Sodium and potassium regulate endothelial phospholipase C-γ and Bmx

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1Division of Nephrology, Department of Medicine, Nephrology Research and Training Center, Center for Free Radical Biology, Center for Aging, and Department of Cell, Developmental and Integrative Biology, University of Alabama at Birmingham, Birmingham, Alabama; and 2Department of Veterans Affairs Medical Center, Birmingham, Alabama

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Ying W, Aaron KJ, Sanders PW. Sodium and potassium regulate endothelial phospholipase C-γ and Bmx. Am J Physiol Renal Physiol 307: F58–F63, 2014. First published April 30, 2014; doi:10.1152/ajprenal.00615.2013.—The amount of Na+ and K+ in the diet promotes significant changes in endothelial cell function. In the present study, a series of in vitro and in vivo experiments determined the role of Na+ and K+ in the regulation of two pleckstrin homology domain-containing intracellular signaling molecules, phospholipase C (PLC)-γ1, and epithelial and endothelial tyrosine kinase/bone marrow tyrosine kinase on chromosome X (Bmx), and agonist-generated Ca2+ signaling in the endothelium. Extracellular K+ concentration regulated the levels of activated PLC-γ1, Bmx, and carbachol-stimulated intracellular Ca2+ mobilization in human endothelial cells. Additional experiments confirmed that high-conductance Ca2+-activated K+ channels and phosphatidylinositol 3-kinase mediated these effects. The content of Na+ and K+ in the diet also regulated Bmx levels in endothelial cells and activated PLC-γ1 levels in rats in vivo. The effects of dietary K+ on Bmx were more pronounced in rats fed a high-salt diet compared with rats fed a low-salt diet. These experiments elucidated an endothelial cell signaling mechanism regulated by electrolytes, further demonstrating an integral relationship between endothelial cell function and dietary Na+ and K+ content.

dietary salt; potassium; endothelium; phospholipase c-γ; Tec kinase; bone marrow tyrosine kinase

EPITHELIAL AND ENDOTHELIAL TYROSINE KINASE/bone marrow tyrosine kinase on chromosome X (termed Bmx in this report), which is a member of the Tec kinase family, and phospholipase C (PLC)-γ1 are expressed in hematopoietic and endothelial cells (ECs) and are important constituents of multiple signaling cascades (16, 21, 35). Among other functions, Bmx and PLC-γ1 are involved in the mobilization of intracellular Ca2+ stores (5, 23, 27, 30, 31). Targeting PLC-γ to the plasma membrane through the production of phosphatidylinositol 3,4,5-trisphosphate (PIP3) is sufficient to catalyze the production of inositol trisphosphate (IP3) (1). The production of IP3 induces the release of Ca2+ from intracellular stores (30). Evidence from multiple laboratories has also demonstrated a critical role for Tec kinases in catalyzing the activation of PLC-γ and the generation of PLC-γ-dependent intracellular Ca2+ signaling pathways (5, 27, 31). Tec kinases serve as important cofactors that facilitate PLC-γ activation through multiple mechanisms that include functions as a scaffolding molecule as well as tyrosine phosphorylation of linker proteins and PLC-γ (23, 27). The level of protein expression of Bmx is an important determinant of cytoprotection and cell proliferation (3, 11, 13).

Phosphatidylinositol 3-kinase (PI3K) and phosphatase and tensin homolog deleted on chromosome 10 (PTEN), a lipid phosphatase that directly antagonizes 3-kinase activity of the class I PI3K family, regulate intracellular levels of PI(3,4,5)P3 (6, 18, 28, 33, 34). PI(3,4,5)P3 promotes the recruitment to the plasma membrane of pleckstrin homology (PH) domain-containing intracellular molecules (22, 29), permitting their activation and initiation of cell signaling pathways. PI3K and PTEN therefore regulate the function of PI(3,4,5)P3, which bind to PI(3,4,5)P3, including PKB (Akt) (18, 28, 29, 33, 34), isoforms of PLC-γ1, including PLC-γ1 (1, 36), and Bmx (22, 25, 27).

Previous studies have demonstrated the involvement of PI3K and PTEN in determining EC function during changes in dietary salt intake (40, 43). While increased dietary salt intake, but not extracellular Na+ concentration per se, increased endothelial PI(3,4,5)P3 through activation of PI3K (40), PTEN levels decreased in a dose-dependent fashion with decreasing extracellular K+ concentration (43). The latter mechanism was mediated through transforming growth factor-β and activation of the Smad signaling pathway, which regulate intracellular PTEN levels in the endothelium (43). Based on these previous studies, the hypothesis of the present study was that Na+ and K+ also regulated PLC-γ1 and Bmx and agonist-generated Ca2+ signaling in the endothelium.

METHODS

Human umbilical vein EC incubation experiments. Primary cultures of macrovascular ECs [human umbilical vein ECs (HUVECs)] were obtained commercially (Life Technologies, Grand Island, NY) and grown at 37°C in medium 200 (Life Technologies) and 5% CO2/95% air. Medium was exchanged at 48-h intervals, and cells were not used beyond 25–30 passages. Monolayers of HUVECs in 96- or 6-well plates were incubated in medium 200 that was produced without K+ by the manufacturer. This medium permitted the addition of KCl to final concentrations between 0 and 5 meq/l; choline chloride (Sigma-Aldrich, St. Louis, MO) at 0–5 meq/l replaced the KCl. Replacing KCl with choline chloride permitted no changes in extracellular osmolality among the groups. During these experiments, the medium was also supplemented with Low Serum Growth Supplement (Life Technologies), which resulted in a final concentration of 2% (vol/vol) FBS. Plates were incubated in these conditions for 24 h at 37°C before experiments. Some experiments also included the addition of iberiotoxin (Sigma-Aldrich) at 100 nM or vehicle as the medium K+ concentration was changed. Iberiotoxin served as a selective and reversible inhibitor of large-conductance Ca2+-activated K+ (BKca) channels (7). In additional experiments, 2-(4-morpholino)-8-phenyl-4H-1-benzopyran-4-one (LY-294002) at 10 μM was used to inhibit PI3K (37). Cell lysates were obtained for analysis of Bmx, phospho-PLC-γ1 (Y783), which indicated activation of PLC-γ1 (14), and GAPDH.

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Intracellular Ca\(^{2+}\) assay. Changes in cytoplasmic Ca\(^{2+}\) levels were determined using a kit (Molecular Probes Fluor-4 NW Calcium Assay, Life Technologies). On the day of the assay, the growth medium was removed from HUVECs, and 100 μl of the dye-loading solution containing the fluorescent Ca\(^{2+}\) indicator (Fluo-4 NW) were added into each well. Cells were incubated at 37°C for 30 min and then at room temperature for an additional 30 min. To demonstrate an effect on intracellular Ca\(^{2+}\) mobilization, HUVECs were then treated with carbachol (200 nM), which is an acetylcholinesterase-resistant acetylcholine analog that activates the M1 muscarinic acetylcholine receptor and increases intracellular Ca\(^{2+}\) through PLC-dependent production of IP\(_3\) (17). Fluorescence was determined between 0 and 5 min after addition of carbachol using an excitation wavelength of 494 nm and emission wavelength of 516 nm (Spectramax M2e microplate reader, Molecular Devices, Sunnyvale, CA).

Animal and tissue preparation. This study was carried out in accordance with recommendations in the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The Institutional Animal Care and Use Committee of the University of Alabama at Birmingham approved the project. Experiments were conducted using 32 male Sprague-Dawley rats (Harlan Laboratories, Indianapolis, IN) that were 28 days of age at the start of the study. Rats were housed under standard conditions and permitted free access to water. In the first series of experiments, rats were fed formulated diets (AIN-76A, Dyets) that contained different amounts of NaCl and KCl. Two of the diets contained 0.3% (wt/wt) NaCl and either 0 or 1.99% (wt/wt) KCl (AIN-76A, Dyets, Bethlehem, PA) that contained a standard amount [0.95% (wt/wt)] of K\(^{+}\) but differed in the content of NaCl, which was either 0.3% or 8.0% (wt/wt) NaCl. In the second series of experiments, rats were divided into four groups and given one of four formulated diets (AIN-76A, Dyets) that contained different amounts of NaCl and KCl. The other two diets contained 8.0% (wt/wt) NaCl and either 0% or 1.99% (wt/wt) K\(^{+}\). Diets were prepared specifically to be identical in protein composition and differed only in Na\(^{+}\), K\(^{+}\), and sucrose content. By replacing the electrolytes, the sucrose content was therefore the highest in the 0.3% NaCl-0% K\(^{+}\) diet and lowest in the 8.0% NaCl-1.99% K\(^{+}\) diet. On the final day of the study, rats were anesthetized with 2% isoflurane. Aortae were harvested under sterile conditions, and aortic EC lysates were obtained as previously described (41, 44). Sera were harvested at the termination of the study for the determination of concentrations of Na\(^{+}\) and K\(^{+}\) (Nova 16 Clinical Analyzer, Nova Biomedical, Waltham, MA).

Western blot analysis. Cell pellets were suspended in 300 μl of modified radioimmunoprecipitation assay buffer that contained the following: 10 mM Tris-HCl (pH 7.4), 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.5% sodium deoxycholate, 1% Triton X-100, 10% glycerol, 0.1% SDS, 20 mM sodium pyrophosphate, 2 mM Na\(_2\)VO\(_4\), 1 mM NaF, 1 mM PMSF, and protease inhibitor mixture. The total protein concentration of the lysates was determined using a kit (BCA Protein Assay Reagent kit, Thermo Scientific Pierce Protein Biology Products, Rockford, IL). Samples containing 25 μg total protein were processed for Western blot analysis, as we have previously described (39, 40, 42). The primary antibodies, diluted 1:1,000, specifically recognized Bmx (BD Biosciences, BD Transduction Laboratories, San Jose, CA), phospho-PLC-γ1 (Y783; Abcam, Cambridge, MA), and GAPDH (Abcam), which served as a loading control. Membranes were developed in standard fashion (SuperSignal West Dura Chemiluminescent Substrate, Thermo Scientific Pierce Protein Biology Products). Density of the bands was quantified using Quantity One software (Bio-Rad Laboratories, Hercules, CA).

Statistical analysis. Data are expressed as means ± SE. Significant differences were determined by ANOVA with post hoc testing, as appropriate. Data that were not normally distributed were analyzed using nonparametric ANOVA (Kruskal-Wallis test). For some experiments, data were modeled using factorial ANOVA, and mean differences were determined with Tukey’s post hoc testing (Proc GLM). P values of <0.05 were assigned statistical significance.

RESULTS

Extracellular K\(^{+}\) concentration determined levels of activated PLC-γ1 and Bmx in HUVECs, and the effect was mediated through BKCa channels. Tyrosine phosphorylation of PLC-γ1 (Y783) has been shown to indicate activation of this enzyme (14). Compared with HUVECs incubated overnight in medium containing 0 mM KCl, HUVECs in medium containing 5 mM KCl demonstrated reductions in phospho-PLC-γ1 (Y783) and Bmx; this effect was lost with the addition of iberiotoxin (100 nM) to cells in medium containing 5 mM KCl (Fig. 1). A dose-dependent effect of extracellular K\(^{+}\) concentration on phospho-PLC-γ1 (Y783) and Bmx was demonstrated (Fig. 2). In these experiments, choline chloride was added to maintain constant extracellular osmolality and Cl\(^{-}\) concentration.

Extracellular K\(^{+}\) concentration determined the level of intracellular Ca\(^{2+}\) mobilization in response to carbachol. Changes in cytoplasmic Ca\(^{2+}\) levels were determined after an overnight incubation of HUVECs in medium containing either 0 or 5 mM KCl. On the day of the assay, carbachol-induced mobilization of intracellular Ca\(^{2+}\) was reduced in cells incubated in the medium containing 5 mM KCl compared with cells incubated...
in the medium containing 0 mM KCl (Fig. 3). In these experiments, the concomitant addition of iberiotoxin abrogated the differences in intracellular Ca\textsuperscript{2+} mobilization generated by extracellular K\textsuperscript{+} concentration. To determine the involvement of PI3K, cells were incubated overnight in medium containing 5 mM KCl with or without iberiotoxin. Experiments were then performed 4 h after the addition of LY-294002 or vehicle. The observed effects of iberiotoxin on the increase in intracellular phospho-PLC-\gamma\textsubscript{1} (Y783) and Bmx were inhibited by the addition of LY-294002 (Fig. 4). Ca\textsuperscript{2+} mobilization experiments using cells preincubated in medium containing LY-294002 demonstrated the loss of the differences in intracellular Ca\textsuperscript{2+} mobilization generated by extracellular K\textsuperscript{+} concentration (Fig. 5).

In the second series of experiments, the effect of dietary K\textsuperscript{+} was examined (Fig. 6). In these experiments, serum K\textsuperscript{+} concentration, but not serum Na\textsuperscript{+} concentration, differed among the four groups (Table 1). Endothelial Bmx levels differed \((P < 0.0001)\) among the four dietary groups. In post hoc
with pretreatment with LY-294002.

Fig. 5. Pretreatment of HUVECs with LY-294002 resulted in similar degrees of carbachol-induced intracellular Ca\(^{2+}\) mobilization. The previously identified reduction in carbachol-induced intracellular Ca\(^{2+}\) mobilization observed in HUVECs incubated in medium containing 5 mM K\(^+\) concentration was lost with pretreatment with LY-294002. \(n = 16\) experiments/group.

analyses, the intake of dietary salt and K\(^+\), as well as their interaction, predicted Bmx levels \((P < 0.001\) for all analyses; Table 2). For endothelial phospho-PLC-\(\gamma_1\) \((Y783)\) levels, the model main effects for dietary salt and K\(^+\), but not their interaction, were statistically significant \((P < 0.005)\). Post hoc testing demonstrated that the dietary salt and K\(^+\) content predicted phospho-PLC-\(\gamma_1\) \((Y783)\) levels \((P < 0.01;\) Table 2).

DISCUSSION

The dietary content of Na\(^+\) and K\(^+\) modulates EC function through a series of signaling events that center primarily on PI3K, PTEN, and the generation of PIP\(_3\) \((40\text{–}43)\). Previous studies have demonstrated specifically that extracellular K\(^+\) concentration directly regulated cellular function \((42, 43)\), but similar increases in Na\(^+\) concentration had no effect and instead dietary salt altered endothelial cell function in vivo through a mechanism(s) reminiscent of shear forces \((39, 40, 42, 43, 46)\). The novel findings of the present series of in vitro and in vivo experiments build on these studies and include 1) extracellular K\(^+\) concentration regulated the levels of activated PLC-\(\gamma_1\), Bmx, and agonist-stimulated intracellular Ca\(^{2+}\) mobilization in human ECs and these effects were mediated through BK\(_{Ca}\) channels; 2) the dietary content of Na\(^+\) and K\(^+\) regulated EC PLC-\(\gamma_1\) and Bmx in rats in vivo; and 3) the effects of reduced dietary K\(^+\) intake on Bmx were more pronounced in rats fed a high-salt diet compared with rats fed a low-salt diet. These documented increases in Bmx and active PLC-\(\gamma_1\) after an increase in dietary salt intake, as well as through modulation by extracellular K\(^+\) concentration, were anticipated by previous studies that demonstrated the involvement of a PI3K/PTEN interaction, which regulated the activity of Akt, another PH-containing enzyme \((43)\). In those experi-

![Graph showing the effects of dietary Na\(^+\) and K\(^+\) on Bmx and PLC-\(\gamma_1\) levels](image)

Fig. 6. Dietary Na\(^+\) and K\(^+\) regulated levels of phospho-PLC-\(\gamma_1\) \((Y783;\) top) and Bmx \((middle)\) in the endothelium in vivo. The findings of the experiments \((n = 6\) animals/group) were summarized in the graph \((bottom)\), which was aligned with the Western blots to match the groups. See text for details.

Table 1. Comparison of serum electrolytes of rats on the four diets

<table>
<thead>
<tr>
<th>Dietary Electrolyte Composition</th>
<th>Serum Electrolytes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Na(^+), % (wt/wt)</td>
</tr>
<tr>
<td>Low NaCl</td>
<td>0.3</td>
</tr>
<tr>
<td>Low NaCl + Iberio</td>
<td>0.3</td>
</tr>
<tr>
<td>High NaCl</td>
<td>8.0</td>
</tr>
<tr>
<td>High NaCl + Iberio</td>
<td>8.0</td>
</tr>
</tbody>
</table>

*Significantly different \((P < 0.05)\) than the means of the groups on diets containing the corresponding identical salt content and increased \((1.99\%\) K\(^+\).

Table 2. Results of two-way ANOVA of the effects of dietary NaCl and dietary K\(^+\) on endothelial Bmx and PLC-\(\gamma_1\) in vivo

<table>
<thead>
<tr>
<th></th>
<th>Bmx</th>
<th>PLC-(\gamma_1)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Least square mean</td>
<td>(P) value</td>
</tr>
<tr>
<td>Main effect of dietary NaCl</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low dietary NaCl</td>
<td>0.24</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>High dietary NaCl</td>
<td>0.73</td>
<td>0.54</td>
</tr>
<tr>
<td>Main effect of dietary K(^+)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No K(^+)</td>
<td>0.66</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>2\times K(^+)</td>
<td>0.31</td>
<td>0.56</td>
</tr>
<tr>
<td>Dietary NaCl (\times) K(^+) interaction</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low NaCl (\times) no K(^+)</td>
<td>0.37</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Low NaCl (\times) 2\times K(^+)</td>
<td>0.11</td>
<td>0.24</td>
</tr>
<tr>
<td>High NaCl (\times) no K(^+)</td>
<td>0.96</td>
<td>0.60</td>
</tr>
<tr>
<td>High NaCl (\times) 2\times K(^+)</td>
<td>0.51</td>
<td>0.47</td>
</tr>
</tbody>
</table>

Bmx, endothelial tyrosine kinase/bone marrow tyrosine kinase on chromosome X; PLC, phospholipase C.
ments, extracellular $K^+$ concentration but not $Na^+$ concentration promoted the effects on this pathway.

$Bmx$, a member of the Tec kinase family, is expressed in ECs (16, 21, 35). While mice lacking the $Bmx$ gene grow normally and lack a phenotype (21), $Bmx$ is critically important in endothelial and vascular remodeling in pathological states (11). Current evidence supports an integral role of Tec kinases in signal transduction mechanisms that involve PLC-$\gamma$ (5, 27, 31). PI3K products activate $Bmx$ through the PH domain, which permits localization at sites in the plasma membrane where this protein can catalyze the activation of PLC-$\gamma$ (25). Consistent with the literature, the findings in the present study demonstrated that the amount of dietary salt, as well as $K^+$, intake regulate $Bmx$ protein expression in ECs.

The two isoforms of PLC-$\gamma$ have traditionally been associated with signal transduction involving receptor and nonreceptor tyrosine kinases. However, recent studies have shown that phosphoinositide products of PI3K (1) as well as G protein-coupled receptors (10, 24, 35, 36) may also activate PLC-$\gamma$. Intracellular $Ca^{2+}$ mobilization in response to agonists, such as acetylcholine, is a critically important feature of EC function (15). In the present study, lowering extracellular $K^+$ concentration, which has been shown to increase PI3K activity in HUVECs (43), increased $Bmx$ and activated PLC-$\gamma$ and promoted $Ca^{2+}$ mobilization after muscarinic receptor stimulation. A role for PI3K was demonstrated since inhibition of PI3K abrogated these findings (Figs. 4 and 5). These observations are also consistent with other studies that demonstrated synergistic interactions between G protein-coupled receptors and PI3K (19, 32). In response to increased salt intake, levels of $Bmx$ and active PLC-$\gamma_1$ in ECs increased; the involvement of these molecules in intracellular $Ca^{2+}$ mobilization should facilitate, for example, endothelial nitric oxide production, which has previously been documented to occur in young rats in response to increased salt intake (4, 39, 40, 43).

The findings of the present study suggest that dietary $Na^+$ and $K^+$ intake interacted to promote an interesting coordinated effect on the signaling molecules $Bmx$ and PLC-$\gamma_1$ in ECs. Although not directly confirmed, the inhibitory effect of dietary $K^+$ on dietary salt-induced $Bmx$ levels is likely mitigated by the concomitant inhibition of transforming growth factor-$\beta$ production (42, 43, 47). However, isolated inhibition of $Bmx$ might promote hypertension. A recently developed Bruton tyrosine kinase (Btk) inhibitor is also a highly effective inhibitor of other Tec kinases, including $Bmx$ (12). In a recent trial involving the use of this synthetic irreversible Btk inhibitor in chronic lymphocytic leukemia, 18% of patients developed hypertension as an adverse event (2). The potential role of $Bmx$ in the development of hypertension or, more specifically, the blood pressure response to increased dietary salt intake, especially with concomitantly low $K^+$ intake, bears additional scrutiny in future studies.

The present study also demonstrated that the $Bk_{Ca}$ channel would have effects on cell types that include vascular smooth muscle and the connecting tubule epithelium (8, 9), the present experiments, combined with previous studies (42, 43), further support an integral function of $Bk_{Ca}$ channels in determining EC responses to the effects of dietary salt and $K^+$ and associated changes in arterial function. Targeted genetic deletion studies would be required to determine how expression of $Bk_{Ca}$ subunits on these cell types affects blood pressure and the response to salt and $K^+$ intake.

GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


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