Detection of apical Na\(^+\)/H\(^+\) exchanger activity inhibition in proximal tubules induced by acute hypertension

KAY-PONG YIP,1 ANTHONY J. WAGNER,2 AND DONALD J. MARSH2
1Department of Physiology and Biophysics, University of South Florida, Tampa, Florida 33612; and 2Department of Molecular Pharmacology, Physiology and Biotechnology, Brown University, Providence, Rhode Island 02912

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Yip, Kay-Pong, Anthony J. Wagner, and Donald J. Marsh. Detection of apical Na\(^+\)/H\(^+\) exchanger activity inhibition in proximal tubules induced by acute hypertension. Am J Physiol Regulatory Integrative Comp Physiol 279: R1412–R1418, 2000.—We previously showed that acute arterial hypertension induces an inhibition of fluid and NaCl reabsorption in proximal tubules of Sprague-Dawley rats, which is associated with a rapid reversible internalization of apical Na\(^+\)/H\(^+\) exchanger in brush border. To determine whether there is a corresponding inhibition of apical Na\(^+\)/H\(^+\) exchanger activity in proximal tubules to account for the reduced tubular reabsorption, an instrument capable of measuring intracellular pH (pHi) ratiometrically and repeatedly on the surface of kidney with high temporal resolution is required. We report the design and validation of such a fluorimetric system based on two ultraviolet nitrogen-pulsed lasers and a photomultiplier. pHi of proximal tubules in situ was measured with pH-sensitive fluorescence dye 2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein at 5 Hz. Using the initial rate of change of pHi (dpHi/dt) during luminal Na\(^+\) removal as an index of apical Na\(^+\)/H\(^+\) exchanger activity, the exchanger activity was found to be reduced by 52 ± 11% (n = 14, P < 0.05) compared with the baseline after 20 min of induced acute hypertension. The inhibition of Na\(^+\)/H\(^+\) exchange activity was alleviated when the blood pressure was returned to prehypertensive level. These observations indicate that acute changes in arterial pressure can reversibly inhibit apical Na\(^+\)/H\(^+\) exchanger activity, which might contribute to pressure natriuresis in proximal tubule.

Intracellular pH, 2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein, pressure natriuresis, microperfusion

AN ACUTE BLOOD PRESSURE INCREASE inhibits fluid and NaCl reabsorption by rat proximal tubules (5, 6) and is associated with a rapid inhibition of basolateral Na\(^+\)-K\(^+\)-ATPase activity in fractionated membranes of renal cortex (20). These observations imply that blood pressure can regulate proximal tubular reabsorption by modulating the activity of Na\(^+\) transporters. This response probably contributes to the natriuresis and diuresis that occurs when blood pressure rises, as well as provides the error signal required by tubuloglomerular feedback for renal autoregulation (5). Regulation of basolateral Na\(^+\)-K\(^+\)-ATPase is certainly a means for regulating tubular reabsorption, but there must be an associated change in the flux of Na\(^+\) across the brush-border membrane to maintain a constant cell volume. Ninety percent of NaCl transcellular reabsorption and 70% of NaHCO\(_3\) reabsorption in proximal tubules is mediated by apical Na\(^+\)/H\(^+\) exchangers (3). We hypothesize that inhibition of proximal reabsorption should involve not only the activity of basolateral Na\(^+\)-K\(^+\)-ATPase but also the apical Na\(^+\)/H\(^+\) exchangers. We tested this hypothesis by using 2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF) to measure the proximal tubular intracellular pH (pHi) and using the initial rate of change (dpHi/dt) during luminal Na\(^+\) removal as an index of apical Na\(^+\)/H\(^+\) exchange activity. The dpHi/dt is an index of apical Na\(^+\)/H\(^+\) exchange activity because removal of luminal Na\(^+\) inhibits the transporters and acidifies the cell. To make reliable measurements of apical Na\(^+\)/H\(^+\) exchange activity repeatedly and to perform calibration on the same proximal tubule, there is a need for an instrument to measure the pHi ratiometrically with high temporal resolution and minimal photobleaching. We have developed such a system based on two ultraviolet (UV) nitrogen-pulsed lasers and a photomultiplier interfaced with two analog-to-digital (A/D) converter boards.

The work reported here has involved the design and validation of a pulse laser-based system to measure pHi in proximal tubules in situ. In vivo studies using this device indicate the apical Na\(^+\)/H\(^+\) exchange activity is reversibly inhibited in proximal tubules during acute arterial hypertension.

METHODS

Animal preparation. Experiments were performed in male Sprague-Dawley rats, 250–300 g body wt. All rats were purchased from Harlan. The rats had free access to food and tap water before the experiments. Anesthesia was induced by placing each rat in a chamber containing 5% halothane administered in 25% oxygen and 75% nitrogen through a Fluotec Mark-3 vaporizer. A tracheostomy was performed.
and the rats were placed on a servocontrolled heated operating table that maintained body temperature at 37°C. The tracheostomy tube was connected to a small animal respirator (Harvard model 683) adjusted to maintain arterial blood pH between 7.35 and 7.45 with a mixture of 25% oxygen-75% nitrogen. Tidal volume ranged from 1.9 to 2.5 ml, depending on body weight, with a frequency of 57–60 breaths/min. The final concentration of halothane needed to maintain sufficient anesthesia was approximately 1%. A polyethylene catheter (PE-50) was placed in the right jugular vein for infusions. After a priming dose of smooth muscle vasorelaxant (Pancuronium, 1 mg/kg body wt) in 1 ml 0.9% saline, a continuous infusion of Pancuronium (1 mg·kg⁻¹·hr⁻¹) in 0.9% saline was given at 20 μl/min. The left kidney was exposed through a flank incision, immobilized with a Lucite ring, and superfused with saline preheated at 37°C. The renal capsule was left intact. Arterial pressure was measured in the left carotid artery with a Statham-Gould P23dB pressure transducer connected to a transducer amplifier (TBM4, WPI).

A bolus of Lissamine Green dye was injected intravenously to identify early segments of proximal tubules. Early proximal tubules were selected for observation only if they had a long segment that ran entirely on the surface, which permitted insertion of a perfusion pipette for orthograde and retrograde perfusion. After a suitable proximal tubule was identified, the tubule was loaded with a pH-sensitive fluorescence dye BCECF/AM (50 μg/ml, Molecular Probes) in synthetic tubular fluid at 20 nl/min for 20 min with a micropipette (3- to 5-μm outer diameter) coupled to a micropump. The loading solution was perfused into the ambient tubular flow. Experiments started after a waiting period of 30 min for deesterification of the dye.

An acute increase in blood pressure was induced by increasing total peripheral vascular resistance, as suggested by Roman and Cowley (13) and Yip et al. (16). Silk ligatures were placed around the superior mesenteric and celiac arteries and around the abdominal aorta caudal to the left renal artery. Ligatures were released after the induction of acute hypertension when arterial pressure was required to be returned to the baseline level.

Detection system. The detection setup is shown schematically in Fig. 1. Excitation and emission to and from the kidney surface were through a Zeiss Ultrafluar objective (×100/0.25) mounted on a modified Leitz Stophot compound microscope equipped for epifluorescence. Fluorescence excitation was via two computer-controlled pulsed nitrogen lasers (Laser Science) that emit at 337 nm and have a pulse duration of 3 ns. Output from each laser is first coupled to a tunable dye control module holding a quartz cuvette filled with UV laser dye (Excite 435 and 485, Exiton). The light emerging from the tunable dye control modules has peaks at 435 nm and 500 nm, respectively, with a bandwidth of 5 nm. The intensity of each laser is adjustable. Mounted directly to the dye control modules were adjustable fiber-optic mounts with collimators that couple the light into a fused silica optical fiber with a core size of 400 μm. The two optical fibers combine into a single common transmission fiber with a core size of 600 μm. The optical fiber is coupled to a fused silica focusing lens attached to the epifluorescence housing on the Leitz microscope. The focusing lens passes the light to a dichroic mirror (510 DCLP, Omega Optical) and focuses a laser spot at the back focal plane of the objective, which focuses the laser spot onto the selected loop of proximal tubules. The size of the laser spot on the kidney is adjustable via a diaphragm in front of the focusing lens. A laser spot size ~100 μm was routinely used. Fluorescence emission from the kidney surface passes back through the dichroic mirror and a band-pass filter (530/25 nm, Omega Optical). The intensity of emission from each excitation pulse was collected with a Hamamatsu photomultiplier sequentially, and the current in the photomultiplier was integrated numerically and stored digitally. The emission ratio (500/435) is calculated after background subtraction at each wavelength. Emission ratio was acquired at 5 Hz in the present study. The duration of each laser pulse is 3 ns. The time delay between the 435 and 500 nm excitation pulses is 4.1 ns. The current on the photomultiplier triggered by each excitation laser pulse was sampled at 380 kHz for 39.5 μs and integrated numerically. The duration and intensity of emission pulses were monitored continuously in a digital oscilloscope (Tektronix).

Computer interface. All software was written in the C programming language. The primary task of the program is to coordinate the firing of the lasers, sampling the output of photomultiplier and arterial blood pressure, and displaying of these variables on a monitor. In brief, the program performs sequential digital-to-analog conversion to trigger the firing of the lasers and A/D conversion to sample the current on the photomultiplier with a DT2839 A/D board (Data Translation). The DT2839 board has internal timers and is capable of sampling a single channel at up to 1 MHz. The program also drives a DT2801 A/D board (Data Translation), which samples the arterial pressure and the timing of luminal perfusion. All digitized variables are stored in the same data file. The emission ratio and arterial pressure are displayed in real time on a monitor. All A/D boards are housed in a 66-MHz microcomputer.

Experiment protocols. Three series of studies were performed. The first set of experiments was to validate the ratiometric pH measurement. Synthetic tubular fluid containing 25 mM NH₄Cl was perfused in a retrograde direction (40 nl/min) into the proximal tubules to alkalize the cells rapidly. The emission intensity at 435 (pH insensitive) and 500 nm (pH sensitive) excitation was monitored. The second set of experiments was designed to test whether this system is sensitive enough to detect the intracellular acidification during luminal Na⁺ removal and whether the acidification is reproducible. Luminal Na⁺ was removed repeatedly by retrograde perfusion of Na⁺-free synthetic tubular fluid (40 nl/min). The third set of experiments was to determine whether apical Na⁺/H⁺ exchange activity is inhibited during acute hypertension and whether the inhibition is reversible.
Change in pHi during Na\textsuperscript{+} luminal removal was determined in the same tubule during the prehypertensive, acute hypertensive, and posthypertensive period. Measurements were made in the control period, 10 and 20 min after induced acute hypertension, and 10 and 20 min after the relief of acute hypertension. Only one tubule was studied from each rat. Acute hypertension was induced only once in each rat. Calibration of pHi was performed in the same tubule at the end of each experiment using the high-potassium nigericin technique (14, 17).

**Data analysis and statistics.** The emission ratios corrected for background fluorescence were converted to pH value according to the calibration parameters obtained in the same tubule. The initial rate of change in pH\textsubscript{i} (dpHi/dt) induced by luminal removal of Na\textsuperscript{+} was calculated by fitting the time course of decrease in pH\textsubscript{i} into a first-order differential equation with a nonlinear algorithm using BMDP statistical package, program 3R (7). The initial pHi before luminal Na\textsuperscript{+} removal and the minimum of pH\textsubscript{i} during luminal Na\textsuperscript{+} removal were also determined from each time course. Results are reported as means ± SE. Paired Student’s t-test was used to determine whether dpHi/dt measured after acute hypertension is significantly different from that measured at control period. Values were considered significant at P < 0.05. Similar paired t-tests were performed for mean blood pressure and other variables.

**Solutions.** Synthetic proximal tubular fluid used contains (in mM) 127 NaCl, 25 NaHCO\textsubscript{3}, 3 KCl, 1 MgSO\textsubscript{4}·7H\textsubscript{2}O, 1 K\textsubscript{2}HPO\textsubscript{4}, 5 urea, and 1.8 CaCl\textsubscript{2}. Sodium was replaced with choline in Na\textsuperscript{+}-free synthetic proximal tubular fluid. The solution to establish an NH\textsubscript{3} gradient was prepared by replacing 25 mM NaCl with 25 mM NH\textsubscript{4}Cl (14). The calibration solution was composed of (in mM) 140 KCl, 2 K\textsubscript{2}HPO\textsubscript{4}, 1 MgSO\textsubscript{4}·7H\textsubscript{2}O, 1 CaCl\textsubscript{2}, 10 glucose, 10 HEPES, and 10 μM nigericin (14). pH of the calibration solution was adjusted to 6.8, 7.2, and 7.6 using N-methyl-glucamine.

**RESULTS**

A rapid alkalization in proximal tubular cells followed by a slow recovery phase of acidification was detected when 25 mM NH\textsubscript{4}Cl was infused into the proximal tubules (Fig. 2A). Stopping infusion of NH\textsubscript{4}Cl containing synthetic tubular fluid led to an acidification overshoot as reported by other investigators (14). The increase of the pH\textsubscript{i} was reflected by the corresponding increase of the 500- to 435-nm emission ratio. The increase of ratio is due to the increase of emission at the pH-sensitive excitation (500 nm). The emission at 435 excitation remains largely unchanged as expected (Fig. 2B). These observations confirmed that our pulsed laser system is capable of measuring pH ratio-metrically. Infusion of NH\textsubscript{4}Cl-free tubular fluid into the proximal tubule did not elicit a change in the emission ratio (data not shown).

On removal of luminal Na\textsuperscript{+}, the pHi of the proximal tubule was acidified immediately and reached the minimal pH\textsubscript{i} within 10 s (Fig. 3). The pH\textsubscript{i} started to recover from the minimal pH\textsubscript{i} despite continuous infusion of Na\textsuperscript{+}-free synthetic tubular fluid. The immediate acidification indicates that apical Na\textsuperscript{+}/H\textsuperscript{+} exchange activity was inhibited. The transient accumulation of H\textsuperscript{+} caused the intracellular acidification as predicted. The initial dpHi/dt is used as an index of the apical Na\textsuperscript{+}/H\textsuperscript{+} exchange activity in vivo. Stopping the retrograde perfusion and allowing the ambient tubular flow to resume led to an immediate alkalinization, which returned the pH\textsubscript{i} to the baseline level. These observations suggest that the inhibition of apical Na\textsuperscript{+}/H\textsuperscript{+} exchange activity by luminal removal of Na\textsuperscript{+} is reversible, and the recovery process is complete within minutes after the resumption of ambient tubular flow. Luminal Na\textsuperscript{+} was removed a second time after 1 min of ambient tubular flow was resumed in the same tubule (Fig. 3). Similar profiles of

![Fig. 2. Time course of changes in pH\textsubscript{i} (A) and intensity of emission fluorescence at each excitation wavelength (B) from a proximal convoluted tubule when 25 mM NH\textsubscript{4}Cl was introduced into the lumen. C: mean time course of changes in pH\textsubscript{i} from 5 tubules. The bottom tracing in each graph indicates the on and off of the intratubular perfusion, 40 nl/min. Dotted lines in C indicate SE.](http://ajpregu.physiology.org/Download)
The proximal tubule is known to play a major role in initiating pressure natriuresis when arterial pressure is increased acutely (5, 10). It has been shown that the basolateral Na\(^+\)/K\(^+\)-ATPase activity of the proximal tubule is inhibited by acute hypertension in fractionated membranes (20). We hypothesized that the apical Na\(^+\)/H\(^+\) exchange activity is also inhibited in acute hypertension. We tested this hypothesis by developing a pulse laser-based fluorometry system to measure the initial dpH/dt in proximal tubules ratiometrically during luminal removal of Na\(^+\) and used it as an index of the apical Na\(^+\)/H\(^+\) exchange activity in vivo. The dpH/dt during luminal Na\(^+\) removal has been measured using BCECF by monitoring the pH-sensitive emission only (2, 12). It was demonstrated that the rapid acidification during luminal Na\(^+\) removal is due to an inhibition of Na\(^+\)/H\(^+\) exchanger and that the acidification is amiloride sensitive (2, 12). In the present study, we successfully developed a fluorometry system based on pulsed lasers to measure pH at 5 Hz ratiometrically by monitoring the emission of BCECF at both pH-sensitive and -insensitive wavelengths. Ratiometric measurement is preferred to the nonratiometric measurement because the former is independent of changes in fluorescence dye concentration. Changes of fluorescence dye concentration might occur due to changes in cell volume, photobleaching, and leaking of the fluorescence dye. Using pulsed laser as the source of excitation (3 ns/pulse) not only provides sufficient temporal resolution for ratiometric measurement but also minimizes the photobleaching of BCECF during data acquisition. Ratiometric measurement is particularly important for the present study because Na\(^+\)/H\(^+\) exchange activity was measured up to five times over a period 45 min, plus the calibration procedure at the end of the experiment. To the authors’ best knowledge, this is the first report of ratiometric measurement of tubular pH\(_i\) in vivo with this level of temporal resolution.

The mean dpH/dt and ΔpH\(_i\) in the hypertensive state were decreased compared with the prehypertensive baseline (Table 1), which were consistent with our hypothesis that acute hypertension inhibits the apical Na\(^+\)/H\(^+\) exchange activity. Returning the blood pressure to prehypertensive level by releasing arterial ligatures restored the dpH/dt and ΔpH\(_i\) induced by luminal Na\(^+\) removal, which indicates the inhibition is reversible. Although there was no difference in the mean dpH/dt and ΔpH\(_i\), the time course of cellular acidification induced by luminal Na\(^+\) removal after the release of hypertension seemed to be a two-phase process: an initial fast component followed by a slow component. Similar observations were found in each tubule. The mean dpH/dt, ΔpH\(_i\), and blood pressure at different time points were tabulated in Table 1.
The application of this fluorometry system to measure pH$_i$ in proximal convoluted tubules was validated by using NH$_4$Cl to induce alkalinization and acidification in the tubules. The observation is comparable to other studies reported in rat proximal tubules (12, 14). There is a transient decrease of emission at 435-nm excitation when NH$_4$Cl solution was introduced into the lumen (Fig. 2). Similar observation was also detected in rabbit cortical collecting duct by using confocal fluorescence microscopy (17). It is probably a reflection of a change in cell volume as NH$_3$ entered the proximal tubular cells. Changes in cell volume could also be due to the retrograde intratubular perfusion. However, no decrease of emission intensity occurred when NH$_3$-free perfusate was infused into tubule (data not shown).

One assumption in the present study is that the dpH$_i$/dt induced by luminal Na$^+$ removal can be measured repeatedly. The pH$_i$ of tubules recovered within minutes to the baseline when the ambient tubular flow was assumed after luminal Na$^+$ removal. Similar dpH$_i$/dt was found when luminal Na$^+$ removal was

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**Fig. 5.** Time course of changes in pH$_i$ of a proximal convoluted tubule during luminal Na$^+$ removal. Control period (A), 10 min (B) and 20 min (C) after induced acute hypertension, and 10 min (D) and 20 min (E) after the relief of acute hypertension. The bottom tracing in each plot indicates the duration of luminal Na$^+$ removal. The corresponding mean arterial blood pressures are 102, 131, 130, 103, and 101 mmHg, respectively.
applied repeatedly to the same tubules (Fig. 3). These observations indicate that dpH/dt can be estimated repeatedly and is a robust measurement of the apical Na⁺/H⁺ exchange activity in proximal tubules. Therefore any difference in dpH/dt observed between the prehypertensive baseline and acute hypertension is not due to the effects of multiple removal of luminal Na⁺ but to the effects of acute hypertension. By using dpH/dt as an index of apical Na⁺/H⁺ exchange activity, we found that Na⁺/H⁺ exchange activity is indeed reduced by acute hypertension by ~40% after 10 min of hypertension. This inhibition is not a transient response. Similar inhibition was also detected after 20 min of hypertension. The magnitude of inhibition in dpH/dt during removal of luminal Na⁺ is significantly less in acute hypertension than prehypertensive control, which indicates that the decrease in dpH/dt is due to an inhibition of Na⁺/H⁺ exchange activity but not to an increase of intracellular buffering capacity. Theoretically, an increase of buffering capacity can reduce dpH/dt. However, changes in cell-buffering capacity have no effect on the steady-state cellular pH (1). The inhibition of Na⁺/H⁺ exchange activity induced by acute hypertension can be due to degradation of the proteins, covalent modification, or protein trafficking (18, 20). The apical Na⁺/H⁺ exchange activity in proximal tubules is mediated mainly by the Na⁺/H⁺ exchanger isoform 3 (NHE3) (15). Western immunoblotting in fractionated membranes isolated from the renal cortex did not detect any significant difference in the total amount of NHE3 between the control rats and rats with induced acute hypertension (20). Twenty minutes of acute hypertension induces a redistribution of NHE3 from the brush-border plasma membrane to subcellular regions behind the brush border (18). All these observations suggest that acute hypertension inhibits apical Na⁺/H⁺ exchange activity by triggering a trafficking of NHE3 from the brush-border plasma membrane to a putative intracellular store. The apical Na⁺/H⁺ exchange activity is reduced because fewer NHE3 molecules reside on the brush border, although change of Na⁺/H⁺ exchange activity due to additional covalent modification of NHE3 cannot be excluded (8). If the inhibiting of apical Na⁺/H⁺ exchange activity is a physiological regulatory process in pressure natriuresis, it is expected that the inhibition should be reversible. It is well-established that mammalian pressure is not steady but fluctuates spontaneously over a wide range of frequency and amplitude (9, 11). Acute hypertension does occur spontaneously in the daily fluctuations of arterial blood pressure. Our observations indicate that releasing the arterial ligatures not only relieves acute hypertension, but it also restores apical Na⁺/H⁺ exchange activity. The apical Na⁺/H⁺ exchange activity was fully recovered at 10 min after the relief of hypertension. The recovery of exchange activity is not a transient phenomenon but longlasting. Similar dpH/dt was found at 20 min after the relief of hypertension (Table 1). The reversible inhibition of apical Na⁺/H⁺ exchange activity found in situ is consistent with the observations from fractionated membranes (19), in which redistribution of NHE3 from brush border into putative intracellular stores induced by acute hypertension was reversed by relieving the hypertension. These observations indicate that acute hypertension inhibits apical Na⁺/H⁺ exchange activity by removing apical NHE3 from brush border, which is reinserted into the brush border when hypertension is relieved.

To test whether the inhibition of Na⁺/H⁺ exchange activity induced by acute hypertension will result in acidification in nonobstructed proximal convoluted tubules, we monitored the pH of proximal convoluted tubules continuously during the induction of acute hypertension in four rats. There was no change in pH before or after the induction of hypertension (data not shown). It is probably because the onset of acute hypertension to inhibit the Na⁺/H⁺ exchange activity is slower than that of luminal Na⁺ removal. The rapid compensatory action of other pH regulatory mechanisms conceals the acidification. Proximal tubular cells are capable of regulating their pH very effectively (2). Recovery of pH was observed even in the presence of 25 mM NH₄Cl (Fig. 2) or in the absence of Na⁺ in lumen (Fig. 3).

In summary, we developed a fluorometry system based on a pair of UV nitrogen-pulsed lasers to measure the apical Na⁺/H⁺ exchange activity of proximal tubules in vivo. We found that apical Na⁺/H⁺ exchange activity is reversibly inhibited during induced acute hypertension, which is consistent with the notion that Na⁺/H⁺ exchange activity is regulated via protein trafficking of NHE3 by blood pressure. The present study...
confirms that there is a parallel regulation of the activity of apical Na$^+/H^+$ exchange and basolateral Na$^+-K^+$-ATPase in proximal tubules in acute hypertension. These cellular responses in proximal tubules will contribute to the increased delivery of NaCl to the distal nephron and initiate the process of pressure natriuresis.

**Perspectives**

It has been proposed that pressure natriuresis is mediated by elevations of renal interstitial pressure, which reduces the net tubular reabsorption of sodium and water (4). The present study demonstrates that pressure natriuresis also involves active regulation of apical Na$^+/H^+$ exchange activity in proximal tubules. Parallel regulation of basolateral Na$^+-K^+$-ATPase activity has been reported in fractionated membranes (20). It is a logical guess that pressure natriuresis regulates not only Na$^+$ transporters in proximal tubules but also in the distal nephron. The increased delivery of NaCl from the proximal tubules should be accompanied with corresponding adjustment of activity in other Na$^+$ transporters distal to the proximal tubule. Otherwise the increased delivery of NaCl will be concealed by a compensatory increase in reabsorption distal to the proximal tubule. To identify these Na$^+$ transporters will be the next step to gain a better understanding of pressure natriuresis.

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