Dominant-Negative c-Jun NH$_2$-terminal Kinase 2 Sensitizes Renal Inner Medullary Collecting Duct Cells to Hypertonicity-induced Lethality Independent of Organic Osmolyte Transport*

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The c-Jun NH$_2$-terminal protein kinases (JNKs), as well as the extracellular signal-regulated protein kinases (ERKs) and p38 mitogen-activated protein kinase, are activated in renal cells in response to extracellular hypertonicity. To determine whether activation of JNKs by hypertonicity is isoform-specific, renal inner medullary collecting duct cells were stably transfected with cDNA's encoding hemagglutinin (HA)-tagged JNK1 and JNK2 isoforms, and the expressed kinases were immunoprecipitated with an anti-HA antibody. Whereas both recombinant kinases were equivalently expressed, only immunoprecipitates from the HA-JNK2 cells displayed hypertonicity-inducible JNK activity. Furthermore, expression of dominant-negative JNK2 (HA-JNK2-APF) in stable clones inhibited hypertonicity-induced JNK activation by 40–70%, whereas expression of dominant-negative JNK1 (HA-JNK1-APF) had no significant inhibitory effect. Independent HA-JNK2-APF (but not HA-JNK1-APF) clones displayed greatly reduced viability relative to neomycin controls after 16 h of exposure to 600 mosM/kg hypertonic medium with percent survival of 20.5 ± 2.7 and 31.5 ± 7.3 for two independent HA-JNK2-APF clones compared with 80.1 ± 1.0 for neomycin controls (p < 0.001, n = 5, mean ± S.E.). However, neither JNK mutant blocked either regulatory volume increase or hypertonicity-induced enhancement of uptake of inositol, an organic osmolyte putatively involved in long term adaptation to hypertonicity. These results define JNK2 as the primary hypertonicity-activated JNK isoform in IMCD-3 cells and demonstrate its central importance in cellular survival in a hypertonic environment by a mechanism independent of acute regulatory volume increase as well as regulation of organic osmolyte uptake.

The cells of the inner medulla of the mammalian nephron are uniquely exposed to large fluctuations in extracellular tonicity due to the changes that occur during diuretic and antidiuretic states. One means of adaptation to a hypertonic environment that has been well described is the intracellular accumulation of “non-perturbing” osmolytes that occurs either by uptake via sodium-coupled transporters (1) or by the generation of sorbitol through the action of aldose reductase on glucose (2). This process involves increased transcription of transporter genes (3, 4) mediated by the osmotic response element that resides in the promoter region of these genes (5). The signaling pathways that impinge upon the osmotic response element remain undefined.

Transcriptional regulation is often mediated by mitogen-activated protein (MAP) kinase pathways (6), which are stimulated by diverse extracellular signals including hypertonicity (7, 8). In this regard, cells of renal origin display osmotic activation of multiple members of the MAP kinase family, including the extracellular signal-regulated kinases (ERKs), c-Jun NH$_2$-terminal kinases (JNKs), and p38 MAP kinases (9–11). However, we have recently shown that induction of osmolyte transport by hypertonicity is not significantly impacted by pharmacologic inhibition of the ERK pathways (11). The marked activation of the JNKs and more modest activation of p38 MAP kinase (11), which is the mammalian counterpart of the osmoregulated HOG-1 in yeast (12), highlights the JNK pathway as being of significance with regard to osmoregulation in the kidney. However, the existence of three distinct JNK genes with several splice variants produced from each gene hampers the clear dissection of the role of JNK in osmoregulation in eukaryotic cells, since different isoforms may play different physiological roles. For example, in small cell lung cancer cells both JNK1 and JNK2 are activated by exposure to UV light but only the JNK1 isoform appears to regulate UV-induced apoptosis (13). Additionally, whereas JNK activation has been linked to induction of apoptosis in some cell types (14), its physiological role in the cellular response to osmotic challenge has not yet been elucidated. The present study was therefore undertaken to define the JNK isoforms regulated by hypertonicity in renal inner medullary collecting duct cells and to determine whether such activation plays a role in cellular adaptation to a hypertonic environment.

**EXPERIMENTAL PROCEDURES**

**Materials—**Cell culture media and serum were from Life Technologies, Inc. Recombinant GST-c-Jun (1–79) and ATF-2 (1–254) were expressed in *Escherichia coli* and purified using glutathione-agarose (Sigma) and Ni$_2^+$-nitrilotriacetic acid-agarose (Qiagen, Studio City, CA), respectively, as described previously (15). Anti-JNK antisera were from Santa Cruz Biotechnology. Radioisotopes were from NEN Life Science Products. The osmolarity of all solutions used was checked with an Advanced Instruments Model 3MO Micro-Osmometer.

**Cell Culture—**The established inner medullary collecting duct cell line IMCD-3 is an immortalized line generously provided by Dr. Steve Gullans (Boston, MA) (16). The cells were routinely propagated in a 1:1 mixture of Dulbecco’s modified Eagle’s medium (DMEM) and Ham’s F-12 nutrient mixture, supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin.

* The abbreviations used are: MAP, mitogen-activated protein; ERK, extracellular signal-regulated kinase; JNK, c-Jun NH$_2$-terminal kinase; IMCD, inner medullary collecting duct cell; HA, hemagglutinin; DMEM, Dulbecco’s modified Eagle’s medium; ATF-2NT, NH$_2$-terminal domain of ATF-2.

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Sensitization to Hypertonicity by Dominant-Negative JNK2

Generation of JNK1 and JNK2 Stable Transfectants—The HA-JNK1, HA-JNK1-APF, HA-JNK2, and HA-JNK2-APF cDNAs (17–19) were ligated between the HindIII and Hpal sites of the retroviral vector pLNCX (20) and packaged into replication-defective retrovirus using 293T cells and the retrovirus component expression plasmids SV-α–MMLV, MLV–SV-α–env–MLV as described previously (21, 22). IMCD-3 cells were cultured for 24 h in virus-containing conditioned medium that had been filtered through a 0.45 μm filter and supplemented with 8 μg/ml polybrene. Positive infectants were selected in 500 μg/ml G418, cloned, and further characterized by Western blots using anti-HA antisera, as well as by kinase activity assay (see below). Experiments were performed on cells that had been passaged less than 6 times.

Assay of JNK Kinase Activity by Immunoprecipitation—To determine activity of transfected HA-JNKs, IMCD-3 cells in 100-mm tissue culture dishes were incubated at 37 °C for 10 min in DMEM:F-12 medium alone or medium supplemented with 150 mM NaCl (600 mosM final). They were then washed three times in isosmotic phosphate-buffered saline, and lysed in 0.2–0.5 ml of lysis buffer (50 mM β-glycero- phosphate (pH 7.2), 0.5% Triton X-100, 1 mM sodium vanadate, 2 mM MgCl₂, 1 mM EGTA, 1 mM dithiothreitol, 2 μg/ml leupeptin, 4 μg/ml aprotinin). The lysate was centrifuged at 4 °C for 10 min (10,000 × g) to remove nuclei and cell debris, and the supernatants were adjusted to 100–200 μg of protein in 0.5 ml, to which was added 5 μl of mouse monoclonal antiserum directed against the influenza HA epitope (Boehringer Mannheim), and 100 μl of 10% protein G-Sepharose (Pharmacia). After 2 h of rocking incubation at 4 °C, the immunoprecipitates were washed three times in lysis buffer and resuspended in 40 μl of 50 mM β-glycero-phosphate (pH 7.2), 0.1 mM sodium vanadate, 20 mM MgCl₂, 200 μM [γ-32P]ATP (5000 cpm/pmol), and 100 μg/ml recombinant NH₂-terminal domain of ATF-2 (ATF-2NT) (6) for 20 min at 30 °C. The reaction was stopped by the addition of SDS sample buffer, and the lysates were heated in a boiling water bath for 5 min and subsequently subjected to SDS-polyacrylamide gel electrophoresis on a 10% polyacrylamide gel and followed by autoradiography. The bands corresponding to phosphorylated ATF-2NT were excised and counted in a liquid scintillation counter.

Assessment of kinase inhibition by HA-JNK-APF constructs was carried out similarly, except that 100 μl of 10% GST-c-Jun-agarose beads were used in place of anti-HA antiserum and ATF-2 peptide, and the final ATP concentration was 20 μM.

Assay by Mono Q Fast Performance Liquid Chromatography of Dominant-Negative JNK Inhibition of Endogenous JNK Activity—JNK activity was also assessed following fractionation on Mono Q fast performance liquid chromatography as described previously (11). Portions of cell lysates prepared as described above (0.5 ml; 1.0–1.5 mg of protein) were applied to a Pharmacia HR5/5 Mono Q anion exchange column equilibrated in 50 mM β-glycero-phosphate (pH 7.2), 0.1 mM sodium vanadate, 1 mM EGTA, and 1 mM dithiothreitol, and eluted with a 30-ml gradient of 0–600 mM NaCl in the same buffer. Fractions (1 ml) were collected, and 20-μl aliquots were mixed with 20 μl of 50 mM β-glycero-phosphate (pH 7.2), 0.1 mM sodium vanadate, 20 mM MgCl₂, 20 mM β-glycero-phosphate (pH 7.2), 0.1 mM sodium vanadate, 20 mM MgCl₂, 200 μM [γ-32P]ATP (5000 cpm/pmol), and 100 μg/ml recombinant NH₂-terminal domain of ATF-2 (ATF-2NT) (6) for 20 min at 30 °C. The reaction was assessed following SDS-polyacrylamide gel electrophoresis and Western blot analysis with 12CA5 antibody. B, kinase activity in 12CA5 immunoprecipitates from cells expressing epitope-tagged JNK1 or JNK2 treated for 10 min in either 300 or 600 mosM/kg medium was measured using ATP-2NT peptide as substrate (see “Experimental Procedures”). Representative data from three individual experiments is shown, WT, wild type.

Measurement of Mean Cell Volume—Cell volume regulation was studied by observing the mean cell volume at various times after hypertonic treatment. Approximately 10 million cells were trypsinized, resuspended in DMEM to inactivate trypsin, centrifuged for 1 min at 2,000 × g, and resuspended in 4 ml of buffer E, consisting of 10 mM HEPES (pH 7.3), 140 mM NaCl, 4 mM KCl, 0.5 mM CaCl₂, 2 mM MgCl₂, 1 mM KH₂PO₄, and 300 mM glucose and having an osmolality of approximately 300 mosM. After 30 min of equilibration, sufficient NaCl was added to bring the osmolality to 600 mosM/kg, and the mean cell volume of 10,000 cells per time point was measured using a Coulter Multisizer, Coulter Sample Stand II, and Multisizer AccuComp version 1.19 software, utilizing an aperture tube diameter of 100 μm. Changes in cell volume over time are expressed as relative volume by normalizing to mean cell volume measurements taken on cells before addition of hypertonic NaCl.

RESULTS AND DISCUSSION

Selective Osmotic Regulation of JNK2—To ascertain whether the activation of JNK by hypertonicity is isoform specific, we prepared IMCD-3 cell clones stably expressing HA-tagged JNK1 and JNK2. The expression of both HA-tagged JNK isoforms was confirmed by Western blot (Fig. 1A). However, whereas neither the neomycin control (devoid of HA-tagged JNK) nor HA-JNK1 was stimulated by hypertonicity, the HA-JNK2 showed a consistent activation in each of three experiments; one is shown in Fig. 1B. A similar specificity was noted for activation of HA-JNK2 and not HA-JNK1 by UV light.

Inhibition of JNK2 Signaling Sensitizes IMCD-3 Cells to Hypertonicity-induced Lethality—To characterize the role of

2 P. Wojtaszek and T. Berl, unpublished observations.
JNK2 in response to osmotic stress, we developed stably transfected IMCD-3 cell clones expressing nonphosphorylatable mutants of JNK1 (JNK1-APF) and JNK2 (JNK2-APF). In these mutants, the phosphorylation site, Thr-Pro-Tyr (TPY), is altered to Ala-Pro-Phe (APF), rendering the expressed kinase incapable of being phosphorylated, and hence incapable of being activated (18, 24). These constructs are predicted to behave as competitive inhibitors of activation of cellular JNK1 and JNK2, respectively, by competing for binding of upstream MAP kinase kinases which normally phosphorylate and thereby activate the JNKs.

Fig. 2A shows the expression of these constructs in two clones each of HA-JNK1-APF and HA-JNK2-APF. To demonstrate whether these constructs inhibit hypertonicity-stimulated JNK activity, GST-c-Jun was employed in JNK activity assays of lysates of cells exposed to hypertonicity. Utilization of this substrate measures activity of all JNK isoforms.

Activation of GST-c-Jun phosphorylation in HA-JNK1-APF clones was not significantly different from that of the neomycin controls (Fig. 2B), which is the predicted result if JNK1 is not osmotically regulated, as the results in Fig. 1 suggest. In contrast, HA-JNK2-APF clones consistently displayed a significant inhibition of hypertonicity-induced GST-c-Jun phosphorylation. Two independent stable HA-JNK2-APF clones displayed mean inhibitions of 42 ± 15% and 48 ± 17%, respectively, as illustrated in Fig. 2B (n = 3, mean ± S.E.).

It was important to confirm that the apparent inhibition of JNK activity observed by immunoprecipitation truly reflected inhibition of endogenous JNK activity and was not simply the result of an excessive amount of dominant-negative JNK saturating the GST-c-Jun beads used in the JNK binding assay. Therefore, lysates from cells subject to hypertonicity were also fractionated by Mono Q fast performance anion exchange liquid chromatography, and the JNK activity in the fractions was measured. Consistent with the percent inhibition by JNK2-APF observed in the GST-c-Jun binding assay, Fig. 2C shows a 50% inhibition of hypertonicity-stimulated ATF-2NT phosphorylation in the JNK2-APF clone 9, but not in the neomycin control or the JNK1-APF clone 5.

These experiments thus support the above observation that JNK2 is responsible for the majority of hypertonicity-induced JNK activity. If JNK1 activation played a significant role in this response, it would be expected that HA-JNK1-APF would at least partially block phosphorylation of GST-c-Jun in lysates from hypertonically exposed cells. However, only HA-JNK2-APF expression resulted in inhibition of hypertonicity-stimulated phosphorylation of GST-c-Jun, suggesting that JNK2 is the primary source of hypertonicity-stimulated JNK activity. Interestingly, in yeast lacking HOG1 MAP kinase, which is required for growth on hypertonic medium, expression of JNK1 but not JNK2 rescues hypertonic growth (19). This suggests that functions of different JNK isoforms may not be dictated by amino acid sequence similarity per se but rather by another factor such as differential affinity for specialized upstream MAP kinase kinases or downstream targets.

To determine if JNKs play a protective role in cellular adaptation to hypertonicity, we investigated the possibility that the presence of the dominant-negative JNK could impact on cell survival after osmotic challenge. Fig. 3 summarizes the survival data in which we employed two distinct stable clones each of HA-JNK1-APF and HA-JNK2-APF and assayed cell survival after hypertonic challenge using trypan blue exclusion assay.
The adaptive response to hypertonicity involves increases in cellular content of organic osmolyte transporters leading to enhancement in the uptake of solute (23). We tested whether dominant-negative JNK2 may confer sensitivity to hypertonic challenge by interfering with hypertonicity-stimulated organic osmolyte uptake. However, HA-JNK2-APF stable transfec-
tants displayed initial rates of inositol uptake after 16 h in a hypertonic environment at a level comparable to that of neomycin controls (Fig. 4). These studies were done at 500 mosM/kg in view of the sensitivity of the transfected cells to hypertonicity-stimulated increase in inositol uptake. The cells were then thoroughly washed in phosphate-buffered saline and lysed in lysis buffer. The lysate was centrifuged at 10,000 \( \times g \), and the supernatant was counted in a liquid scintillation counter. Data shown are mean \( \pm S.E. \) (n = 3).

Finally, staining with acridine orange:ethidium bromide revealed that the process leading to cell death was clearly necro-
sis and not apoptosis. The adaptive response to hypertonicity involves increases in cellular content of organic osmolyte transporters leading to enhancement in the uptake of solute (23). We tested whether dominant-negative JNK2 may confer sensitivity to hypertonic challenge by interfering with hypertonicity-stimulated organic osmolyte uptake. However, HA-JNK2-APF stable transfec-
tants displayed initial rates of inositol uptake after 16 h in a hypertonic environment at a level comparable to that of neomycin controls (Fig. 4). These studies were done at 500 mosM/kg in view of the sensitivity of the transfected cells to prolonged incubations at higher tonic-
ity (see above and Fig. 3).

Furthermore, inositol uptake measurements were normalized per milligram of cell protein, and the culture dishes were vig-
oro usly washed three times with isotonic buffer before lysis to avoid interference by protein from nonviable cells in the protein determination assay. These results do not entirely rule out the role for JNK activation in initiating the transcription of sodium myo-inositol transporter genes, as the residual JNK activity

\footnote{3 P. Wojtaszek, P. Squier, J. J. Cohen, and T. Berl, unpublished observations.}
port, the present experiments suggest that the JNK pathway is not involved in regulation at any step in the process.

Another characteristic of cell adaptation to hypertonicity is the regulatory volume increase, by which a cell recovers from osmotic shrinkage and regains the volume it displayed before hypertonic treatment. To determine if the JNK2 pathway might contribute to this process, control and JNK-APF cells were subjected to 600 mosM/kg, and their cell volume was monitored with a Coulter Multisizer. Fig. 5 shows that both neomycin and JNK2-APF cells regained their original volume by 20 min, and that the JNK2-APF cells were not significantly different from controls in either time course or extent of regulatory volume increase. Similar data were obtained with the JNK1-APF clones, as well as with neomycin, JNK1-APF, and JNK2-APF cells treated with the ERK inhibitor PD098059 or the p38 kinase inhibitor SB203580.2 These data suggest that the mechanism by which JNK2-APF sensitizes cells to killing by hypertonicity does not involve the cell's ability to carry out regulatory volume increase, and in addition, that neither the JNK, ERK, or p38 pathways play a significant role in this process.

Whereas these experiments address primarily volume-regulatory mechanisms by which a cell responds to hypertonic stress, it is likely that other systems also play a role in survival of hypertonicity. For example, several groups (26, 27) have described members of the heat shock protein 70 superfamily that are up-regulated by exposure to hypertonic NaCl. The hypothesis that these heat shock proteins carry out an osmoprotective function, and that the JNK2 pathway may be involved in their regulation, is presently under investigation. Alternatively, JNK2 may activate as yet unidentified factors that stabilize the cellular machinery to the intracellular ionic changes that occur during osmoregulation.

In summary, we have for the first time determined that osmotic activation of JNK activity in renal inner medullary collecting duct cells is isoform-specific, with the major regulated form identified as JNK2. We have also dissociated JNK2 from the adaptive response that recruits sodium myo-inositol transporters and from the ability to carry out regulatory volume increase, but have defined a crucial role for JNK2 in the survival of inner medullary collecting duct cells in a hypertonic environment.

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