PKC-α contributes to high NaCl-induced activation of NFAT5 (TonEBP/OREBP) through MAPK ERK1/2

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PKC-α contributes to high NaCl-induced activation of NFAT5 (TonEBP/OREBP) through MAPK ERK1/2. Am J Physiol Renal Physiol 308: F140–F148, 2015. First published November 12, 2014; doi:10.1152/ajprenal.00471.2014.—High NaCl in the renal medullary interstitial fluid powers the concentration of urine but can damage cells. The transcription factor nuclear factor of phosphatase 1; urinary concentration; nuclear factor of phosphatase 1; serine-591-phosphorylated Src homology 2 domain—through SHP-1-S591.

High NaCl-dependent activation of NFAT5 through ERK1/2 but not cells affected SHP-1-S591-P. We conclude that PKC-/H9251 knockout of PKC-/H9251 has been previously shown to increase SHP-1-S591-P, which raised the possibility that SHP-1-S591-P in the inner medulla. PKC-/H9251 contributes to the activation of NFAT5. PKC-/H9251 protein abundance was greater in the renal medulla than in the cortex. Knockout of PKC-/H9251 reduced NFAT5 protein abundance and expression of its target genes in the inner medulla. In human embryonic kidney (HEK)-293 cells, high NaCl increased PKC-α activity, and small interfering RNA-mediated knockdown of PKC-α attenuated high NaCl-induced NFAT5 transcriptional activity. Expression of ERK1/2 protein and phosphorylation of ERK1/2 were higher in the renal inner medulla than in the cortex. Knockout of PKC-α decreased ERK1/2 phosphorylation in the inner medulla, as did knockdown of PKC-α in HEK-293 cells. Also, knockdown of ERK2 reduced high NaCl-dependent NFAT5 transcriptional activity in HEK-293 cells. Combined knockdown of PKC-α and ERK2 had no greater effect than knockdown of either alone. Knockdown of either PKC-α or ERK2 reduced the high NaCl-induced increase of NFAT5 transactivating activity. We have previously found that the high NaCl-induced increase of phosphorylation of Ser591 on Src homology 2 domain-containing phosphatase 1 (SHP-1-S591-P) contributes to the activation of NFAT5 in cell culture, and here we found high levels of SHP-1-S591-P in the inner medulla. PKC-α has been previously shown to increase SHP-1-S591-P, which raised the possibility that PKC-α might be acting through SHP-1. However, we did not find that knockout of PKC-α in the renal medulla or knockdown in HEK-293 cells affected SHP-1-S591-P. We conclude that PKC-α contributes to high NaCl-dependent activation of NFAT5 through ERK1/2 but not through SHP-1-S591.

inner medulla; hypertonicity; Src homology 2 domain-containing phosphatase 1; serine-591-phosphorylated Src homology 2 domain-containing phosphatase 1; urinary concentration; nuclear factor of activated T cells 5; toxicity-responsive enhancer-binding protein; osmotic response element-binding protein

RENAL MEDULLARY CELLS are normally exposed to high NaCl concentrations in their interstitial fluid, which powers urinary concentration. Such high NaCl concentrations can damage and even kill cells. The survival and function of renal medullary cells depend on the transcription factor nuclear factor of activated T cells 5 (NFAT5; also called toxicity-responsive enhancer-binding protein or osmotic response element-binding protein), which activates the expression of osmoprotective genes that code for proteins, such as aldose reductase (AR) and betaine/glycine transporter 1 (BG1). AR and BG1 cause the cellular accumulation of protective organic osmolytes sorbitol and glycine betaine, respectively (2). NFAT5 also plays an important role in salt-induced or -associated inflammation (29), hypertension (24), and experimental autoimmune encephalomyelitis (19, 42). In addition, NFAT5 is involved in biological processes and diseases that are not obviously associated with high NaCl, such as cardiac development (26), muscle differentiation and atherosclerosis (11, 12), brain injury (25, 45), cancer metastasis (10), T lymphocyte development (1), leishmaniasis (3, 50), and proliferation of human immunodeficiency virus (32).

PKC-α is a member of the PKC family. This family of serine/threonine kinases can be divided into three categories based on their structure and biochemical properties: classical or conventional PKCs, including PKC-α, PKC-βI, PKC-βII, and PKCγ; novel PKCs, including PKC-δ, PKC-ε, PKC-η, and PKC-θ; and atypical PKCs, including PKC-ζ and PKC-λ. PKCs play critical roles in a wide variety of physiological and pathophysiological processes, such as cell growth, inflammation, cancer metastasis, and cardiac hypertrophy (33). Knockout of PKC-α impairs urinary concentration (44), thereby lowering urinary osmolality (18). The effect of PKC-α on urinary concentration is mediated in mouse inner medullary collecting ducts by its contribution to high NaCl-induced increases of phosphorylation of urea transporters (UTs) (18) and urea permeability (41). These observations led us to question whether PKC-α also contributes to the activation of NFAT5 in the renal inner medulla.

High NaCl activates MAPK ERK1/2 by increasing phosphorylation mediated by its upstream kinase MEK1/2 (51). MEK1/2 is activated by prooncogene serine/threonine kinases Ras and Raf as well as PKCs, including PKC-α (6). ERK1/2 activation is necessary for high NaCl-enhanced expression of TNF-α in LPS- or PMA-activated THP-1 cells (5), monocyte chemoattractant protein-1 in NRK52E rat renal proximal tubular cells (20), cyclooxygenase-2 expression in mIMCD-K2 cells (43), aquaporin-1 in renal medullary collecting duct cells (40), and the renal outer medullary K+ channel in cultured mouse thick ascending limb cells (9). Although NFAT5 is a major transcription factor activated by high NaCl and is responsible for high NaCl-dependent activation of gene expression, it is not clear whether ERK1/2 is involved in high NaCl-induced activation of NFAT5. ERK1/2 contributes to high NaCl-induced activation of NFAT5 in nucleus pulposus cells (38) but apparently not to high NaCl-dependent activation of NFAT5 in primary splenocytes (27).

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Despite extensive studies, how high NaCl activates NFAT5 remains incompletely understood. Furthermore, none of the signaling molecules identified in cultured cells have been tested directly in the kidney inner medulla. Phosphorylation is a prominent signaling mechanism underlying high NaCl-dependent activation of NFAT5 (2). We undertook the present study to investigate the possible role of PKC-α and ERK1/2 in transducing high NaCl-induced activation of NFAT5 in the kidney inner medulla and human embryonic kidney (HEK)-293 cells. We found that PKC-α and ERK1/2 contribute to high NaCl-induced activation of NFAT5 and that the effect of PKC-α on NFAT5 is mediated by ERK1/2.

**MATERIALS AND METHODS**

**Animals.** C57BL/6 mice (8 wk old), purchased from Jackson Laboratory, were handled according to procedures approved by the Uniformed Services University Institutional Animal Care and Use Committee. PKC-α knockout mice were bred in house according to procedures approved by the Emory University Institutional Animal Care and Use Committee. Mice were allowed to access food and water ad libitum.

**Cells.** HEK-293 cells (American Type Culture Collection) were incubated in Eagle’s minimal essential medium plus 10% FBS in 5% CO2-95% air at 37°C. HEK-293 cells were used between passages 38 and 48. HEK-293 cells stably expressing ORE luciferase reporter or mutated ORE luciferase reporter (47) were used between passages 40 and 45. The basal osmolality was 290 mosmol/kg (control). All experiments were performed on subconfluent cells.

**Small interfering RNAs, transfections, and luciferase activity.** Small interfering (si)RNAs against human PKC-α (SI00301308, designated as #1 siRNA, and SI00605927, designated as #2 siRNA, Qiagen) were used either separately or in combination. siRNAs against human ERK2 were purchased from New England Biolabs and Qiagen (SI000300762, SI000300748, SI000300755, and SI00605983).

The control siRNA has been previously described (14). siRNAs were transfected with Lipofectamine 2000 using the recommended amount of siRNA and transfection reagent (Invitrogen). For cotransfection of PKC-α and ERK2 siRNAs, the total amounts of siRNAs and Lipofectamine 2000 were doubled. All transfections were accomplished by adding cell suspensions to a plated complex of siRNAs and transfection reagent. Luciferase activity was measured as previously described (47).

**Quantitative PCR.** Kidney inner medullas were sonicated in an ice-cold RNAzol RT kit (Molecular Research Center). The extracted total RNA was measured using a NanoDrop 8000 (Thermo Scientific). cDNAs were synthesized with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). cDNAs (160–240 ng of total RNA per reaction) were quantified with a SYBR Green PCR kit (QuantiTast, Qiagen) in an Applied Biosystems 7900HT. The primers for each gene were as follows: AR, forward 5’-CTATTTCCACG-GATGCGT-3’ and reverse 5’-TTTCCACCAACTTCATCACA-3’; BGI, forward 5’-TTGTTTTGTGTCCTTCG-3’ and reverse 5’-GACCTGACTGACACTTCCA-3’; and NFAT5, forward 5’-GAGTCATGCTGAATGTTG-3’ and reverse 5’-ATCATGTTGAGA-GGGTGGTCT-3’. 18S rRNA was used to control the amount of cDNA used in each analysis, but mRNA abundance was not normalized to 18S rRNA. The fold difference in RNA abundance between conditions was calculated as previously described (8).

**PKC-α activity assay.** HEK-293 cells were plated in a 10-cm dish for at least 22 h and then treated with control or high NaCl medium (500 mosmol/kg) for 60 min. At the end of treatment, cells were washed once with ice-cold PBS (adjusted to the experimental NaCl concentration) and collected in lysis buffer containing 50 mM Tris HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, 2.0 µM NaF, 2.0 µM Na2VO4, and a protease inhibitor tablet (catalog no. 11697498011, Roche). The renal cortex and outer and inner medullas of C57BL/6 mice were homogenized in 10 mM triethanolamine (pH 7.4) and 250 mM sucrose plus 2.0 µM NaF, 2.0 µM Na2VO4, and a protease inhibitor tablet (catalog no. 11697498011, Roche). The supernatant was cleared with agarose-conjugated rabbit IgG at 4°C for 45 min and then incubated with agarose-conjugated rabbit anti-PKC-α antibody (catalog no. sc-208, Santa Cruz Biotechnology) at 4°C for 2 h. After incubation, the agarose beads were gently washed once with ice-cold lysis buffer and once with the kinase assay dilution buffer provided with the PKC kinase activity kit (ADI-ESK-420A, Enzo Life Sciences). The total activity of immunoprecipitated PKC-α was determined according to the manufacturer’s instructions. After measurement of absorption at 450 nm, proteins were stripped from the beads with 1× SDS loading buffer at 95°C for 5 min and analyzed by immunoblot analysis. The specific PKC-α activity was expressed by readings at 450-nm absorbance normalized to immunoprecipitated PKC-α.

**Western blot analysis and antibodies.** After treatment, cells were washed once with ice-cold PBS adjusted to the osmolality of the medium by the addition of NaCl. To measure phosphorylation, samples were collected with Phosphosafe buffer (EMD Chemicals) or M-PER buffer (Pierce) plus 2.0 µM NaF and 2.0 µM Na2VO4. To measure NFAT5 protein abundance in HEK-293 cells, samples were collected with 1% Triton X-100, 150 mM NaCl, and 50 mM Tris-HCl (pH 7.4) plus a protease inhibitor tablet (Roche). For Western blot analyses of kidney tissues, samples were homogenized in 10 mM triethanolamine (pH 7.4) and 250 mM sucrose plus 2.0 µM NaF, 2.0 µM Na2VO4, and a protease inhibitor tablet (Roche). Expression of NFAT5 (175 kDa), PKC-α (75 kDa), and AR (37 kDa) in the kidney could be probed in the same membrane because they have different molecular sizes. Western blot results of both HEK-293 cells and kidney tissues were quantified by infrared imaging (Odyssey, Li-Cor). Antibodies against NFAT5 (catalog no. sc-13035, rabbit), brahma-related gene-1 (Brp-1; catalog no. sc-17796, mouse), PKC-α (catalog no. sc-208, rabbit), actin (catalog no. SC1615, goat), and Src homology 2 domain-containing phosphatase 1 (SHP-1; catalog no. sc-287, rabbit) were from Santa Cruz Biotechnology. Antibody against Ser901 phosphorylated SHP-1 (SHP-1-S901-P; catalog no. sc-1391, rabbit) was from ECM Biosciences. To measure the phosphorylation of SHP-1-S901-P in HEK-293 cells, mouse anti-SHP-1 (catalog no. 610126, BD Biosciences) was combined with rabbit anti-SHP-1-S901-P (ECM Biosciences), and the antibodies were identified simultaneously by secondary anti-rabbit and anti-mouse antibodies conjugated with different wavelengths of Alexa fluorophores. Mouse β-tubulin antibody (catalog no. T8660) was from Sigma. Rabbit antibodies against ERK1/2 (catalog no. 9102, phospho-ERK1/2 (catalog no. 9101), and GAPDH (catalog no. 2118) were from Cell Signaling.

**NFAT5 transactivating activity and nuclear localization.** NFAT5 transactivating activity was measured in HEK-293 cells stably expressing a yeast binary GAL4 reporter assay system (8). To measure NFAT5 nuclear localization, HEK-293 cells were transfected with control, PKC-α, or ERK2 siRNAs for 22 h, divided into two dishes, and then incubated for an additional 24 h. After incubation, the medium was replaced either with fresh control culture medium or medium at 500 mosmol/kg (NaCl added) for 2 h, and cells were then washed once with ice-cold PBS adjusted to the osmolality of the medium by the addition of NaCl. The NE-PER kit (Pierce) was used to extract cytoplasmic and nuclear proteins. NFAT5 in each fraction was measured by Western blot analysis, and the nuclear-to-cytoplasmic ratio was calculated as previously described (7). The cytoplasmic marker protein α-tubulin and nuclear marker protein brahma-related gene-1 were monitored in each extract to exclude the possibility that the ratio was affected by inadequate separation of nuclear and cytoplasmic proteins.

**Statistics.** Data are expressed as means ± SE. For data from the kidney, the reading from the first sample in the control group was set to 1, and the rest of the data were normalized to this value. For data...
PKC-α contributes to high NaCl-induced activation of nuclear factor of activated T cells 5 (NFAT5). A: the protein level of PKC-α increases from the cortex to inner medulla (IM) in the kidney of C57BL/6 mice. OM, outer medulla. *P < 0.05 vs. the cortex (by repeated-measures ANOVA, n = 3). B: knockout (KO) of PKC-α reduces mRNA abundance in the kidney IM of transcriptional targets of NFAT5, namely, aldose reductase (AR) and betaine/glycine transporter 1 (BGT1), and also reduces AR protein. *P < 0.05 vs. wild type (WT; by unpaired t-test, n = 4). C and D: knockdown of PKC-α decreases NFAT5 transcriptional activity at 290 and 500 mosmol/kg. Human embryonic kidney (HEK)-293 cells stably expressing a luciferase reporter containing the NFAT5-targeted ORE DNA element (C) or a mutated ORE DNA sequence to prevent NFAT5 binding (D) were transfected with control or separately with two different PKC-α small interfering (si)RNAs for 24 h at 290 mosmol/kg, and the medium was then replaced with an identical one or with one in which osmolality was increased to 500 mosmol/kg (NaCl added) for an additional 24 h before luciferase activity was measured. *P < 0.05 vs. the respective control (by repeated-measures ANOVA, n = 3).

RESULTS

Effects of PKC-α on the expression of NFAT5-targeted genes in the kidney inner medulla and on NFAT5 transcriptional activity in HEK-293 cells. The high NaCl in the renal medullary interstitial fluid elevates the expression of NFAT5 (35). Renal interstitial NaCl has been previously found to increase from the cortex to medulla in parallel with increased expression of the NFAT5-targeted gene Na\(^+\)-dependent myo-inositol transporter (4). In the present study, we found that PKC-α protein increases from the cortex to inner medulla (Fig. 1A). In addition, knockout of PKC-α significantly reduced mRNA expression of the NFAT5 targets AR and BGT1 and expression of AR protein in the kidney inner medulla (Fig. 1B). Furthermore, siRNA-mediated knockdown of PKC-α in HEK-293 cells attenuated high NaCl-induced NFAT5 activity, as measured with a luciferase reporter containing the NFAT5 DNA-binding sequence. As a control, the same treatment had no significant effect on the activity of a mutated reporter to which NFAT5 cannot bind (Fig. 1, C and D). We conclude that PKC-α contributes to high NaCl-induced activation of NFAT5.

Specific activity of PKC-α in the kidney and the effect of high NaCl on specific PKC-α activity in HEK-293 cells. We measured the specific activity of immunoprecipitated PKC-α from the kidney cortex and outer and inner medullas with a spectrophotometric method. We found that the specific activity of the kinase was not significantly higher in the inner medulla than in the cortex and outer medulla (Fig. 2A). Therefore, PKC-α activity is apparently higher in the inner medulla because its protein abundance is greater. We also examined the effect of acute elevation of extracellular NaCl on PKC-α

Fig. 1. PKC-α contributes to high NaCl-induced activation of nuclear factor of activated T cells 5 (NFAT5). A: the protein level of PKC-α increases from the cortex to inner medulla (IM) in the kidney of C57BL/6 mice. OM, outer medulla. *P < 0.05 vs. the cortex (by repeated-measures ANOVA, n = 3). B: knockout (KO) of PKC-α reduces mRNA abundance in the kidney IM of transcriptional targets of NFAT5, namely, aldose reductase (AR) and betaine/glycine transporter 1 (BGT1), and also reduces AR protein. *P < 0.05 vs. wild type (WT; by unpaired t-test, n = 4). C and D: knockdown of PKC-α decreases NFAT5 transcriptional activity at 290 and 500 mosmol/kg. Human embryonic kidney (HEK)-293 cells stably expressing a luciferase reporter containing the NFAT5-targeted ORE DNA element (C) or a mutated ORE DNA sequence to prevent NFAT5 binding (D) were transfected with control or separately with two different PKC-α small interfering (si)RNAs for 24 h at 290 mosmol/kg, and the medium was then replaced with an identical one or with one in which osmolality was increased to 500 mosmol/kg (NaCl added) for an additional 24 h before luciferase activity was measured. *P < 0.05 vs. the respective control (by repeated-measures ANOVA, n = 3).

Fig. 2. The specific activity of PKC-α is not elevated in the kidney IM (A) but is increased when extracellular osmolality is raised in HEK-293 cells by the addition of NaCl to the medium (B). Total activity of PKC-α immunoprecipitated from the renal cortex, OM, and IM or from HEK-293 cells was measured with a PKC kinase activity kit (Enzo Life Sciences), and specific PKC-α activity was calculated by normalization to the amount of immunoprecipitated PKC-α, n = 4. HEK-293 cells were incubated at 290 or 500 mosmol/kg (NaCl added, 60 min) before immunoprecipitation (IP) was performed. *P < 0.05 (by paired t-test, n = 3).
activity in HEK-293 cells. Elevation of osmolality to 500 mosmol/kg by the addition of NaCl for 60 min increased the specific activity of PKC-α by 125% (Fig. 2B).

Effects of PKC-α on phosphorylation and protein expression of ERK1/2 in the kidney inner medulla and on high NaCl-induced phosphorylation of ERK1/2 in HEK-293 cells. Phosphorylation (phospho-ERK1/2) and protein abundance of ERK1/2 were significantly higher in the renal inner medulla than in the outer medulla or cortex (Fig. 3A). Knockout of PKC-α significantly reduced phospho-ERK1/2 in the inner medulla without significantly affecting ERK1/2 protein abundance (Fig. 3B). In HEK-293 cells, siRNA-mediated knockdown of PKC-α reduced high NaCl-induced ERK1/2 phosphorylation (Fig. 3C). We conclude that PKC-α mediates high NaCl-induced phosphorylation (activation) of ERK1/2.

Lack of additional effect of knockdown of PKC-α on inhibition of high NaCl-induced NFAT5 activity by knockdown of ERK2 in HEK-293 cells. siRNA-mediated knockdown of ERK2 (with a pool of siRNA that knocks down ERK2 but not ERK1) reduced NFAT5 transcriptional activity, as measured with a luciferase reporter containing intact NFAT5-binding sites (Fig. 4, A and C), but not if the sites were mutated to prevent binding of NFAT5 (Fig. 4, B and D). siRNA-mediated knockdown of PKC-α inhibited NFAT5 activity to the same extent as did knockdown of ERK2, provided that the binding sites are intact (Fig. 4, C and D). Importantly, combined knockdown of ERK2 and PKC-α had no greater effect than knockdown of either individually (Fig. 4, C and D). We conclude that PKC-α and ERK2 contribute to high NaCl-induced activity of NFAT5 via the same pathway and that, considering that knockdown of PKC-α inhibits ERK2 (Fig. 3), PKC-α is upstream of ERK2.

Effect of PKC-α and ERK2 on NFAT5 protein abundance, transactivating activity, and nuclear localization. To identify the mechanism(s) by which PKC-α and ERK2 contribute to high NaCl-induced activation of NFAT5, we examined the effects in the kidney inner medulla of knockout of PKC-α on NFAT5 protein levels and in HEK-293 cells of knockdown of PKC-α or ERK2 on high NaCl-dependent increase of NFAT5 protein abundance, transactivating activity, and nuclear localization. NFAT5 has been previously observed to increase from the renal cortex to medulla (4), which is in parallel with the increase of PKC-α protein levels (Fig. 1A). Knockout of PKC-α significantly reduced NFAT5 protein in the kidney inner medulla without significantly affecting its mRNA level (Fig. 5A). However, siRNA-mediated knockdown of PKC-α or ERK2 had no significant effect on the high NaCl-induced increase of NFAT5 protein abundance in HEK-293 cells (Fig. 5B), which raises the question of the mechanism by which PKC-α contributes to the protein abundance of NFAT5 in the kidney inner medulla without affecting its mRNA. Knockdown of PKC-α did not reduce ERK2 protein abundance in HEK-293 cells (Fig. 5C). Knockdown of ERK2 increased PKC-α protein abundance, possibly due to a compensatory feedback (Fig. 5D). Knockdown of PKC-α or ERK2 reduced the high NaCl-induced increase of NFAT5 transactivating activity in HEK-293 cells but did not significantly affect high NaCl-induced NFAT5 nuclear localization (Fig. 5, E and F). High NaCl increased ERK1/2 nuclear localization, but PKC-α was apparently not involved (Fig. 5F). We conclude that PKC-α contributes to increase NFAT5 protein expression in the kidney inner medulla and that PKC-α and ERK1/2 augment NFAT5 transactivating activity in HEK-293 cells.

Lack of effect of PKC-α on phosphorylation of SHP-1-S591 in the kidney inner medulla and HEK-293 cells. We have previously demonstrated that high NaCl inhibits the protein

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**Fig. 3.** PKC-α contributes to high NaCl-induced phosphorylation of ERK1/2. A: ERK1/2 protein and phosphorylation are greater in the IM than in the cortex (Cor) of C57BL/6 mice kidneys. *P < 0.05 vs. the cortex; **P < 0.05 vs. the OM (by repeated-measures ANOVA, n = 3). B: knockout of PKC-α reduces phosphorylation of ERK1/2 in the kidney IM. *P < 0.05 vs. WT (by unpaired t-test, n = 4). C: knockdown of PKC-α reduces high NaCl-induced phosphorylation of ERK1/2 in HEK-293 cells. HEK-293 cells were transfected with control or PKC-α siRNAs at 290 mosmol/kg for 25.5 h, divided into two dishes, and then incubated for an additional 22 h before one dish was treated with the fresh medium at 290 mosmol/kg (control) and the other dish at 500 mosmol/kg (NaCl added) for 30 min. Western blot analyses were performed with anti-ERK1/2 antibody and with antibody specific for phosphorylated ERK1/2 (ERK1/2-P). *P < 0.05 compared with the control at 500 mosmol/kg (by paired t-test, n = 3).
DISCUSSION

Previously published evidence suggests that PKC family proteins are involved in the regulation of high NaCl-induced activation of NFAT5. Thus, high NaCl increases total PKC activity (51), PKC inhibitors reduce NFAT5 transcriptional activity in mIMCD3 (46) and NIH3T3 cells (22), and siRNA-mediated knockdown of PKC-α, a kinase closely related to PKC family kinases (36), inhibits high NaCl-induced expression of heat shock protein 70, a target of NFAT5 in NIH3T3 cells (22). Our present study of mouse kidneys and HEK-293 cells indicates that PKC-α contributes to high NaCl-induced activation of NFAT5 in those tissues. Thus, PKC-α activity is greater in the renal inner medulla than in the cortex because PKC-α protein abundance is higher in the medulla (Figs. 1A and 2A), associated with the higher interstitial NaCl in the medulla. Furthermore, knockout of PKC-α decreases expression of NFAT5-targeted genes (Fig. 1B) and NFAT5 protein abundance (Fig. 5A). In HEK-293 cells, high NaCl increases specific PKC-α activity (Fig. 2B) and siRNA-mediated knockdown of PKC-α reduces NFAT5 transcriptional activity (Fig. 1, C and D) and transactivating activity (Fig. 5E) without significantly affecting NFAT5 nuclear localization (Fig. 5F) or protein abundance (Fig. 5B). It is not surprising that NFAT5 transactivating activity is affected independent of NFAT5 nuclear localization and protein abundance, because the measurement of transactivation uses a yeast binary GAL4 assay system in which the amino terminus of NFAT5, which has the nuclear localization signal and DNA-binding domain (23), is replaced by GAL4 DNA binding and nuclear localization domains (8). The NFAT5 transactivating activity assay is also independent of endogenous NFAT5 protein. We cannot determine whether knockout of PKC-α reduces NFAT5 transactivating activity in the kidney inner medulla, since there is no method available to make the measurement in vivo. More than a dozen kinases have been demonstrated to contribute to high NaCl-dependent activation of NFAT5 in cell culture (49), but PKC-α is the first kinase directly shown to be involved in the regulation of NFAT5 in the kidney medulla in vivo.

PKC-α also supports function of the renal medulla through mechanisms in addition to its effects on NFAT5. High levels of urea and NaCl in the renal medullary interstitial fluid provide an osmolar gradient for water absorption from the tubule fluid. PKC-α signals the high NaCl-induced increase of phosphorylation of UTs (18), which increases urea permeability in the mouse inner medullary collecting duct (41), elevating urea concentration in the medullary interstitial fluid. PKC-α may also directly support NaCl transport driven by Na\(^{+}\)-K\(^{+}\)-Cl\(^{-}\) cotransporter 2 (NKCC2), which is responsible for raising NaCl in the medullary interstitial fluid, considering that there are multiple phylogenetically conserved potential PKC phosphorylation sites in NKCC2 (13). Furthermore, PKC-α apparently also regulates Na\(^{+}\)-K\(^{+}\)-ATPase in the rat medullary thick ascending limb, which powers NaCl transport there (39). Our present demonstration that PKC-α contributes to high NaCl-induced activation of NFAT5 adds to multiple other known mechanisms by which it contributes to urinary concentration.

![Graph showing knockdown of ERK2 in HEK-293 cells](image-url)

**Fig. 4.** Knockdown of ERK2 in HEK-293 cells significantly decreases high NaCl-induced NFAT5 transcriptional activity, and combined knockdown of PKC-α and ERK2 is not additive. A and B: cells were treated as described in Fig. 1, C and D. *P < 0.05 compared with the control at 500 mosmol/kg (by paired t-test, n = 3). C and D: cells were treated as in A and B except that the total amounts of siRNAs and Lipofectamine 2000 were doubled. In cells transfected with PKC-α or ERK2 siRNAs alone, control siRNA was added to give a total amount equal to the combined siRNAs. *P < 0.05 vs. the control at 500 mosmol/kg (by repeated-measures ANOVA, n = 3).

Tyrosine phosphatase SHP-1 by increasing inhibitory phosphorylation of SHP-1 at Ser\(^{591}\) (SHP-S591-P) and that this contributes to the activation of NFAT5 (48, 49). Since PKC-α phosphorilates SHP-1-S591 under other conditions (30), we questioned whether PKC-α is involved in high NaCl-induced phosphorylation of SHP-1-S591. In agreement with that possibility, SHP-1-S591-P was higher in the renal inner medulla than in the outer medulla or cortex (Fig. 6A). However, neither knockout of PKC-α in the inner medulla (Fig. 6B) nor knockdown of PKC-α in HEK-293 cells (Fig. 6C) affected the phosphorylation of SHP-1-S591. We conclude that PKC-α is not responsible for the high NaCl-induced increase of phosphorylation of SHP-1-S591 and that SHP-1-S591 is not involved in the activation of NFAT5 by PKC-α.
PKC-α knockout mice have reduced urinary osmolality (18, 44), associated with reduced NFAT5 protein abundance (Fig. 5A) and expression of NFAT5-targeted genes (Fig. 1B). UT-A1/3 has been previously identified as one of the NFAT5 transcriptional targets in inner medullary collecting duct cells (28). We could not find a significant reduction of UT-A1/3 mRNA in the inner medulla of PKC-α knockouts (data not shown). However, UT-A transcription is not controlled only by NFAT5; it is also activated by the transcription factor Kruppel-like factor 12 (37) and possible other transcription factors. It is not clear whether knockout of PKC-α activates these transcription factors as a compensatory mechanism. If the knockouts have reduced renal medullary interstitial NaCl, which seems plausible, we cannot distinguish whether the reduction of NFAT5 protein is secondary to the reduced renal medullary interstitial NaCl or vice versa. The lack of effect of PKC-α
PKC-α contributes to the activation of NFAT5 through ERK1/2

Previous investigations of the possible involvement of ERK1/2 in the activation of NFAT5 by high NaCl are difficult to interpret. PKC-α contributes to high NaCl-dependent expression of heat shock protein 70, but PD-98059 (20 μM), an inhibitor of ERK1/2, does not inhibit the effect (22). Similarly, PD-98059 (10 μM) does not reduce NFAT5 transcriptional activity in primary splenocytes (27). In contrast, U-0126, another inhibitor of ERK1/2, inhibits high salt-dependent protein expression of aquaporin-1, which is a target of NFAT5 (21), in mIMCD3 cells (40). On the other hand, ERK2 siRNA, a dominant negative mutant of ERK2 and PD-98059 (10 μM), reduces high NaCl-dependent NFAT5 transcriptional activity in nucleus pulposus (38). Presumably, the differences come from the different model systems that were studied. In the present study, we found that siRNA-mediated knockdown of ERK2 decreases high NaCl-induced NFAT5 transcriptional activity (Fig. 1C). Since we tested different ERK2 siRNAs from both New England Biolabs and Qiagen and received similar results (data not shown), off-target effects are unlikely. Furthermore, we found that PKC-α contributes to high NaCl-dependent activation of NFAT5 through ERK1/2 in HEK-293 cells (Fig. 4, C and D) and that knockout of PKC-α reduces phosphorylation of ERK1/2 in the kidney inner medulla (Fig. 3B). However, we cannot use an available transgenic model of ERK1/2 knockout to determine whether ERK1/2 regulates NFAT5 activity in the kidney inner medulla in vivo, because global knockout of ERK1 or ERK2 is embryonically lethal (31, 34) and kidney epithelium-specific ERK1 and ERK2 knockouts are not available.

We have previously found that in HEK-293 cells, SHP-1 inhibits NFAT5 activity and that high NaCl inhibits SHP-1 by phosphorylation of SHP-1-S591, contributing to the activation of NFAT5 (48, 49). In the present study, we found that SHP-1-S591 is heavily phosphorylated in the kidney inner medulla, which suggests a similar mechanism. However, despite the previous observation that PKC-α is responsible for the phosphorylation of SHP-1-S591 in thrombin receptor-activated human platelets (17), knockout or knockdown of PKC-α has no significant effect on high NaCl-dependent phosphorylation of SHP-1-S591 in the renal inner medulla or HEK-293 cells (Fig. 6). Thus, the role of PKC-α in high NaCl-induced activation of NFAT5 is not mediated by SHP-1-S591. These results are also consistent with our previous observation that SHP-1 is not involved in high NaCl-dependent phosphorylation of ERK1/2 (48).

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DISCLOSURES

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

Fig. 6. PKC-α is not involved in the high NaCl-induced increase of phosphorylation of Ser591 (S591-P) on Src homology 2 domain-containing phosphatase 1 (SHP-1). A: phosphorylation of Ser591 on SHP-1 (SHP-1-S591) is much higher in the C57BL/6 mouse kidney IM than in the cortex or OM. *P < 0.05 vs. the cortex or OM (by repeated-measures ANOVA, n = 3). B: Knockout of PKC-α does not significantly affect phosphorylation of SHP-1-S591 in the mouse kidney IM. C: Knockdown of PKC-α does not significantly affect phosphorylation of SHP-1-S591 in HEK-293 cells. Cells were transfected with control or PKC-α siRNAs for 22 h at 290 mosmol/kg, divided, and then incubated with the identical medium for an additional 25 h. The medium was then replaced with fresh identical medium or with otherwise identical medium at 500 mosmol/kg (NaCl added) for 60 min before samples were collected with M-PER buffer for Western blot analysis.

Knockdown on NFAT5 protein levels in HEK-293 cells (Fig. 5B) is not necessarily inconsistent with reduced NFAT5 protein in medullas of PKC-α knockout mice (Fig. 5A) but presumably indicates that the effect in vivo is not direct and that other factors are at work.

Knockout of PKC-α does not affect NFAT5 mRNA abundance in the inner medulla despite a significant reduction of NFAT5 protein abundance (Fig. 5A). Knockdown of 14-3-3β and/or 14-3-3ε has also been shown to reduce NFAT5 protein abundance in HEK-293 cells without affecting the level of NFAT5 mRNA (15). Both results suggest important posttranscriptional, in addition to transcriptional (16), regulation of NFAT5 protein abundance.
REFERENCES


