Shear stress-mediated NO production in inner medullary collecting duct cells

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Cai, Zheqing, Jingdong Xin, David M. Pollock, and Jennifer S. Pollock. Shear stress-mediated NO production in inner medullary collecting duct cells. Am J Physiol Renal Physiol 279: F270–F274, 2000.—Recent evidence suggests that nitric oxide (NO) within the inner medullary collecting duct (IMCD) functions to regulate sodium and water reabsorption. Because fluid shear stress has been shown to increase NO production in endothelial and vascular smooth muscle cells, experiments were designed to determine whether a similar mechanism exists in IMCD cells. Cultured IMCD-3 cells derived from murine IMCD were subjected to 60 min of pulsatile shear stress. Nitrite production (2,3-diaminonaphthalene fluorometric assay) increased 12-, 16-, and 23-fold at 3.3, 10, and 30 dyn/cm², respectively, compared with static control cultures. Preincubation with the non-isoform-specific NO synthase inhibitor nitro-L-arginine methyl ester reduced nitrite production by 83% in response to 30 dyn/cm². Western blotting and immunofluorescence analysis of static IMCD-3 cell cultures revealed the expression of all three NO synthase isoforms (NOS-1 or neuronal NOS, NOS-2 or inducible NOS, and NOS-3 or endothelial NOS) in IMCD-3 cultures. These results indicate that NO production is modulated by shear stress in IMCD-3 cells and that fluid shear stress within the renal tubular system may play a role in the regulation of sodium and water excretion by control of NO production in the IMCD.

The mechanisms that regulate NO production in collecting duct cells are not known. Because fluid shear stress is an important mechanism for stimulating NOS-3 to produce NO in endothelial cells (3, 10), we hypothesized that the same mechanism may exist within the renal tubular system, especially the inner medullary collecting ducts. Fluid shear stress is a parallel friction force produced by flowing fluid. In a tubular system, fluid shear stress is directly dependent on fluid viscosity and flow and indirectly related to the radius. On the basis of values obtained from the literature (2, 4, 5, 9, 15), we estimate that shear stress for tubular fluid in the collecting duct is roughly the same as that with blood in small resistance vessels (0.2–20 dyn/cm²). Recently, investigators have reported that shear stress response elements, which modulate gene expression in endothelial cells, are also active in renal epithelial cells (11). Therefore, the present study was designed to investigate whether fluid shear could provide a mechanism for regulating NO production in inner medullary collecting duct cells.

A ROLE FOR NITRIC OXIDE (NO) in the regulation of sodium and water reabsorption within the renal medulla has been supported by evidence from numerous investigators (12). Of particular importance, infusion of the nitric oxide synthase (NOS) inhibitor nitro-L-arginine methyl ester (L-NAME) directly into the renal medullary interstitium, increases arterial pressure in association with retention of sodium and water (17). Urinary NO₂⁻ and NO₃⁻, metabolites of NO, were positively correlated with the excretion of sodium and water (22). Recent data from Western blot and RT-PCR analysis indicate that all three isoforms of NOS, termed NOS-1 or neuronal NOS, NOS-2 or inducible NOS, and NOS-3 or endothelial NOS, are constitutively expressed in the renal medulla of normal rats (1, 18, 24, 25, 27). Our laboratory has reported immunohistochemical evidence that NOS-3 expression is upregulated in collecting duct cells of salt-loaded, hypertensive rats compared with placebo control animals (2). One possible mechanism of action for renal medullary NO is suggested by in vitro studies demonstrating that NO inhibits solute and water reabsorption in renal tubular epithelium (8, 13, 23).

METHODS

Cell culture and shear stress apparatus. A cell line derived from mouse inner medullary collecting duct (IMCD-3; obtained from American Type Culture Collection) were cultured in DMEM containing 10% fetal calf serum, penicillin/streptomycin, and 2 mM glutamine. At passages 4–8, cells were cultured on glass slides (5.5 × 7.0 cm) to confluence and placed into a parallel-plate flow chamber (CytoDyne, San Diego, CA). The flow chamber consists of a machine-milled polycarbonate plate, a rectangular Silastic gasket, and the glass slide with the attached cells. These were held together by a vacuum maintained at the periphery of the slide, forming a channel of parallel-plate geometry. The use of a vacuum ensured a uniform channel depth. The depth of the channel formed was 220 μm, and the area of cells exposed to shear...
was 16 cm². The polycarbonate plate has two manifolds through which medium enters and exits the channel. The entry port is larger than the exit port and serves as a bubble trap. A valve opposite the entry port allows for the removal of the bubbles. Medium is added to the top reservoir, filling the bottom reservoir as well, and flooding the chamber. Then, the slide with the cultured cells is inverted over the flooded chamber, and clamped. A pump (Bio-Rad) was used to drive the recirculating fluid (20 ml) consisting of phenol red-free culture media (DMEM, 10% fetal calf serum, penicillin/streptomycin, and 2 mM glutamine). The flow was set at different rates to administer various shear stress (static or no flow, 3.3, 10, 30 dyn/cm²). Samples (200 µl) were taken at 15-min intervals during a 1-h treatment period for analysis of nitrite and 30 dyn/cm²). The concentration of nitrite was determined by trypan blue exclusion. Each experiment was repeated three times.

Nitrite determination by 2,3-diaminonaphthalene assay. Concentrations of nitrite were measured in perfused culture media by using 2,3-diaminonaphthalene (DAN; nonfluorescent; Aldrich Chemical) to react with nitrite to form 1-H-naphthotriazole, a highly fluorescent product, immediately after the experiment. Each sample was assayed in duplicate. In a white 96-well microtiter plate (Dynatech), 10 µl of freshly prepared DAN (0.5 mg/ml in 0.62 N HCl) were added to 100 µl of sample. After a 10-min incubation at room temperature, 10 µl of 1.4 N NaOH were used to neutralize the acid in each well and stop any further reaction. The intensity of fluorescence was detected with excitation at 360 nm and emission at 450 nm (CytoFluor Series 4000, PerSep tive Biosystems). Nitrite concentration was determined relative to a standard curve from 40 to 2,500 nM. The sensitivity of the assay is ~80 nM.

Western blot analysis. To determine the expression of NOS isoforms in nonstressed cultures, IMCD-3 cells were har vested and lysed in buffer containing protease inhibitors (1% SDS, 10 mM Tris · HCl, pH 7.4, 1 µM leupeptin, 1 µM pepstatin A, 1 mM phenylmethylsulfonyl fluoride). The cell lysates were separated on 7.5% SDS-polyacrylamide mini gels. Lysates from rat brain homogenates, activated RAW 264.7 macrophages, and bovine aortic endothelial cells were obtained to serve as positive control cultures for NOS-1, NOS-2, and NOS-3, respectively. The protein was transferred to a nitrocellulose membrane by using a wet blotting apparatus for 60 min. The membranes were allowed to air dry for 30 min and blocked with 5% nonfat dry milk diluted in Tris-buffered saline (blocking buffer) for 1 h at room temperature. The blots were incubated with the primary antibody for NOS-1 (SA-227, rabbit polyclonal, Bio-Mol), NOS-2 (SA-200, rabbit polyclonal, Bio-Mol), and NOS-3 (H32, mouse monoclonal, Bio-Mol) diluted 1:1,000 in blocking buffer at 4°C overnight. Blots were then incubated with horseradish peroxidase-conjugated secondary antibody (goat anti-mouse or anti-rabbit; 1:3,000; Amersham) for 1 h at room temperature followed by four washes with Tris-buffered saline. The specific bands were detected by using an enhanced chemiluminescence system (Amersham).

Cellular immunofluorescent staining. To compare the cellular localization of the NOS isoforms, IMCD-3 cells were cultured on glass slides and fixed by treating with cold acetone-methanol (1:1) for 30 min. The fixed cells were blocked with 0.2% bovine serum albumin in PBS in a humidified chamber for 30 min. Using separate slides, NOS isoform-specific antibodies (SA-227, SA-200, and H32) were diluted in blocking buffer (1:50, 1:800, 1:200, respectively). After an overnight incubation at room temperature, cells were washed three times for 5 min with PBS. Specific reaction products were detected with FITC-conjugated anti-mouse or anti-rabbit secondary antibody (1:1,000, Sigma Chemical) incubated for 1 h at room temperature, followed by three 5-min washes with PBS. Slides were mounted, covered, and air dried. IMCD-3 cells were viewed and photographed with an Olympus BX40 fluorescence microscope. Staining in the absence of the primary antibody served as a negative control.

Statistical analysis. A two-factor analysis of variance was used to determine differences in mean nitrite concentrations within the various time points between different shear rates compared with static control cultures. Dunnett’s test was
used to determine whether the differences in mean nitrite concentrations existed between static control and other shear rates within each time point. A P value of 0.05 or less was considered statistically significant.

RESULTS

Fluid shear stress stimulated nitrite production in IMCD-3 (Fig. 1). The magnitude of the increase in nitrite production was directly dependent on the level of fluid shear stress (P < 0.0001). Compared with the initial time point (time 0), a significant increase in nitrite production was observed after 15-min exposure to 10 (P < 0.05) and 30 dyn/cm² (P < 0.005) shear stress and after 60 min of exposure to 3.3 dyn/cm² (P < 0.005). After 60 min of applied fluid shear, nitrite production was increased 12-, 16- and 23-fold at 3.3, 10, and 30 dyn/cm², respectively, compared with static control cultures. All of these increases in nitrite production after shear stress were significantly greater than in static control cultures (P < 0.005).

The non-isoform-specific NOS inhibitor L-NAME completely inhibited shear stress-induced nitrite production (Fig. 2). Nitrite production was inhibited by 83% after 60-min exposure to 30 dyn/cm² shear stress compared with 30 dyn/cm² in the absence of L-NAME. The two groups were significantly different at all time points after initiation of shear stress (P < 0.01).

Western blots of IMCD-3 lysates demonstrated NOS-1, NOS-2, and NOS-3 immunoreactivity (Fig. 3) was present in nonstressed cultures. Positive immunoreactivity for NOS-1 in IMCD-3 cells corresponds to rat brain homogenates, which served as a positive control. In the same preparations, NOS-2 and NOS-3 immunoreactivity was observed and corresponded to activated macrophage and bovine aortic endothelial cell homogenate control, respectively.

Immunofluorescent staining of IMCD-3 cultures demonstrated positive expression of all three NOS iso-
forms (Fig. 4). Staining for NOS-1 was somewhat diffuse throughout the cytosol, whereas NOS-2 and NOS-3 staining show distinct particulate staining within the cell.

**DISCUSSION**

Our findings are the first to demonstrate shear-dependent responses of NO production in cultured renal epithelial cells. Endothelial and vascular smooth muscle cells have also been reported to have shear-induced response mechanisms for releasing NO (3, 6, 7, 14, 19, 20, 26, 28). In the range of physiological flow rates reported for the inner medullary collecting duct, we estimated shear stress to be in the range of 0.2–20 dyn/cm². This estimation was based on information obtained from the literature for tubular flow rates and diameters. Tubular flow rates were estimated by dividing urine flow by the number of tubules at a given level within the inner medulla. “Normal” and “high” urinary flow rates were based on 24-h urine collections in conscious placebo and DOCA-salt treated rats, respectively (2). Normal urine flow was reported to be ~10 ml/day, whereas high urine flow was ~100 ml/day. Han and colleagues (9) have reported the number of tubules within the inner medulla at 1,000 farthest from the tip of the papilla and 30–300 tubules near the tip (9). The diameter of the tubules is estimated to range from 22 μm farthest from the tip to 50 μm near the tip of the papilla (4, 15). Thus we can use Poiseuille’s law to calculate shear: \[ \tau = \frac{4\eta Q}{\pi r^4}, \]
where \( \tau \) is shear stress in dyn/cm², \( \eta \) is viscosity in dyn·s⁻¹·cm⁻², \( Q \) is flow rate in cm³/s, and \( r \) is radius in cm. By assuming tubular fluid viscosity to be the same as water at 37°C (25a), we can obtain estimates of shear stress at normal flow to be in the range of 0.2 to 2.0 dyn/cm² and at high flow to be in the range of 2.0 to 20 dyn/cm². In this range of shear stress, we observed significant, shear-dependent increases in nitrite production. Because L-NAME significantly reduced the increase in NO produced by shear stress, we can conclude that NO production in IMCD-3 cells is due to activation of NOS. L-NAME is a non-isoform-specific NOS inhibitor; thus, at present, these studies cannot discern which NOS isoform is responsible for the shear-dependent mechanism.

In contrast to results in the present study, NO production in endothelial and smooth muscle cells has been shown to display a two-phase response (6, 7, 14, 20). The initial phase of NO production is rapid and peaks within 15 min, which is followed by a slow, sustained increase. It has been determined that the initial burst is Ca²⁺ dependent and the sustained increase is Ca²⁺ independent in endothelial cells. These two phases are not apparent in IMCD cells, indicating differences in response time and suggesting a single mechanism for sensing changes in shear stress in the IMCD cells.

Using both Western blot analysis and immunofluorescence, we observed that IMCD-3 cells express all three NOS isoforms. These findings agree with recent results in vivo that identified all three isoforms in the IMCD and other tubular segments (1, 18, 24, 25, 27). Further studies will be necessary to determine the specific isoform or isoforms responsible for NO production in IMCD cells. In endothelial cells, shear stress activates NOS-3 (6, 7, 14), whereas in smooth muscle NO production is mediated by NOS-1 (20).

Endothelial cells exposed to chronic high shear stress have increased NOS-3 expression in vitro (28) and in vivo (19, 26). A shear stress response element has been described in the promoter region for several shear-dependent genes (3, 10). NOS-3 is among those genes that have been shown to be induced by shear stress. Our laboratory recently has shown that NOS-3 is increased in the renal medulla of DOCA-salt hypertensive rats in which urine flow is roughly 10-fold higher than normal (2). We also have preliminary evidence that NOS-3 is elevated in the renal medulla during other states of high urine flow in the rat, i.e., streptozotocin-induced diabetes and chronic diuresis induced by 10% glucose in the drinking water (21). It is possible that chronic increases in tubular fluid flow may upregulate all isoforms in IMCD cells in vivo either indirectly or directly. It was reported that expression of all three NOS isoforms in the renal medulla was increased when rats were fed a high-salt diet, which is associated with chronic elevations in tubular fluid flow through the IMCD (16).

Our findings have led us to hypothesize that fluid shear stress may participate in arterial pressure regulation and sodium and water excretion by control of renal NO production. It is also possible that shear-induced NO production participates in medullary vasodilation associated with pressure natriuresis. We conclude from the present study that NO production is modulated by shear stress in IMCD-3 cells. Fluid shear stress within the renal tubular system may play a role in the regulation of sodium and water excretion via one or all three NOS isoforms present in the renal medullary collecting duct. We suggest that this may be a mechanism for regulating renal medullary function and may participate in the control of extracellular fluid volume and arterial pressure.

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**REFERENCES**


