Interleukin-6 stimulates epithelial sodium channels in mouse cortical collecting duct cells

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1Georgia Prevention Institute, Department of Pediatrics, Medical College of Georgia, Augusta, Georgia; 2Medical College, Wuhan University, Wuhan, Hubei, People’s Republic of China; and 3Tulane University and Tulane Hypertension and Renal Center of Excellence New Orleans, Louisiana

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Li K, Guo D, Zhu H, Hering-Smith KS, Hamm LL, Ouyang J, Dong Y. Interleukin-6 stimulates epithelial sodium channels in mouse cortical collecting duct cells. Am J Physiol Regul Integr Comp Physiol 299: R590–R595, 2010. First published May 26, 2010; doi:10.1152/ajpregu.00207.2009.—The aim of this study is to elucidate the effects of interleukin-6 (IL-6) on the expression and activity of the epithelial sodium channel (ENaC), which is one of the key mechanisms underlying tubular sodium reabsorption. M-1 cortical collecting duct cells were treated with IL-6 (100 ng/ml) for 12 h. Real-time polymerase chain reaction and immunoblotting were employed to examine the mRNA and protein abundance. Transepithelial voltage (VTE) and resistance (RET) were measured with an ohm/voltmeter (EVOM, WPI). The equivalent current was calculated as the ratio of VTE to RET. Treatment with IL-6 (n = 5) increased the mRNA abundance of α-ENaC by 11 ± 7% (P = not significant), β-ENaC by 78 ± 14% (P = 0.01), γ-ENaC by 185 ± 38% (P = 0.02), and prostasin by 29 ± 5% (P = 0.01), all normalized by β-actin. Treatment with IL-6 increased the protein expression of α-ENaC by 19 ± 3% (P = 0.001), β-ENaC by 89 ± 21% (P = 0.01), γ-ENaC by 36 ± 12% (P = 0.02), and prostasin by 33 ± 6% (P = 0.02). The amiloride-sensitive sodium current increased by 37 ± 5%, from 6.0 ± 0.4 to 8.2 ± 0.3 μA/cm² (P < 0.01), in the cells treated with IL-6 compared with controls (P = 0.01). Aprotinin (28 μg/ml), a prostasin inhibitor, reduced the amiloride-sensitive sodium current by 61 ± 5%, from 6.1 ± 0.3 to 3.7 ± 0.2 μA/cm² (P = 0.01). The magnitude of the IL-6-induced amiloride-sensitive sodium current in the presence of aprotinin dropped by 57 ± 2%, from 8.6 ± 0.2 to 4.9 ± 0.2 μA/cm² (P < 0.01). This study has identified a novel function of IL-6, namely, IL-6 may activate ENaC. Therefore, renal inflammation mediated by IL-6 likely contributes to impaired pressure natriuresis.

INTERLEUKIN-6 (IL-6), a pleiotropic cytokine produced by numerous cell types, plays an important role in the inflammation cascade. IL-6 exerts multiple effects on infiltrated inflammatory cells and on structural cells in a variety of tissues and organs, including kidneys (8, 24, 44, 48). Data suggest that IL-6-involved inflammatory processes in the kidney contribute to the pathogenesis of nephropathy (28, 40). IL-6-deficient mice were resistant to HgCl₂-induced acute kidney injury compared with wild-type mice (42). Renal function decline in patients with type 1 diabetes was correlated with elevated excretion of IL-6 in urine (67). Renal cortical mRNA expression of IL-6 was 3.4-fold higher in diabetic than in nondiabetic rats, which was significantly associated with urinary albumin excretion (41). In patients with diabetic nephropathy, IL-6 mRNA was expressed by glomerular resident cells and interstitial cells in the renal tissue (56). IL-6 was also shown to be linked to hypertensive glomerular injury (31, 47). Biopsy studies using indirect immunofluorescence microscopy showed that, in patients with kidney disease, IL-6 expression was increased in the area of glomerular and tubular inflammation and tubular atrophy (18, 19).

Furthermore, using mice with knockout of IL-6, Lee et al. (33) found that angiotensin II (ANG II)-induced hypertension was significantly dependent on IL-6. The kidneys of the IL-6 knockout mice were more capable of eliminating a salt load during ANG II infusion than were the kidneys of the wild-type mice. The hypertension caused by a high dose of ANG II and a high-salt diet was attenuated in IL-6 knockout mice (34). These data suggest that there is a powerful effect of IL-6 in mediating the rightward shift in the renal pressure-natriuresis relationship caused by ANG II and high salt intake, providing evidence for an important long-term blood pressure effect of IL-6. In addition, prenatal exposure to IL-6 resulted in hypertension, alterations in the renal and circulatory renin-angiotensin system, and decreased mean urinary sodium excretion in rats (49). However, the mechanisms whereby IL-6 regulates sodium handling remain unknown.

The epithelial sodium channel (ENaC) consists of at least three subunits (α-ENaC, β-ENaC, and γ-ENaC) and is thought to regulate the final adjustment of renal sodium reabsorption in the distal tubules (51). Therefore, sodium homeostasis, extracellular volume, and, ultimately, blood pressure are maintained by precise regulation of ENaC activity. ENaC activity is controlled by aldosterone, other hormones such as vasopressin, and a network of intracellular factors and multiple accessory regulatory proteins that are not completely understood. Prostasin, a glycosylphosphatidylinositol-anchored serine protease expressed in the distal nephron, has been shown to regulate the proteolytic processing and activation of ENaC subunits in Xenopus oocytes and a mouse cortical collecting duct (CCD) cell line (M-1 cells) (1, 13, 14, 25, 26, 30, 46, 59–61, 63, 64). Prostasin is thought to induce cleavage of an inhibitory peptide from γ-ENaC to activate the channel fully in the cell (9, 10, 25). Because of the impact of ENaC on sodium homeostasis and blood pressure control, the present study was conducted to evaluate the effects of IL-6 on the gene and protein expression of ENaC subunits and the activity of ENaC in CCD cells. In addition, we hypothesize that IL-6 might affect prostasin. We chose M-1 cells, a mammalian cell line derived from microdissected CCD of a mouse transgenic for the early region of

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SV40, which retains a number of the characteristics of the parental CCDs, including the expression of ENaC, high trans-epithelial electrical resistance ($R_{te}$), and amiloride-sensitive sodium currents (6, 55). Thus, M-1 cells have been valuable tools for studying ENaC and related regulatory proteins (29, 35, 38, 39, 58, 65).

**METHODS**

*Cell culture.* The M-1 cells were obtained and cultured as previously described (38). Briefly, the cultures were initially maintained in a defined medium consisting of equal amounts of Ham’s F-12 and low-glucose Dulbecco’s modified Eagle’s medium (Sigma), supplemented with 2 mmol/l l-glutamine, 50 U/ml penicillin, 50 mg/ml streptomycin, 5% fetal bovine serum, growth-promoting factors (6.25 mg/ml transferrin, insulin, and sodium selenite), and 100 nmol/l dexamethasone. After the cells reached confluence, they were passaged to permeable membranes (Transwells, Costar). Cells were synchronized by serum deprivation for 24 h and treated with IL-6 (100 ng/ml) for 12 h. Finally, cells were harvested for mRNA and protein extraction. The Alamar blue viability assay showed that the cell survival rate was $\approx$85%, after treatment with 100 ng/ml IL-6 for 12 h.

*Real-time PCR.* Prescribed TaqMan gene expression assays for mouse α-ENaC (Mm00803386_m1), β-ENaC (Mm00441215_m1), γ-ENaC (Mm00441228_m1), prostasin (Mm00504792_m1), and β-actin (Mm00607939_s1) were purchased from Applied Biosystems (Foster City, CA). Total RNA was extracted from M-1 cells, and 5 μg of total RNA were reverse-transcribed to cDNA with oligo(dT) primers using Superscript III. Real-time PCR was performed with the ABI 7500 fast real-time PCR system (Applied Biosystems). The M-1 cells were used for electrophysiological transepithelial measurements.

**RESULTS**

*Effects of IL-6 on the gene expression of ENaC subunits and prostasin.* Treatment with IL-6 (100 ng/ml) for 12 h increased the mRNA abundance of α-ENaC by $11 \pm 7\%$ ($P = 0.01$), β-ENaC by $78 \pm 14\%$ ($P = 0.01$), γ-ENaC by $185 \pm 38\%$ ($P = 0.02$), and prostasin by $29 \pm 5\%$ ($P = 0.01$; Fig. 1).

*Effects of IL-6 on the protein expression of ENaC subunits and prostasin.* Treatment with IL-6 (100 ng/ml) for 12 h increased the protein expression of α-ENaC by $19 \pm 3\%$ ($P = 0.001$), β-ENaC by $89 \pm 21\%$ ($P = 0.01$), γ-ENaC by $36 \pm 13\%$ ($P = 0.01$), and prostasin by $16 \pm 5\%$ ($P = 0.01$).

**Statistical analyses.** Values are means ± SE. Statistical analyses were performed with SPSS 15.0 (SPSS, Chicago, IL). For Western blotting and gene expressions, Student’s $t$-test was used to determine statistical significance between two groups before and after experimental manipulations. For electrophysiological transepithelial measurements, ANOVA was used to determine the statistical significance among groups before and after experimental manipulations. Results were considered significant if $P < 0.05$.

**Fig. 1.** Effects of IL-6 on mRNA expression of epithelial sodium channel (ENaC) α-, β-, and γ-subunits and prostasin. M-1 cells, which were serum-deprived for 12 h, were treated with 100 ng/ml IL-6 for 12 h. At 12 h after treatment, total RNA was extracted from M-1 cells, and 5 μg of total RNA were reverse-transcribed to cDNA with oligo(dT) primers. Abundance of each mRNA was normalized to β-actin and compared with control. Values [means ± SE (n = 5)] are expressed as fold increase over control. *$P < 0.05$ vs. control.
higher doses, 150 and 200 ng/ml, however, appeared to de-
ting (10% SDS-PAGE).
with 100 ng/ml IL-6, and protein expression was determined by immunoblot-
expressed as fold increase over control. Values are means 
IL-6 (100 ng/ml) significantly increased \( I_{eq} \) by 37 ± 5% compared with nontreated cells. Amiloride almost completely inhibited IL-6-induced \( I_{eq} \), and preincubation of IL-6 for 12 h did not counterbalance the inhibitory effect of amiloride on \( I_{eq} \). Values are means ± SE (n = 3). *P < 0.05 vs. control.

Amiloride-sensitive \( I_{eq} \) in response to IL-6. IL-6 concentrations of 10, 100, 150, and 200 ng/ml were used to test the potential dose dependency of \( I_{eq} \) (n = 3 for each dose). The amiloride-sensitive \( I_{eq} \) was increased only ~5% by 10 ng/ml IL-6 (P > 0.05) and ~37% by 100 ng/ml IL-6 (P < 0.01). The higher doses, 150 and 200 ng/ml, however, appeared to de-
crease the amiloride-sensitive \( I_{eq} \) ~30% (P = 0.04) and ~40% (P = 0.01), respectively. Thus, 100 ng/ml was used to test the effects of IL-6 on amiloride-sensitive \( I_{eq} \) in the M1-cells. Next, the effects of 100 ng/ml IL-6 were evaluated at 30 min, 1 h, 6 h, 12 h, 24 h, and up to 48 h (n = 3 for each time point). The amiloride-sensitive \( I_{eq} \) remained unchanged at 30 min, 1 h, and 6 h (P > 0.05). At 24 and 48 h, amiloride-sensitive \( I_{eq} \) was decreased up to 42% (P = 0.001) and 56% (P = 0.04), respectively. However, at 12 h, the amiloride-sensitive \( I_{eq} \) significantly increased (Fig. 3). As such, in the following experiments, M-1 cells were treated with IL-6 at 100 ng/ml for 12 h.

Amiloride, an ENaC inhibitor at a concentration of 10 \( \mu \)mol/l, almost completely abolished \( I_{eq} \). Conversely, aldoste-
rone (1 \( \mu \)mol/l) increased the amiloride-sensitive \( I_{eq} \) by 66 ± 5%. The aldosterone-induced \( I_{eq} \) was inhibited by 31 ± 4% by aprotinin (28 \( \mu \)g/ml), a prostasin inhibitor. The amiloride-
sensitive \( I_{eq} \) increased by 37 ± 5%, from 6.0 ± 0.4 to 8.2 ± 0.3 \( \mu \)A/cm\(^2\) (P < 0.01), in the cells treated with IL-6 (100 ng/ml) compared with controls. Amiloride almost completely inhibit-
ed IL-6-induced \( I_{eq} \) and IL-6 did not counterbalance the inhibitory effect of amiloride on \( I_{eq} \) (Fig. 3). After 12 h of incubation, aprotinin (28 \( \mu \)g/ml), a prostasin inhibitor, reduced the amiloride-sensitive \( I_{eq} \) by 61 ± 5%, from 6.4 ± 0.2 to 3.4 ± 0.3 \( \mu \)A/cm\(^2\) (P < 0.01), by reducing \( R_{te} \) and \( V_{te} \) (Table 1). In contrast, IL-6 did not alter \( R_{te} \) but increased \( V_{te} \) from 8.4 ± 0.2 to 12.3 ± 0.8 mV and, in turn, increased \( I_{eq} \). The magnitude of the IL-6-induced amiloride-sensitive \( I_{eq} \) in the presence of aprotinin dropped by 57 ± 2%, from 8.4 ± 0.2 to 4.9 ± 0.2 \( \mu \)A/cm\(^2\) (P < 0.01). Furthermore, IL-6 did not alter the amiloride-sensitive \( I_{eq} \) in the cells pretreated with aprotinin (Fig. 4).

**DISCUSSION**

The present study is one of the first to examine the distal tubular effects of IL-6, and the major finding is that exogenous IL-6 appears to augment ENaC expression and activity in M-1 CCD cells in vitro. M-1 cells treated with IL-6 exhibit an increase in the mRNA and protein levels of ENaC subunits and prostasin. The significant increase in \( I_{eq} \) in response to IL-6 is abolished by amiloride, indicating that the electrophysiological induction likely is of ENaC origin. Furthermore, the IL-6 induced \( I_{eq} \) is aprotinin-sensitive, suggesting that prostasin could contribute to the IL-6-related ENaC pathway. Our data imply that IL-6 might play a key role in sodium handling in the

**Table 1. Effects of IL-6 on \( R_{te} \) and \( V_{te} \)**

<table>
<thead>
<tr>
<th>Group</th>
<th>( R_{te} ), ( \Omega ) cm(^{-2} )</th>
<th>( V_{te} ), mV</th>
<th>( I_{eq} ), ( \mu )A/cm(^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>292.3 ± 7.5</td>
<td>8.4 ± 0.2</td>
<td>6.4 ± 0.2</td>
</tr>
<tr>
<td>Aprotinin</td>
<td>262.0 ± 20.4*</td>
<td>4.0 ± 0.3*</td>
<td>3.4 ± 0.3*</td>
</tr>
<tr>
<td>IL-6</td>
<td>307.3 ± 6.7*</td>
<td>12.3 ± 0.8*</td>
<td>8.9 ± 0.4*</td>
</tr>
<tr>
<td>IL-6 + aprotinin</td>
<td>250.7 ± 6.8*</td>
<td>5.6 ± 0.4*</td>
<td>4.9 ± 0.2*</td>
</tr>
<tr>
<td>Aprotinin + IL-6</td>
<td>254.0 ± 15.9*</td>
<td>4.2 ± 0.8*</td>
<td>3.6 ± 0.5*</td>
</tr>
</tbody>
</table>

Values are means ± SE. Treatment with aprotinin (28 \( \mu \)g/ml) for 12 h decreased transepithelial resistance (\( R_{te} \)) and voltage (\( V_{te} \)). Treatment with IL-6 (100 ng/ml) for 12 h did not alter \( R_{te} \) but markedly increased \( V_{te} \). In cells pretreated with IL-6 for 12 h (IL-6 + aprotinin), aprotinin decreased \( R_{te} \) and \( V_{te} \); in cells pretreated with aprotinin for 12 h (aprotinin + IL-6), IL-6 did not alter \( R_{te} \) or \( V_{te} \). \( I_{eq} \), transepithelial equivalent current. *P < 0.05 vs. control.
Inflammation and proinflammatory cytokines have been demonstrated to affect the function of ENaC. Transforming growth factor-β1 (TGF-β1) was shown to antagonize the action of aldosterone on sodium transport by the rat inner medullary collecting ducts in primary cell culture (27, 54). Specifically, TGF-β1 decreased the endogenous prostasin mRNA and protein expression by 50% ± 12% and 44% ± 12%, respectively, and the amiloride-sensitive \( \Delta^{22} \text{Na} \) uptake by 35.9 ± 2% after 12 h of incubation in aprotinin. IL-6 did not alter \( l_{eq} \) in cells pretreated with aprotinin for 12 h. Values are means ± SE (n = 3). *\( p < 0.05 \) vs. control.

Physiological stimuli for the synthesis of IL-6 include norepinephrine, ANG II, other cytokines, and even IL-6 itself (62). Whether IL-6 originates locally or systemically, IL-6 is the main circulating cytokine in linking systemic inflammation with local pathology (43). High salt intake, aldosterone, and ANG II have been shown to increase circulatory IL-6 level in Dahl-salt sensitive rats, hypertensive rats, diabetic rats, and normotensive humans (7, 36, 53, 57). High circulating levels of IL-6 have been correlated with increased blood pressure in spontaneously hypertensive rats and apparently healthy men and might be an independent risk factor for future myocardial infarction in humans (5, 11, 45, 50). It was suggested that IL-6 could be a determinant of diastolic blood pressure in children with obesity-related hypertension (21). Our results suggest that IL-6 might impair natriuresis via ENaC activation, which could partly contribute to salt sensitivity in subclinical inflammatory conditions such as diabetes, obesity, hypertension, and kidney diseases.

Fig. 4. Inhibition of IL-6-induced \( l_{eq} \) stimulation. Aprotinin (28 μg/ml), a proteasome inhibitor, reduced the amiloride-sensitive \( l_{eq} \) by 61 ± 5%. Magnitude of the IL-6-induced \( l_{eq} \) dropped by 57 ± 2% after 12 h of incubation in aprotinin. IL-6 did not alter \( l_{eq} \) in cells pretreated with aprotinin for 12 h. Values are means ± SE (n = 3). *\( p < 0.05 \) vs. control.

TNF exposure led to distal tubule sensitization that permitted acute TNF-induced activation of ENaC in diabetic rats (16). On the contrary, in cultured A6 distal nephron cells, inhibition of ENaC by TNF-α was mediated by ceramide through protein kinase C (2). The mRNA expressions of α-ENaC, β-ENaC, and γ-ENaC were depressed after treatment of mice and cultured M-1 cells with TNF-α and IL-1β, respectively (52). Interferon-γ also altered ENaC function in vitro and in vivo (52). Furthermore, lipopolysaccharide-induced downregulation of ENaC expression was inhibited in knockout mice with a deficiency for TNF-α, IL-1 receptor-1, or interferon-γ. In addition, TNF-α and IL-1β inhibited electrogenic sodium absorption in rat distal colon by mRNA expression regulation of β-ENaC and γ-ENaC (3). The effects of IL-2, IL-4, and IL-13 on sodium absorption, possibly via ENaC, were also shown in colon or bronchial epithelium (4, 20), although their impact on renal ENaC has not been studied. Masilamani et al. (37) demonstrated that elevated circulating aldosterone (due to dietary salt restriction or aldosterone infusion) markedly increased the abundance of α-ENaC protein without increasing the abundance of β-ENaC and γ-ENaC. In the present study, the responses of the gene and protein expression of ENaC subunits to IL-6 also seem to be heterogeneous, although they could be attributable to antibodies from different commercial sources in terms of the Western blot data. IL-6 consistently and markedly enhanced the mRNA and protein expression of β-ENaC, but the increase in the protein expression of γ-ENaC and α-ENaC was relatively small. IL-6 did not alter the gene expression of α-ENaC. The increase in the gene and protein expression of prostasin was also marginal, such that the biological impact of these subtle changes is in question. The aldosterone-induced \( l_{eq} \) (66 ± 5%) was more than twice as high as the IL-6-induced \( l_{eq} \) (37 ± 5%), suggesting that aldosterone is a more powerful ENaC regulator than is IL-6. Unlike aprotinin, IL-6 did not seem to inhibit the R\(_{sc}\). Instead, treatment with IL-6 resulted in the increment of voltage and, subsequently, the elevation of \( l_{eq} \), indicating that IL-6 might interact with ENaC to increase amiloride-sensitive sodium current. More than half (57 ± 2%) of the IL-6-induced \( l_{eq} \) was reduced by the inhibition of aprotinin, indicating that the stimulatory effect of IL-6 on ENaC activity involves the proteolytic processing. However, cautions are needed in the data interpretation, because aprotinin, also known as bovine pancreatic trypsin inhibitor, is a nonspecific inhibitor of prostasin (69). Several other serine proteases, such as kallikrein and trypsin, are also inhibited by aprotinin, so that other serine proteases may be induced by IL-6. The involvement of prostasin in the IL-6-ENaC conduit needs to be further studied in vitro and in vivo. It is likely that IL-6 could affect ENaC via signal transduction pathways, such as the NF-κB cascade (66, 32), which may be also involved in prostasin activity (58, 65). Furthermore, our data with a single dose (100 ng/ml) at a single time point (12 h) need to be interpreted with caution. In addition, we did not separate the immature from the mature form of the ENaC subunits, which is another limitation, since channel processing and trafficking may play an important role in the response of ENaC to the stimuli in the kidney (22, 23).

We speculate that, apart from ENaC, IL-6 may regulate other renal sodium channels, such as the sodium/hydrogen exchanger (NHE3) and sodium-chloride cotransporter in the proximal tubules, which collectively contribute to the tubular
sodium reabsorption. In particular, prostatin is believed to be expressed in the proximal tubules (68) and may affect these sodium transporters.

**Perspectives and Significance**

Taken together, we have demonstrated that exposure of M-1 cells to IL-6 might upregulate ENaC expression. Exogenous IL-6 increases amiloride-sensitive cells to IL-6 might upregulate ENaC expression. Exogenous expressed in the proximal tubules (68) and may affect these sodium reabsorption. In particular, prostasin is believed to be increased in hypertension, diabetes, and obesity, all of which demonstrate impaired sodium handling. Although further studies are required to fully elucidate the detailed regulatory mechanisms and biological implications of IL-6-induced ENaC activation in cells, animal models, and human subjects, our data could provide a potential mechanism linking sodium homeostasis derangement with chronic subclinical inflammatory conditions, including diabetes and diabetic nephropathy, obesity, hypertension, and kidney disease.

**GRANTS**

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the authors.

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