Hydrogen Sulfide Inhibits Plasma Renin Activity

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ABSTRACT
The development of renovascular hypertension depends on the release of renin from the juxtaglomerular (JG) cells, a process regulated by intracellular cAMP. Hydrogen sulfide (H2S) downregulates cAMP production in some cell types by inhibiting adenylyl cyclase, suggesting the possibility that it may modulate renin release. Here, we investigated the effect of H2S on plasma renin activity and BP in rat models of renovascular hypertension. In the two-kidney-one-clip (2K1C) model of renovascular hypertension, the H2S donor NaHS prevented and treated hypertension. Compared with vehicle, NaHS significantly attenuated the elevation in plasma renin activity and angiotensin II levels but did not affect plasma angiotensin-converting enzyme activity. Furthermore, NaHS inhibited the upregulation of renin mRNA and protein levels in the clipped kidneys of 2K1C rats. In primary cultures of renin-rich kidney cells, NaHS markedly suppressed forskolin-stimulated renin activity in the medium and the intracellular increase in cAMP. In contrast, NaHS did not affect BP or plasma renin activity in normal or one-kidney-one-clip (1K1C) rats, both of which had normal plasma renin activity. In conclusion, these results demonstrate that H2S may inhibit renin activity by decreasing the synthesis and release of renin, suggesting its potential therapeutic value for renovascular hypertension.


Renovascular hypertension is a common secondary hypertension and the most prevalent form of curable hypertension.1 Although presently used antihypertensive agents have been shown to reduce the incidence of cardiovascular events, achievement of blood pressure (BP) control continues to be a worldwide public health problem. Hence, newer antihypertensive agents are needed to expand therapeutic options, increase treatment efficacy, decrease side effects, and enhance patient adherence.

Hydrogen sulfide (H2S) is considered as a novel gasomodulator besides nitric oxide and carbon monoxide.2,3 It is produced by cystathionine γ-lyase (CSE), cystathionine β-synthase (CBS),4 and a newly identified enzyme, 3-mercaptopyruvate sulfurtransferase.5 The expression of these enzymes has been identified in many human and other mammalian cells, including those from liver, kidney, brain, and blood lymphocytes.6 We and other groups previously report H2S plays important roles in numerous physiologic and pathologic processes such as cardioprotection, neurotransmission, inflammatory processes, etc.6–9 In vascular vessels, it was reported that H2S is a physiologic vasodilator.10–12 Recently, it was reported that inhibition of endogenous H2S production by knockout of cystathionine γ-lyase (CSE−/−) may cause hypertension in mice.13

BP is controlled by the renin-angiotensin system (RAS). Excessive activity of RAS can result in hypertension and disorders of fluid and electrolyte homeostasis. Pharmacologic blockade of RAS has
been available for almost 25 years. There is extensive documentation of its effectiveness in the treatment of hypertension. The renin-angiotensinogen reaction is the first and rate-limiting step in the production of angiotensin II (Ang II) and thus could be a therapeutic target for the treatment of hypertension. Renin is predominantly regulated by intracellular cAMP in juxtaglomerular (JG) cells. We previously reported that H2S may downregulate cAMP production via inhibition of adenylyl cyclase (AC) activity in the heart and vascular smooth muscle cells.12,14 We therefore hypothesized that H2S may also regulate BP by inhibition of renin release.

Among the experimental models of hypertension, the renovascular model has brought considerable insights to studies of the pathophysiology of hypertension.15–17 The two-kidney-one-clip (2K1C) model is an ideal animal model of renovascular hypertension. The reduction in pressure caused by stenosis of the renal artery decreases the GFR, which in turn causes the quantity of filtered solutes to fall.18 Macula densa cells present in the JG apparatus are able to detect these changes and induce the nearby granular cells to release renin, which in turn induces hypertension. In contrast, the development and maintenance of hypertension in the one-kidney-one-clip (1K1C) model is mainly related to sympathetic drive,15,19 local angiotensin converting enzyme (ACE) activity (particularly in organs regulating hypertension such as the aorta and heart),20 and Na⁺/H⁺ retention.21,22 The transient elevation of renin (within the first day after clipping) is only responsible for the onset of development of hypertension in the 1K1C model.23 The maintenance of hypertension has been considered to be independent of the circulating RAS with normal or low plasma renin activity (PRA).24

The study presented here was designed to examine the effect of H2S on RAS and its role in renovascular hypertension with 2K1C and 1K1C rat models. The underlying mechanism was investigated in in vivo and in vitro studies.

RESULTS

Preventive and Therapeutic Effects of Sodium Hydrosulfide on Hypertension in 2K1C Renovascular Hypertensive Rats

To examine the preventative effect of H2S on the development of renovascular hypertension in 2K1C rats, sodium hydrosulfide (NaHS; an H2S donor) was given daily from day 3 after surgery until the end of the 4-week experiment in the 2K1C/NaHS group. As shown in Figure 1A, the systolic BP (SBP) in 2K1C rats was significantly elevated starting from the first week after surgery, and it continuously increased during the whole 4 weeks of observation. Treatment with NaHS (5.6 mg/kg per d, intraperitoneally) attenuated the development of hypertension starting from the second week to the end of fourth week. At 1.68 to 5.6 mg/kg per d (30 to 100 µmol/kg per d), NaHS significantly reduced the elevated BP after 4 weeks of treatment (Figure 1B).

To confirm the antihypertensive effect of NaHS, the right carotid artery was catheterized to monitor BP at the end of 4 weeks. As shown in Table 1, systolic, diastolic, and mean artery pressures were all significantly elevated in 2K1C rats compared with those in sham-operated rats. NaHS treatment attenuated the elevation of BP in all of these parameters. This is consistent with the data measured with the noninvasive tail-cuff system (Figure 1A). Taken together, these data clearly suggest that NaHS treatment produces significant antirenovascular hypertensive effects.

We also examined the therapeutic effect of H2S after development of renovascular hypertension in 2K1C rats. One week
after surgery, BP significantly increased from 104.7 ± 2.3 mmHg to 141.6 ± 7.6 mmHg (P < 0.01). Rats were then separated into two groups and received saline or NaHS (5.6 mg/kg per d, intraperitoneally) injection from the second day after grouping. As shown in Figure 1C, NaHS significantly suppressed the elevation of BP from the second week to the fourth week of treatment. These data suggest that NaHS treatment was also able to decrease BP even after the development of hypertension in renovascular rats.

**Effect of NaHS on RAS in 2K1C Rats**

To examine the mechanism for the antihypertensive effects of NaHS, we investigated the involvement of RAS. As shown in Figure 2A, PRA was significantly elevated to 71.9 ± 12.3 ng/ml per h in the plasma of 2K1C rats, which was approximately 5-fold of that in the sham rat (14.0 ± 2.7 ng/ml per h). NaHS treatment dramatically reversed the elevated PRA to 26.1 ± 10.1 ng/ml per h.

We next examined the effect of NaHS on ACE activity. Incubation of normal rat aortic tissue with NaHS (1 to 1000 μmol/L) for 30 minutes failed to affect ACE activity. In contrast, captopril (1 μmol/L), an ACE inhibitor, abolished ACE activity in the aorta (Figure 2B). ACE activity in the aortic tissues was markedly elevated in 2K1C rats compared with that in sham rats (Figure 2C). The observation is consistent with previous reports.

Neither chronic treatment with NaHS (5.6 mg/kg per d) for 4 weeks nor acute treatment with NaHS (10–1000 μmol/L) for 30 minutes influenced the upregulated ACE activity in 2K1C rats. However, captopril (1 μmol/L) abolished ACE activity in aortic tissues.

The plasma level of Ang II was also investigated. As shown in Figure 2D, the Ang II level was elevated to 42.6 ± 12.9 pg/ml in the plasma of 2K1C rats, which was approximately 5-fold that of sham operated rats (8.4 ± 3.7 pg/ml). Treatment with NaHS for 4 weeks reduced the Ang II level to 14.0 ± 3.3 pg/ml.

**Effect of NaHS on Protein and mRNA Levels of Renin in 2K1C Rats**

We further examined the renin level in the kidneys. Preimmune mouse IgG was used as a negative control to show the background of immunostaining (Figure 3A). As shown in Figure 3, immunostaining revealed that renin level was largely increased in the nearby renal glomeruli and tubular epithelia in the clipped kidney of 2K1C rats compared with that in the unclipped kidney (Figure 3F) and that in the sham group (Figure 3, B and E). Application of NaHS significantly attenuated the accumulation of the renin level in the clipped kidney of 2K1C rats (Figure 3, D and G).

The result was further confirmed by Western blotting. Renin protein level was

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**Table 1. Effect of NaHS treatment on body weight and carotid BP in 2K1C rats**

<table>
<thead>
<tr>
<th>Sample Size</th>
<th>Body Weight (g)</th>
<th>SBP (mmHg)</th>
<th>Diastolic BP (mmHg)</th>
<th>MAP (mmHg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 Days</td>
<td>14 Days</td>
<td>28 Days</td>
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<tr>
<td>Sham</td>
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<tr>
<td>2K1C</td>
<td></td>
<td></td>
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<tr>
<td>NaHS</td>
<td>8</td>
<td>244.9 ± 5.7</td>
<td>376.8 ± 9.9</td>
<td>400.6 ± 10.3</td>
</tr>
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MAP, mean arterial pressure.

<sup>a</sup>P < 0.01 versus sham.

<sup>b</sup>P < 0.01 versus 2K1C.
significantly increased in the clipped (Figure 4A) but not in the unclipped (Figure 4B) kidney of 2K1C rats. NaHS treatment for 4 weeks significantly attenuated the elevated renin protein level in the clipped kidney (Figure 4A).

We also examined the effect of NaHS on the mRNA level of renin in the clipped kidneys of 2K1C rats. As shown in Figure 4, C and D, NaHS treatment markedly attenuated the upregulation of renin expression in clipped kidneys. (E through G) There was no significant change of renin expression in unclipped kidneys of 2K1C rats among (E) sham, (F) vehicle, and (G) NaHS treatment groups. The photos were taken at ×40 magnification.

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Figure 3. NaHS decreased renin expression in clipped kidneys of 2K1C rats. (A) Negative control stained by preimmune mouse IgG. Renin expression increased dramatically in (C) clipped kidneys compared with those in (B) the sham group. (D) NaHS treatment markedly attenuated the upregulation of renin expression in clipped kidneys. (E through G) There was no significant change of renin expression in unclipped kidneys of 2K1C rats among (E) sham, (F) vehicle, and (G) NaHS treatment groups. The photos were taken at ×40 magnification.

Effect of NaHS on cAMP Level in the Clipped and Unclipped Kidneys

cAMP is the best-established second messenger responsible for regulating renin release in JG cells.29 To examine the mechanism underlying H2S-suppressed PRA, we observed cAMP production in clipped and unclipped kidney with and without NaHS treatment. As shown in Figure 6, cAMP was markedly elevated in the 2K1C clipped kidney compared with that in the 2K1C unclipped kidney or the kidneys from sham rats. NaHS treatment for 4 weeks significantly inhibited the elevated cAMP levels in the clipped kidney in 2K1C rats.

Effect of NaHS on Renin Activity in the Culture Medium and cAMP Level in Primary Cultured Renin-Rich Kidney Cells

The effect of NaHS was further confirmed in JG cells. As shown in Figure 7A, NaHS at 100 µmol/L markedly attenuated forskolin-stimulated renin activity from 0.05 ± 0.01 ng/ml per h to 0.025 ± 0.005 ng/ml per h in the medium of primary cultured renin-rich kidney cells. cAMP production was determined in the cell lysates. As shown in Figure 7B, the stimulated cAMP production by forskolin (1.98 ± 0.08 pmol/mg protein) was significantly attenuated by H2S (1.64 ± 0.10 pmol/mg protein). In a separate experiment, we found that NaHS at 1000 µmol/L did not induce cell injury and produce toxic effects (data not shown). These data suggest that the effect of H2S on renin production is mediated by downregulation of cAMP production.

Effect of NaHS on Renin Level, PRA, and BP in 1K1C Rats

To exclude the possibility that the antihypertensive effect of H2S was from mechanisms other than inhibition of PRA, we observed the effect of H2S on the development of hypertension in 1K1C rats, a renin-independent hypertensive model.24 Consistent with previous reports, there was no significant change in renin protein level in the single kidney (Figure 8A) and PRA (Figure 8B) in 1K1C rats. However, BP increased from approximately 100 to 180 mmHg at the end of second week of surgery (Figure 8C). Interestingly, administration of NaHS (5.6 mg/kg per d, intraperitoneally) from day 3 after surgery failed to suppress the hypertension in 1K1C rats.

Effect of Endogenous and Exogenous Application of H2S on BP and Renin Activity in Normal Rats

As shown in Figure 9A, systemic administration of NaHS for 4 weeks increased the plasma H2S level from 28.2 ± 0.4 µmol/L in control rats to 34.4 ± 2.7 µmol/L, whereas application of hydroxylamine hydrochloride, an inhibitor of CSE and CBS, decreased the plasma H2S level to 25.4 ± 0.8 µmol/L. However, both treatments had no significant effect on BP and PRA.

H2S Inhibited Acute Renal-Artery-Stenosis-Induced Venous PRA Elevation

To exclude the possibility that the suppressive effect of NaHS on PRA is from the chronic effect on angiogenesis, we also examined whether NaHS can regulate venous PRA induced by acutely decreasing blood supply. As shown in Figure 5, after 30 minutes of vessel occlusion, PRA in renal venous blood raised to 43.67 ± 4.87 ng/ml per h, which was approximately 3-fold of that in the control group (14.0 ± 2.7 ng/ml per h). Intravenous injection of NaHS (40 nmol/min) 15 minutes before and during occlusion significantly attenuated the elevated PRA to 28.72 ± 3.99 ng/ml per h.

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in normal rats (Figure 9, B and C). Our data suggest that H2S may only regulate PRA when RAS is overactivated.

**DISCUSSION**

The renin-angiotensinogen reaction is the first and rate-limiting step in the production of Ang II and thus could be a therapeutic target for treatment of hypertension. The most important finding in this study is that H2S inhibits PRA, thus producing preventive and therapeutic effects on renovascular hypertension in 2K1C rats.

With the 2K1C renovascular hypertensive model, we found that H2S may have marked therapeutic value for hypertension induced by renal artery stenosis. The elevations of SBP and diastolic BP were significantly attenuated by NaHS treatment for 4 weeks. Furthermore, application of NaHS also markedly ameliorates the disease's aggravation, even after development of renovascular hypertension. All of these beneficial effects of H2S are attributed to the decreased PRA and Ang II production in plasma. These data suggest that H2S exerts its antihypertensive role by suppressing the excessively activated RAS.

Perfusion pressure to the kidney is the most important regulator of renin release. Stenosis of the renal artery decreases blood flow and GFR, thus enhancing renin secretion. To confirm whether the antihypertensive effect of H2S is specific secondary to the inhibition of renal renin release, we observed the venous PRA after acute renal stenosis for 30 minutes. We found in this study that NaHS treatment significantly suppressed lower perfusion-rate-induced venous PRA. However, because renin has a long half-life and recirculates in the blood, we cannot conclude that H2S inhibits renin secretion/release without measurement of the renin levels in the renal artery and vein. The most accurate way to determine the effect of H2S on renin secretion is to subtract the renin level in the artery from that in the renal venous blood. Nonetheless, our above data at least exclude the possibility that the effect of H2S on renin is secondary to systematic reaction or angiogenesis.

We also examined the underlying mechanism for the inhibitory effect of H2S on PRA. Among the intracellular signaling pathways involved in the regulation of renin secretion, the cAMP pathway signaling cascade appears to be the central and stimulatory pathway for the exocytosis of renin. All maneuvers that increase cellular cAMP levels in JG

**Figure 4.** NaHS reduced the protein and mRNA levels of renin in the clipped kidneys of 2K1C rats. NaHS treatment inhibited the upregulated renin expression in (A) clipped kidney but had no action on unchanged renin level in (B) unclipped kidney (n = 5). (C and D) NaHS suppressed the upregulated renin mRNA level in the clipped kidney of 2K1C rats (n = 4). **P < 0.01 and ***P < 0.001 versus sham rats. *P < 0.05 and **P < 0.01 versus 2K1C rats.

**Figure 5.** Perfusion with NaHS (40 nmol/min) significantly inhibited stenosis-stimulated venous PRA (n = 9 to 10). **P < 0.001 versus control and *P < 0.05 versus the vehicle group.

**Figure 6.** NaHS treatment (5.6 mg/kg per d) reduced cAMP production in clipped kidney of 2K1C rats (n = 6 to 9). **P < 0.01 versus sham, and ***P < 0.001 and *P < 0.05 versus the 2K1C clipped group.
cells (e.g., activation of AC, inhibition of cAMP-phosphodiesterases, and addition of membrane-cAMP analogs) stimulate renin secretion. We found in the study presented here that cAMP content in the clipped kidney of 2K1C rats was much higher than that in the unclipped one. The elevated cAMP level was downregulated by NaHS treatment. The same effects were found in the primary cultured renin-rich kidney cells. NaHS incubation suppressed forskolin-stimulated cAMP level in the renin-rich kidney cells. This finding is consistent with what we found in other tissues (e.g., rat aortic smooth muscle cells and cardiac myocytes).12,14 In the culture medium of renin-rich kidney cells, we found that NaHS treatment decreased forskolin-stimulated renin activity. These data imply that H2S may inhibit renin release by decreasing intracellular cAMP levels in JG cells.

We also examined the effect of NaHS on renin synthesis by measuring mRNA and protein levels of renin in the clipped kidney in 2K1C rats. It was found that systemic treatment with NaHS inhibited the elevated mRNA and protein levels of renin in the clipped kidney tissues. These data suggest that H2S may also inhibit renin synthesis.

The 1K1C model is considered to be a renin-independent renovascular hypertension model.24 In the 1K1C model, renin is only transiently increased within the first day after clipping and is therefore only responsible for the onset of development of hypertension.23 This was also confirmed with our results that renin was not elevated in our 1K1C rats. Interestingly, application of NaHS 3 days after surgery failed to affect hypertension in the 1K1C rats. Similarly, exogenous application of NaHS or suppressing endogenous H2S production failed to change BP and PRA in normal rats. The above data suggest that the antihypertensive effect of H2S is more potent/specific to hypertension with higher PRA. This is also supported by our data that H2S only decreased the upregulated renin activity and renin protein in the clipped kidney in 2K1C rats but had no effect on normal renin protein level in the unclipped kidney in 2K1C and 1K1C rats.

In summary, our findings may provide a potential therapeutic approach for treatment of renovascular hypertension by suppressing the excessively activated RAS. It is promising that H2S-based antihypertensive therapies may one day be developed. These may offer the following advantages over ACE inhibitors and angiotensin receptor blockers:

**Figure 7.** NaHS markedly suppressed the forskolin (1 μmol/L) stimulated elevation of (A) renin activity in culture medium (n = 5 to 11) and (B) cAMP production in renin-rich kidney cells (n = 9 to 12). #*P < 0.01 and #**P < 0.001 versus control, and *P < 0.05 versus forskolin.

**Figure 8.** NaHS did not affect renin protein expression in the kidney, PRA, and BP in 1K1C rats. (A) Renin protein expression in the left kidneys of sham rats and 1K1C rats with and without NaHS treatment (n = 6). (B) PRA in sham and 1K1C rats with or without NaHS treatment (n = 4 to 5). (C) NaHS failed to affect elevated BP in 1K1C rats (n = 7 to 8). ***P < 0.001 versus sham.
1. Because renin-angiotensinogen is the rate-limiting step to produce Ang II, inhibition of renin release would reduce Ang II production from the source.

2. Because angiotensinogen is the only known renin substrate, direct inhibition of renin release should have minimal side effects.

3. H2S has high bioavailability. This may provide better antihypertensive effects than aliskiren.

4. As demonstrated by different groups including ours, H2S produces direct protective effects on the heart, brain, and kidneys.

5. H2S or its donors should have better cost-effectiveness.

CONCISE METHODS

The Institutional Animal Care and Use Committee of the National University of Singapore approved the experimental protocol.

Animal Model and Experimental Procedure

Seven-week-old male Sprague–Dawley rats were anesthetized with ketamine (75 mg/kg, intraperitoneally) and xylazine (10 mg/kg, intraperitoneally). In the 2K1C and 2K1C+NaHS groups, the left kidney was exposed through a lumbar incision and the left renal artery was dissected free and clipped by a rigid U-shaped silver clip with a 0.25-mm slit. The sham procedure was performed including the entire surgery, with the exception of arterial clipping. For the 1K1C model, the contralateral kidney was removed after clipping of the left kidney. The rats were kept in cages after surgery with constant temperature (23°C) and humidity. They were exposed to a 12:12-hour light-dark cycle and had unrestricted access to tap water and food. NaHS (0.56, 1.68, and 5.6 mg/kg per d or 10, 30, 100 μmol/kg per d) was administered daily to rats via intraperitoneal injection starting from day 3 after surgery in the 2K1C+NaHS group, and NaHS (5.6 mg/kg per d) was applied in the 1K1C+NaHS group. Sham group, 2K1C, and 1K1C control rats received vehicle (saline) treatment. To examine the therapeutic effect of H2S after development of renovascular hypertension, NaHS (5.6 mg/kg per d) was given 8 days after surgery. To investigate the effect of H2S on BP in normal rats, NaHS (5.6 mg/kg per d) or hydroxylamine hydrochloride (0.5 mg/kg) were applied daily to normal rats via intraperitoneal injection.

BP Measurement

SBP was measured in calm, conscious rats using a tail-cuff transducer connected to a Powerlab system running Chart 5 software (Powerlab, AD Instruments). SBP was measured in each rat immediately before and weekly after surgery for the following 4 weeks in all groups. SBP of normal rats was tested before treatment to determine the baseline and once a week after treatment for 4 weeks. Before each measurement, the rats were prewarmed to 35°C for 10 minutes in a cupboard. The average of three pressure readings was recorded for each measurement. After 4 weeks, SBP and diastolic BP were recorded at the right carotid artery with a catheter (PE-50) connected with a transducer and Powerlab system. Animals were anesthetized with ketamine (75 mg/kg, intraperitoneally) and xylazine (10 mg/kg, intraperitoneally). Powerlab system software automatically calculated mean arterial pressure.

Histologic Staining and Immunohistochemistry

Kidney tissues were fixed in 10% neutral formalin for 24 hours and then embedded in paraffin. Samples were sectioned (5 μm) using a microtome. Immunostaining was performed with a commercial kit [EnVision + Dual Link System-HRP (DAB +; Dako Cytomation]]. Briefly, kidney tissue sections were deparaffinized in xylene and dehydrated in a gradient concentration of ethanol. Antigen retrieval was performed by microwave with citrate buffer (trisodium citrate 10 mM, 0.05% Tween 20, pH 6.0) for 5 minutes, followed by incubation with normal rabbit serum for 30 minutes at room temperature. These tissue sections were then incubated with the renin primary antibody (1:100; AnaSpec) for 2 hours at room temperature. After the sections were incubated with secondary antibody (anti-mouse, 1:10000) for 1 hour at room temperature, immunostaining was visualized by using substrate-chromogen solution for 10 minutes. Sections were then counterstained with hematoxylin and ammonia. Preimmune mouse IgG was used as a negative control.
Acute Low-Renal-Blood-Flow Experiment
Rats were anesthetized with ketamine (75 mg/kg, intraperitoneally) and xylazine (10 mg/kg, intraperitoneally). The left kidney was exposed through an abdominal incision 1.5 cm below the xiphoid process, and the left renal artery was cautiously dissected free from the vein to avoid dragging the blood vessel. The left kidney arteries were clamped with a rigid U-shaped silver clip with a 0.25-mm slit for 30 minutes. NaHS (100 μM) or saline were perfused into the right femoral vein by a butterfly needle (27 gauge, 0.4 × 10 mm) at a speed of 400 μl/min 15 minutes before and during the whole process of artery occlusion. At the end of the 30-minute occlusion, blood samples were collected from the renal vein with a 27-gauge needle (0.4 × 12 mm) for renin activity determination.

Measurement of Renin Activity
Renin activity was measured at the National University Hospital of Singapore by radioactive immunoassay with quantitative determination of angiotensin I. Briefly, blood samples or culture medium (preincubated with excess renin substrate) were collected by centrifuging at 2000 × g for 10 minutes. A five-hundred-microliter sample of supernatant, 10 μl of phenylmethylsulfonyl fluoride, and 50 μl of angiotensin I generation buffer were added into noncoated generation tubes to generate angiotensin I generation buffer were added into noncoated generation tubes to generate angiotensin I. After incubation for 90 minutes at 37°C, the generation tubes were immediately placed in an ice bath. The following assay was performed at room temperature. Fifty microliters of sample or calibrator and 500 μl of tracer were added to the bottom of tubes that were coated with the 125I-labeled hormone, BSA, phosphate buffer, stabilizers, preservatives, and an inert red dye. Radioactivity was 81 kBq (2 μCi). The contents of tubes were mixed with a vortex and incubated for 3 hours at room temperature. The incubation mixture was carefully aspirated and a Gamma counter suitable for counting the radionuclide I measured the radioactivity of tubes (counter window setting, 15 to 80 keV; counter efficiency, 70%; counting time, 1 minute). PRA was calculated as nanograms angiotensin I generated per milliliter per hour [PRA = (ng 37°C − ng 4°C) × 1.12/h of incubation].

Ang II Measurement
Ang II level in plasma was examined with an Ang II immunoassay kit (Spibio). Briefly, blood samples were collected in EDTA containing tubes in ice. After centrifugation at 3000 × g for 20 minutes at 4°C, the supernatants (2 ml) were collected and passed through the phenyl cartridge, which was precleaned with methanol and 1 ml of water. Absorbed angiotensin peptides were eluted with 0.5 ml of methanol and then were evaporated by vacuum centrifugation. Samples were dissolved in 0.5 ml of enzyme immunoassay buffer and centrifuged at 3000 × g for 10 minutes at 4°C. Supernatants (100 μl) were dispensed to the appropriate antibody-coated wells. After incubation at room temperature for 1 hour, 50 μl of glutaraldehyde were added to each well and incubated for 5 minutes, followed by addition of borane-trimethylamine (50 μl) and incubation for another 5 minutes at room temperature with gentle agitation. After incubation at 4°C overnight with anti-Ang II IgG tracer and dispensing Ellman’s reagent, the level of Ang II was read at a wavelength of 405 nm.

ACE Activity Assay
A fluorescence-based protocol was used to quantify ACE activity.31 Briefly, tissue samples (50 μl) were mixed with 150 μU ACE substrate working solution (Sigma, A-6778) and 0.45 mM O-aminobenzoyl-glycyl-p-nitro-l-phenylalanyl-l-proline (200 μl, Bachem, E-2920) in a 96-well microplate and incubated at 37°C for 30 minutes. The fluorescence signals before and after 30-minute incubation were obtained using a microplate fluorometer (Thermo Electron) at excitation and emission wavelengths of 365 and 415 nm, respectively. The differences in fluorescence signals between 0 and 30 minutes were used to represent ACE activity.

Western Blotting
Tissue samples were homogenized in tissue lysis buffer (1:10, w/v; Sigma). Protein concentrations were determined by the Lowry method. Protein samples (30 μg) were separated by 10% SDS/PAGE and transferred onto a nitrocellulose membrane (Amersham Biosciences). After blocking at room temperature in 10% milk with TBST buffer (10 mM Tris-HCl, 120 mM NaCl, 0.1% Tween-20, pH 7.4) for 1 hour, the membrane was incubated with renin (1:500, AnaSpec) and β-actin (1:1000, Santacruz) primary antibodies at 4°C overnight. Membranes were then washed 3 times in TBST buffer, followed by incubation with 1:10,000 dilutions of horseradish-peroxidase-conjugated anti-rabbit IgG at room temperature for 1 hour and washing 3 times in TBST. Visualization was carried out using an ECL (advanced chemiluminescence) kit (GE Healthcare). The density of the bands on Western blots was quantified by densitometry analysis of the scanned blots using ImageQuant software.

Reverse-Transcription PCR Analysis
Reverse-transcription PCR analysis was performed by LightCycler (Roche Diagnostics). Gene expression was normalized to the endogenous control glyceraldehyde-3-phosphate dehydrogenase mRNA in each sample. The following primers were used: for renin, sense 5’-CCCCGTGCTTTGACACAT-3’ and antisense 3’-CGCACAGCC TTCTTCACAT-5’; for glyceraldehyde-3-phosphate dehydrogenase, sense 5’-TGACGGAGCTCACTGG-3’ and antisense 5’-TCCACCCCTGTGCTGTA-3’. Reverse-transcription PCR was performed at 50°C for 30 minutes and at 95°C for 15 minutes for reverse transcription, followed by 30 cycles of PCR reaction consisting of 94°C (45 seconds) for denaturation, 58°C (45 seconds) or 52°C for renin) for annealing, and 72°C (45 seconds) for extension. A final extension was performed at 72°C for 10 minutes. Afterwards the PCR products were separated by electrophoresis on a 1.5% agarose gel.

Isolation of Renin-Rich Kidney Cells
Male Sprague–Dawley rats weighing 180 to 250 g were used as described in previous publications.32,33 Briefly, kidneys were aseptically removed and perfused with enzymes in Krebs buffer consisting of (in mM) 111 NaCl, 5.0 KCl, 1.0 NaH2PO4, 11.2 glucose, 25 NaHCO3, 0.54 MgCl2, 2.5 CaCl2, 0.1% BSA, 0.1% collagenase (type 1), and 0.017 mg/ml DNase (type IV). The kidneys were then decapsulated and the papilla was removed. Each kidney was cut into longitudinal halves and cortices were minced with a scalpel blade to obtain tissue pieces approximately 1 mm3 in size. The minced tissue was suspended...
and incubated in the enzyme solution at 37°C for 90 minutes, and enzyme activity was terminated by dilution (1:10) with 20% FBS. Suspension was filtered with a 105-μm nylon mesh. Cell suspension was centrifuged at 250 × g for 2 minutes and resuspended in a 40% Percoll solution (Sigma) followed by centrifugation at 27,000 × g for 25 minutes at 4°C. Typically four bands with accumulated cells were developed in the gradient, and the third band from the top with a density of 1.07 g/ml was then harvested by aspiration. Cells were washed 3 times with enzyme-free Krebs buffer to ensure cells were free from Percoll and then cultured in medium containing 10% FBS.

Intracellular cAMP Assay
A direct cAMP enzyme immunoassay kit (Cayman Chemical) was used to examine the involvement of cAMP.12,14 Briefly, cells were incubated in Krebs solution containing NaHS (100 μmol/L) for 5 minutes, followed by application of forskolin (1 μmol/L), an AC activator. After 30 minutes of incubation, culture medium was harvested for renin activity test and the cells were lysed in 0.1 mol/L HCl for 20 minutes. For measurement of the cAMP level in kidney tissues, the samples were homogenized in 5% TCA solution (1:10 w/v) and centrifuged at 1000 × g for 10 minutes. TCA was extracted from the supernatant using water-saturated ether. Fifty microliters of samples were added into a 96-well plate followed by incubation with cAMP acetylcholine esterase tracer and cAMP antiserum for 18 hours at 4°C. Each sample was developed by Ellman’s reagent and the plate was read at a wavelength of 405 nm. cAMP concentration was calculated according to the cAMP standard and the protein was examined by dissolving the pellets.

Measurement of H₂S Level
H₂S concentration in plasma was measured essentially as described in our previous publications.7–9 Briefly, aliquots (100 μL of plasma) were mixed with potassium phosphate buffer (pH 7.4, 350 μL) and zinc acetate (1% w/v, 250 μL) followed by incubation with N,N-Dimethyl-p-phenylene diamine sulfate (20 mmol/L, 133 μL) in 7.2 mol/L HCl and FeCl₃ (30 mmol/L, 133 μL) in 1.2 M HCl. Reactions were terminated by TCA (10% w/v, 250 μL) after 15-minute color development. The resulting solutions (300 μL) were transferred to a 96-well plate and the absorbance of the mixture (670 nm) was measured. H₂S was calculated against a calibration curve of NaHS (0.01 to 100 μmol/L).

Statistical Analysis
All data are presented as mean ± SEM. Statistical significance was assessed with one-way ANOVA followed by a post hoc (Tukey) test for multiple group comparison. Differences with P < 0.05 were considered statistically significant.

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DISCLOSURES
None.

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