite (Figure 4B). In the setting of acute salt loading, there was a trend of differences in urinary nitrate/nitrite excretion between the 2 strains of mice but no interaction was detected using repeated measures ANOVA (Figure 4C).

mRNA levels of all 3 NOS isoforms in the inner medulla of mPGES-1+/+ mice was significantly increased in response to chronic high-salt loading as previously described in rats.27 mPGES-1−/− mice exhibited a significant inhibition of high salt–induced increases in gene expression of all 3 NOS isoforms, whereas these mice had increased basal levels of NOS III but not NOS I and NOS II (Figure 5).
Regulation of Renal mPGES-1 Gene Expression by High Salt

The importance of mPGES-1 for the natriuretic response suggests the possibility that the renal expression of this enzyme might be responsive to high-salt intake. Indeed, by immunohistochemistry, low levels of basal expression of mPGES-1 protein were detected in the CD and were remarkably induced in the same tubular cells following chronic high-salt loading (Figure 6A, left). The CD localization was confirmed by immunostaining of consecutive kidney sections with respective mPGES-1 and AQP2 antibodies (Figure 6A, right). Following chronic high-salt loading, total abundance of mPGES-1 protein, as assessed by immunoblotting, exhibited an 8-fold increase in the cortex and a 2-fold increase in the inner medulla (Figure 6B). In contrast, mPGES-1 mRNA assessed by real-time RT-PCR was increased in the cortex but not the inner medulla (Figure 6C).

To study the cellular mechanism of this phenomenon, we compared the direct effects of high salt on mPGES-1 mRNA expression and PGE2 release in primary cultures of mPGES-1+/+ and mPGES-1−/− CD cells. Exposure of mPGES-1+/+ CD cells to hypertonic NaCl for 24 hours at 550 mosmol/kg significantly inhibited mPGES-1 mRNA expression and PGE2 release. The increases in PGE2 release were significantly inhibited in mPGES-1−/− CD cells (Figure 7A and 7B). We compared the effects of sodium chloride and choline chloride at equal osmolalities and found that choline chloride exhibited a similar stimulatory effect on mPGES-1 expression and PGE2 release as sodium chloride, ruling out sodium as a direct stimulus for mPGES-1 expression in cultured CD cells (Figure 7C and 7D).

Ang II Infusion Experiments

To obtain additional evidence for the antihypertensive function of mPGES-1, we determined the responsiveness of mPGES-1−/− mice to Ang II infusion. Ang II was infused at 0.7 mg/kg per day via osmotic pump, and changes of MAP were monitored by telemetry. During a 4-day Ang II infusion, mPGES-1+/+ mice maintained almost constant MAP levels. In a sharp contrast, this treatment induced significant elevations of MAP in mPGES-1−/− mice (Figure 8A). The rise of MAP in mPGES-1+/+ mice was associated with increased urinary excretion of PGE2 that was blocked in mPGES-1−/− mice (Figure 8B).

Renal Morphology and Function in mPGES-1−/− Mice

Analysis of hematoxylin and eosin–stained kidney sections revealed normal renal morphology in mPGES-1−/− mice (Figure 8C). In addition, these mice appeared to have normal renal function, as reflected by normal plasma blood urea nitrogen levels (wild type [WT], 18±0.89 mg/dL; KO, 20±1.0 mg/dL, n=5 to 6; P<0.05)

Discussion

The present study was undertaken to evaluate the role of mPGES-1 in regulation of sodium balance and Bp. mPGES-1−/− mice on a high-salt diet for 1 week developed severe and progressive hypertension associated with positive sodium balance. Consistent with this finding, these mice exhibited a significantly impaired natriuretic response to an acute salt load. These mice had remarkably suppressed urinary excretion of PGE2 as well as NO during salt loading. mPGES-1 expression in the CD was significantly increased following salt loading. These findings represent compelling evidence for mPGES-1 as a natriuretic factor acting through PGE2 stimulation of NO production in the distal nephron. Furthermore, we provided evidence that mPGES-1−/− mice were prone to Ang II–induced hypertension. Taken together, this study characterized an overall natriuretic and antihypertensive role of mPGES-1. The importance of this observation is 2-fold: (1) it provides evidence indicating possible cardiovascular consequences of mPGES-1 inhibitors; and (2) it contributes to a better understanding of the roles of PGE2 in the control of sodium balance and Bp.
COX inhibitors are among the most commonly used of all drugs and are associated with many adverse effects. COX-2-selective inhibitors, which are coxibs including celecoxib, rofecoxib, and valdecoxib, have dominated the prescription drug market for nonsteroidal antiinflammatory drugs, with approximately $10 billion sales worldwide. Unfortunately, cardiovascular consequences have emerged as major side effects associated with coxibs, especially rofecoxib. There is ever-increasing demand for alternatives of COX-2 inhibitors.

mPGES-1 is critically important for inflammation and thus represents a novel target for antiinflammatory drugs. However, the observation that mPGES-1−/− mice are prone to hypertension induced by high salt and Ang II infusion raises a concern that, like coxibs, mPGES-1 inhibitors may cause perturbation of cardiovascular and renal functions.

Urinary excretion and renal medullary synthesis of PGE2 was significantly increased in rodents on a high-salt diet. In cultured renal medullary epithelial and interstitial cells, hypertonically, mPGES-1 mRNA expression was determined by real-time RT-PCR and PGE2 release by enzyme immunoassay. Statistical analysis was performed using paired or unpaired Student’s t test. N=4 in each group.
hypertonic NaCl exerted a direct stimulatory effect on PGE2 release.\textsuperscript{31,32} The present study has provided compelling evidence to support mPGES-1 as a major source of PGE2 during high-salt loading. First, renal expression of mPGES-1 was highly inducible by high-salt loading. Second, increased urinary excretion of PGE2 in response to acute and chronic high-salt loading was almost completely abolished in mPGES-1 $-/-$ mice. Finally, hypertonic NaCl exerted a direct stimulatory effect on mPGES-1 mRNA expression in cultured CD cells. These observations establish an essential role of mPGES-1 in high salt–stimulated renal synthesis of PGE2 but do not directly rule out the contribution of other PGE isosforms such as mPGES-2 or cPGES.

We demonstrated that mPGES-1 $-/-$ mice were salt sensitive and prone to hypertension on a high-salt diet. The hypertension in these animals appeared to be the result of abnormalities in renal sodium handling. In the setting of chronic salt loading, mPGES-1 $-/-$ mice exhibited abnormalities in urinary sodium excretion and sodium balance that returned to normal after 24 to 48 hours, possibly because of the reestablishment of sodium balance at the expense of increased Bp. Furthermore, mPGES-1 $-/-$ mice exhibited significant impairment of the natriuretic response to an acute salt load. These observations strongly support mPGES-1 as a natriuretic and antihypertensive factor. Because the induction of mPGES-1 expression after high-salt loading is restricted to the CD, it is likely that the distal nphreron will be the site for mPGES-1–mediated natriuresis. PGE2 produced from the CD may act in an autocrine fashion to directly inhibit tubular transport\textsuperscript{13,33,34} and/or in a paracrine fashion to augment renal blood flow,\textsuperscript{33} leading to increased sodium excretion. The specific EP receptor subtypes involved are unclear but could be related to the EP\textsubscript{3} receptor as EP\textsubscript{3} $-/-$ mice develop salt-sensitive hypertension, similar to mPGES-1 $-/-$ mice.\textsuperscript{36}

Our results are somewhat inconsistent with the report by Cheng et al who were unable to observe an elevated Bp in mPGES-1 KO mice on a high-salt diet.\textsuperscript{37} The exact reason for the disparities between the 2 studies is unclear. One possibility might be related to differences in experimental protocols. Despite the same sodium content (8% NaCl in both studies), the formulation of the high-salt diet was different: Cheng et al used a solid chow diet, whereas we used a gelled diet. The gelled formulation will stimulate sodium intake. In addition, we used saline as drinking fluid, whereas Cheng et al used tap water. The use of gelled formulation of high-salt diet in conjunction with saline as drinking fluid in the current study may make sodium loading more efficient. Intensive salt loading appeared necessary to overcome the compensatory mechanism to induce hypertensive phenotype in mPGES-1 $-/-$ mice in the present study. A second possibility for the disparities might be related to differences in mouse genetic backgrounds. The mPGES-1 KO mice originally were generated on the DBA/1lacJ genetic background. It appeared that Cheng et al maintained the colony on a mixed DBA/1lacJ×C57/BL6 genetic background, whereas we maintained it on a mixed DBA/1lacJ×C57/BL6×129/Sv genetic background. It is unclear whether the presence of 129/Sv genetic background in our study may contribute to a salt sensitive phenotype in mPGES-1 $-/-$ mice. In line with this possibility, 129/Sv strain exhibits increased sensitivity to deoxycorticosterone acetate (DOCA)-salt–induced hypertension as compared with C57/BL6 mouse.\textsuperscript{38} To address this issue, future studies are needed to breed the KO mice into inbred genetic backgrounds.

The data demonstrated that mPGES-1–derived PGE2 acts via NO in the setting of salt loading. Following salt loading, urinary NO excretion along with the cGMP levels were remarkably elevated in WT animals but were completely
suppressed in mPGES-1 −/− mice, suggesting possible dependence of NO production on mPGES-1–derived PGE2 in the setting of chronic salt loading. The suppression of urinary NO excretion appeared to be attributable to reduced renal NO synthesis because high salt–induced stimulation of all 3 NOS isoforms in the renal medulla was significantly inhibited in mPGES-1 −/− mice. Interestingly, upregulation of basal gene expression in mPGES-1 −/− mice was observed only for NOS III but not NOS I and NOS II, suggesting unique interactions between mPGES-1 and various isoforms of NOS.

It is well recognized that vascular PGE2 is a component of the buffering system that mitigates Ang II–induced vasoconstriction. The presence of mPGES-1 in the endothelial cells and vascular smooth muscle cells raises the possibility that mPGES-1 accounts for the vasodilator effect of PGE2. In support of this hypothesis, mPGES-1 −/− mice exhibited an exaggerated hypertensive response to Ang II infusion as compared with the WT controls. This finding is consistent with the observation that COX-2 deletion or inhibition exaggerates the vasoconstrictive response after Ang II infusion.39

It is somewhat surprising that Bp, determined by telemetry, was not significantly increased in WT mice following chronic infusion of Ang II at 0.7 mg/kg per day, a dose known to induce hypertension in mice as evaluated by tail cuff plethysmography.40,41 Similarly, fructose feeding increased Bp as assessed with tail cuff plethysmography but did not affect Bp, as determined by telemetry.44 It is likely that the increases of Bp as assessed with tail cuff plethysmography may reflect the influence of stress associated with physical restraint and tail cuff inflation.

In summary, we demonstrated that mPGES-1 −/− mice developed severe and progressive hypertension on a high-salt diet. These animals had impaired natriuretic responses during acute sodium loading. Unexpectedly, the mice also had remarkably suppressed NO production. mPGES-1 expression in the distal nephron was highly inducible by high-salt loading. In addition, mPGES-1 −/− mice were prone to Ang II–induced hypertension. These findings represent compelling evidence for the natriuretic and antihypertensive function of mPGES-1. Thus, mPGES-1 inhibition may not be devoid of cardiovascular sequences associated with COX-2 inhibition.

Acknowledgments
We thank Pfizer for providing mPGES-1 KO mice; Mark A. Knepper (NIH/National Heart, Lung, and Blood Institute) for providing AQP2 antibody; Noel Carlson and Kenneth Hill (Salt Lake Veterans Affairs Medical Center) and John Kircher (University of Utah) for technical assistance; and Chris Baylis (University of Florida) for helpful discussions.

Sources of Funding
This work was supported by NIH grants HL079453, DK066592, DK069460, and DK064981 (to T.Y.).

Disclosures
None.

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_Circ Res._ 2006;99:1243-1251; originally published online November 9, 2006;
doi: 10.1161/01.RES.0000251306.40546.08
_Circulation Research_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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