Pressure activates epidermal growth factor receptor (EGFR) leading to the induction of iNOS via NFκB and STAT3 in human proximal tubule cells (HKC-8)

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Running head: Pressure and EGF upregulate iNOS expression in renal cells.

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Ureteral obstruction (UO) leads to increased pressure and inducible nitric oxide synthase (iNOS) expression. This study examined the involvement of epidermal growth factor receptor (EGFR), nuclear factor kappa B (NFκB) and signal transducers and activators of transcription 3 (STAT3) in iNOS induction in human proximal tubule cells (HKC-8) in response to pressure or epidermal growth factor (EGF.) HKC-8 cells were subjected to 60 mmHg pressure or EGF from (0 - 36 hrs). iNOS was rapidly induced in response to EGF, earlier than that of pressure. The addition of EGFR, NFκB and STAT3 inhibitors significantly suppressed pressure or EGF stimulated iNOS mRNA and protein expression. Analysis of the activated states of EGFR, NFκB p65 and STAT3 following exposure to both stimuli demonstrated phosphorylation within 2.5 min. Anti-EGF antibody inhibited iNOS induction in pressurized HKC-8 cells, providing evidence that endogenous EGF mediates the response to pressure. In UO, when pressure is elevated, pEGFR was detected in the apical surface of the renal tubules, validating the *in vitro* findings. These data indicate that EGFR, NFκB and STAT3 are required for human iNOS gene induction in response to pressure or EGF, indicating a similar mechanism of activation.

Keywords: Epidermal Growth Factor Receptor (EGFR)

Epidermal Growth Factor (EGF)

Inducible nitric oxide synthase (iNOS)

Nuclear Factor kappa B (NFκB)

Signal transducers and activators of transcription (STAT)

Cytokine mix (CM)

Cycloheximide (CHX)

Introduction

Pressure is an important component of the mechanical environment that regulates gene expression (15, 21, 26, 36, 59) with significant impact on the function and remodeling of tissue (8, 41, 51, 55). Overexpression of the iNOS gene has been shown to coincide with increased pressure in many chronic and acute diseases, including glaucoma (35, 36), osteoarthritis and rheumatoid arthritis (15, 26), bladder outlet obstruction (7) and ureteral obstruction (24, 28, 56). In obstruction, pressure may also affect the expression of other genes; for example, Woolf and colleagues demonstrated increased PAX2 overexpression following in utero ureteral obstruction (59). Increased expression of other genes associated with proliferation, fibrosis and EMT has also been demonstrated following obstruction (10, 30, 40).

Ureteral obstruction results in the distension of the kidney caused by the increased pressure due to the obstructed flow of urine (10). Pressures of up to 48 - 60 mmHg have been documented in the obstructed ureter (6, 14). Nitric oxide (NO) produced by iNOS has been shown to have beneficial effects in the obstructed kidney (16, 23, 24, 27). The signal transduction pathways that govern iNOS gene expression as a result of pressure in proximal tubule epithelial cells have not been characterized. Hence, elucidation of these pathways would provide insight into novel therapeutic strategies to modulate iNOS expression in renal disease, with broader implications in other organ systems affected by pressure.

Activation and/or inhibition of nuclear factor kappa B (NFκB) and Janus kinase and signal transducers and activators of transcription (JAK/STAT) pathways are thought to be the central mechanism behind iNOS expression (31, 32, 38, 49, 53). Increased NFκB and STAT3 activation has been demonstrated following experimental ureteral obstruction (33, 39, 42). While a prominent role for NFκB-mediated signaling in the transcriptional control of iNOS expression has been suggested (30), STAT3 activation may also be affecting iNOS induction in obstructed nephropathy.

Pressurization of the human optic nerve head astrocytes (ONHA) and human bronchial epithelial cells (NHBE) lead to the phosphorylation of epidermal growth factor receptor (EGFR) (36, 54), with subsequent iNOS induction (36). EGFR has been shown to activate NFκB (20, 22) and STATs (11, 13, 37, 46, 50). Epidermal growth factor (EGF) stimulation of EGFR induces NFκB activation in human cancer and proximal tubule cells (20, 22), while EGFR/STAT3 interaction in the nucleus leads to transcriptional activation of iNOS (37). We and others have recently reported that pressurization of human kidney epithelial cells (HKC-8) and ONHA lead to the increased expression of iNOS via an NFκB dependent mechanism (4, 43).

Studies were performed to examine the signaling pathways activated by pressure in comparison to EGF in HKC-8 cells. We examined the role that EGFR, NFκB and STAT3 play in the transcriptional activation of human iNOS gene. HKC-8 cells were pressurized or exposed to EGF and iNOS expression examined. To investigate the pathways involved in pressure-induced or EGF-induced iNOS expression in HKC-8 cells, pharmacological modulations of EGFR, NFκB and STAT3 were utilized. In addition *in vivo* UUO studies were preformed and iNOS and EGFR expression were investigated.

The observations suggest that pressure and EGF contribute directly to the activation of EGFR and subsequent iNOS expression in the proximal tubule epithelial cells via a similar mechanism of action.

Methods

Bovine serum albumin (BSA), human recombinant interleukin-1β (IL1β), tumor necrosis factor α (TNFα), interferon γ (IFNγ) and epidermal growth factor (EGF) were purchased from (Sigma, St. Louis, Missouri). NFκB inhibitors, MG-132 (Z-Leu-Leu-Leu-H) (MG), a proteasome inhibitor that prevents the degradation of IκB and Bay 11-7058 (E)-3-((4-t-Butylphenylsulfonyl)-2-propenenitrile (BAY)), which prevents the phosphorylation of IκBα, were purchased from BIOMOL (Plymouth Meeting, PA). EGFR inhibitors, AG 1478 (4-(3-Chloroanilino)-6,7-dimethoxyquinazoline) and AG 183 (2-Amino-4-(3′,4′,5′-trihydroxyphenyl)-1,1,3-tricyanobuta-1,3-diene Tyrphostin A51), STAT3 inhibitor, AG 490 (N-Benzyl-3,4-dihydroxybenzylidenecyanoacetamide) and p38 MAP kinase inhibitor, SB 202190 (FHPI 4-(4-Fluorophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl)1H-imidazole) were purchased from Calbiochem (Darmstadt, Germany). Translation inhibitor, cycloheximide (CHX) was purchased form Sigma (St. Louis, MO). Beta actin (BA), EGFR, NFκB p65, STAT1 and STAT3 rabbit polyclonal antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Phosphorylated form of NFκB p65 was purchased from Santa Cruz Biotechnology. Phosphorylated EGFR (Tyr 1068), STAT1 (Tyr 701) and STAT3 (Tyr 705) rabbit polyclonal antibodies were purchased from Cell Signaling Technology (Danvers, MA). EGFR mouse monoclonal was purchased from Abcam (Cambridge, MA). An antibody blocking EGF function was purchased from R&D Systems (Minneapolis, MN). Dulbecco's modified eagle medium (DMEM) with or without phenol red was obtained from Invitrogen (Carlsbad, CA); fetal bovine serum (FBS) was obtained from Gemini-Bio Products (Woodland CA). A broad

spectrum inhibitor of matrix metalloprotease (MMP) activity, GM 6001 was purchased from Calbiochem (Darmstadt, Germany). All other chemicals were of reagent grade.

Pressure Apparatus

To study the effects of pressure on cells *in vitro*, a motorized pressure apparatus originally designed was used. The entire system was placed in a CO_2 incubator to maintain constant temperature, atmosphere and humidity. Continuous exchange of the incubator atmosphere is facilitated via inlet and outlet valves. This novel system can reproducibly apply pressures of 20 to 200 mmHg. (1mmHg = 1.3595 cm H_2O).

Cultures

Human kidney epithelial cells, HKC-8 were obtained from Lorraine Racusen (John Hopkins University, Dept of Pathology). Cultures were grown in a humidified atmosphere of 5% CO_2 and 95% air at 37°C in DMEM (containing 1,000 mg/L D-glucose, L-glutamine, 25mM 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 mm^2 flasks. When cells reached 60 - 80 % confluence, the medium was replaced with fresh serum free medium. For cells requiring 12 - 36 hrs stimuli, cultures were at 60% confluency; in all other experiments (5 min - 4 hrs), cultures were at 80% confluency at the start of the experiment. Pressures of 0 mmHg (control) or 60 mmHg was applied, for varying times as described below. A known stimulus for iNOS induction, a cytokine mixture (CM)

composed of human recombinant IL1β (1 ng/ml), TNFα (25 ng/ml), and IFNγ (400 U/ml) was used as a positive control (48).

Cell Death.

Cell viability was assessed using the trypan blue exclusion method. HKC-8 cells exposed to the various inhibitors (AG 1478, AG 183, AG 490, BAY, MG and SB 202190 at 10μM and 100μM concentration) for 120 min in the absence and presence of 60 mmHg pressure or EGF or CM were assessed. Cell death was measured at 24 hrs in the presence and absence of the inhibitors - AG 1478, AG 490, BAY, SB 202190 (10 μM) and CHX (10 μg/ml) following 60 mmHg pressure, EGF or CM treatment. Analysis at 36 hrs following 60 mmHg pressure, EGF or CM treatment was also conducted. Cells were detached in a solution of 0.25% trypsin-EDTA (GIBCO) in phosphate buffered saline (PBS), pH 7.4. Cells were counted using a hemocytometer with the addition of trypan blue. Less than 8% cell death occurred at 120 min and less than 15 % cell death occurred with experiments conducted at 24 and 36 hrs.

Reverse transcriptase (RT) –PCR

Reverse transcriptase (RT) was used to measure the steady-state levels of mRNA using primers for human iNOS and GAPDH (see table 1). HKC-8 cells were exposed to a dose increase of EGF (0, 10, 50, 100 and 500 nM) for 60 min and EGF (10 nM) over time (0, 5, 30, 60 and 120 min). HKC-8 cells exposed to the inhibitors of EGFR, NFκB, STAT3 and p38 MAP kinase were allowed to incubate for 30 min before the application of 60 mmHg pressure, EGF or CM for 120 min. Inhibitors, AG 1478, AG 183, AG 490,

BAY and MG, were at 10 μM and 100 μM concentration. Rat kidneys were also examined for the expression of iNOS, endothelial nitric oxide synthase (eNOS), soluble guanylyl cyclase (sGC), EGFR and GAPDH (see Table 1 for primer sequence).

Total RNA was extracted using the Trizol ®-Chloroform extraction procedure. mRNA was purified using the Oligotex mRNA extraction kit (Qiagen, Valencia, California) according to manufacturer's instructions. mRNA concentration and purity was determined by measuring absorbance at 260 nm. RT-PCR was preformed using Qiagen One-step PCR kit (Qiagen, Valencia, California) using equal (20-100 ng) of transcript: PCR was preformed in an automated Thermal 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 sec at 94°C, annealing for 30 sec at 60°C, extension for 60 sec 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 GAPDH primer was designed according to the literature (44). iNOS primer was designed using Primer 3 program. HKC-8 cells were subjected to 60 mmHg pressure, EGF (10 nM) or CM over time (0, 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 transcription and PCR to take place. The following reverse-transcriptase was employed using 500 ng of RNA: denaturation 5 min at 65^{o}C, 10 - 20 min at 4^{o}C, cDNA Synthesis 50 min at 50°C,

termination of reaction 5 min at 85°C, removal of RNA with the addition of 1µl RNaseH 20 min at 37°C. qPCR protocol was employed using 2µl of the RT product: Reverse transcription for 2 min at 50°C, initial activation step (for HotStar Taq DNA polymerase activation) for 2 min at 95°C, denaturation for 15 sec at 95°C, annealing for 30 sec at 60°C, extension for 30 sec at 72°C; 35 rounds of amplification was 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 manufacturer's protocol. A melting step by slow heating from 65° C to 95° C with a rate of 0.2°C/sec 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 $2^{-\Delta\Delta CT}$ method (19).

iNOS ELISA

iNOS ELISA was conducted on HKC-8 cells incubated with EGF and CM for 4, 12, 24 and 36 hrs as well as on HKC-8 cells following 60 mmHg pressure, EGF, CM for 24 hrs in the absence and presence of inhibitors. Inhibitors AG 1478, AG 183, AG 490, BAY, MG, SB 202190, or GM 6001 at 10 µM concentration, and CHX or anti-EGF at 10ug/ml, were added to HKC-8 cells for 60 min before 60 mmHg pressure, EGF (10 nM) or CM applied for 24 hrs. Cells were washed two times with PBS. Cells were lysed and iNOS protein expression assessed using Human iNOS Quantikine kit (R&D Systems,

Minneapolis, MN) according manufacturer's instruction. Data were normalized using BSA assay to determine total protein concentration.

EGF ELISA

EGF ELISA was conducted on HKC-8 cells following 60 mmHg pressure for 5, 30, 60 and 120 min. Supernatants were collected and assayed according to Human EGF Quantikine kit (R&D Systems, Minneapolis, MN) manufacturer's instruction. BSA assay was used to determine total protein concentration. Data were normalized to total protein concentration.

Immunoblot

Following 60 mmHg pressure or EGF for 0, 2.5, 5, 10, 15, 20 and 30 min as well as in the presence and absence of EGFR inhibitor AG 1478 (10μM) for 30 min. Cells were collected and lysed using RIPA buffer (Pierce biotechnology)**.** Cellular proteins were separated on SDS-polyacrylamide gel (7.5% and 12%) electrophoresis (50 and 25 ug protein per lane) and then transferred onto a polyvinylidene difluoride membrane. The immobilized proteins were visualized by subsequent incubation with polyclonal rabbit antibody against human EGFR, pEGFR, NFκB p65, pNFκB p65 STAT1, pSTAT1, STAT3 and pSTAT3. A polyclonal horse radish peroxidase- conjugated goat anti-rabbit IgG (Bio Rad) were used as secondary antibody and staining was preformed with BM chemiluminescence's blotting kit (Bio Rad). Initial analysis for EGFR was also preformed in resting HKC-8 cells.

Animals

Sprague Dawley (SD) rats underwent left ureteral ligation at the end of lower ureter, just above the ureterovesical junction with 4-0 silk suture. A midline abdominal incision was made under sterile conditions. Animals were anesthetized with ketamine /xylazine cocktail. Both obstructed and unobstructed kidneys were harvested after 30, 60 and 120 min. Kidneys were perfused with 1x PBS for 10 min then sectioned and stored accordingly for RT-PCR and immunohistochemistry. For immunohistochemistry tissue was placed in formalin (36 hrs), paraffin embedded, sectioned and stained for EGFR, pEGFR and iNOS. Animal treatment adhered to approved institutional guidelines. We also sectioned formalin-fixed, paraffin-embedded kidney tissue remaining from the published study (28) and stained for EGFR and iNOS.

Immunohistochemistry: (120 min and 24 hrs Obstruction),

Paraffin-embedded kidney sections were cut at 5 μm. Slides were deparaffinized in CitriSolvent (Fisher Scientific) and alcohol, with endogenous peroxidase activity quenched with hydrogen peroxide for 10 min. Slides were incubated in primary antibody for iNOS, EGFR and pEGFR (Santa Cruz Biotechnology and Cell Signaling Technology) in concentrations of 1:200, 1:25 and 1:100 respectively overnight at $4^{o}C$; negative control slides were incubated in BSA antibody buffer without secondary antibody. Slides were washed in PBS (three times, 5 min/ wash) and incubated for 30 min at room temperature in 1:1000 anti-rabbit secondary antibodies (Vector labs). Slides were washed in PBS (three times, 5 min/ wash) and incubated for a further 30 min at room temperature in 1:1000 tertiary antibodies (Vector labs). Slides were developed in DAB and counter

stained with 10% hematoxylin. Additionally, immunofluorescence was also conducted using EGFR and Alexa Fluor® antibody (Invitrogen) as per manufacturer's instructions.

Statistics

All data were expressed as mean ± S.E.M. Statistical comparison were by t-test and paired t-test, with statistical significance taken as $p < 0.05$ (two-tailed).

Results

EGFR is expressed in HKC-8 cells.

To ensure that HKC-8 cells express EGFR, they were evaluated for presence of the receptor using immunoblot. We identified EGFR protein to be expressed in quiescent HKC-8 cells (Figure 1).

EGF elicits an earlier increase in iNOS mRNA expression as compared to pressure.

EGFR signals the induction of iNOS in human ONHA and carcinoma cells in response to both pressure and EGF, or EGF, respectively (36, 37, 43). To determine whether EGF/EGFR induces iNOS mRNA expression in HKC-8 cells, cells were treated with increasing concentrations of EGF (0 to 500 nM). iNOS mRNA expression was increased with 1 - 10 nM EGF; however at the higher concentration of 100 and 500 nM no further increase was detected (Figure 2A).

To compare iNOS mRNA expression in response to either EGF (10 nM) or 60 mmHg pressure, HKC-8 cells were stimulated over time (0 to 120 min). CM was used as a positive control. A time dependent increase in iNOS mRNA expression was detected when cells were treated with 10 nM EGF (Figure 2B and 2C). HKC-8 cells treated with EGF demonstrated a significant early increase of iNOS mRNA at 5 min with increasing expression up to 120 min (control: 1.02 ± 0.09 vs. 5 min: 1.44 ± 0.13, $p<0.05$, 120 min: 2.22 ± 0.47, $p<0.05$, mean fold change in gene expression) (Figure 2C). HKC-8 cells subjected to 60 mmHg pressure had a significant increase in iNOS expression at 30 min with increasing expression up to 120 min (control: 1.08 ± 0.16 vs 30 min: 2.06 ± 0.21, $p< 0.05$, 120 min: 2.44 ± 0.37, $p< 0.05$, mean fold change in gene expression) (Figure 2C).

CM induced a significant increase in iNOS expression at 120 min (control: 1.14 ± 0.43 vs 120 min: 11.82 ± 2.67, $p < 0.05$, mean fold change in gene expression) (Figure 2C). A dose of 10 nM EGF and a 60 mmHg pressure were used for the rest of the experiments.

iNOS protein expressed is increased in response to EGF or CM.

We next examined whether HKC-8 cells express iNOS protein in response to EGF. HKC-8 cells were treated with EGF for 4, 12, 24 and 36 hrs. As a positive control, cells were treated with CM. As shown in Figure 3A, EGF significantly increased iNOS protein expression at 12 and 24 hrs. iNOS expression in CM treated HKC-8 cells was 3 times more at 12 hrs compared to EGF (Figure 3A). The protein expression of iNOS was decreased by 36 hrs, whereas iNOS expression in CM treated cells was still elevated compared to control (control: 1.00 ± 0.19 vs. 36 hrs: 3.60 ± 0.07, $p<0.005$, increase relative to control) (Figure 3A).

To ensure that new protein synthesis was occurring as a result of these stimuli, the translation inhibitor CHX was employed. Cells were incubated for 60 min with CHX followed by 24 hrs of 60 mmHg pressure, EGF or CM. As shown in Figure 3B, CHX inhibited the increase in iNOS resulting from 60 mmHg pressure or EGF to basal level (pressure: 3.35 ± 0.39 vs. CHX + pressure: 1.14 ± 0.12, $p<0.01$, EGF: 4.52 ± 0.83 vs. CHX + EGF: 1.18 ± 0.28, $p< 0.01$, increase relative to control) (Figure 3B). Similarly, CHX significantly inhibited CM induced iNOS expression, but not to basal levels (control: 1.00 ± 0.18 vs CHX ± CM: 2.52 ± 0.60, $p = 0.063$) (Figure 3B). CHX did not affect the basal level of iNOS protein expression.

Inhibition of EGFR suppresses iNOS expression in response to pressure or EGF, but not CM.

Inhibitors of EGFR suppressed iNOS expression following stimulation of ONHA with pressure or EGF, but not CM (36, 43). We therefore examined whether EGFR is involved in the induction of iNOS in HKC-8 cells in response to 60 mmHg pressure or EGF. CM was used as positive control. Using two different inhibitors of EGFR (AG 1478 and AG 183), we demonstrated that the increase in iNOS protein expression resulting from 24 hrs of 60 mmHg pressure or EGF was inhibited (pressure: 3.02 ± 0.75 vs. AG 1478 + pressure: 0.32± 0.39, $p< 0.05$, increase relative to control) or (EGF: 6.02 ± 0.80 vs. AG 1478 + EGF: 1.32 ± 0.34, $p< 0.0001$, increase relative to control) (Figure 4A). CM induction of iNOS protein expression was not affected by either EGFR inhibitors (Figure 4A). iNOS mRNA expression was suppressed to basal levels in response to 60 mmHg pressure or EGF in the presence of the EGFR inhibitors (see Supplement 1 A). Only AG 1478 at the higher concentration was effective at suppressing CM induced iNOS mRNA expression to basal levels (Supplement 1 A). EGFR inhibitors did not affect the basal level of iNOS (mRNA or protein) expression.

Inhibition of NFκB suppresses iNOS expression in response to pressure, EGF or CM

EGF or stimulation of EGFR leads to the activation NFκB in a variety of cells (20, 22, 52), while inhibition of NFκB suppressed both pressure and CM induced iNOS expression in ONHA (43). To determine if a similar response occurs with pressure and EGF, HKC-8 cells were treated with the two NFκB inhibitors, MG, and BAY (see methods). As shown in Figure 4B, both inhibitors suppressed iNOS protein expression in

response to 60 mmHg pressure, EGF or CM. iNOS mRNA expression was inhibited in the presence of the NFκB inhibitors following EGF or CM (Supplement 1B). NFκB inhibitors did not affect the basal level of iNOS (mRNA or protein) expression.

Inhibition of STAT3 suppresses iNOS expression in response to pressure, EGF or CM.

EGFR/STAT3 interaction results in nuclear translocation and transcriptional activation of iNOS (37). We sought to examine the role of STAT3 in iNOS induction in HKC-8 cells. AG 490 was used to selectively inhibit STAT3 activation. Increased iNOS protein expression as a result of 24 hrs of 60 mmHg pressure, EGF or CM was significantly inhibited in the presence of AG 490 (Figure 4C). AG 490 inhibited iNOS mRNA expression to basal levels following 60 mmHg pressure, EGF or CM (Supplement 1C). AG 490 did not affect the basal level of iNOS (mRNA or protein) expression.

Increased phosphorylation of EGFR, NFκB p65 and STAT3 in response to pressure or EGF.

Activation and/or nuclear localization of EGFR, NFκB, STAT1 and STAT3 have been demonstrated following pressurization and/or EGF treatment (11, 20, 22, 36, 37, 50, 54). In addition, following obstruction NFκB and STAT3 are activated (33, 39, 42). To further confirm the involvement of EGFR, NFκB, STAT1 and STAT3, immunoblots were used to assess the activation of these proteins following 60 mmHg pressure or EGF over time (2.5- 30 min). As shown in Figure 5A, both 60 mmHg pressure or EGF activated EGFR, NFκB p65 and STAT3 within 2.5 min with increased phosphorylation up to 30 min. STAT1 expression was not affected with either stimulus.

We next examined EGFR, NFκB p65 and STAT3 activation with or without EGFR inhibitor (AG 1478) pretreatment. In the presence of the EGFR inhibitor, the increased phosphorylation of EGFR, NFκB p65 and STAT3 as a result of 60 mmHg pressure or EGF was inhibited (Figure 5B). Total EGFR, NFκB p65 and STAT (1 and 3) expression were constant throughout all conditions in the experiments conducted (see the unphosphorylated forms in Figure 5).

Inhibition of p38 MAP kinase suppresses iNOS expression in response to pressure, EGF or CM

p38 MAP kinase has been shown to mediate transcriptional activation of iNOS (1, 43, 47) and internalization of EGFR (12, 57, 63). Endocytosis of the EGFR/STAT3 has been shown to be essential for STAT3 nuclear translocation and gene expression (2). To further explore the mechanism involved in pressure induced regulation of EGFR, we selectively inhibited p38 MAP kinase activity with SB 202190. As shown in Figure 6, SB 202190 effectively inhibited pressure induced iNOS protein expression (pressure: 3.12 ± 0.72 vs. SB 202190 + pressure: 0.53 ±0.35, $p<0.05$, increase relative to control). Similar effects were seen in the presence of EGF or CM (Figure 6). SB 202190 did not affect the basal level of iNOS protein expression.

Growth factor shedding into the extracellular space may be responsible for the induction of iNOS expression.

Pressure can lead to growth factor shedding into the extracellular space triggering cellular signaling via autocrine/paracrine binding to EGFR (54). The kidney is a major

site for EGF production (3, 62). Since physiological concentrations of EGF elicit an increase in iNOS expression, we first used ELISA to measure the EGF concentration in culture medium obtained following 60 mmHg pressure for 5, 30, 60 and 120 min. No change in EGF concentration was detected following application of pressure (Figure 7A). Using a broad-spectrum inhibitor of MMP activity (GM 6001) to inhibit ectodomain shedding of transmembrane EGF family members (54), we obtained data showing that iNOS expression is governed by growth factor shedding in pressurized HKC-8 cells (Figure 7B). GM 6001 did not affect the basal level of iNOS protein expression (Figure 7B).

To identify if EGF was responsible for this effect, we pressurized cells in the presence and absence of neutralizing antibody to EGF. As seen in Figure 7C, anti-EGF inhibited the pressure induced iNOS protein expression (pressure: 4.35 ± 0.46 vs. anti-EGF + pressure: 0.52 ± 0.5, $p<0.001$). The basal level of iNOS protein expression was not affected by anti-EGF (Figure 7C).

iNOS and sGC expression are increased following ureteral obstruction. pEGFR is present on the apical cell surface of the renal tubules of the obstructed kidney.

iNOS and EGFR expression are increased following UUO (3, 24, 28, 56). We examined iNOS, eNOS and EGFR expression at 30, 60 and 120 min following UUO. In addition we examined a downstream target of NO, sGC expression that has been demonstrated *in vitro* to increase with pressure (4). Expression and localization of EGFR and iNOS were also examined at 120 min and 24 hrs following UUO. There was a significant increase in iNOS mRNA expression at 30 and 60 min (30 min, unobstructed:

0.19 ± 0.03 vs. obstructed: 0.31 ± 0.05, $p<0.05$, 60 min, unobstructed: 0.13 ± 0.03 vs. obstructed: 0.31 ± 0.05, $p<0.01$, normalized to GAPDH) (Figure 8A and 8B). There was an increase in the expression of sGC and EGFR at 60 min following UUO (Data not shown). eNOS expression did not change following UUO (Data not shown). In the unobstructed kidneys minimal expression of iNOS was seen in the cortical tubular cells. iNOS staining was increased predominantly in the renal tubules of the obstructed kidney following 120 min and 24 hrs UUO (Figure 8C). No change in the intensity of EGFR expression was evident in the obstructed kidney as compared to the unobstructed contralateral kidney. However, in both the 24 hrs obstructed and contralateral kidneys EGFR was predominantly localized apically in the tubular cells (Figure 8D). In the sham operated animals, mRNA expression of iNOS, eNOS, sGC and EGFR were not different between left and right kidneys, confirming that the experimental trauma of the surgery was not affecting the gene expression examined (Figure 8A).

Immunohistochemistry was also used to investigate the presence of pEGFR at 120 min following UUO. pEGFR was not apparent in the contralateral kidney (Figure 8D). In contrast, immunohistochemistry labeling for pEGFR was detected in the obstructed kidney. The distribution of pEGFR in the obstructed kidney was apparent in the apical cell surface of the renal tubules (Figure 8D).

Discussion

Sustained pressure is an important contributor to pathologic insults in a number of organs (8, 41, 51, 55). Identification of the mechanisms by which signals are transduced by pressure is important in identifying targets which may be manipulated to therapeutic advantage. This report presents data showing that pressure can lead to the activation of EGFR and downstream targets NFκB and STAT3 leading to an increase in iNOS expression in human kidney epithelial cells (HKC-8). This conclusion is consistent with the observation that cells exposed to inhibitors of EGF, EGFR, NFκB or STAT3 in the presence of pressure or EGF prevented iNOS induction. Figure 9 depicts a model of pressure induced EGF/EGFR activation that illustrates our findings. In addition, our results show an extremely rapid increase in iNOS mRNA expression in response to exogenous EGF compared to pressure. *In vivo* findings corroborated our *in vitro* data.

To date, the mechanism underlying iNOS expression in response to pressure remains unknown. EGFR has been implicated in this signaling pathway (36, 43). Phosphorylated EGFR is detected following pressurization as early as 5 min in ONHA and NHBE cells (36, 54). Nuclear localization has been identified within 10 min of pressurization of ONHA cells (36), while stimulation of human epithelial carcinoma cell (A431) with EGF resulted with phosphorylation and nuclear localization within 1 min (34). Inhibition of EGFR has been shown to suppress iNOS expression resulting from pressurization (43) and we have demonstrated similar findings. Phosphorylated EGFR was detected 2.5 min following pressure or EGF treatment.

Previously, heparin-binding epidermal growth factor (HB-EGF) shedding has been shown to be upstream of pressure stimulated EGFR activation (54). In our system,

data suggest that EGF is the growth factor upstream of EGFR. Exogenous EGF mimicked the effects of pressure on iNOS expression with an earlier significant increase in iNOS mRNA expression at 5 min. Time taken for pro-EGF to be shed in response to pressure may be responsible for differences in initial iNOS induction. Assaying the medium of pressurized HKC-8 failed to detect an increase in EGF. Dilution of EGF in the conditioned medium may have occurred. However, in the presence of the broad specific inhibitor of MMP (GM 6001) or neutralizing antibody against EGF, the increase in iNOS protein expression resulting from pressure was inhibited. These data suggest that the pro-EGF can be cleaved under pressure and is accountable for the observed EGFR signaling and iNOS expression in HKC-8 cells. We did not identify the MMP that leads to the cleavage of pro-EGF as a result of pressure.

Activation of transcription factors STATs and NFκB downstream of EGFR has been demonstrated (11, 20, 22, 37, 52). STAT and NFκB are thought to be the major pathways involved in iNOS induction (20, 31, 37). The iNOS promoter of human, rat and mouse contains multiple binding sites for NFκB and STAT (17, 31). Lo et al have provided conclusive evidence that EGF/EGFR/STAT3 growth factor pathway activates iNOS gene expression and subsequent NO synthesis in human tumor cells (37). Similarly, activation of NFκB has been shown to occur via EGF/EGFR pathway (20, 22, 45). Inhibition of NFκB in pressure stimulated OHNA and HKC-8 cells inhibited iNOS expression (4, 43). Using inhibitors specific for EGFR, STAT3 or NFκB, we demonstrated their effectiveness in blocking iNOS expression in HKC-8 cells in response to pressure, EGF or CM. Immunoblot confirmed the activation of EGFR, NFκB p65 and STAT3 within 2.5 min following pressure or EGF. Inhibition of EGFR suppressed this

activation, suggesting that NFκB p65 and STAT3 are downstream of EGFR. STAT1 was not affected, but we cannot rule out the possibility that it could have been activated later, after 30 min. Studies by Yu et al have demonstrated STAT3/ NFκB p65 interaction decreases the transcription of iNOS (60, 61), thus controlling the magnitude of iNOS induction and possibly explains the lesser induction as seen in pressure or EGF stimulated cells in comparison to the more potent induction seen with CM. Involvement of TNFα/NFκB and INFγ/JAK2/STAT1α pathway in iNOS induction has been demonstrated in murine and human cells (31). Also in addition to activating INFγ, IL1 and TNF receptors, CM has been known to activate receptor tyrosine kinases (31). TNFα and IFNγ have been shown to activate EGFR (5, 25) and probably accounts for the portion of CM-induced iNOS which is blocked by EGFR inhibitors.

We observed an increase in iNOS expression as a result of p38 MAPK signaling. The consistent inhibition of iNOS protein production by a specific inhibitor of p38 MAPK (SB 202190) in response to pressure, EGF or CM highlights the relevance of p38 MAPK in iNOS induction. Increase in iNOS expression via p38 MAPK activation has been demonstrated (1, 43, 47). MAPK inhibition has been shown to decrease CM-stimulated increase in iNOS protein expression in human kidney epithelial and ONHA cells (43, 47), while having no effect on iNOS expression in pressurized ONHA cells (43). Differences in cell types and amount of pressure may explain the discrepancies in the literature. Transient phosphorylation of EGFR by p38 MAPK has been shown to lead to internalization via a clathrin mediated process (57, 63). Further studies are needed to clarify the role of p38 MAPK activation, EGFR internalization and nuclear localization in the induction of iNOS following pressure.

Our *in vivo* study demonstrated significant increase in iNOS expression in the obstructed kidneys and confirms data presented by other investigators (16, 24, 28). In HKC-8 cells, pressure induces a time-dependent increase in iNOS expression that is not observed in the obstructed kidney. Maximum increase of iNOS expression was documented at 60 min *in vivo*. An increase in transforming growth factor β (TGFβ) may be affecting iNOS expression following UUO (40). TGFβ has been shown to be a negative regulator of iNOS expression; iNOS and NO were increased in the kidney of TGFβ -/- mice (58). eNOS mRNA expression remained unchanged following obstruction and is consistent with our *in vitro* data (4). EGFR was apically localized in the 24 hrs obstructed and contralateral kidneys. Kiley et al have demonstrated the change in EGFR localization resulting from changes in the polarization of tubular cells as a result of obstruction (29). In normal kidneys EGFR is present in the basolateral membrane of tubular cells (9, 18, 29) while apical membrane localization is seen in the dilated tubules of obstructed kidneys (29). Phosphorylated EGFR was evident in the apical surface of the renal tubules of the obstructed kidney (120 min) and not in the contralateral unobstructed kidney, indicating activation of EGFR *in vivo* at a time when pressure increases in response to obstruction.

The results presented clarify the mechanism whereby pressure mediates the activation of the EGF/EGFR cascade leading to iNOS induction. In addition, our data lead us to speculate that there might be a functional significance of EGFR relocation. Relocation and activation of EGFR may provide a mechanism for regulating either the time course or spatial distribution of NO production resulting in distinctly different consequences for the cell. Further experiments with this system should provide insight

into the relevance of pressure-EGFR mediated iNOS induction in a number of physiological and pathophysiological conditions.

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Titles and Legends

Figure 1: <u>Protein expression of EGFR in HKC-8 cells.</u> EGFR and beta actin proteins were detected using immunoblot. Gels were loaded with 25 μg protein. Anti-EGFR and beta actin antibodies were used to detect the respective proteins.

Figure 2: <u>iNOS mRNA expression in response to 60 mmHg, EGF and CM.</u> ***A:*** Representative gel of iNOS mRNA expression in response to increasing EGF concentration (1, 10, 100, 500 nM) for 60 min. ***B:*** Representative gel of iNOS mRNA expression in response to EGF (10nM) over time (0, 5, 30, 60 and 120 min). HKC-8 iNOS mRNA levels were detected using RT-PCR. ***C:*** iNOS expression in response to 60 mmHg, EGF and CM over time (5, 30, 60 and 120 min). iNOS expression was detected using qPCR. ** $p < 0.01$, * $p < 0.05$ compared with control , # $p < 0.05$ compared with 60 mmHg or EGF ($N \geq 4$). Data are expressed as means ± SE. Black: 60 mmHg, Checkered: EGF and Grey: CM.

Figure 3: <u>iNOS protein expression in response to 60 mmHg, EGF or CM and in the presence of translational inhibitor, CHX.</u> ***A:*** iNOS protein expression in response to EGF and CM over time. ** $p < 0.005$, * $p < 0.05$ vs. control ($N \geq 4$). Data are expressed as means ± SE. Checkered: EGF and Grey: CM. ***B:*** iNOS protein expression in response to 60 mmHg, EGF and CM in the absence and presence of CHX for 24 hrs. iNOS protein was detected using ELISA. Expressed as increase relative to control. ** $p < 0.001$, * $p < 0.01$ vs control , ## $p < 0.01$, # $p < 0.05$ vs. individual treatment group ($N \geq 5$). Data are expressed as means ± SE.

Figure 4: <u>iNOS protein expression in the presence of EGFR, NFκB and STAT3 inhibitors</u>. ***A:*** iNOS protein expression in the absence and presence of the EGFR inhibitors, AG 1478 and AG183 (10 μM), followed by 60 mmHg, EGF or CM for 24 hrs. ** $p < 0.001$, * $p < 0.05$ vs control, ## $p < 0.0001$, # $p < 0.05$ vs. individual treatment group ($N \geq 4$). ***B:*** iNOS protein expression in the absence and presence of the NFκB inhibitors, MG and BAY (10 μM), followed by 60 mmHg, EGF or CM for 24 hrs. ** $p < 0.001$, * $p < 0.05$ vs. control, ## $p < 0.005$, # $p < 0.05$ vs. individual treatment group ($N \geq 4$). ***C*:** iNOS protein expression in the absence and presence of the STAT3 inhibitor, AG 490 (10 μM), followed by 60 mmHg, EGF or CM for 24 hrs. ** $p < 0.001$, * $p < 0.05$ vs. control, ## $p < 0.005$, # $p < 0.05$ vs. individual treatment group ($N \geq 4$). iNOS protein was detected using ELISA. Expressed as increase relative to control. Data are expressed as means ± SE.

Figure 5: <u>Activation of EGFR, NFκB and STAT.</u> Representative immunoblots of phosphorylated EGFR, NFκB p65, STAT1 and STAT3 ***A*:** in response to 60 mmHg or EGF. ***B:*** in response to 60 mmHg pressure or EGF in the presence of EGFR inhibitor AG 1478 (10 μM). Anti-pEGFR, pNF-kB p65, pSTAT1, pSTAT3 and were used to detect the proteins. N = 2. C: control, P: 60 mmHg, A: AG 1478(10μM) and E: EGF (10nM).

Figure 6: <u>iNOS protein expression in the presence of p38 MAPK inhibitor.</u> iNOS protein expression in the absence and presence of the p38 MAPK inhibitor (10 μM), followed by 60 mmHg, EGF or CM for 24 h. iNOS protein was detected using ELISA. Expressed as fold increase relative to control. * $p < 0.05$ vs. control, # $p < 0.05$, vs. individual treatment group ($N \geq 4$)). Data are expressed as means ± SE.

Figure 7: <u>Identification of the growth factor responsible for iNOS induction:</u> ***A:*** EGF protein expression in the absence and presence of 60 mmHg over time (5, 30, 60 and 120 min). EGF protein was detected using ELISA. Expressed as increase relative to control. (N ≥5). Data are expressed as means ± SE. ***B:*** iNOS protein expression in the absence and presence of MMP inhibitor, GM6001. **P* < 0.01, compared with control (N = 4). ***C:*** iNOS protein expression in the absence and presence of anti-EGF inhibitor. iNOS protein was detected using ELISA. Expressed as increase relative to control. ***P* < 0.01, **P* < 0.05, compared with control. (*N= 6*). Data are expressed as means ± SE.

Figure 8: <u>iNOS, eNOS, sGC and EGFR expression and activation following UUO.</u> ***A:*** Representative gels of iNOS, eNOS, sGC, and EGFR mRNA expression in response to 30, 60 and 120 min UUO (O: obstructed, U: unobstructed) and 120 min sham operation (L: left kidney, R: right kidney). ***B:*** iNOS mRNA expression following 30, 60 and 120 min UUO. mRNA levels were detected using RT-PCR. Densitometric analysis obtained with NIH Image J software, mRNA expression normalized to GAPDH. $^{**}P < 0.01$, $^{*}P < 0.05$, compared with control ($N \geq 8$). White: unobstructed, Black: obstructed. ***C:*** Immunohistochemistry of iNOS protein expression following 120 min and 24 hrs UUO. Top left:120 min unobstructed contralateral kidney, Top right: 120 min obstructed kidney, Bottom left: 24 hrs unobstructed contralateral kidney, bottom right: 24 hrs obstructed kidney. Anti-iNOS antibody was used to identify iNOS-positive cells (brown stain). Magnification 10X. ***D:*** Immunohistochemistry of EGFR and pEGFR protein expression following 120 min and 24 hrs UUO. Top left: 120 min unobstructed contralateral kidney, Top right: 120 min obstructed kidney, Middle left: 24 hrs unobstructed contralateral kidney, Middle right: 24 hrs obstructed kidney, Bottom left*:*120 min unobstructed contralateral kidney, Bottom right: 120 min obstructed kidney. Anti-EGFR antibody was used to identify EGFR-positive cells (brown stain). Magnification 10X. Anti-pEGFR antibody and was used to identify pEGFR positive cells. Labeling for pEGFR (arrow) in obstructed kidney. Magnification 20X.

Figure 9. Model of iNOS induction: Renal proximal tubular cells response to pressure. Our findings favor a model where pressure promotes the early shedding of the EGFR ligand, EGF that is proteolytically cleaved by matrix metalloproteases (MMP). Once released, ligand binding to the receptor occurs via an autocrine/paracrine event. EGFR signals to NFκB and STAT3 promoting nuclear translocation and transcriptional activation of the iNOS gene and subsequent translation of the protein. In the disease state of UUO where EGFR levels are elevated and apically located, NFκB and STAT3 are recruited to the receptor. Upon activation, NFκB and STAT3 translocate to the nucleus to activate iNOS transcription. *In vivo*, other EGFR ligand/s may be involved in this process.

Supplementary Figure 1: **<u>iNOS mRNA expression in the presence of EGFR, NFκB and STAT3 inhibitors</u>.** ***A:*** iNOS mRNA expression in the absence and presence of the EGFR inhibitors, AG 1478 and AG 183 (10 and 100 μM), following 60 mmHg, EGF or CM for 120 min. ***B:*** iNOS mRNA expression in the absence and presence of the NFκB inhibitors, MG and BAY (10 and 100 μM), following EGF or CM for 120 min. ***C:*** iNOS mRNA expression in the absence and presence of the STAT3 inhibitor, AG 490 (10 and 100 μM), following 60 mmHg, EGF or CM for 120 min.

iNOS mRNA levels were detected using RT-PCR. Densitometric analysis obtained with NIH Image J software, for iNOS expression normalized to GAPDH. ** $p < 0.01$, * $p < 0.05$ vs. control , ## $p < 0.01$, # $p < 0.05$ vs. individual treatment group ($N \geq 4$). Data are expressed as means ± SE. Black: 60mmHg, Checkered: EGF and Grey: CM

Tables

Table 1: Primer sequence used for RT-PCR and qPCR

GENE NAME	5'- 3' SEQUENCE	SIZE: BP
Human		
iNOS	F: GACTTCTGTGACCTCCA R: GGTGATGCTCCCAGACAT	257
GAPDH	F: ACGGGAAGCTTGTCATCAAT R: AGTTGTCATGGATGACCTTGG	311
Rat		
iNOS	F: GACTTCTGTGACCTCCA R: GGTGATGCTCCCAGACAT	
eNOS	F: CGCTCCTGCAAAGAAAAACT R: TTCTGGCAAGACCGATTACAC	478
sGC	F: TCCATTTTCCCTGGTGATGT R: TTGCTTGCCAGAGTGACATT	401
EGFR	F: GTGCAAATAGCAACAGTTCCA R: TGTTGAGATACTCAGGGTTGC	299
GAPDH	F: ACCACAGTCCATGCCATCAC R: TCCACCACCCTGTTGCTGTA	451

iNOS; inducible nitric oxide synthase, eNOS; endothelial nitric oxide synthase, sGC; soluble guanylyl cyclase, EGFR; epidermal growth factor receptor, GAPDH; glyceraldehyde-3-phosphate dehydrogenase