Flash photolysis of caged nitric oxide inhibits proximal tubular fluid reabsorption in free-flow nephron

Kay-Pong Yip

1Department of Physiology and Biophysics, University of South Florida, Tampa, Florida

Submitted 8 September 2004; accepted in final form 20 March 2005

Yip, Kay-Pong. Flash photolysis of caged nitric oxide inhibits proximal tubular fluid reabsorption in free-flow nephron. Am J Physiol Regul Integr Comp Physiol 289: R620–R626, 2005. First published March 24, 2005; doi:10.1152/ajpregu.00610.2004.—A nonobstructing optical method was developed to measure proximal tubular fluid reabsorption in rat nephron at 0.25 Hz. The effects of uncaging luminal nitric oxide (NO) on proximal tubular reabsorption were investigated with this method. Proximal fluid reabsorption rate was calculated as the difference of tubular flow measured simultaneously at two locations (0.8–1.8 mm apart) along a convoluted proximal tubule. Tubular flow was estimated on the basis of the propagating velocity of fluorescent dextran pulses in the lumen. Changes in local tubular fluid flow induced by intratubular perfusion were detected simultaneously along the proximal tubule, indicating that local tubular fluid flow can be monitored in multiple sites along a tubule. The estimated tubular reabsorption rate was 5.52 ± 0.38 nl min⁻¹ mm⁻¹ (n = 20). Flash photolysis of luminal caged NO (potassium nitrosylpentachlororuthenate) was induced with a 30-Hz UV nitrogen-pulsed laser. Release of NO from caged NO into the proximal tubule was confirmed by monitoring intracellular NO concentration using a cell-permeant NO-sensitive fluorescent dye (DAF-FM). Emission of DAF-FM was proportional to the number of laser pulses used for uncaging. Photolysis of luminal caged NO induced a dose-dependent inhibition of proximal tubular reabsorption without activating tubuloglomerular feedback, whereas uncaging of intracellular cGMP in the proximal tubule decreased tubular flow. Coupling of this novel method to measure reabsorption with photolysis of caged signaling molecules provides a new paradigm to study tubular reabsorption with ambient tubular flow.

METHODS

Animal preparation. Experiments were carried out in accordance with guidelines for the care and use of research animals. All experiments were performed under protocols approved by the Institutional Animal Care and Use Committee at the University of South Florida, in accordance with Public Health Service Policy on Human Care and Use of Laboratory Animals. Experiments were performed in male Sprague-Dawley rats (220–280 g body weight). 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 servo-controlled heated operating table, which 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 flow is then equivalent to the fluid reabsorption rate at that segment of tubule. To date, there are two nonobstructing optical techniques available to measure tubular flow, namely, the fluorescent photobleaching recovery technique and the dual-slit method. Flamion et al. (5) perfused isolated inner medullary collecting duct with fluorescein sulfonate (an impermeant fluorophore) and induced localized photobleaching with an argon laser. They demonstrated that the recovery rate of fluorescent photobleaching is a direct function of perfusate velocity. However, this technique has not been evaluated in vivo. The only optical method that has been applied successfully in vivo to measure proximal tubular flow was developed by Chou and Marsh (3). This technique measures the propagating velocity of fluorescent dextran, which is injected periodically into the ambient tubular flow with a micropipette, by monitoring the time delay of fluorescence transients between two adjacent slits.

In the present study, the dual-slit technique was modified to measure local tubular flow at two locations simultaneously along a convoluted proximal tubule by employing two micropetites to independently inject fluorescent dextran pulses. This novel method was applied in conjunction with flash photolysis of caged nitric oxide (NO) in luminal fluid to examine the important role of NO in the regulation of proximal tubular reabsorption (4, 13). Our results demonstrate that it is feasible to monitor proximal tubular fluid reabsorption continuously in a nephron with ambient tubular flow at 0.25 Hz and that controlled release of caged signaling molecules with the use of a UV nitrogen-pulse laser can be applied to intact renal epithelium without interrupting data acquisition.

Address for reprint requests and other correspondence: K.-P. Yip, Dept. of Physiology and Biophysics, College of Medicine, Univ. of South Florida, MDC 8, 12901 Bruce B. Downs Blvd., Tampa, FL 33612 (E-mail: dyip@usc.edu).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
were anesthetized with Inactin [5-ethyl-5-(L-methylpropyl)-2-thiobarbituric acid, 100 mg/kg ip] instead of halothane in experiments designed for examining the effects of NO on tubular fluid reabsorption. No pancuronium was administered when Inactin was used as anesthetic. 

Measurement of tubular flow and reabsorption rate. A bolus of lissamine green dye was injected intravenously to identify early segments of proximal tubules. Proximal tubules were selected for observation only if they had a long segment (>1 mm) that ran on the downstream sites were synchronized with the use of a signal generator (CCD) video camera (IC-300; PTI) through a long-distance connection. The firing frequency and number of laser pulses in each injection site was then induced with a UV nitrogen-pulse laser (337 nm, 4 ns/pulse; Laser Science 337ND) through a quartz optical fiber of 1 mm in diameter. The firing frequency and number of laser pulses in each burst were controlled by an analog-to-digital board (Data Translation DT-2801A) housing on a Pentium computer.

Flash photolysis of caged NO in cultured renal vascular smooth muscle cells. The efficiency of the UV nitrogen-pulse laser in uncaging NO was first tested in vitro. A primary culture of renal vascular smooth muscle cells was prepared using the iron oxide method as described previously (2). Vascular smooth muscle cells of second passage in a collagen-coated coverslip were loaded with 10 μM 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate (DAF-FM diacetate), a cell-permeant NO-sensitive fluorescent dye (Molecular Probes) for 30 min, washed, and incubated with Hanks’ balanced salt solution (HBSS) for 15 min for decetylation. The cells were then bathed with fresh HBSS containing 2-μM caged NO (potassium nitrosylpentachlororhenumate; Molecular Probes). Emission of DAF-FM was measured at 0.5 Hz by exciting DAF-FM at 488 nm with an argon laser and collecting emission at 522 nm via a band-pass filter (bandwidth 22 nm) with a Bio-Rad confocal scanning unit (MRC-1000) at ×20 (N.A. 0.75; Zeiss). Flash photolysis of caged NO was then induced with a UV nitrogen-pulse laser (337 nm, 4 ns/pulse; Laser Science 337ND) through a quartz optical fiber of 1 mm in diameter. The firing frequency and number of laser pulses in each burst were controlled by an analog-to-digital board (Data Translation DT-2801A) housing on a Pentium computer.

Flash photolysis of caged NO in proximal tubule. To test whether photolysis of luminal caged NO increases NO concentration ([NO]) in proximal tubules, we first loaded proximal tubular cells with DAF-FM via intraluminal perfusion (10 μM in synthetic tubular fluid, 10 nM/min for 30 min). Luminal perfusate was then switched to synthetic tubular fluid containing 2 μM caged NO. DAF-FM in proximal tubule was excited at 490 nm via the objective lens (×20, N.A. 0.25) with a UV nitrogen-pulse laser (Laser Science 337) coupled to a laser dye module (22). Emission from DAF-FM was collected by a photomultiplier through a band-pass filter of 522 nm (bandwidth 32 nm) at 2 Hz. Flash photolysis of caged NO was initiated by shining a burst of UV laser pulses on the surface of kidney via a quartz optical fiber (1 mm in diameter). The firing frequency and number of laser pulses in each burst were controlled by an analog-to-digital board (Data Translation DT-2801A) housing on a Pentium computer. The trigger of excitation and collection of emission was controlled by an analog-to-digital board (Data Translation DT-2813) housing on another Pentium computer as described previously (22).
To test the effects of uncaging NO in proximal tubular reabsorption, we used two dextran-injecting micropipettes to measure tubular flow at 0.25 Hz in the same convoluted proximal tubule as described above. Caged NO (2 μM) was included in the dextran solution of the upstream injection pipette. The holding pressure of the upstream injection pipette was then set at <2 lb./in.², which was just sufficient to overcome the intratubular hydrostatic pressure and provide a continuous leaking of fluorescent dextran into the lumen. Photolysis of caged NO was induced by delivering a burst of UV laser pulses on the kidney surface while tubular flow was simultaneously monitored on two locations along the tubule. Caged NO was introduced into the lumen solely by the upstream injection pipette.

Flash photolysis of caged cGMP in the proximal tubule. To test the effects of uncaging intracellular cGMP in proximal tubular flow, we loaded caged cGMP [guanosine 3′,5′-cyclic monophosphate, P-1-(2-nitrophenyl)ethyl ester, a cell-permeant form of caged cGMP; Calbiochem] into proximal tubular cells by luminal perfusion. Caged cGMP (0.4 mM) was perfused intraluminally at 10 nl/min for 30 min. Photolysis of caged cGMP was then induced by a UV nitrogen-pulse laser while proximal tubular flow was monitored at a site proximal to the cGMP loading site. It has been suggested that NO inhibits proximal tubular reabsorption through a cGMP-linked pathway (4).

Solutions. The synthetic proximal tubular fluid used contained (in mM) 127 NaCl, 25 NaHCO₃, 3 KCl, 1 MgSO₄·7H₂O, 1 K₂HPO₄, 5 urea, and 1.8 CaCl₂.

Statistics. Paired t-tests were used wherever applicable. Results are reported as means ± SE. Only one proximal tubule was studied in each rat.

RESULTS

Proximal tubular flow in halothane-anesthetized rats is known to oscillate at about 2 cycles/min because of the operation of TGF (7). When tubular flow was monitored simultaneously at two sites along a convoluted proximal tubule, synchronized oscillations were found, as expected (Fig. 2). Activation of TGF by intraluminal perfusion of tubular fluid distal to both dextran injection sites triggered synchronous reduction in tubular flow (Fig. 2). The decrease in tubular flow was proportional to the intraluminal perfusion rate (5–20 nl/min at 5 nl/min step). The reduction in tubular flow was due to TGF-mediated vasoconstriction in afferent arterioles. Conversely, inhibition of TGF by intraluminal perfusion of furosemide-containing perfusate (0.4 mM, 10 nl/min) increased proximal tubular flow (Fig. 3). Intraluminal perfusion of furosemide distal to both dextran injection sites elicited a synchronous increase in tubular flow (Fig. 3A). When intraluminal perfusion of furosemide was applied between two dextran injection sites, there was an immediate increase of flow at the downstream site because of luminal perfusion, followed by a synchronous increase of flow on both sites because of TGF inhibition (Fig. 3B). Termination of intraluminal perfusion resulted in an immediate decrease of tubular flow at the downstream site, followed by a synchronous decrease of flow on both sites. These observations established the fact that local tubular flow could be monitored simultaneously at two different locations along a proximal tubule every 4 s. Therefore, proximal tubular fluid reabsorption rate could be monitored continuously at 0.25 Hz without interrupting ambient tubular flow. However, the resolution in measuring tubular flow is not constant but is a nonlinear function of fluid stream velocity, or the transit time delay between two sampling windows. The resolution of flow measurement at a specific time delay was defined as the increase in tubular flow when the time delay is reduced by 1/60 s. Figure 4 is a plot of resolution of flow measurement as a function of transit time delay in a typical tubule with a diameter of 30 μm and a distance of 150 μm between sampling windows. The resolution was 3.5 nl/min when the time delay was 10/60 s, whereas the resolution was 0.6 nl/min when the time delay was 25/60 s. The resolution deteriorated very...
rapidly when the time delay was <10/60 s. Typical transit time delay was ~10/60 s at the rising phase of tubular flow oscillations in halothane-anesthetized rats (Figs. 2 and 3). In Inactin-anesthetized rats, typical transit time delay varied between 15/60 and 25/60 s in each oscillatory cycle. Therefore, Inactin was the choice of anesthetic in measuring tubular flow and tubular reabsorption rate. The mean proximal tubular reabsorption rate in Inactin-anesthetized rats was 5.52 ± 0.38 nl·min⁻¹·mm⁻¹ (n = 20) in the present study. The calculated reabsorption rate ranged from 4 to 8 nl·min⁻¹·mm⁻¹. The distance between dextran injection sites ranged from 0.8 to 1.8 mm. Proximal fluid reabsorption measured using microperfusion and fluid recollection in rats with similar body weights was in the range of 2.5–5.2 nl·min⁻¹·mm⁻¹ (14, 21).

To examine whether the laser pulses delivered through a 1-mm-diameter optical fiber (~150 μJ/pulse measured by laser meter) are capable of triggering photolysis of caged NO, we first tested the uncaging capability of the laser system in cultured renal vascular smooth muscle cells using confocal fluorescence microscopy. The [NO] in cultured smooth muscle cells was monitored with a cell-permeant NO-sensitive fluorescent dye, DAF-AM. In the presence of 2-μM caged NO in the bathing solution, 30 pulses of UV laser at 30 Hz were sufficient to induce a detectable increase of DAF-FM fluorescence (Fig. 5). A graded accumulative increase of DAF-FM fluorescence was detected when the number of laser pulses was increased in each successive burst. These observations indicate that 30 pulses of UV laser delivered within 1 s are sufficient to initiate photolysis of caged NO and that the amount of NO released can be controlled by the number of pulses in each laser burst.

To further examine whether photolysis of caged NO in luminal fluid could raise the intracellular [NO] of proximal tubule in situ, we microperfused the tubule with luminal perfusate containing 2-μM caged NO and monitored intracellular [NO] in proximal tubular cells with DAF-FM. The time course of changes in DAF-FM emission intensity from a proximal tubule during photolysis is shown in Fig. 6A. Sixty pulses of laser triggered a detectable spike of DAF-FM emission intensity from cultured renal vascular smooth muscle cells. Flash photolysis of caged nitric oxide (NO) was induced by bursts of 30-Hz UV laser pulses. Each arrow indicates a burst of laser pulses; 30, 60, 90, 120, and 240 pulses of UV laser were applied sequentially. Dotted lines represent SE (n = 30 cells/3 coverslips).
R624

NITRIC OXIDE INHIBITS PROXIMAL TUBULAR REABSORPTION

Fig. 7. Effects of photolysis on variations of tubular flow rates measured simultaneously at 2 sites on a convoluted proximal tubule with 2 μM caged NO in the lumen (A) and a timed control (B). Horizontal bars indicate duration of 30-Hz laser burst; 1,200, 2,400, and 3,600 pulses of UV laser were applied sequentially.

Fig. 8. Changes in mean normalized proximal tubular fluid reabsorption rate when an increasing number of laser pulses was used to induce photolysis of caged NO in luminal fluid. The mean baseline reabsorption rates are 5.23±0.46 (n=6) and 5.53±0.69 nl·min⁻¹·mm⁻¹ (n=8) for the timed control and NO uncaging, respectively. *P<0.05, significant inhibition.

Table 1. Mean normalized proximal tubular flow at upstream and downstream sites after exposure to uncaging laser pulses and absence of caged NO in the lumen

<table>
<thead>
<tr>
<th>Location</th>
<th>Luminal Caged NO</th>
<th>n</th>
<th>Mean Baseline Tubular Flow, nl/min</th>
<th>Normalized Mean Tubular Flow</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Baseline 300 Laser Pulses</td>
</tr>
<tr>
<td>Upstream</td>
<td>Absent</td>
<td>6</td>
<td>27.1±4.5</td>
<td>1 1.02±0.01</td>
</tr>
<tr>
<td>Downstream</td>
<td>Absent</td>
<td>6</td>
<td>21.3±4.8</td>
<td>1 1.12±0.07</td>
</tr>
<tr>
<td>Upstream</td>
<td>Present</td>
<td>8</td>
<td>28.7±3.2</td>
<td>1 1.03±0.04</td>
</tr>
<tr>
<td>Downstream</td>
<td>Present</td>
<td>8</td>
<td>20.3±3.1</td>
<td>1 1.04±0.03</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = no. of tubules. Only 1 tubule was studied in each rat. *P<0.05, significant change in tubular flow compared with baseline.

DISCUSSION

The present study successfully implemented a novel approach to measure proximal tubular fluid reabsorption in vivo at 0.25 Hz without interrupting tubular flow. This was achieved by measuring tubular flow at two locations along a proximal convoluted tubule based on the method originally developed by Chou and Marsh (3). A major challenge of applying this new method was to locate a segment of convoluted proximal tubule that permitted insertion of two micropipettes to inject fluorescent dextran without interfering with each other. A separation of 0.8 mm between two injection sites was in general far enough to avoid cross-detection at the downstream site due to...
Injection from the upstream site. The validity of the method was indicated by the detection of differential increases and decreases in local tubular flow along a proximal convoluted tubule (Fig. 3). These observations indicate that this method has sufficient temporal and spatial resolution to detect local changes in tubular flow.

The transit time delay detected between two sampling windows was not continuous but discrete because of the CCD camera framing rate. The resolution in measuring tubular flow deteriorated rapidly when the transit time delay was <10/60 s (Fig. 4). Typical transit time delay was ~10/60 s at the rising phase of tubular flow oscillations in halothane-anesthetized rats. The amplitude of TGF-mediated oscillations is smaller in Inactin-anesthetized rats than in halothane-anesthetized rats because of the difference in proximal tubular compliance (12). The transit time delays in Inactin-anesthetized rats were in the range of 15/60–25/60 s in each oscillatory cycle, values that were in the more linear range of Fig. 4. The estimated mean proximal tubular fluid reabsorption rate was 5.52 ± 0.38 nl·min⁻¹·mm⁻¹ in the present study, consistent with values reported in the literature (14, 21). The proximal tubular fluid reabsorption rates measured with microperfusion and fluid recollection (using radioactive inulin as volume marker) in rats with similar body weight are in the range of 2.5–5.2 nl·min⁻¹·mm⁻¹ (14, 21). Proximal tubular fluid reabsorption rate measured using the perfusion/recollection method depends on the tubular perfusion rate used. In the optical method, the tubular perfusion rate is not an experimental variable because ambient tubular flow is not disturbed. The temporal resolution of the perfusion/recollection method is on the order of minutes, whereas that of the optical method is on the order of seconds.

A silicon rubber cast of the proximal tubule is required to determine the tubular length for the normalization of reabsorption rate in the perfusion/recollection method, whereas the tubular length is determined from the digitized image in the optical method. Therefore, the optical method not only provides a means to measure native tubular fluid reabsorption but also improves the temporal resolution in monitoring tubular reabsorption.

Flash photolysis of caged signaling molecules allows rapid manipulation of intra- and extracellular environment with minimal disturbance in data acquisition. Conventional flash photolysis is induced by a flash lamp through the objective lens, which limits the field of illumination, interrupts image acquisition, and imposes a finite time delay between consecutive flashes. These issues were alleviated in the present study by using a UV nitrogen-pulse laser coupled to an optical fiber to induce photolysis (23). One advantage of using the UV pulse laser is the ability to apply consecutive flashes over a long uncaging period with relatively short laser dwelling time. Each laser pulse lasts only 4 ns. The laser dwelling time for 1 min of uncaging at 30 Hz is 7.2 μs, and thus the potential tissue damage due to UV radiation is minimized. With the use of confocal fluorescence microscopy, it was found that 30 pulses of UV laser were sufficient to initiate photolysis of caged NO and that the extent of uncaging could be controlled by the number of laser pulses in each burst (Fig. 5). The imaging system used for detecting DAF-FM emission from proximal tubule is similar to that used for detecting BCECF emission described previously (20, 22). Uncaging of NO in luminal perfusate was achieved by delivering the laser pulses to the kidney surface via an optical fiber. The laser spot on the kidney surface was positioned with a visible light source through the optic fiber before uncaging. Similar to the observations in cultured vascular smooth muscle cells, DAF-FM emission intensity from proximal tubule was proportional to the number of laser pulses used for uncaging.

Uncaging of NO in luminal perfusate inhibited tubular reabsorption. The degree of inhibition was increased as the number of laser pulses used for uncaging was increased (Fig. 8). Luminal application of sodium nitroprusside (0.1–1 mM) has been shown to inhibit proximal tubular reabsorption with the use of the shrinking split-droplet technique (4), which is consistent with the present study. Inhibition of proximal tubular reabsorption in free-flow nephron increases distal NaCl delivery, which activates TGF-mediated vasoconstriction and decreases tubular flow. Therefore, pharmacological inhibition of proximal tubular reabsorption will not manifest as an increase of tubular flow unless TGF is deactivated. This prediction has been verified using EIPA to inhibit apical Na+/H⁺ exchanger in the proximal tubule of free-flow nephron. Intratubular perfusion of EIPA into the proximal tubule decreases proximal tubular flow when TGF is intact, and the same maneuver increases proximal tubular flow when TGF is inhibited by furosemide (20). cGMP is the mediator of NO to inhibit proximal tubular reabsorption (4). Uncaging of intracellular cGMP within the proximal tubule triggered reduction of tubular flow, which indicated inhibition of proximal tubular reabsorption and activation of TGF. However, the NO-induced inhibition of fluid reabsorption did not trigger TGF-mediated vasoconstriction. One plausible explanation is that intracellular cGMP released by photolysis is membrane impermeant, whereas diffusion of NO from the uncaging site is very rapid and far reaching (9).

NO production is an integral part of the TGF signaling event at the macula densa to buffer excessive TGF-mediated vasoconstriction (8, 18). There are three potential mechanisms by which uncaged NO can reach the macula densa and afferent arterioles to inhibit TGF. Uncaged NO from convoluted proximal tubules might diffuse directly to the macula densa and renal arteriole. NO has a high diffusion constant (3,300 μm²/s) and is very widely diffusible (15, 19). The range of NO

---

**Fig. 9.** Mean normalized time course of changes in proximal tubular flow induced by photolysis of intracellular caged cGMP. Photolysis was induced by a burst of 30-Hz UV laser pulses for 120 s. Horizontal bar indicates duration of uncaging. Dotted lines represent SE (n = 5 tubules/5 rats).
Mediated vasoconstriction. Inhibits proximal tubular reabsorption without activating TGF-
flow and to deliver luminal NO via photolysis reveals that NO
approach to measure tubular reabsorption with ambient tubular
environment of intact renal epithelium. The unique ap-
caged signaling molecules to rapidly manipulate the intracel-
cells by flash photolysis demonstrates the potential of using
ulation of intracellular [NO] and [cGMP] of proximal tubular
at 0.25 Hz without interrupting ambient tubular flow. Manip-
lumen of early distal tubule.

Finally, uncaging of NO might occur in close
proximity to the macula densa if the UV laser penetrates into
the cortex and there is a sufficient amount of caged NO in the
lumen of early distal tubule.

In summary, an optical technique was successfully developed and implemented to measure proximal fluid reabsorption at 0.25 Hz without interrupting ambient tubular flow. Manipulation of intracellular [NO] and [cGMP] of proximal tubular cells by flash photolysis demonstrates the potential of using caged signaling molecules to rapidly manipulate the intracellular environment of intact renal epithelium. The unique approach to measure tubular reabsorption with ambient tubular flow and to deliver luminal NO via photolysis reveals that NO inhibits proximal tubular reabsorption without activating TGF-mediated vasoconstriction.

 ACKNOWLEDGMENTS


GRANTS

This study was supported by National Institutes of Health Grants HL-59156
and DK-60501.

REFERENCES

9. Lancaster JR Jr. Simulation of the diffusion and reaction of endog-
22. Yip KP, Wagner AJ, and Marsh DJ. Detection of apical Na\(^{+}/H\(^{+}\) exchange activity inhibition in proximal tubules induced by acute hyper-
23. Zhang WM, Yip KP, Lin MJ, Shimoda LA, Li WH, and Sham JS. ET-1 activates Ca\(^{2+}\) sparks in PASMC: local Ca\(^{2+}\) signaling between inositol trisphosphate and ryano

Received from http://ajpregu.physiology.org/ by 10.220.33.3 on April 28, 2017