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# Osmolar regulation of endothelin-1 production by the inner medullary collecting duct

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ABSTRACT

*Aims:* Endothelin-1 (ET-1) is an autocrine inhibitor of collecting duct (CD) $Na^+$ and water reabsorption. CD ET-1 production is increased by a high salt diet and is important in promoting a natriuretic response. The mechanisms by which a high salt diet enhances CD ET-1 are being uncovered. In particular, elevated tubule fluid flow, as occurs in salt loading, enhances CD ET-1 synthesis. Tubule fluid solute content and interstitial osmolality can also be altered by a high salt diet, however their effect on CD ET-1 alone, or in combination with flow, is poorly understood.

*Main methods:* ET-1 mRNA production by a mouse inner medullary CD cell line (mIMCD3) in response to changing flow and/or osmolality was assessed.

*Key findings:* Flow or hyperosmolality (using NaCl, mannitol or urea) individually caused an ~2-fold increase in ET-1 mRNA, while flow and hyperosmolality together increased ET-1 mRNA by ~14 fold. The hyperosmolality effect alone and the synergistic effect of flow + hyperosmolality was inhibited by chelation of intracellular $Ca^{2+}$, however were not altered by blockade of downstream $Ca^{2+}$-signaling pathways (calcineurin or NFATc), inhibition of cellular $Ca^{2+}$ entry channels (purinergic receptors or polycystin-2), or blockade of the epithelial $Na^+$ channel. Inhibition of NFAT5 with rottlerin or NFAT5 siRNA greatly reduced the stimulatory effect of osmolality alone and osmolality + flow on mIMCD3 ET-1 mRNA levels.

*Significance:* Both flow and osmolality individually and synergistically stimulate mIMCD3 ET-1 mRNA content. These findings may be relevant to explaining high salt diet induction of CD ET-1 production.



## 1. Introduction

Collecting duct (CD) endothelin-1 (ET-1) is an important regulator of arterial pressure and urinary $Na^+$ and water excretion. ET-1 is a potent autocrine inhibitor of CD $Na^+$ and water reabsorption [1,2]; CD-specific knockout of ET-1 causes $Na^+$ and water retention and hypertension [3–5]. ET receptor antagonist-induced fluid retention, a major factor in clinical trials [6,7], is substantially due to inhibition of CD ET receptors [8].

Increased salt intake increases CD ET-1 production; this response is important in maintaining body fluid volume (BFV) homeostasis [9]. The mechanism(s) by which elevated salt intake enhances CD ET-1 synthesis are beginning to be understood. Circulating hormones (aldosterone, atrial natriuretic peptide, angiotensin II and vasopressin) do not appear to be involved [10–12], suggesting a role for local factors. Recent studies by our group showed that tubule fluid flow, which is increased by high salt intake, stimulates both cortical CD (CCD) [13,14] and inner medullary CD (IMCD) [14] ET-1 mRNA accumulation. The stimulatory effect of flow on CCD ET-1 synthesis is mediated, at least in part, by $Na^+$ delivery and the epithelial $Na^+$ channel (ENaC) [14], however flow-stimulated IMCD ET-1 production is independent of ENaC [14]. Since the IMCD produces and binds more ET-1 than any other cell type in the kidney [9,15], we sought to further explore how IMCD ET-1 is regulated. One possible IMCD ET-1 regulatory factor, in addition to flow, is osmolality. Renal interstitial and tubule fluid NaCl concentration can be increased by a high salt diet. Increased renal medullary interstitial NaCl concentration has been shown to enhance thick ascending limb ET-1 production [16]. Increased media osmolality has also been reported to stimulate IMCD ET-1 production [17–19], however other studies have not observed this and/or have described a dependence upon the specific solute studied [9,19]. Furthermore, the effect of osmolality on the IMCD ET-1 response to flow is unknown. Consequently, the current study was undertaken to investigate whether osmolality alone, or together with flow, can affect IMCD ET-1. We hypothesized that since both flow and osmolality are increased by high salt intake, that these factor work together to augment CD ET-1 biosynthesis.

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## 2. Materials and methods

### 2.1. Reagents

Calcineurin inhibitory peptide and cyclosporine A were obtained from Tocris Bioscience (Ellisville, MO). All other drugs and chemicals were obtained from Sigma (St. Louis, MO) unless stated otherwise.

### 2.2. Cell culture

The mouse IMCD cell line, mIMCD3, was used for all studies. Mouse IMCD3 cells with polycystin-2 knockdown were provided by Drs. Rajeev Rohatgi and Luca Gusella at the Icahn School of Medicine at Mount Sinai, NY [20]. Cells were grown to confluence on 10-$cm^2$ plastic culture plates in a 5% $CO_2$ incubator at 37 °C; 50:50 DMEM/F-12 supplemented with 10% fetal bovine serum, 1 mg/ml penicillin and 1 mg/ml streptomycin was used as growth medium. For the control studies done under stationary conditions, cells were grown in 12-well plates under identical conditions. In each study, sample size (N) refers to separate plates. No more than 2 plates came from a given cell split.

### 2.3. Flow and osmolality studies

Rectangular parallel plate polycarbonate flow chambers (Catalog number 31-010, Glycotech, Gaithersburg, MD) were attached to 10-cm cell culture plates containing confluent mIMCD3 cells using vacuum and Silastic gaskets to form a channel with the dimensions: 0.25 mm depth, 1 cm width and 5.9 cm in length, having a total surface area of 5.9 $cm^2$. The flow chamber has two ports through which perfusate enters and exits the channel. The liquid was pumped with a peristaltic pump (Ismatec, Glattburg, Switzerland). HBSS (pH 7.4) was used as the perfusate for control experiments and was supplemented with drugs and/or chemicals for additional experiments. RNA was extracted from cells exposed to flow and from control no-flow cells. All experiments were performed at 37 °C.

Previous studies by our group determined that a 2 h exposure to 2 dyne/$cm^2$ shear stress optimally stimulated ET-1 mRNA accumulation in mIMCD3 cells [14], hence all experimental periods lasted 2 h and all shear stress was 2 dyne/$cm^2$. All pharmacologic agents were added 30 min before, and continued throughout, the experimental period. Similarly, studies on hyperosmolality involved increasing perfusate osmolality 30 min before and throughout the experimental period. Each experimental period involved exposing cells to either stationary conditions or to flow. For stationary studies, cells were placed in flow chambers in a manner identical to flow studies, however the chambers were only filled with perfusate and not exposed to flow.

### 2.4. RNA analysis and real-time PCR

RNA from cultured cells was isolated using the RNeasy Mini Kit and reverse transcribed with Omniscript RT Kit (Qiagen, Valencia, CA). ET-1, GAPDH and NFAT5 mRNA levels were determined by real-time PCR (StepOne Plus, Applied Biosystems, Foster City, CA) using the Taqman Gene Expression Assay with ET-1 (catalog number Mm00438656_m1), GAPDH (catalog number Mm03302249_m1) and NFAT5 (catalog number Mm00467257_m1) primers, respectively.

### 2.5. siRNA studies

Mouse NFAT5 siRNA (catalog number MSS225643) and negative controls (scrambled siRNA sequences) were purchased from Invitrogen (Waltham, MA). Cells were grown on 25 mm glass coverslips (Warner Instruments, Hamden, CT), transfection was carried out for 48 h using Lipofectamine RNAiMax as the transfection agent (Invitrogen), and flow studies were performed in polycarbonate flow chambers (catalog number 64-1860, Warner instruments, Hamden CT) according to conditions described above. RNA was extracted from cells exposed to flow or no flow conditions. All experiments were performed at 37 °C.

### 2.6. Statistics

Data are represented as mean ± SE. One-way ANOVA was used to compare the differences between groups. The Shapiro–Wilk test was used to evaluate for normal distribution. $P < 0.05$ was considered significant.

## 3. Results

### 3.1. Effect of flow and osmolality on mIMCD3 ET-1 mRNA

ET-1 mRNA very closely reflects ET-1 protein levels [9]; since ET-1 protein levels were below the level of detection in the flow system, only ET-1 mRNA values are reported. As previously described [14], flow increased mIMCD3 ET-1 mRNA ~2-fold as compared to cells under stationary (no flow) conditions (Fig. 1). Increasing media osmolality to 450 mOsm with mannitol augmented ET-1 mRNA ~2-fold in cells under stationary conditions, while hyperosmolality and flow synergistically increased ET-1 mRNA by ~14-fold (Fig. 1). Increasing osmolality with urea or NaCl to 450 mOsm gave a similar ET-1 mRNA response to hyperosmolality ± flow as that seen with mannitol (Fig. 2).

### 3.2. Role of ENaC or $Ca^{2+}$ pathways in flow- and osmolality-stimulated mIMCD3 ET-1 mRNA

As previously described [14], ENaC inhibition did not affect the mIMCD3 ET-1 flow response (Fig. 3). In addition, ENaC blockade had no effect on osmolality-stimulated ET-1 mRNA levels (Fig. 3). Chelation of intracellular $Ca^{2+}$ with BAPTA-AM inhibited the ET-1 response to flow, hyperosmolality and flow + hyperosmolality (Fig. 4). Since $Ca^{2+}$ appears to play a central role, the effect of modifying various $Ca^{2+}$-signaling pathways and $Ca^{2+}$ channels that have been implicated as having a role in flow-stimulated mIMCD3 ET-1 mRNA production [14] was assessed. Inhibition of calcineurin with cyclosporine A prevented the flow stimulated ET-1 mRNA increase (Fig. 5), however it had no effect on hyperosmolality or hyperosmolality + flow stimulated ET-1 mRNA. Since nuclear factor of activated T-cells (NFAT) 1–4 (NFATc) has been implicated in ET-1 regulation in IMCD cells [21], the effect of inhibition of NFATc (VIVIT peptide) was examined; VIVIT peptide had no effect on flow and/or hyperosmolality stimulation of ET-1 mRNA (Fig. 6). The effect of blockade of two $Ca^{2+}$ cellular entry pathways (polycystin-2 and purinergic receptors) was examined since both pathways have been implicated in modulation of IMCD ET-1

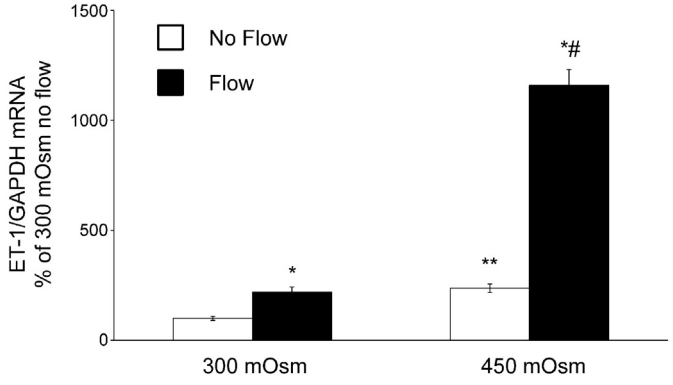


**Fig. 1.** Effect of hyperosmolality (mannitol) on stationary and flow stimulated ET-1 mRNA content in mIMCD3 cells. n = 10–12. *p < 0.05 vs. no flow control same conditions; **p < 0.05 vs. no flow control 300 mOsm: #p <0.05 vs. flow 300 mOsm.

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