H$_2$O$_2$ generated by NADPH oxidase 4 contributes to transient receptor potential vanilloid 1 channel-mediated mechanosensation in the rat kidney

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Submitted 18 August 2014; accepted in final form 17 June 2015

Lin CS, Lee SH, Huang HS, Chen YS, Ma MC. H$_2$O$_2$ generated by NADPH oxidase 4 contributes to transient receptor potential vanilloid 1 channel-mediated mechanosensation in the rat kidney. Am J Physiol Renal Physiol 309: F369–F376, 2015. First published July 1, 2015; doi:10.1152/ajprenal.00462.2014.—The presence of NADPH oxidase (Nox) in the kidney, especially Nox4, results in H$_2$O$_2$ production, which regulates Na$^+$ excretion and urine formation. Redox-sensitive transient receptor potential vanilloid 1 channels (TRPV1s) are distributed in mechanosensory fibers of the renal pelvis and monitor changes in intrapelvic pressure (IPP) during urine formation. The present study tested whether H$_2$O$_2$ derived from Nox4 affects TRPV1 function in renal sensory responses. Perfusion of H$_2$O$_2$ into the renal pelvis dose dependently increased afferent renal nerve activity and substance P (SP) release. These responses were attenuated by cotreatment with catalase or TRPV1 blockers. In single unit recordings, H$_2$O$_2$ activated afferent renal nerve activity in response to rising IPP but not high salt. Western blots revealed that Nox2 (gp91$^{phox}$) and Nox4 are both present in the rat kidney, but Nox4 is abundant in the renal pelvis and originates from dorsal root ganglia. This distribution was associated with expression of the Nox4 regulators p22$^{phox}$ and polymerase 6-interacting protein 2. Coimmunoprecipitation experiments showed that IPP increases polymerase 6-interacting protein 2 association with Nox4 or p22$^{phox}$ in the renal pelvis. Interestingly, immunofluorescence labeling demonstrated that Nox4 localizes with TRPV1 in sensory fibers of the renal pelvis, indicating that H$_2$O$_2$ generated from Nox4 may affect TRPV1 activity. Stepwise increases in IPP and saline loading resulted in H$_2$O$_2$ and SP release, sensory activation, diuresis, and natriuresis. These effects, however, were remarkably attenuated by Nox inhibition. Overall, these results suggest that Nox4-positive fibers liberate H$_2$O$_2$ after mechanostimulation, thereby contributing to a renal sensory nerve-mediated diuretic/natriuretic response.

renal nerves; mechanoreceptors; hydrogen peroxide; diuresis/natriuresis; NADPH oxidase

THE CA$^{2+}$-PERMEABLE transient receptor potential vanilloid type 1 channel (TRPV1) is expressed mainly in A6-fibers and unmyelinated C-fibers of sensory nerves in the renal pelvic wall of the rat kidney (8, 45). Renal pelvic TRPV1 functions as a mechanoreceptor that senses rises in intrapelvic pressure (IPP), leading to substance P (SP) release and neurokinin-1 receptor activation, thus increasing afferent renal nerve activity (ARNA) (8). The enhanced ARNA plays an important role in the maintenance of Na$^+$ and fluid homeostasis by triggering an inhibitory renorenal reflex, which reduces efferent renal sympathetic nerve activity (ERNA) and leads to diuresis and natriuresis in both kidneys (8, 19–22, 27–30, 45). Blockade of TRPV1 abolishes the renal mechanosensory ability and subsequent reflex response and may cause excretory impairment when responding to saline loading (8). The possible function of TRPV1 in mechanosensing has been demonstrated in hypertensive Dahl salt-sensitive rats fed a high-salt diet; these rats showed defective TRPV1 expression in mesenteric arteries and renal tissues (43). The loss of TRPV1 makes it difficult to sense fluid accumulation in the vascular system caused by salt loading and to increase fluid clearance by the kidney (43).

ROS are a group of molecules produced in the cell after O$_2$ consumption and include highly reactive free oxygen radicals, such as the superoxide anion, and stable “diffusible” nonradical oxidants, such as H$_2$O$_2$ (11). ROS are conventionally considered cytotoxic byproducts of abnormal metabolism (12). Recent studies have shown that ROS are essential in cellular signaling and the regulation of cell function (10, 46).

In the kidney, H$_2$O$_2$ and superoxide have been suggested to maintain the overall salt/water balance via regulation of microvascular function and net tubular ion transport under physiological conditions (7, 13, 15, 24, 35, 46). The enzymatic activity of NADPH oxidase (Nox) is a major source of ROS generation in various signaling pathways (24, 46). One of the most abundant and widely expressed Nox isoforms is Nox4 (26). Originally identified in the renal cortex, Nox4 (also termed renal oxidase or Renox) is abundant in the kidney in the distal tubules, collecting ducts, renal papilla epithelium, and mesangial cells (34, 37).

The catalytic moiety of Nox4 is homologous to gp91$^{phox}$ (or Nox2), which is found in phagocytes (34, 40). Most Nox activation requires an association with cytosolic p47$^{phox}$, p67$^{phox}$, or the small GTPase Rac (34, 40). Nox4, however, is constitutively active in a reconstituted system containing p22$^{phox}$ and produces H$_2$O$_2$ (32, 40). Moreover, a recent study (26) showed that polymerase 6-interacting protein 2 (Poldip2) interacts with p22$^{phox}$ and increases superoxide and H$_2$O$_2$ generation facilitated by Nox4 in vascular smooth muscle cells, indicating that Nox4 activity can be induced by Poldip2. Intriguingly, the mRNAs of Nox4 and p22$^{phox}$, as well as those of other Nox proteins, are expressed in dorsal root ganglion neurons at T1–L2 (4), the spinal levels that contain the cell bodies of renal afferent nerves (19). Because renal sensory nerves act as important regulators in the reflex control of salt and water balance, we queried whether Nox4 and its regulators are present in the renal pelvis to generate H$_2$O$_2$ and mediate ARNA.

Considerable evidence has demonstrated that ROS enhance the sensory function of vagal afferents or C-fibers in the heart,
lung, and bladder; these sensory nerves are always capsaicin sensitive (2, 31, 39). Under pathophysiological conditions, the H$_2$O$_2$-mediated thermal hyperalgesia is attenuated in TRPV1 knockout mice (17), indicating that H$_2$O$_2$ sensitizes the threshold of sensory neurons and that TRPV1 is involved in a downstream pathway responsible for this sensitization. These observations led us to hypothesize that H$_2$O$_2$ derived from Nox4 in the renal pelvis may stimulate sensory nerves via TRPV1. Therefore, we tested the direct effects of H$_2$O$_2$ on ARNA and further distinguished the mechanosensitive or chemosensitive ARNA responses that can be activated by H$_2$O$_2$. We also examined the presence of Nox in the renal pelvis to determine the possible distribution of Nox4 in sensory nerve fibers. Finally, we examined the role of H$_2$O$_2$ on the induction of SP release and renal sensory activation in response to mechanical stimuli or saline load by Nox inhibition.

**MATERIALS AND METHODS**

**Animals.** Female Wistar rats (Biolasco, Taipei, Taiwan) weighing 200–220 g were used in these experiments. The experimental protocols were approved by the Institutional Animal Care and Use Committee of Fu-Jen Catholic University. All animal experiments and care were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Renal pelvic perfusion and recording of renal nerve activity. Rats were anesthetized with pentobarbital sodium (50 mg/kg ip) and cannulated for the recording of arterial blood pressure and heart rate. The rectal temperature of the experimental animals was maintained at 37°C with a servo-null heating pad controlled by a temperature controller (model TC-1000, CWE, Ardmore, PA). The left kidney was then exposed via a left flank incision. Both ureters were cannulated, but the left ureter was cannulated with a combination of two heat-pulled tips of polyethylene (PE)-10 tubing in one PE-50 catheter near the pelvis to allow renal pelvic perfusion, the collection of effluent, and the recording of IPP. The techniques for recording and expressing mechanical stimuli or saline load by Nox inhibition.

**Effects of H$_2$O$_2$ on the sensory response.** In multiunit ARNA recordings, 1, 30, or 100 µmol/l H$_2$O$_2$ (Wako Pure Chemical, Osaka, Japan) were freshly prepared and perfused randomly via one PE-10 tube into the renal pelvis over a 3-min period at rate of 10 µl/min, bracketed by a 10-min baseline and a 10-min recovery period of saline perfusion ($n = 8$ for each dose). Because the test concentrations of H$_2$O$_2$ at 1, 30, and 100 µM have pH values of ~6.9, 6.7, and 6.4, respectively, the saline solutions were adjusted with HCl to maintain the baseline and recovery period in an attempt to control for the acidic effect of H$_2$O$_2$ solution on the renal sensory responses. The other PE-10 tube was used for saline perfusion at the same rate and pH values and then switched to perfusion of the ROS scavenger catalase (5 U/ml, Sigma) or the TRPV1 inhibitors capsazepine (10 µmol/l) and SB-366791 (1 µmol/l) to test the role of H$_2$O$_2$ in TRPV1-mediated ARNA activation and renorenal reflex responses ($n = 8$ for each test). A 3-min experimental period was chosen because long-term treatment with H$_2$O$_2$ may desensitize ARNA.

Functional evidence for the effect of H$_2$O$_2$ on mechanosensitive or chemosensitive ARNA responses was collected by single unit recordings ($n = 6$). Renal pelvic stimulation was performed by raising the PE-50 catheter to increase IPP by 20 mmHg or to perform a perfusion with 0.9 mol/l NaCl for 3 min. H$_2$O$_2$ (30 µmol/l) was then perfused into the pelvis for 3 min. Each test was bracketed by a 10-min baseline and a 10-min recovery period with saline perfusion.

**Detection of Nox and associated proteins in renal tissues.** After the ARNA response had been tested, the kidneys were removed after transcardial perfusion of PBS (pH 7.4) (8, 27–30). Another group of rats with a bilateral dorsal rhizotomy (DRX) at T$_0$–L$_4$ or sham-operated rats after a 3-wk induction period ($n = 6$ rats/group each) were prepared as previously described (28) to test whether Nox proteins are of sensory origin. To examine the protein interaction between Podlip2 with Nox4 or p22phox, another group of rats was anesthetized and prepared for mechanostimulation, as described above. After 1 h of stabilization, IPP was increased to 10 mmHg for 5 min ($n = 4$), whereas IPP in control rats was not exposed to elevation. Renal pelvic tissues were then rapidly excised after transcardial perfusion of PBS and stored at −80°C for immunoprecipitation analysis.

Western blot analysis was performed to analyze protein expression using antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) against rat Nox2 (sc-5827, 1:1,000), Nox4 (sc-28180, 1:2,500), p22phox (sc-11712, 1:2,000), and Podlip2 (sc-82998, 1:500), or actin (sc-8432, 1:1,000) (8, 27–30). Positive control samples were prepared from cell lysates of leukocytes isolated from arterial blood (for Nox2) or from the aorta (for Nox4, p22phox, and Podlip2).

**Indirect immunofluorescence** was used to examine the distribution of Nox4 and the colocalization of Nox4, SP (sc-58591), or TRPV1 (sc-28579) in the renal pelvis (8, 28). Briefly, the kidneys were postfixed, embedded, and prepared as 5-µm sections on a cryostat (Microm, Heidelberg, Germany). After being blocked with 5% skim milk in PBS, sections were incubated overnight at 4°C with anti-Nox4 antibody (1:500 in 5% normal goat serum in PBS) and then for 1 h at room temperature with a FITC-conjugated antibody (Jackson ImmunoResearch, West Grove, PA, 1:100 in 5% skim milk). After detection of Nox4, tissue sections were incubated overnight at 4°C with anti-SP antibody (1:400 in PBS) or anti-TRPV1 antibody (1:500 in PBS) followed by an incubation for 1 h at room temperature with...
the corresponding rhodamine red-conjugated antibody (Jackson Immunoresearch, 1:500 in 5% skim milk). Sections were then examined on an inverted microscope (Leica, Wetzlar, Germany) equipped with a fluorescence image analytic system (Diagnostic Instruments). Nuclei were counterstained using 4’,6-diamidino-2-phenylindole. For the negative control, antibodies specific for Nox4, SP, and TRPV1 were incubated with the corresponding blocking peptides (Santa Cruz Biotechnology) for 30 min at 37°C before being used for labeling. Because no fluorescent signal was detected in these negative control images, the images of Nox4 versus SP and Nox4 versus TRPV1 were therefore merged as one control image.

Coimmunoprecipitation was performed by incubating the protein samples with 2 μg primary antibody overnight at 4°C (25), and the immunoprecipitates were harvested using protein A/G-agarose beads (Santa Cruz Biotechnology). After an extensive wash, immunoprecipitates were eluted by boiling the beads for 5 min in SDS-PAGE sample buffer [62.5 mM Tris·HCl (pH 6.8), 2% SDS, 40 mM DTT, and 10% glycerol] and characterized by Western blot analysis with the appropriate antibodies, as described above.

Quantitation of H2O2 or SP in renal tissues or the renal pelvic effluent. Tissue production and pelvic effluent content of H2O2 were measured using Amplex red (Molecular Probes, Eugene, OR) (7). SP was quantified using ELISA (8, 27–30).

Statistical analysis. Numeric data are presented as means ± SE. Friedman two-way ANOVA was used to examine differences in responses within groups in terms of changes in renal neural responses, renal excretion, release of H2O2 and SP after stimulation or saline loading, and intrapelvic Nox protein expression. The Mann-Whitney U-test was used to examine differences between groups in the effects of catalase, TRPV1 inhibition, Nox blockers, and DRX on Nox protein expression. Differences were regarded as significant at the level of P < 0.05.

RESULTS

H2O2 induces mechanosensory activation. Perfusion of H2O2 into the renal pelvis increased ARNA (Fig. 1A, top) and SP release (Fig. 1A, bottom) in a dose-dependent manner. Cotreatment with the ROS scavenger catalase or the TRPV1 inhibitors capsazepine or SB-366791 attenuated H2O2-induced increases in ARNA and release of SP. In the catalase or TRPV1 inhibitor cotreatments, significant increases in ARNA and SP release were only seen at the highest dose (100 μmol/l) of H2O2 used. H2O2 did not affect arterial blood pressure, heart rate, or IPP (data not shown).

Activation of the renal sensory nerve by 30 μmol/l H2O2 elicited an inhibitory renorenal reflex (Fig. 1B, left). H2O2 resulted in an increase in ARNA and a decrease in ERNA in the left kidney, accompanied by increases in the urine and urinary Na+ excretion in the contralateral (right) kidney. In contrast, after capsazepine or SB-366791 treatment (Fig. 1B, middle and right), no contralateral diuresis/natriuresis was seen with the same H2O2 treatment.

Because multunit ARNA activation during mechanostimulation may also include the activity of renal chemoreceptors (33), the response to H2O2 was next tested in single unit ARNA recordings. Representative tracings of single unit ARNA recordings in response to IPP elevation (20 mmHg), high salt (0.9 mol/l NaCl), or H2O2 (30 μmol/L) are shown in Fig. 1C, and the results are shown in Table 1. Table 1 clearly demonstrates that H2O2 leads to significant increases in single unit ARNA in response to increases in IPP (mechanostimulation) but not in response to perfusion of high NaCl (chemostimulation).

Expression of Nox and associated proteins in renal tissues. Nox2, Nox4, p22phox, and Poldip2 were all present in the rat kidney, as demonstrated by Western blot analysis (Fig. 2A). Nox2 was more abundant in the renal cortex and medulla than in the pelvis. In contrast, Nox4, p22phox, and Poldip2 were most abundant in the renal pelvis, with 2.6-, 1.7- and 2.2-fold
Mechanostimulation was induced by raising the intrapelvic pressure (IPP) to 20 mmHg. Intrapelvic infusion of 0.9 mol/l NaCl was used to induce renal chemostimulation \(\times 10^{-10} \text{mol/l}\) after IPP. NaCl (0.9 mol/l) and NaCl (3.0 mol/l) were compared between groups. Baseline values were 4.2 ± 0.5 and 4.1 ± 0.5 \(\times 10^{-10} \text{mol/l}\) after NaCl-infusion, respectively. Nox inhibition significantly also decreased the urinary flow rate at 5–30 min relative to control kidneys (Fig. 5C). The cumulative urinary output in apocynin- or DPI-treated kidneys was not affected by an increase in IPP (20 mmHg) 3.9 ± 0.3 vs. 2.4 ± 0.3 \(\times 10^{-10} \text{mol/l}\) after NaCl-infusion. The increases in Poldip2 after coimmunoprecipitation were not due to more Poldip2 content because the total content of Poldip2 in tissue lysates (input) between groups was similar.

Moreover, immunoprecipitation of tissue lysates with nonspecific goat IgG revealed no expression of Poldip2, Nox4, or p22phox. Because increased IPP induces \(\mathrm{H}_2\mathrm{O}_2\) release, these results suggest that mechanostimulation induces more Poldip2 to associate with Nox4 and p22phox, further enhancing Nox4 activity and \(\mathrm{H}_2\mathrm{O}_2\) production.

Nox inhibition reduces renal excretion after saline loading. Saline loading induced transient increases in the mean arterial blood pressure and IPP of the left kidney (Fig. 5, A and B). Neither vehicle nor Nox blocker had any effect on the pressor responses or rise in IPP due to saline loading. Interestingly, saline loading not only increased ARNA but also enhanced urinary excretion of \(\mathrm{H}_2\mathrm{O}_2\); both of these responses were lowered by Nox inhibition at 5–45 min compared with controls. Nox inhibition significantly also decreased the urinary flow rate at 5–30 min relative to control kidneys (Fig. 5C).

Table 1. Responses of single unit afferent renal nerve activity to mechanostimulation or chemostimulation or to \(\mathrm{H}_2\mathrm{O}_2\)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Basal</th>
<th>During Treatment</th>
<th>Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>IPP (20 mmHg)</td>
<td>3.9 ± 0.2</td>
<td>18.9 ± 2.4*</td>
<td>3.8 ± 0.3</td>
</tr>
<tr>
<td>(\mathrm{H}_2\mathrm{O}_2) (3.0 (\mu)mol/l) after IPP</td>
<td>4.0 ± 0.3</td>
<td>26.3 ± 4.1*</td>
<td>4.6 ± 0.5</td>
</tr>
<tr>
<td>NaCl (0.9 mol/l)</td>
<td>7.5 ± 0.5</td>
<td>32.3 ± 5.0*</td>
<td>7.9 ± 0.4</td>
</tr>
<tr>
<td>(\mathrm{H}_2\mathrm{O}_2) (3.0 (\mu)mol/l) after NaCl</td>
<td>7.4 ± 0.6</td>
<td>9.5 ± 0.8</td>
<td>8.2 ± 0.6</td>
</tr>
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Values are afferent renal nerve activity (ARNA; in counts/10 s) ± SE. Mechanostimulation was induced by raising the intrapelvic pressure (IPP) to 20 mmHg. Intrapelvic infusion of 0.9 mol/l NaCl was used to induce renal chemostimulation \(\times 10^{-10} \text{mol/l}\).

For the treatment groups, positive control samples from leukocytes or the aorta. \(\text{NaCl} (0.9 \text{ mol/l}) \times 10^{-10} \text{mol/l}\) after IPP. NaCl (0.9 mol/l) and NaCl (3.0 mol/l) were compared between groups. Baseline values were 4.2 ± 0.5 and 4.1 ± 0.5 \(\times 10^{-10} \text{mol/l}\) after NaCl-infusion, respectively. Nox inhibition significantly also decreased the urinary flow rate at 5–30 min relative to control kidneys (Fig. 5C). The cumulative urinary output in apocynin- or DPI-treated kidneys was not affected by an increase in IPP (20 mmHg) 3.9 ± 0.3 vs. 2.4 ± 0.3 \(\times 10^{-10} \text{mol/l}\) after NaCl-infusion. The increases in Poldip2 after coimmunoprecipitation were not due to more Poldip2 content because the total content of Poldip2 in tissue lysates (input) between groups was similar.

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Nox inhibition reduces renal excretion after saline loading. Saline loading induced transient increases in the mean arterial blood pressure and IPP of the left kidney (Fig. 5, A and B). Neither vehicle nor Nox blocker had any effect on the pressor responses or rise in IPP due to saline loading. Interestingly, saline loading not only increased ARNA but also enhanced urinary excretion of \(\mathrm{H}_2\mathrm{O}_2\); both of these responses were lowered by Nox inhibition at 5–45 min compared with controls. Nox inhibition significantly also decreased the urinary flow rate at 5–30 min relative to control kidneys (Fig. 5C). The cumulative urinary output in apocynin- or DPI-treated kidneys was not affected by an increase in IPP (20 mmHg) 3.9 ± 0.3 vs. 2.4 ± 0.3 \(\times 10^{-10} \text{mol/l}\) after NaCl-infusion. The increases in Poldip2 after coimmunoprecipitation were not due to more Poldip2 content because the total content of Poldip2 in tissue lysates (input) between groups was similar.

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DPI-treated kidneys was 64.7 ± 9.8% and 56.2 ± 8.1%, respectively, of that in control kidneys (all P < 0.05).

**DISCUSSION**

The present study demonstrated that the presence of the H₂O₂-producing enzyme Nox4 in the renal pelvis is derived from sensory neurons in the dorsal root ganglia and contributes to ARNA activation when IPP increases. As shown in the model in Fig. 6, H₂O₂ augments SP release by enhancing the activity of Ca²⁺-permeable TRPV1 channels. Both TRPV1 and neurokinin-1 receptor activation contribute to increases in ARNA after mechanostimulation. The function of Nox4 in renal mechanosensitive fibers has a profound effect on the reflex control of urinary excretion, because Nox inhibition attenuates H₂O₂ production and diuretic/natriuretic responses in the renal reflex and in response to saline loading.

Using stable and sensitive horseradish peroxidase-based measurements, our results demonstrated that basal levels of H₂O₂ in the pelvic effluent before IPP stimulation and in the urine before saline loading were 15.2 and 53.6 μmol/l, respectively. These levels are comparable with previous findings showing that urinary H₂O₂ is ~25–36 μmol/l (15, 18). Interestingly, changes in the excretion of H₂O₂ are always associated with the diuretic/natriuretic response or the presence of high intrarenal hydrostatic pressure. In other studies, urinary excretion of Na⁺ increased in rats fed a high-salt diet (4% NaCl) for 4 wk and was associated with elevated urinary excretion of H₂O₂ (15). Stepwise elevation of renal perfusion pressure caused parallel increases in the urinary excretion of Na⁺ and H₂O₂ (15). Increased H₂O₂ excretion has been found not only in animal experiments but also in healthy volunteers after salt loading tests (23). Our results are consistent with these findings, as increases in intrarenal pressure were induced by elevating IPP or by saline loading, stimulating renal H₂O₂ excretion (Figs. 3 and 4). We therefore speculate that H₂O₂ triggers diuresis and natriuresis under conditions of high intrarenal pressure.

Basal release of H₂O₂ in the pelvic effluent was not affected here by Nox inhibition (Fig. 3C). This observation raises the

![Figure 3](http://ajprenal.physiology.org/) **Fig. 3.** Effects of Nox inhibition on mechanosensory response. A: original traces of raw ARNA, IPP, and integrated multiunit ARNA of one rat in response to IPP elevations in the presence of vehicle solution (0.05% DMSO, left) or 100 μg/ml apocynin (right). B: grouped data for the effect of IPP on ARNA in the presence or absence of Nox inhibitor [apocynin or diphenyleneiodonium (DPI), n = 8 in each test]. C: the release of H₂O₂ and SP was measured in the pelvic perfusate of the IPP-stimulated (left) kidney during the basal, experimental, and recovery periods. D: the renorenal reflex response was determined by changes in the urinary flow rate and urinary Na⁺ excretion rate in the contralateral (right) kidney. *Significant difference (P < 0.05) compared with the basal period; †significant difference (P < 0.05) compared with the vehicle treatment.

![Figure 4](http://ajprenal.physiology.org/) **Fig. 4.** Poldip2 associates with Nox4 and p22<sub>phox</sub>. A and B: tissue lysates isolated from the rat renal pelvis were used to measure expression (input) of the indicated proteins and immunoprecipitated (IP) with nonspecific goat IgG (as a negative control), Nox4 (A), or p22<sub>phox</sub> antibody and immunoblotted (IB) with Poldip2, Nox4, or p22<sub>phox</sub> antibodies. Representative blots (left) show statistical results for four experiments as the ratio of the indicated proteins to input, Nox4, or p22<sub>phox</sub> in DU. *Significant difference (P < 0.05) compared with the group without IPP treatment.
stains) or before the loss of preloaded Cr51 (14). Moreover, before the loss of membrane integrity (as measured by vital and cytoskeleton remodeling. All of these processes occur close to the renal pelvis. Superoxide is rapidly degraded by superoxide dismutase to form H2O2. Superoxide or H2O2 may therefore also be the source of H2O2 generation and water conservation (15, 46). The renal cortex and medulla have higher superoxide generation by Nox and water conservation (15, 46). The renal cortex and medulla are known to cause vasoconstriction and antinatriuresis, which contribute to salt and water conservation (15, 46). The renal cortex and medulla may therefore also be the source of H2O2 generation and release into the urine at baseline. Contrary to previous findings, the present results demonstrated a diuretic/natriuretic effect of H2O2. This finding can potentially be explained by H2O2 generated from different sources, leading to different tissue responses.

One may ask whether H2O2 can freely penetrate the cell membrane. The present study did not test this possibility, but a previous study (14) showed that H2O2 diffuses freely into cells, whereupon it induces several biological effects, including intracellular ATP production, redox reactions, Ca2+ utilization, and cytoskeleton remodeling. All of these processes occur before the loss of membrane integrity (as measured by vital stains) or before the loss of preloaded Cr51 (14). Moreover, another previous study (9) examining the mechanism underlying H2O2 diffusion showed that Nox-induced H2O2 easily diffuses across the cell membrane via aquaporins after dismutation from superoxide. Various types of aquaporins are present in the mammalian kidney, where they regulate water reabsorption and maintain body fluid balance. Furthermore, aquaporins are also expressed in the peripheral nerve fibers that project into the dorsal root and are possibly involved in pain sensation (3). Taken together, these findings suggest that H2O2 can leach out of sensory nerve endings in the renal pelvis. However, it is unclear whether H2O2 affects the renal sensory response via aquaporins.

Nox4, first described as Renox, requires the p22#phox membrane subunit for constitutive ROS production (34). The low expression of Nox2 (gp91#phox) and abundance of Nox4, as well as its regulatory components p22#phox and Poldip2, indicate that the dominant isof orm responsible for H2O2 production in the renal pelvis is Nox4. The reason for the reduction in the expression of Nox4 and associated proteins after DRX is probably that these proteins are produced in the cell bodies of sensory neurons at the dorsal root ganglion. It is possible that these proteins are then transported to the peripheral ends of renal sensory nerves and into the renal pelvis via an axonal transport pathway. Studies have shown that both Nox4 and p22#phox are present at dorsal root ganglia, where they colocalize with sensory markers, such as calcitonin gene-related peptide or SP (5, 16, 21). A previous study (20) has also demonstrated that DRX reduces the basal level of SP or the PGF2-induced release of SP from an isolated renal pelvic wall preparation. Because decreases in the expression of Nox4 and associated proteins were observed after DRX treatment (Fig. 2C), we speculate that these proteins in the renal pelvis originate from sensory neurons located at dorsal root ganglia. However, our results show that DRX does not affect the expression of Nox2 (Fig. 2C); this is possibly due to the absence of Nox2 from the peripheral ends of renal sensory nerves. Further studies are required to examine the exact location of Nox2 in the renal pelvic wall.

Nox proteins have been proposed to be sensitive to mechanical stimuli or to act as mechanotransducers (1). The most typical example is that endothelial Nox4 can be activated by...
oscillatory mechanical stress, which generates ROS to regulate vascular tone (10). In the kidney, Nox present in tubular cells also responds to increased luminal flow and generates ROS (13). Mechanical strain applied to mesangial cells isolated from glomeruli activates Nox within 30 s (44). Consistent with these results, IPP elevation was shown here to stimulate renal pelvic Nox4, which may act as a mechanosensor. The increased activity/function of Nox4 was previously thought to be upregulated by cytokines or ANG II (34, 36, 40). Recently, Lyle et al. (26) provided elegant evidence that Nox4 activity decreased by cortical actin increased cytoskeletal remodeling and cell adhesion (32). The study presented a consistent result, whereby Poldip2 physically associated with Nox4 and/or p22phox in the renal pelvis (Fig. 4). After mechanostimulation (caused here by IPP elevation), the enhanced association of Poldip2 with p22phox went on to activate Nox4. This sequence of events may have resulted in H2O2 generation for ARNA activation. Renal pelvic mechanostimulation, however, did not affect Nox4 or p22phox expression when the two factors were coimmunoprecipitated with each other, suggesting that p22phox constitutively interacts with Nox4.

Rising IPP elicited a similar response in terms of increases in ARNA and SP release as that induced by perfusing H2O2 directly into the renal pelvis (Figs. 1A and 3C). The effect of H2O2 on the sensory response was first confirmed by catalase treatment to determine the effect of oxygen radicals and then by Nox inhibition to inhibit the production of H2O2 (Figs. 1A and 3). This indicates that SP release and ARNA activation caused by rising IPP or H2O2 perfusion are Nox dependent. The functional link between Nox/H2O2 and the sensory response was further confirmed by the colocalization of Nox4 and the sensory neuronal marker SP (Fig. 2B). Because SP release is Ca2+-dependent (6), an increase in intracellular Ca2+ may be downstream of H2O2 in ARNA activation.

We previously showed that Ca2+-permeable TRPV1 is present in the renal pelvis and that inhibition of TRPV1 completely abolished SP release (8). H2O2 is therefore suggested to increase TRPV1 activity, which may, in turn, cause Ca2+ influx and stimulate SP release. The colocalization of TRPV1 and H2O2-producing Nox4 in the renal pelvic wall (Fig. 2B) as well as the TRPV1 inhibition-mediated attenuation of H2O2 production and SP release (Fig. 1A) support this hypothesis. However, the mechanism by which H2O2 potentiates TRPV1 activity for renal sensory activation was not explored in the present study. In TRPV1-transfected human embryonic kidney-293T cells, H2O2 was reported to strongly potentiate heat-evoked activation of TRPV1 (38). Moreover, previous investigations have demonstrated that TRPV1 is a redox-sensitive molecule (41, 42). Together, these results suggest that the activity of TRPV1 channels in the renal pelvis can be enhanced by H2O2.

In conclusion, our results clearly show that Nox4 responds to changes in hydrostatic pressure in the renal pelvis by producing H2O2 to stimulate TRPV1 for increases in SP release and ARNA. H2O2-induced ARNA activation is essential for the renal mechanoreceptor-mediated renorenal reflex that controls the excretory response during extracellular fluid expansion. Understanding the role of H2O2 in the detection of pressure changes by renal afferent nerves may reveal why the kidneys become unresponsive to fluid retention and provide a novel therapeutic strategy to relieve abnormal fluid retention.

GRANTS
This work was supported by Chi-Mei Medical Center Grant CMFJ10107, Cardinal Tien Hospital Grants CTH-99-1-2A42 and 700179, and Ministry of Science and Technology in Taiwan Grant NSC98-2314-B-030-004-MY3.

DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS
Author contributions: C.-S.L., S.-H.L., H.-S.H., Y.-S.C., and M.-C.M. conceived and designed the research; C.-S.L., S.-H.L., and M.-C.M. performed the experiments; C.-S.L., S.-H.L., H.-S.H., Y.-S.C., and M.-C.M. contributed new reagents or analytic tools; C.-S.L., S.-H.L., H.-S.H., Y.-S.C., and M.-C.M. analyzed the data; C.-S.L., S.-H.L., H.-S.H., Y.-S.C., and M.-C.M. wrote the manuscript; C.-S.L., S.-H.L., H.-S.H., Y.-S.C., and M.-C.M. edited and revised the manuscript.

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