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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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 (24, 46). The catalytic moiety of Nox4 is homologous to gp91phox (or Nox2), which is found in phagocytes (34, 40). Most Nox activation requires an association with cytosolic p47phox, p67phox, or the small GTPase Rac (34, 40). Nox4, however, is constitutively active in a reconstituted system containing p22phox and produces H$_2$O$_2$ (32, 40). Moreover, a recent study (26) showed that polymerase 6-interacting protein 2 (Poldip2) interacts with p22phox 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 p22phox, as well as those of other Nox proteins, are expressed in dorsal root ganglion neurons at T13–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,
TRPV1. Therefore, we tested the direct effects of H$_2$O$_2$ on Nox4 in the renal pelvis may stimulate sensory nerves via PE-10 tube was used for saline perfusion at the same rate and pH

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.

Effects of Nox inhibition on the mechanosensory response and renorenal reflex. Raising IPP elicits mechanosensory activation of ARNA and the renorenal reflex (8, 27–30). IPP was consecutively elevated to 5, 10, 20, 40, and 60 mmHg for 1 min at each pressure. The experimental treatment was bracketed by a 10-min baseline and a 10-min recovery period. Vehicle (0.05% DMSO, Sigma), cell-permeable apocynin (100 μg/ml, Sigma), or diphenyleneiodonium (DPI; 20 μg/ml, Sigma) was administered from the initial baseline throughout the test ($n = 8$ for each test). Pelvic effluent was collected to measure the release of H$_2$O$_2$ and SP. The contralateral urine was also collected to determine the urinary flow rate and Na$^+$ excretory rate (27–30). The doses for the blockers were chosen based on their IC$_{50}$ values.

Saline loading. Saline was perfused intravenously in amounts equal to 3% of the body weight over a 10-min period to produce a mild volume expansion (27–30). Apocynin (100 μg/ml) or DPI (20 μg/ml) was perfused via the intrapelvic route, as described above, at 10 min before saline loading and throughout the experiment ($n = 8$). Renal pelvic effluent from the left kidney and urine from the contralateral kidney were collected to determine the urinary H$_2$O$_2$ excretory rate and flow rate, respectively.

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$_9$–L$_1$ 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 p22$^{phox}$, 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-21860, 1:2,500), p22$^{phox}$ (sc-11712, 1:2,000), Poldip2 (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, p22$^{phox}$, and Poldip2).

Indirect immunofluorescence was used to examine the distribution of Nox4 and the colocalization of Nox4, SP (sc-58591), or TRPV1 (sc-28759) in the renal pelvis (8, 28). Briefly, the kidneys were postfixed, embedded, and prepared as 5-μm sections on a cryostat (Micron, 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, 1:1,000 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 multuniunit 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
edly increased the release of SP and H$_2$O$_2$, but this observation was not shown). DRX did not affect Nox2 expression in the renal pelvic wall.

Basic data, such as body weight and daily intake of food and water, were similar between DRX and sham-operated rats (data not shown). DRX did not affect Nox2 expression in the renal pelvis but significantly attenuated the expression of Nox4, p22$^{phox}$, and Poldip2 (Fig. 2C).

**Nox inhibition attenuates the mechanosensory response.** A typical ARNA recording in response to consecutive increases in IPP is shown in Fig. 3A. The nerve firing and integrated ARNA response increased by elevating IPP were substantially attenuated by the presence of apocynin compared with the vehicle control treatment. IPP-mediated ARNA increases were completely blocked by apocynin or DPI at IPP in the range of 5–20 mmHg, and significant increases were only seen at 40 and 60 mmHg (Fig. 3B). The consecutive elevations in IPP markedly increased the release of SP and H$_2$O$_2$, but this observation was greatly attenuated by Nox inhibition (Fig. 3C). Elevation of IPP in the left kidney caused diuresis and natriuresis in the contralateral kidney; however, this reflex was abolished by Nox blockers (Fig. 3D).

**Poldip2 association with Nox4 and p22$^{phox}$ in the renal pelvis.** Previous work showed that Nox4 activation requires Poldip2 association with p22$^{phox}$ in vascular smooth muscle cells (26). Therefore, we examined a potential association of Nox proteins for constitutive Nox4 activation in the renal sensory response. Lysates of renal pelvis tissues were immunoprecipitated with Nox4- or p22$^{phox}$-specific antibodies and then immunoblotted with an antibody against Poldip2. The results showed that Poldip2 coimmunoprecipitated with Nox4 (Fig. 4A) and p22$^{phox}$ (Fig. 4B) and that the expression of Poldip2 was higher in renal pelvic tissues exposed to an increase in IPP than in tissues not exposed to IPP. However, expression of Nox4 or p22$^{phox}$ was not affected by an increase in IPP. The decreases 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 p22$^{phox}$. Because increased IPP induces H$_2$O$_2$ release, these results suggest that mechanostimulation induces more Poldip2 to associate with Nox4 and p22$^{phox}$, further enhancing Nox4 activity and H$_2$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 H$_2$O$_2$ (Fig. 5B). Perfusion of apocynin or DPI into the renal pelvis significantly attenuated the increases in ARNA and urinary excretion of H$_2$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 5–10% less than that in saline-infused sham kidneys (Fig. 5D). Moreover, measurement of urinary excretion of H$_2$O$_2$ revealed this effect.

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**Table 1. Responses of single unit afferent renal nerve activity to mechanostimulation or chemostimulation or to H$_2$O$_2$**

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<th>Treatment</th>
<th>Basal</th>
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<td>IPP (20 mmHg)</td>
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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 (n = 6 in each test). *Significant difference (P < 0.05) compared with basal activity.

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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 renorenal 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

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**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.

**Fig. 4. Poldip2 associates with Nox4 and p22phox.** 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 p22phox (B) antibody and immunoblotted (IB) with Poldip2, Nox4, or p22phox antibodies. Representative blots (left) show statistical results for four experiments as the ratio of the indicated proteins to input, Nox4, or p22phox in DU. *Significant difference (P < 0.05) compared with the group without IPP treatment.
possibility that basal H2O2 release may be derived from Nox activity in tissues upstream of the renal pelvis. An earlier fluorescence spectrophotometric assay revealed that the renal cortex and medulla have higher superoxide generation by Nox than what is found in the renal papilla (46), a tissue region close to the renal pelvis. Superoxide is rapidly degraded by superoxide dismutase to form H2O2. Superoxide or H2O2 generation in 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 p22phox membrane subunit for constitutive ROS production (34). The low expression of Nox2 (gp91phox) and abundance of Nox4, as well as its regulatory components p22phox and Poldip2, indicate that the dominant isoforum 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 p22phox 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 PGE2-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 originated 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

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Fig. 5. Effects of Nox inhibition on the renal response to saline loading. Saline was infused intravenously for 10 min, as indicated by the horizontal bars. Apocynin (left) or DPI (right) was infused by the intrapelvic route at 10 min before saline loading. A and B: changes in mean arterial blood pressure (MABP; A); IPP, multiunit ARNA (calculated as a percentage of the time 0 level), and urinary H2O2 excretion rate (UH2O2V; B) were determined. C: urine was collected to determine UV. Significant difference (P < 0.05) between the two treatments.

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Fig. 6. Schematic diagram depicting Nox4-mediated signaling mechanisms in renal sensory activation. Upon mechanostimulation through an increase in IPP, activation of Nox4 in the sensory nerve terminals generates H2O2, which, in turn, activates TRPV1 and increases SP release into the pelvic space, possibly via a Ca2+ influx. SP then activates neurokinin (NK)-1 receptors and generates ARNA.
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 may be downstream of H2O2 in 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-activated evocation 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 renalorex 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.

REFERENCES

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