Early upregulation of iNOS mRNA expression and increase in NO metabolites in pressurized renal epithelial cells

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Am J Physiol Renal Physiol 293: F1877–F1888, 2007. First published September 19, 2007; doi:10.1152/ajprenal.00238.2007.—Pressure is an important physiological regulator, but under abnormal conditions it may be a critical factor in the onset and progression of disease in many organs. In vivo, proximal tubular epithelial cells are subjected to pressure as a result of ureteral obstruction, which may influence the production of nitric oxide (NO), a ubiquitous multifunctional cytokine. To directly explore the effect of pressure on the expression and activity of NO synthase (NOS) in cultured proximal tubular epithelial cells, a novel pressure apparatus was developed. Cells were subjected to pressures of 20–120 mmHg over time (5 min–72 h). RT-PCR demonstrated an increase in inducible NOS (iNOS) and sGC, while endothelial NOS remained unchanged. Real-time PCR (qPCR) confirmed an earlier induction of iNOS transcript subjected to 60 mmHg compared with cytokine mix. iNOS protein expression was significantly increased following 60 mmHg of pressure for 24 h. Use of nuclear factor-κB inhibitors was shown to prevent the increase in iNOS expression following 60 mmHg for 2 h. NO and cGMP were increased with the application of pressure. The addition of the irreversible iNOS inhibitor (1400W) was shown to prevent this increase. We demonstrate that with the use of a simply designed apparatus, pressure led to an extremely early induction of iNOS and a rapid activation of NOS activity to increase NO and cGMP in proximal tubule epithelial cells. The rapid effects of pressure on iNOS may have important implications in the obstructed kidney.

unilateral ureteral obstruction; nitric oxide; cyclic guanosine monophosphate; inducible nitric oxide synthase; cytokine mix

THE GASEOUS FREE RADICAL nitric oxide (NO) is a multifunctional intercellular messenger synthesized from L-arginine by NO synthase (NOS) (21, 23). NOS isoforms of two biochemical types are encoded by three genes. Neuronal NOS (nNOS or NOS I) and endothelial NOS (eNOS or NOS III) are constitutive Ca2+-dependent isoforms. Transient activation of constitutive NOS dimers occurs upon the binding of Ca2+-activated calmodulin. The third NOS isoform, inducible NOS (iNOS or NOS II), is widely expressed in diverse cell types after transcriptional induction by inflammatory mediators, lipopolysaccharide (LPS) and cytokines (21, 23). Activation of the transcription factor nuclear factor-κB (NF-κB) is thought to be essential for the induction of iNOS (31, 34, 37). NF-κB binds specific recognition elements in the promoter regions of inflammatory and stress-induced proteins, including iNOS, thereby activating their transcription (37). Unlike the constitutive enzyme, iNOS is tightly coupled to calmodulin even at basal Ca2+ levels and generates the sustained production of large amounts of NO until the enzyme is degraded (21, 23). One of the major intracellular targets of NO, soluble guanylyl cyclase (sGC), leads to the conversion of guanosine triphosphate to cyclic guanosine monophosphate (cGMP) that mediates a plethora of biological effects (4, 36).

In animal models of unilateral ureteral obstruction (UUO), a marked elevation of ureteral pressure occurs, increasing to 35 to 60 mmHg (3, 6, 8, 20, 38, 44). Sustained obstruction can impart mechanical strain on the renal system resulting in dilation of the collecting system, parenchymal thinning, tubular atrophy, interstitial infiltration and fibrosis, and significant loss in kidney weight and function (17, 44, 47).

Several studies showed that NO has a restorative effect on renal function and injury in obstructed kidneys (6, 15, 18). It has been reported that exogenous nitrates, L-arginine or iNOS gene transfection, restored renal blood flow (RBF), glomerular filtration rates (GFR) and reduced renal injury (15, 17, 18). Recently, Ito et al. (18) and Valles et al. (43) reported increased iNOS expression in animal models of UUO and unilateral ureteropelvic junction obstruction (UPJ) in children, respectively. In addition, Hegarty et al. (15) documented an early increase in iNOS protein expression, at 10 min following UUO.

Increased pressure is also characteristic of diseases in the tendons (12), cartilage (7, 16), eyes (25, 26), heart (40), and kidneys (6, 20). Mechanotransduction of the signal mediates the effects of pressure on NOS (7, 14, 26, 32). Increased amounts of NO are released by articular cartilage, periodontal ligament fibroblasts, and trabecular cells, when stimulated by static and intermittent pressure (7, 26, 32). In a variety of cell types, it has been shown that elevated pressure increases iNOS expression (7, 16, 25, 45). However, it remains unknown whether pressure modulates NO synthesis in renal proximal tubule cells. Furthermore, it is unclear whether mechanical and biochemical stimuli have similar effects on NOS. We developed a simple system to apply pressure directly to cultured cells, which may be applicable to several areas of research. Using this novel apparatus, we investigated the dose response and time course to pressure and determined that pressure has novel effects on NOS in renal proximal tubule epithelial cells. In addition, we present data showing that NF-κB is involved in the induction of iNOS as a result of pressure.
**METHODS**

Reagents. S-ethylisothiourea (hydrobromide), aminoguanidine hemisulfate, N-[3-(aminomethyl) benzyl] acetamidine (1400W), actinomycin D (Act D), penicillin-streptomycin neomycin solution, BSA, human recombinant interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), and interferon-γ (IFN-γ) were purchased from Sigma (St. Louis, MO). NF-κB inhibitors, MG-132 (Z-Leu-Leu-Leu-H) (MG) and Bay 11-7058 (E)-3-[4-(Butylphenylsulfonyl)-2-propenenitrile] (BAY), were purchased from BioMol (Plymouth Meeting, PA). DMEM with or without phenol red was obtained from Invitrogen (Carlsbad, CA); fetal bovine serum (FBS) was obtained from Gemini-Bio Products (Woodland, CA). All other chemicals were of reagent grade.

Pressure apparatus. To study the effects of pressure on cells in vitro, a pressure apparatus was originally designed as a simple closed system (Fig. 1A). A syphymomanometer was used that was retubed retaining the gauge, bulb, and valve, while the armlet band was removed. Valves and rubber stoppers were added to this set up such that pressure was manually applied to flasks of cells. The entire system was placed in a CO2 incubator to maintain constant temperature, atmosphere, and humidity. Although this system provided a simple reproducible means to apply pressure, it also prevented the exchange of air with the flasks. We finished experiments within 120 min, and the flasks used, 25 and 75 mm², had a volume of 60 and 300 ml, respectively. They contained enough oxygen for the cells used during this short period.

A motorized pump and an additional valve (inlet and outlet valves) were later added to this system so as to facilitate continuous exchange of the incubator atmosphere. By adjusting the opening of the outlet valve, pressure could be increased or decreased (Fig. 1B). With this new system, we can reproducibly apply pressures of 20 to 200 mmHg for as long as 72 h. The data presented in the paper are a combination of the old system (Figs. 2A, 3, A–E, see Figs. 7A, 8, 9, and 10) and new system (Figs. 2B, C, D, 3, F and G, see Figs. 5, A and B, and 6, A and B, and 7B). To ensure that the results of studies conducted with the old system over a maximum time of 120 min are not statistically different from that of the new system, the following parameters were compared from both systems: NO, cGMP, pH, and cell death. As documented in Table 1, no significant differences were noted between this system and the one exposed to circulated air for the parameters tested (see Table 1). All the details of the studies are given below.

Cultures. Human and rat kidney epithelial cells, HK-8-4 and NRK-52E, were obtained from L. Racusen (John Hopkins University, Dept. of Pathology) and ATCC, respectively. Cultures were grown in a humidified atmosphere of 5% CO2-95% air at 37°C in DMEM (containing 1,000 mg/d glucose, l-glutamine, 25 mM HEPES buffer, 110 mg/l sodium pyruvate) supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (100 mg/ml). Cells were trypsinized, suspended in completed medium, and cultured in 25- and 75-mm² flasks. When cells reached 60–90% confluence, the medium was replaced with fresh medium or phenol red-free DMEM (containing 1,000 mg/d glucose and 110 mg/l sodium pyruvate without l-glutamine and hormone). For cells requiring 12- to 72-h stimuli, cultures were at 60–70% confluency; in all other experiments (5 min-4 h), cultures were at 80–90% confluency at the start of the experiment. Pressures of 0 mmHg (control) to 120 mmHg were applied, for varying times. A known stimulus for iNOS induction, a cytokine mixture (CM) composed of human recombinant IL-1β (1 ng/ml), TNF-α (25 ng/ml), and IFN-γ (400 U/ml) was used as a positive control (37).

Cell death. Cell viability was assessed using the Trypan blue exclusion method. HK-8-8 cells exposed to increasing pressures (20, 30, 60, and 120 mmHg) over time (5, 30, 60, and 120 min) or 60 mmHg for 4–72 h and NRK-52E cells exposed to increasing pressure (60 and 120 mmHg) over time (60 and 120 min) were detached in a solution of 0.25% trypsin-EDTA (GIBCO) in PBS, pH 7.4. Cells were counted using a hemocytometer with the addition of Trypan blue. Only cells that excluded the dye were counted as viable cells, whereas those that stained blue were counted as dead.

Apoptosis was assessed using The ApopTag Peroxidase In Situ Apoptosis Detection Kit S7100 (Intergen, Purchase, NY). Briefly, monolayers at ambient pressure or subjected to 60 mmHg for 18 h were fixed in 4% paraformaldehyde for 1 h. Following PBS wash (3 times, 5 min/wash), endogenous peroxidase were quenched with 3% H2O2/methanol for 30 min. Nonspecific binding was blocked using 5% BSA for 30 min. Equilibrium buffer was applied to the slides for 10 s, removed, and terminal deoxynucleotidyl transferase (TDT) enzyme was applied for 1 h at room temperature (RT). Enzyme was removed and stop/wash buffer was applied for 10 min. Anti-digoxigenin peroxidase was applied to slides for 30 min. Cells were developed with diaminobenzidene (DAB) and counterstained with 10% hematoxylin.

RT-PCR. RT-PCR was used to measure the steady-state levels of mRNA using primers for human iNOS, e-NOS (R&D Systems, Minneapolis, MN), nNOS (41), and sGC and rat iNOS and GADPH (see Table 2). HK-8 cells were exposed to increasing pressures (20, 30, 60, and 120 mmHg) over time (0, 5, 15, 30, 60, and 120 min). HK-8 cells exposed to NF-κB inhibitors, MG and BAY, were allowed to incubate for 30 min before the application of pressure of 60 mmHg for 120 min. HK-8 cells were exposed to CM over time (5, 30, 60, and 120 min). NRK-52E cells were exposed to pressure of 120

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Fig. 1. Pressure apparatus used in study. A: original system used a modified syphymomanometer. We retained the gauge, bulb, and valve while the armlet bag was removed; rubber stoppers and connecting valves were added to this setup, such that pressure can be manually applied to flasks of cells. Once the desired pressure was attained by squeezing the bulb, the single inlet valve was closed to maintain pressure. B: new system is connected to a motorized pump to maintain the pressure within the flasks. The rubber stopper attached to the flask has 2 holes, allowing the influx of air through one valve, and efflux through the other. Increasing or decreasing the outlet valve changes the magnitude of the pressure. Inset: outlet valve fully open. The entire apparatus was placed into the incubator maintained at 5% CO2, 37°C.
Fig. 2. Effect of pressure on cell viability, proliferation, and apoptosis in HKC-8 and NRK-52 E cells. A: percent dead HKC-8 cells in response to increasing pressure (20, 30, 60, and 120 mmHg) over time (5, 30, 60, and 120 min). Gray bars, 5 min; striped bars, 30 min; open bars, 60 min; stippled bars, 120 min ($n = 3$). B: percent dead NRK-52E cells in response to increasing pressure (60 and 120 mmHg) over time (60 and 120 min). C: apoptotic cells (brown stain) were detected by TUNEL assay. I: HKC-8 cells at ambient pressure. II: HKC-8 cells subjected to 60 mmHg for 18 h. Magnification ×20, inset ×40. D: total live and dead HKC-8 cells in response to 60 mmHg for 4–72 h. ▲, total live cells at ambient pressure; ○, total live cells at 60 mmHg; ▲, total dead cells at ambient pressure; ■, total dead cells at 60 mmHg ($n = 3$). Total cells and Trypan blue-stained cells were counted. *$P < 0.05$, compared with control at the same time point. Data are expressed as means ± SE.
mmHg or CM for 60 or 120 min, respectively, in the absence or presence of transcription inhibitor Act D. Total RNA was extracted using the TRIzol-Chloroform extraction procedure. mRNA was purified using the Oligotex mRNA extraction kit (Qiagen, Valencia, CA) according to the manufacturer’s instructions. mRNA concentration and purity were determined by measuring absorbance at 260 nm.

RT-PCR was performed using Qiagen One-step PCR kit (Qiagen) using equal (20–100 ng) transcript: PCR was performed in an automated Termal Cycler ThermoHybrid, PX2 with an initial activation step (for HotStar Taq DNA polymerase activation) for 15 min at 95°C followed by 35 cycles of denaturation for 45 s at 94°C, annealing for 30 s at 60°C, and extension for 60 s at 72°C. PCR products were separated by a 2% agarose gel electrophoresis. Bands on gels were visualized by ethidium bromide staining and analyzed using NIH Image J densitometric analysis software.

Real-time PCR (qPCR). Housekeeping gene GADPH primer was designed according to the literature (35). iNOS primer was designed...
25-mm² flasks were washed with PBS and treated with 1 ml of fresh reaction 5 min at 85°C, removal of RNA with the addition of 10 –20 min at 4°C, cDNA Synthesis 50 min at 50°C, termination of transcription and PCR to take place. The following reverse-transcriptase inum SYBR Green qPCR SuperMix UDG allows both reverse transcrip-

tion and PCR to take place. The following reverse-transcriptase was employed using 500 ng of RNA: denaturation 5 min at 65°C, activation step (for HotStar RNaseH 20 min at 37°C. qPCR protocol was employed using 2 µl of the RT product: reverse transcription for 2 min at 50°C, initial denaturation for 15 s at 95°C, annealing for 30 s at 60°C, and 120 min. Cells were exposed to 60 mmHg of pressure over time (5, 30, 60, and 120 min). Use of Invitrogen SuperScript III First-Strand Synthesis System for RT-PCR and Platinum SYBR Green qPCR SuperMix UDG allows both reverse tran-
scription and PCR to take place. The following reverse-transcriptase was employed using 500 ng of RNA: denaturation 5 min at 65°C, activation step (for HotStar Taq DNA polymerase activation) for 2 min at 95°C, denaturation for 15 s at 95°C, annealing for 30 s at 60°C, extension for 30 s at 72°C; 35 rounds of amplification were conducted. To ensure an accurate quantification of the desired product, an optional data-acquisition step in a fourth segment of the PCR run was performed according to the manufacturer’s protocol. A melting step by slow heating from 65 to 95°C with a rate of 0.2°C/s was performed at the end of reaction to eliminate nonspecific fluorescence signals. Threshold Cycle (CT) values were acquired by using the DNA Engine Opticon Continuous Fluorescence Detection System (Bio-Rad, Waltham, MA). The specificity of the desired products was determined using high-resolution gel electrophoresis. Quantification for real-time data was determined using the 2^{ΔΔCT} method (13).

Measurement of nitrite and cGMP production. Cells grown in 25-mm² flasks were washed with PBS and treated with 1 ml of fresh fenold red medium or 3–5 ml of fresh phenol red medium for nitrite (NOx) and cGMP measurements, respectively. For NOx experi-

ments, HKC-8 cells were exposed to increasing pressures (20, 30, 60, and 120 mmHg) over time (5, 30, 60, and 120 min). NOx was also measured in the presence of NOS inhibitors after HKC-8 cells were exposed to 60 mmHg of pressure over time (5, 30, 60, and 120 min). Cells were placed in the 37°C incubator (95% O2-5% CO2) for 10–15 min before pressure was applied. Inhibitors were allowed to incubate for 30–60 min before the application of pressure. Supernatants were collected for NO measurement. NO was assessed using a Total Nitric Oxide Assay Kit (Pierce, Rockford, IL) according to the manufacturer’s instructions. Cells were assessed for viability and total number of cells. cGMP was measured following HKC-8 cells exposure to pressure of 60 mmHg over time (5, 30, 60, and 120 min) in the presence and absence of NOS inhibitors. cGMP was assessed using the direct cGMP ELISA kit (Assay Designs, Ann Arbor, MI) according to the manufacturer’s instructions. Briefly, cells were lysed using 1 ml of 0.1 M HCl for 10–15 min after removal of the medium. The acetylated version was conducted to detect low levels of cGMP.

Table 1. Comparison of original system (old system) with new system

<table>
<thead>
<tr>
<th>Parameters Tested</th>
<th>Old System</th>
<th>New System</th>
</tr>
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<tbody>
<tr>
<td>Ambient pressure</td>
<td>1.13±0.07</td>
<td>0.99±0.08</td>
</tr>
<tr>
<td>60 mmHg</td>
<td>1.82±0.11</td>
<td>1.94±0.28</td>
</tr>
<tr>
<td>pH</td>
<td>182±0.26%</td>
<td>193±0.23%</td>
</tr>
<tr>
<td>Cell death</td>
<td>4±0.001%</td>
<td>3±0.005%</td>
</tr>
<tr>
<td>60 mmHg</td>
<td>4±0.009%</td>
<td>4±0.01%</td>
</tr>
</tbody>
</table>

Values are means ± SE. The following parameters were compared using both the old and new systems at the maximum experimental time of 120 min. No significant changes were noted for any of the parameters tested. a, µM/10⁶ cells (n = 4); b, fold increase relative to control, expressed as % (n = 5); c, n = 3; d, total dead cells expressed as a percentage of total cells (n = 4).

Effect of pressure on cell viability, apoptosis, and proliferation. We first examined viability, apoptosis, and proliferation in HKC-8, human proximal tubular cells, subjected to varying pressures over time. Pressure did not affect viability or apoptosis in HKC-8 cells. Cell death at ambient pressure or in cells exposed to elevated pressure of 120 mmHg for 120 min was <10% (Fig. 2A). Continuous pressure of 60 mmHg for 72 h resulted in <15% dead cells (control: 14 ± 0.01% vs. pressure: 13 ± 0.01%). Similarly <10% cell death occurred when NRK-52E cells were exposed to elevated pressure of 120 mmHg for 120 min (Fig. 2B). Compared with HKC-8 cells at ambient pressure, there was no difference in the number of

Table 2. Primer sequences used for RT-PCR and qPCR

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>5'-3' Sequence</th>
<th>Size, bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td></td>
<td></td>
</tr>
<tr>
<td>iNOS</td>
<td>+FW GAATTCGTGACCTCAGCA</td>
<td>257</td>
</tr>
<tr>
<td></td>
<td>+RW GGGATCCGAGCTTACGAGAT</td>
<td>295</td>
</tr>
<tr>
<td></td>
<td>+FW CGGATCCGAGCTTACGAGAT</td>
<td>131</td>
</tr>
<tr>
<td></td>
<td>+RW CCGATCCGAGCTTACGAGAT</td>
<td>876</td>
</tr>
<tr>
<td></td>
<td>+RW GGGATCCGAGCTTACGAGAT</td>
<td>566</td>
</tr>
<tr>
<td>sGC</td>
<td>+FW GGGATCCGAGCTTACGAGAT</td>
<td>969</td>
</tr>
<tr>
<td>BA</td>
<td>+FW GGGATCCGAGCTTACGAGAT</td>
<td>539</td>
</tr>
<tr>
<td>GADPH</td>
<td>+FW GGGATCCGAGCTTACGAGAT</td>
<td>299</td>
</tr>
<tr>
<td></td>
<td>+RW GGGATCCGAGCTTACGAGAT</td>
<td>189</td>
</tr>
<tr>
<td>Rat</td>
<td></td>
<td></td>
</tr>
<tr>
<td>iNOS</td>
<td>+FW GGGATCCGAGCTTACGAGAT</td>
<td>294</td>
</tr>
<tr>
<td></td>
<td>+RW GGGATCCGAGCTTACGAGAT</td>
<td>451</td>
</tr>
</tbody>
</table>

FW, forward primer; RW, reverse primer; iNOS, inducible nitric oxide; nNOS, neuronal nitric oxide; sGC, soluble guanylyl cyclase; BA, β-actin; a, Primers used in RT-PCR; b, Primers used in qPCR.
apoptotic cells when HKC-8 cells were subjected to pressure of 60 mmHg for 18 h (Fig. 2C). Proliferation of HKC-8 was significantly increased with the application of pressure at 60 mmHg for 24 and 36 h (24 h: 1.45 × 10^6 ± 2.25 × 10^5 cells vs. 1.55 × 10^6 ± 1.30 × 10^5 cells, \( P < 0.05 \); Fig. 2D).

Effect of pressure on NOS and sGC expression. A mean ureteral pressure of 60 mmHg and increased iNOS expression have been demonstrated following obstruction (6, 18, 29). The earliest increase in iNOS expression was documented at 10 min following UUO (15). We therefore investigated whether pressure increased NOS and sGC expression. The mRNA expression of NOS and sGC was analyzed by RT-PCR. iNOS and sGC mRNA expression increased in a dose-dependent manner when HKC-8 cells were subjected to increasing pressures of 20, 30, 60, and 120 mmHg (Fig. 3A) and in a time-dependent manner when subjected to elevated pressure of 60 mmHg for 5, 30, 60, and 120 min (Fig. 3B). No change in eNOS expression was detected in cells at ambient pressure vs. those subjected to varying pressures or to a time course at the pressure of 60 mmHg (Fig. 3, A and B). Densitometric analysis (Fig. 3C) revealed that iNOS expression was significantly increased compared with control at 5 min when HKC-8 were subjected to pressure of 20 mmHg (1.00 ± 0.00- vs. 2.47 ± 0.36-fold increase, \( P < 0.05 \)) and at 30 and 60 min when pressure of 60 mmHg was applied [1.00 ± 0.00- vs. 2.36 ± 0.44-fold increase (\( P < 0.05 \); 30 min), 2.40 ± 0.39-fold increase (\( P < 0.01 \); 60 min)]. eNOS expression remained unchanged in the presence of pressure (Fig. 3D). sGC was significantly increased when exposed to pressures of 30, 60, and 120 mmHg for 30 min, and 60 mmHg for 5 min (Fig. 3E). nNOS was not detected (data not shown).

NRK-52E, a rat proximal tubule cell line, was used to determine whether the effect on iNOS was specific to human iNOS. The iNOS transcript was also significantly increased when NK-52E cells were subjected to pressure of 120 mmHg for 60 min compared with ambient pressure. This effect was abolished when the transcription inhibitor Act D was used (Fig. 3, F and G).

To confirm the effects of pressure on iNOS gene expression noted with RT-PCR analysis, qPCR was conducted. There was a significant increase in iNOS expression at 30, 60, and 120 min when HKC-8 cells were subjected to pressure of 60 mmHg [1.08 ± 0.16- vs. 2.06 ± 0.21-fold increase (\( P < 0.05 \); 30 min), 2.23 ± 0.32-fold increase (\( P < 0.05 \); 60 min), 2.44 ± 0.37-fold increase (\( P < 0.05 \); 120 min)]. However, the 5-min time point did not reach statistical significance (data not shown).

Previous studies documented the earliest induction of iNOS transcription to occur at 120 min (5, 19). Unlike rat or murine cells that express iNOS in response to LPS, IL-1\( \beta \), TNF-\( \alpha \), or IFN-\( \gamma \), most human cells require a complex combination of IL-1\( \beta \), TNF-\( \alpha \), and IFN-\( \gamma \) to obtain the maximal transcriptional activity of the iNOS promoter (5, 37). Incubating HKC-8 with a mixture of cytokines (CM), we confirmed that the earliest induction of iNOS occurs at 120 min (0.2 ± 0.06 vs. 1.03 ± 0.1 normalized to GADPH, \( P < 0.001 \); Fig. 4, A and B). Comparison of these two stimuli using qPCR demonstrates that iNOS is significantly increased at 30 and 60 min following application of 60 mmHg of pressure compared with CM [2.06 ± 0.21- vs. 1.40 ± 0.14-fold increase (\( P < 0.05 \); 30 min), 2.23 ± 0.32- vs. 1.24 ± 0.21-fold increase (\( P < 0.05 \); 60 min)]. Although iNOS is significantly increased following 120 min exposure to either stimulus, CM is more potent at inducing iNOS transcription compared with pressure of 60 mmHg at this time point (11.82 ± 2.67- vs. 2.44 ± 0.37-fold increase, \( P < 0.05 \)).

Since iNOS transcript was increased with the application of pressure, protein expression was examined. The expression of iNOS protein was analyzed by ELISA and immunocytochemistry with anti-iNOS antibody. iNOS protein expression was significantly increased following a continuous application of pressure at 60 mmHg for 24 h (100 ± 0.3- vs. 303 ± 0.7-fold increase relative to control, \( P < 0.05 \); Fig. 5A). Very little immunoreactive iNOS was detected in ambient pressurized cells, while an overnight continuous application of pressure at 120 mmHg resulted in more positively stained iNOS expressing cells (see Fig. 5B, I and II).

Effect of NF-\( \kappa \)B inhibition on iNOS expression. From a large body of work conducted using CM, NF-\( \kappa \)B has been shown to be central to the induction of iNOS (38). We therefore examined this pathway using two inhibitors, namely MG which blocks NF-\( \kappa \)B activation by preventing IkB degradation and BAY which irreversibly inhibits I\( \kappa \)B\( \beta \) degradation and blocks NF-\( \kappa \)B activity. Both MG and BAY at 10 \( \mu \)M concentration significantly inhibited the increase in iNOS expression following 120 min of 60 mmHg of pressure [0.74 ± 0.06 vs. 0.45 ± 0.04 (\( P < 0.05 \),
Similarly, increased iNOS protein expression following a continuous application of pressure at 60 mmHg for 24 h was significantly inhibited in the presence of BAY (328 ± 0.77- vs. 119 ± 0.14%-fold increase relative to control, *P < 0.05; Fig. 6B). We also assayed iNOS expression in the presence of the inhibitors alone and no significant differences were observed compared with the control (data not shown).

Effect of pressure on NO and cGMP production. Static and intermittent pressures have been shown to increase NO (7, 26, 32). To explore the effect of pressure on NOS activity, nitrite accumulation (NOx) and cGMP levels were measured following pressurization using Greiss reaction and ELISA, respectively. As shown in Fig. 7A, HKC-8 cells subjected to increasing doses of pressure, 20, 30, 60, and 120 mmHg, had a marked accumulation of NOx in the culture medium. Pressure of 60 mmHg significantly increased NOx production as early as 5 min (0.57 ± 0.16 vs. 3.28 ± 0.84 μM/10^6 cells, *P < 0.05) and remained elevated for 120 min (0.91 ± 0.18 vs. 3.44 ± 0.47 μM/10^6 cells, *P < 0.05; Fig. 7A).

NRK-52E were subjected to pressures of 60 and 120 mmHg for 60 and 120 min, and compared with cell cultures at ambient pressure. The mean concentration of NO metabolites present in the supernatants following pressure of 60 mmHg was 0.84 ± 0.33 μM/10^6 cells (P < 0.05, 60 min) and 2.14 ± 0.39 μM/10^6 cells (P < 0.01, 120 min) vs. control −0.2 ± 0.15 μM/10^6 cells. The elevated pressure of 120 mmHg had a similar effect (Fig. 7B).

cGMP production was significantly decreased after 5 min (591.59 ± 75.40 vs. 268.17 ± 44.73 pM, *P < 0.01; Fig. 8) and increased significantly at 120 min when pressure of 60 mmHg was applied to HKC-8 cells (591.59 ± 75.40 vs. 1,386.59 ± 175.55 pM, *P < 0.01).

Measurement of NO and cGMP was conducted on HKC-8 cells exposed to CM over time (5, 30, 60, and 120 min) and levels were decreased compared with control (data not shown).

Effect of iNOS inhibitors on increased NO and cGMP production as a result of pressure. Since both protein and mRNA expression of iNOS were increased with pressure, the irreversible iNOS inhibitor, 1400W and reversible inhibitors aminoguanidine and s-ethylisothiourea (10, 11, 37) were employed to determine whether iNOS was responsible for the increase in NOx and cGMP as a result of pressure.

With the addition of the inhibitors, NOx was significantly decreased at 5, 30, 60, and 120 min following exposure to pressure of 60 mmHg compared with ambient pressurized cells (Fig. 9). Both reversible and irreversible inhibitors suppressed NO production as early as 5 min [60 mmHg; 4.66 ± 0.29 μM/10^6 cells vs. 1400W; 1.23 ± 0.04 μM/10^6 cells (P < 0.01), aminoguanidine; 1.31 ± 0.26 μM/10^6 cells (P < 0.001), s-ethylisothiourea; 1.01 ± 0.19 μM/10^6 cells (P < 0.001)] and inhibition continued to occur over the 120 min of pressurization.

Both basal cGMP and cGMP elevated as a result of pressure were significantly decreased in the presence of 1400W (Fig. 8). Aminoguanidine significantly decreased the elevated cGMP at
60 min following exposure to pressure of 60 mmHg (781.77 ± 103.58 vs. 444.33 ± 61.08 pM, \( P < 0.05 \)), but failed to suppress the increase at 120 min. Although s-ethylisothiourea decreased the level of cGMP at 120 min, there was no statistical difference between cells subjected to pressure of 60 mmHg vs. those in the presence of the inhibitor (Fig. 8).

**DISCUSSION**

Pressure is instrumental in the function and remodeling of tissues. It represents an important component of the mechanical environment that regulates gene expression in many organs and tissues, including cartilage (7, 16), tendons (12), eyes (25, 26), hearts (40), kidneys (6, 20), bladder (2), and brain (39, 42). Devices to apply pressure in vitro have been used in a variety of experiments. Most systems used by others involved putting a load on the layer of cells or tissue growing at the bottom of culture dishes; closed chambers are utilized which do not
facilitate the circulation or exposure to air, and the limited supply of oxygen is a major drawback (7, 16, 25, 26). Applications of pressure within these devices involve the use of hydraulic or compressive force via the reduction of space in fluid-filled chambers, or hydrostatic pressure is generated in resting fluid as a result of the weight of the fluid in a column. In addition, most of these are costly and cumbersome. We report herein the fabrication of a simple, yet effective device for applying pressure. In the system we developed, atmospheric pressure is elevated in the culture chamber with the use of a pump which pumps incubator atmosphere (95% air-5% CO2); the amount of pressure can be easily changed by increasing or decreasing the opening of the outlet valve. Using this device, we also noted the extremely rapid effects of pressure on induction of iNOS mRNA and activation of NOS. These studies demonstrate the profound effects of pressure on iNOS and suggest that pressure may be an important signaling mechanism in kidney, as well as in other organ systems.

There have been a few previous reports on the effect of pressure on iNOS expression. In human astrocytes, elevated hydrostatic pressure (60 mmHg) increased both mRNA and iNOS protein expression with the earliest increase detected at 12 and 24 h, respectively (25). In addition, Fermor et al. (7) demonstrated that both hydrostatic and intermittent compression augmented iNOS protein expression. Using porcine articular cartilage explants, both static (0.5 MPa) and intermittent compression (0.1 MPa, 0.5 Hz) for 24 h increased iNOS protein with no detectable eNOS or nNOS protein in control or compressed tissue. We present data of a dose response to pressure and a time course showing increased iNOS transcript at 5 min with the earliest significant quantitative induction at 30 min following 60 mmHg in the human renal epithelial cells, HKC-8. In addition, a 24-h application of 60 mmHg significantly increased iNOS protein expression. We did not assay for constitutive NOS proteins. This phenomenon is not limited to human cells, for the rat renal epithelial cells (NRK-52 E) subjected to 120 mmHg for 60 min had significantly increased iNOS mRNA expression. The subcellular mechanism behind this response of cells to pressure is unknown. It has been suggested that pressure-sensitive membrane channels, called curvature-sensitive channels, are responsive to pressure (25). Also, cytoskeletal changes as a result of biomechanical stress are thought to be responsible for the synthesis of adhesion molecules, growth factors, cytokines, opening and closing of...
ion channels, and synthesis and degradation of extracellular matrix macromolecules.

Femor et al. (7) also showed that both hydrostatic and intermittent compression increase NOS activity and NO production. Static (0.1 MPa for 24 h) and intermittent compression significantly increased NO production. NOS activity, evaluated using only intermittent compression, was significantly increased. Use of iNOS-specific inhibitor (1400W) completely inhibited NO release in response to 0.1 MPa, 0.5 Hz for 24 h, confirming the role of iNOS in pressure-induced NO production. In experiments conducted over a shorter time course (400 s), Matsuo (26) and Nakago-Matsuo et al. (32) reported that pressure increased NO levels in human trabecular cells and periodontal ligament fibroblasts, respectively. Trabecular cell intracellular NO levels significantly increased following an application of 30, 40, and 50 mmHg, while the periodontal ligament fibroblasts responded to 75 and 100 mmHg with increased NO. Both authors attributed this increase to the activation of the constitutive NOS, nNOS. However, they did not assay for iNOS, and although N-methyl-L-arginine (L-NMMA) abolished the increase of NO, this broad inhibitor of all three NOS isoforms does not distinguish between the activation of the NOSs. We documented an early increase of NO following pressure –20, 30, 60, and 120 mmHg, at 5 min that remained elevated up to 120 min in the human (HKC-8) renal epithelial cells. A similar increase was noted in the rat (NRK-52 E) cells when subjected to pressures of (60 and 120 mmHg) for 60 and 120 min. In contrast, rat endothelial cells pressurized for 72 h at 150 mmHg had decreased NO levels, increased ANG II, and expression of ANG II receptor 2 (ATR2) (14). ANG II acting via ATR2 is believed to suppress NO in these pressurized endothelial cells.

Rapid release of NO as a result of pressure is thought to occur via Ca$^{2+}$ transients and subsequent activation of constitutive NOS (27, 33). However, we show that iNOS inhibitors suppressed the increase in NO in pressure-stimulated renal epithelial cells as early as 5 min and continue to inhibit the increase up to 120 min, resulting in the release of NO metabolites comparable to ambient pressurized cells. cGMP levels were suppressed below basal levels (with 1400W) or remained at basal level in the presence of inhibitors. However, conflicting results were found when examining cGMP concentrations obtained with the inhibitors at 120 min following 60 mmHg. A likely explanation is due to the selectivity and reversibility of the drugs. 1400W is 5,000-fold more selective for iNOS and has been shown to inhibit enzyme activity for over 120 min (10). Isothioureas and aminoouargine are reversible inhibitors and are 30- to 40-fold more selective for iNOS (11). Twenty-two percent of iNOS activity has been shown to be regained after 180 min of aminoguanodidine exposure (46). The total time of drug exposure (i.e., incubating cells with the drug and maximal experimental time of 120 min) yields a duration of 150–180 min; hence, iNOS can potentially recover its enzymatic activity. Slight increases in NO could potentially lead to the increased cGMP levels as recorded at 120 min with reversible inhibitors. Taken together, these findings suggest that pressure acts directly on iNOS protein in renal epithelial cells without new protein synthesis. Further studies are needed to identify the mechanism behind iNOS activation.

iNOS, thought to be primarily regulated at the transcriptional level, has recently been shown to undergo posttranslational regulation via proteasomal degradation (31), aggresome formation (22), and inhibition of dimerization (23) which affect the stability of the enzyme as part of the cell’s regulatory mechanism. iNOS has been shown to be expressed in proximal tubular epithelial cells (1, 8, 9, 30) and increased in obstructed nephropathy (18, 43). We present data showing that the mechanical stimulus, elevated pressure, can induce renal epithelial cells to express iNOS. Low levels of iNOS mRNA and protein were found in ambient pressurized cells, while 1) a dose-dependent increase of iNOS transcript occurred with increasing pressures, 2) transcript increased over time in cells subjected to 60 mmHg, 3) an earlier induction was found in pressurized cells compared with known stimuli, and 4) a 24-h exposure of 60 mmHg increased the protein expression of iNOS implicating pressure in the de novo synthesis of the enzyme. The intracellular mechanism of the response of renal cells to elevated pressure is unknown. However, from the analysis of iNOS gene expression using cytokines (IL-1β, TNF-α, and IFN-γ), whereby combinations of all three cytokines are required to induce maximal iNOS expression in human cells, the cytokine induction of iNOS has been shown to occur via the activation of NF-κB and Jak-Stat (signal transducer and activator of transcription) pathways (5, 37). Neufeld and Liu (34) showed using inhibitors of NF-κB that the induction of iNOS in response to both cytokines and elevated hydrostatic pressure was blocked. In contrast, only the response to cytokines was blocked by the inhibition of p38 mitogen-activated protein kinase, while only the response to pressure was blocked by inhibition of epidermal growth factor receptor tyrosine kinase.

We present data showing that NF-κB is involved in the increased expression of iNOS as a result of pressure. Under basal conditions, NF-κB is sequestered within the cytoplasm by the IκB family of inhibitory proteins. Proinflammatory mediators indirectly activate the IκB kinase complex to phosphorylate the IκB proteins. Phosphorylated IκB then dissociates from the NF-κB, undergoing degradation by ubiquitin-dependent proteasomes (37). The two inhibitors used namely MG which blocks NF-κB activation by preventing IκB degradation and BAY which irreversibly inhibits IκBα phosphorylation confirmed that NF-κB is involved in expression of iNOS as a result of pressure.

Islam et al. (16) showed that hydrostatic pressure (2.5–5 MPa, 30 min-4 h) increased cell death and apoptosis and reduced proliferation in chondrocytes. Harada et al. (14) recorded no differences in proliferation or apoptosis in pressure-stimulated (150 mmHg, 72 h) endothelial cells compared with those at atmospheric pressure. In this study, the application of 20–120 mmHg had no effect on cell death or apoptosis, while proliferation was significantly increased at 24 and 36 h following 60 mmHg in HKC-8 cells. Differences in methodology, degree of pressurization, and cell types may have contributed to the varying results. In UUO, NO has been shown to reduce cell death (18). The addition of the iNOS-irreversible inhibitor (1400W) significantly increased cell death at 30 and 60 min suggesting that the pressure-stimulated NO release may have prevented cell death.

Although UUO is known to alter the expression of several genes, it has not yet been established whether deformation acts directly or via local paracrine and autocrine factors in response to ureteral pressure. In particular, the local NOS system may play an important role in the adaptation of the kidney to...
pressure and volume overload (15, 43). NO secreted by the kidney is important in maintaining homeostatic regulation, proliferation, transcription, and energy metabolism (24, 28). Many, but not all, of the biological effects of NO are mediated by cGMP (4, 36). In the present study, pressure also increased the expression of sGC and increased cGMP levels. Thus it is likely that the NOS-sGC system is involved in the response to pressure. These data correlate with the in vivo findings, which demonstrate increased hyperemic response following the initial phase (0–90 min) of obstruction thought to be partially mediated by the augmented production of NO resulting in afferent and efferent arteriolar vasodilatation (6).

In conclusion, pressure induces an early induction of iNOS mRNA and also activation of NOS activity in both rat and human renal epithelial cells. As pressure is an important regulator of both physiological and pathophysiological function, elucidation of this mechanism will be important not only in the pathophysiology of obstructive nephropathy, but in other organ systems affected by pressure. Our novel simple pressure device should be very useful in studying pressure in many systems. Finally, these data suggest that pressure is a potent regulator of gene expression, which needs to be further explored.

GRANTS

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REFERENCES


