Osmoregulation of Natriuretic Peptide Receptor Signaling in Inner Medullary Collecting Duct

A REQUIREMENT FOR p38 MAPK

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In the inner medullary collecting duct of the terminal nephron, the type A natriuretic peptide receptor (NPR-A) plays a major role in determining urinary sodium content. This nephron segment, by virtue of its medullary location, is subject to very high levels of extracellular tonicity. We have examined the ability of medium tonicity to regulate the activity and expression of this receptor in cultured rat inner medullary collecting duct cells. We found that NaCl (75 mm) and sucrose (150 mm), but not urea (150 mm), increased natriuretic peptide receptor activity, gene expression, and promoter activity. The osmotic stimulus also activated extracellular signal-regulated kinase (ERK), c-Jun NH2-terminal kinase (JNK), and p38 mitogen-activated protein kinase (p38 MAPK). In the latter instance the β isoform was selectively activated. Inhibition of p38 MAPK with SB203580 blocked the osmotic induction of receptor activity and expression, as well as receptor gene promoter activity, whereas inhibition of ERK with PD98059 had no effect. Cotransfection of p38β MAPK together with the receptor gene promoter resulted in amplification of the osmotic stimulation of the latter, whereas cotransfection of dominant negative MKK6, but not dominant-negative MEK, completely blocked the osmotic induction of receptor promoter activity. Collectively, the data indicate that extracellular osmolality stimulates receptor activity and receptor gene expression through a specific p38β-dependent mechanism, raising the possibility that changes in medullary tonicity could play an important role in the regulation of renal sodium handling in the terminal nephron.

Atrial (ANP)† and brain natriuretic peptide exert potent natriuretic, vasorelaxant, and anti-mitogenic activity by virtue of their interaction with a specific, high affinity, guanylyl cyclase-linked receptor called the type A natriuretic peptide receptor (NPR-A) (1). These receptors are located on the surface of a variety of cells including vascular endothelial and smooth muscle cells as well as epithelial cells lining the inner medullary collecting duct (IMCD), where activation leads to an increase in intracellular cyclic GMP levels, suppression of sodium transport on the luminal surface, and a net increase in urinary sodium excretion (2). The IMCD is involved in handling up to 5% of total filtered sodium load, and because of its location in the terminal segment of the nephron, it plays a critical role in the determination of final urinary sodium concentration.

Although we know a great deal about how the natriuretic receptors function (3), we know very little about their regulation. NPR-A is acutely desensitized following exposure to ANP (4) or phorbol ester (5), reflecting dephosphorylation of the receptor protein. At the level of gene expression, the NPR-A gene has been shown to be regulated by glucocorticoids (6), transforming growth factor-β (7), and chonic gonadotrophin (8). The NPR-A gene also undergoes homologous down-regulation following exposure to its natriuretic peptide ligand or the ligand’s second messenger (cGMP) (9), but the precise mechanism(s) underlying this regulation remains undefined.

By virtue of its location in the inner medulla of the kidney, the IMCD is subject to substantial variation in the level of extracellular tonicity. Although a number of studies have been carried out investigating the osmoregulation of gene expression in various cell types including those of the IMCD (10–12), few have focused on genes involved in sodium handling in this segment of the nephron. For this reason we have undertaken a study to examine the osmoregulation of NPR-A activity and expression in primary cultures of rat IMCD cells. We have found that the activity of NPR-A, the expression of its gene, and the activity of the NPR-A gene promoter are all stimulated by increased extracellular tonicity. This stimulation appears to traffic selectively through the β isoform of p38 MAPK.

EXPERIMENTAL PROCEDURES

Materials—[α-32P]dCTP was purchased from PerkinElmer Life Sciences. ANP was obtained from Phoenix Pharmaceuticals Inc. (Mountain View, CA). RNeasy Minikit was purchased from Qiagen Inc. (Santa Clara, CA). Primer-it® RMT kit, hybridization solution, and Nuctrap bodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Other reagents were obtained through standard commercial suppliers.

Isolation and Culture of IMCD Cells—Animal studies were approved by the University of California at San Francisco Committee on Animal Research. Adult Sprague–Dawley rats were euthanized by CO2 narcosis followed by bilateral thoracotomy. Kidneys were excised and bivalved with a scalpel blade. The inner medullary tissue was dissected free from the outer medulla, minced into 1-mm3 fragments and digested with 1 mg/ml collagenase at 37 °C with gentle agitation during each 30 min cycle. IMCD cells were enriched in the preparation using hypotonic lysis as described previously (13). The cells were resuspended in medi-

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Fig. 1. Effect of increased osmolality on NPR-A activity, gene expression, and promoter activity in IMCD cells. A, IMCD cells were cultured in DMEM (calculated osmolality 330 mosm/kg) containing 150 mM sucrose, 75 mM NaCl, or 150 mM urea (these same concentrations were used for all subsequent experiments) for different time intervals. Osmolalities provided in the figure represent additions above that attributed to DMEM itself. ANP-stimulated cGMP (ANP-s-cGMP) was determined as described under “Experimental Procedures.” Pooled data from 3–4 independent experiments are shown. Control cGMP levels were 152 ± 15 pmol/ml soluble protein. B, IMCD cells at ~80% confluence were incubated in medium containing sucrose, NaCl, or urea for the times indicated. RNA was extracted, size-fractionated on agarose gels, and blot-hybridized with radiolabeled NPR-A cDNA or 18 S rRNA probes as described under “Experimental Procedures.” Data shown were pooled from four independent experiments. *, p < 0.05; **, p < 0.01 versus untreated group.
mM HEPES, pH 7.4, 10 mM MgCl$_2$, 10 mM MnCl$_2$, 1 mM dithiothreitol) without ATP. After being washing, the immunoprecipitates were resuspended in 30 µl of kinase buffer containing 20 µM ATP, 10 µCl of [γ-$^32$P]ATP, and 10 µg of myelin basic protein for measurements of extracellular signal-regulated protein kinase (ERK), p38, and p38β activities, or 5 µg of glutathione S-transferase-c-Jun (GST-c-JUN) for c-Jun NH$_2$-terminal kinase (JNK) activity and incubated at 30°C for 20 min. The incubation mixtures were electrophoresed on 15% SDS-polyacrylamide gels, which were then dried and exposed to x-ray film. Phosphorylation of each substrate protein was quantitated by Scion Image.

Immunoblot Analysis—Cell lysates (30 µg of soluble protein) was subjected to 12.5% SDS-PAGE and transferred onto nitrocellulose membranes (Amersham Biosciences Inc.). The membranes were blocked with 5% nonfat milk in 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Tween 20 and probed with anti-phospho-p38 antibody. A horseradish peroxidase-conjugated second antibody was employed to detect immunoreactive bands using the enhanced chemiluminescence Western blotting detection system (ECL, Amersham Biosciences Inc.).

Statistical Analysis—Data were evaluated using one-way analysis of variance with Newman-Keuls test for significance.

RESULTS

Osmoregulation of NPR-A Activity and Gene Expression—Activity of NPR-A in rat IMCD cells, assessed as ANP-stimulated cyclic GMP (cGMP) generation, proved to be very sensitive to extracellular osmolality. As shown in Fig. 1A, both sodium chloride and sucrose effected significant increments in ANP-stimulated cGMP generation in these cultures, whereas the osmotically inert solute urea added to the culture medium was devoid of activity. The addition of 150 mM sucrose (final calculated tonicity in the medium = 404 mosM/kg H$_2$O) or 75
mM NaCl increased NPR-A activity almost 3-fold over a 24-h incubation period. Equimolar (150 mM) urea was without effect over the same 24-h period.

This increase in NPR-A activity was accompanied by an increase in NPR-A gene expression. As shown in Fig. 1B, there was a time-dependent increase in NPR-A transcript levels following exposure to either sucrose (150 mM) or NaCl (75 mM), which was noted as early as 4 h and reached a maximum, without peaking, after 24 h. This increase in NPR-A mRNA levels was related, at least in part, to increased transcription of the NPR-A gene. Transient transfection of a chimeric NPR-A promoter-luciferase reporter into IMCD cells (Fig. 1C) revealed a sucrose- and NaCl-dependent induction of promoter activity, which began as early as 4 h and reached a maximum after 24 h of incubation. The background vector (pGL3-LUC) was affected minimally by the addition of either sucrose or NaCl (-fold induction relative to control: 0.98 ± 0.12 at 4 h, 1.38 ± 0.2; mean ± S.D., n = 3). Osmotically inactive urea had no effect on either NPR-A gene transcript levels or NPR-A promoter activity.

Osmotic Stimulation of MAPK Activity in IMCD Cells—Members of the extended mitogen-activated protein kinase (MAPK) family are known to be regulated by a variety of biochemical and physical stimuli including, in selected cases, extracellular tonicity (18–22). We examined the ability of NaCl (75 mM) to stimulate ERK, JNK, and p38 MAPK in cultured rat IMCD cells. As shown in Fig. 2A, all three kinases were activated by the osmotic stimulus. ERK was stimulated to the greatest degree (almost 5-fold) 20 min following application of the osmotic stimulus. JNK stimulation followed a similar time course (maximal at 20 min) but reached a maximal induction of only 2.5-fold over control. p38 MAPK activation, assessed through measurements of phospho-p38 MAPK levels in IMCD cell extracts, peaked slightly later (60 min) with maximal induction of ~3-fold. As expected the ERK induction by NaCl was completed abrogated by the MEK (ERK kinase) antagonist PD98059, as was the p38 MAPK induction by SB203580 (Fig. 2B). SB203580, at the concentrations employed here, had no effect on JNK activity (Fig. 2B).

p38 MAPK exists as two major isoforms, p38 MAPK α and β. Importantly, these isoforms can differentially activate independent downstream targets (15). Both isoforms exist in the rat IMCD cell (Fig. 3). Interestingly, it appears to be the β rather than the α isoform that is induced by extracellular tonicity. As shown in Fig. 3A, the β isoform of p38 MAPK was induced ~2.5-fold by NaCl (75 mM), whereas the α isoform was induced little, if at all. Similar to the finding with phospho-p38 MAPK in Fig. 2, the activity of p38 MAPK β was readily suppressed by SB203580 (Fig. 3B).

Inhibition of p38 MAPK Blocks Osmostimulation of NPR-A—Treatment of IMCD cells with the p38 inhibitor SB203580 almost completely blocked the NaCl-dependent (Fig. 4A) and sucrose-dependent (Fig. 4B) stimulation of NPR-A activity, assessed as ANP-dependent cyclic GMP synthesis. The MEK inhibitor PD98059 had no effect on NaCl-dependent activation of NPR-A, and neither inhibitor had any effect on basal activity. This effect on NPR-A activity was mirrored at the level of NPR-A gene expression. As shown in Fig. 5, pretreatment with SB203580, but not PD98059, led to a significant reduction in the osmotic stimulation of NPR-A mRNA levels in IMCD cells. The latter effect was accompanied by a reduction in NPR-A gene transcriptional activity, SB203580 (Fig. 6A), but not PD98059 (Fig. 6B), effected a dose-dependent reduction in NPR-A gene promoter activity following transient transfection of ~1150 NPR-A-luciferase into IMCD cells.

To examine the p38 dependence of the osmotic induction more closely we employed a genetic approach. Cotransfection of an expression vector encoding the β isoform of p38 MAPK together with the NPR-A-luciferase reporter resulted in a 2-fold increment in promoter activity, slightly less than that seen with NaCl (Fig. 7A). Cotransfection of a vector encoding the β isoform mutated at the kinase active site (p38β(AF)) resulted in no net increment in promoter activity. Of note, cotransfection of p38-β into cells that were subsequently exposed to NaCl resulted in a greater-than-additive increment in reporter activity, implying a synergistic interaction between the osmotic stimulus and levels of p38β-MAPK in the IMCD cell. This interaction was not shared with the p38-β AF mutant. Cotransfection of a dominant negative mutant of MKK6, a p38 MAPK kinase that targets both the α and β isoforms of the kinase, resulted in a dose-dependent reduction in NaCl-dependent NPR-A promoter activation, whereas dominant-negative MEK (ERK kinase) was without effect (Fig. 7B).
mutant affected basal promoter activity (i.e. in the absence of the NaCl induction) to a significant degree.

DISCUSSION

This study provides the first evidence for regulation of natriuretic peptide receptor gene transcription by extracellular tonicity. It also suggests that this occurs, in large part, through a mechanism operating downstream of p38 MAPK activation.

The role of p38 MAPK in signaling osmotic activity has been demonstrated in a number of other systems including yeast, where the p38 MAPK homologue HOG1 plays a critical role in signaling an increase in glycerol-3-phosphate dehydrogenase gene expression following exposure to an osmotic stimulus (23). Activation of p38 MAPK has also been linked to the osmotic induction of aldose reductase (22), betaine/-amino-/n/-butyric acid transporter (24), and the serum- and glucocorticoid-inducible protein kinase (21). The selective activation of p38β results in amplification of the hypertrophic response when overexpressed in neonatal rat cardiac myocytes, whereas activation of the α isoform leads to an increase in programmed cell death (25). Preferential signaling of the osmotic stimulus through the β isoform in IMCD cells suggests that independent regulatory circuitry may govern the activity of these two isoforms in this nephron segment. Thus, as in the cardiac myocyte, it is conceivable that differential activation of these two isoforms in the IMCD could result in opposing physiological activities under selected conditions.

It is noteworthy that treatment of our cultures with SB203580 resulted not only in reduction in the end point under examination (e.g. NPR-A mRNA levels or NPR-A promoter activity) but a reduction in levels of phospho-p38 MAPK as well. Since SB203580 blocks the activity of p38 itself, it is not immediately clear why it should exert an effect on the activation/phosphorylation of the kinase. A similar result has been reported with several other systems (26–28). This led to the suggestion that SB203580 may block the biological activity of p38 MAPK by binding to the inactive form of the kinase and reducing its rate of activation (28), although a subsequent study seems to refute this hypothesis (29). One potential explanation for this seemingly paradoxical finding may be found in the structure of the p38 MAPK-SB203580 complex (30). Much as one might predict, the inhibitor binds tightly in the ATP-binding pocket of the kinase providing an obvious mechanism for the reduction in kinase activity. However, one seg-
ment of the inhibitor projects toward the region harboring the tyrosine residue (Tyr182) that is phosphorylated as a prelude to kinase activation (31). This portion of the inhibitor could conceivably result in an alteration in the three-dimensional conformation of the region surrounding this potential phosphorylation site, resulting in a decrease in phosphorylation of the kinase and impairment in its activation.

JNK and ERK activity are known to be activated by osmotic stimuli and, in some cases, these activations have been tied to specific downstream effects (20, 22). In the case of induction of the NPR-A gene, neither JNK nor ERK appears to play a significant role. The MEK inhibitor PD98059 completely blocked ERK activity but had no effect on NPR-A gene promoter activity. SB203580, the p38 MAPK antagonist used in these studies, has also been shown to inhibit JNK activity when used at higher concentrations (>5 × 10^{-6} M) (32). SB203580 completely suppressed both p38 MAPK activity and NPR-A gene promoter activity but, at the concentrations employed in our studies, had no effect on JNK activity. Thus, the data collectively support a role for a p38 MAPK, but not for JNK or ERK, in trafficking the osmotic induction of NPR-A.

Previous studies have shown NPR-A-dependent guanylyl cyclase activity to be either activated (33) or inhibited (34, 35) by increases in extracellular tonicity. Although none of these studies was definitive in terms of elucidating the mechanisms involved, the available data seem to suggest that activation dominates with longer exposure (i.e., 24 h) and inhibition with short term exposure (i.e., minutes) to the osmotic stimulus. Our findings support this model in that all of our experiments were carried out at longer time intervals to maximize the probability of detecting changes in gene expression. The mechanism(s) underlying the transient reduction of NPR-A activity at shorter time intervals remains, as yet, undefined.

Positioned at the most terminal portion of the nephron, the IMCD deals with up to 5% of filtered sodium load and is responsible for the final decision regarding urinary sodium concentration. A variety of local and systemic regulatory fac-
tors act at the level of the IMCD to influence sodium conservation in either a positive or negative fashion (2). Thus, even modest perturbations in NPR-A activity in this nephron segment might be predicted to result in significant changes in urinary sodium excretion. The IMCD, by virtue of its position deep in the renal medulla, is also subject to tremendous variations in extracellular tonicity. The osmotic regulation of urinary sodium excretion through the natriuretic peptide/NPR-A system might be predicted to result in significant changes in either a positive or negative fashion (2). Thus, even modest perturbations in NPR-A activity in this nephron segment could play a significant role in the regulation of sodium balance.

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REFERENCES