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Mitogen-Activated Protein Phosphorylation in Endothelial Cells Exposed to Hyperosmolar Conditions

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Abstract The effect of hyperosmolarity on the induction of the mitogen-activated protein kinases (MAPK) was studied in bovine aortic endothelial cell (EC). Different types of agents were used to differentiate the effects of osmolarity from other variables. Hypertonic treatment with physiologically relevant levels of NaCl (350 mOsm/kg H_2O) significantly increased the level of expression of p38 within 2 min, and ERK-1/2 and JNK after 10 min. The inductions peaked between 30 and 60 min and returned to baseline levels within 2 h. A similar pattern of induction occurred with ionic contrast agent. p38 induction by glucose and mannitol showed a similar pattern, although the level of ERK-1/2 phosphorylation was not as robust, and JNK was not induced by glucose. Urea did not affect the level of induction of the MAPK isoforms. It is concluded that MAPK plays an important role in hyperosmolality-induced signal transduction. Different osmotic agents induce MAPK expression differently. No MAPK induction with urea implies that cell shrinkage may be an important component of hyperosmolality-induced MAPK phosphorylation. J. Cell. Biochem. 76:567–571, 2000.

Key words: MAPK; endothelial cell; signal transduction

A hyperosmolar blood milieu can commonly occur in a number of clinical conditions, such as diabetes mellitus, uremia, and hypernatremia. Endothelial cells (EC) form the interface between the blood and vessel wall and so are inevitably exposed to the continually changing osmotic environment. The EC response to osmotic stress is critically important in maintaining homeostasis. In the presence of increasing extracellular tonicity, EC shrink rapidly due to loss of cell volume [Mazzoni et al., 1989]. Activation of membrane ion transporters, such as the Na-K-Cl cotransporter, has been associated with this volume change and is involved in subsequent restoration of cell volume [O'Donnell, 1993]. However, the process by which the cell senses changes in osmolarity and translates this to a biochemical response is not fully understood.

Mitogen-activated protein kinases (MAPK) are a major intracellular signal transduction pathway that has been demonstrated to play an important role in cell proliferation, differentiation, and stress response [Blenis, 1993; Karin, 1998]. Three members of the MAPK family—extracellular signal regulated kinase-1/2 (ERK 1/2), p38, and c-jun N terminal kinase (JNK)—were initially identified and analyzed. Several studies have demonstrated that osmotic shock results in marked phosphorylation of MAPK (hence activation) of several types of mammalian cells, including fibroblasts, intestinal cells, astrocytes, neutrophils, and glial cells [Krump et al., 1997; Matsuda et al., 1995; Sinning et al., 1997]. MAPK has also been recently reported to play an important role in signal transduction in EC exposed to mechanical stress such as shear [Takahashi and Berk, 1996] and cyclic strain [Ikeda et al., 1999]. The activation of MAPK by osmotic shock in EC has not been fully studied.

The present study was conducted to investigate the characteristics of hypertonicity-induced stimulation of endothelial cell MAPK activity. Specifically, we determined the time course and fold increase of the three major members of the MAPK family by NaCl-induced hyperosmotic stress. We also evaluated the effect of three other different osmotically active compounds on MAPK phosphorylation and acti-

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vation in an attempt to separate the effect of osmolarity from other independent confounding variables.

MATERIALS AND METHODS

Cell Culture

Bovine aortic EC were harvested from calf thoracic aorta and plated on collagen type I-coated polystyrene dishes (Falcon, Becton Dickinson Labware, Franklin Lakes, NJ) and maintained with medium M199 (Sigma Chemical Co., St. Louis, MO) (300 mOsm/kg H_2O, glucose 5.5 mM). Cultures were kept in an incubator with 5% CO_2, 95% air at 37°C. Cells from passages 3–7 were used for experimentation. For these studies, media were made hyperosmotic by addition of a small concentrated volume of NaCl, glucose, mannitol, urea, or contrast agent (Iothalamate meglumine 60%, Conray, Mallinckrodt Medical, St. Louis, MO), to produce a final media osmolality of 350 mOsm/kg H_2O.

Western Blot Analysis

After the cells were exposed to the hyperosmotic conditions for varying times, medium was removed, the plates washed with ice-cold Hank's balanced salt solution (HBSS), and cells lysed with ice-cold buffer containing 100 mM NaCl, 1% Triton X-100, 20 mM NaF, 1 mM Na-EGTA, 10 mM benzamidine, 10 μg/ml aprotinin, 10 μM leupeptin, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 30 mM Na-HEPES, pH 7.5. Lysates were collected, sonicated, and centrifuged at 14,000 rpm for 15 min. Protein concentration was determined by the method of Bradford [1976]. Equal protein amounts were then diluted (1:1) into 2× Laemmli sample buffer, boiled for 5 min, and subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The separated proteins were transferred to nitrocellulose membrane, blots were then blocked with Tris-buffered saline containing Tween 20 and 5% milk or 1% bovine serum albumin (BSA) before incubation with the primary and secondary antibodies [Ikeda et al, 1999]. In the present studies, analysis of MAPK activation was performed using phospho-specific antibodies. We have previously reported that in bovine aortic EC exposed to mechanical forces, use of phospho-specific antibodies correlates strongly with *in vitro* assessment of activity using specific substrates [Ikeda et al, 1999]. Therefore, for this study, anti-active ERK1/2 antibody (Promega, Madison, WI), anti-active JNK and p38 antibodies (New England Biolabs, Beverly, MA) were used. Immunodetection was performed by chemiluminescence (Amersham, Piscataway, NJ) and quantitated using a phosphoimager densitometer (Molecular Dynamics, Sunnyvale, CA).

Statistics

Data were derived from at least three separate experiments. Results are expressed as mean ± SEM. Statistical analysis was performed by using Student's *t*-test or analysis of variance with post hoc testing where appropriate, $P < 0.05$ was considered significant.

RESULTS

The magnitude and time course of induction of MAPK in EC exposed to hyperosmolar NaCl are shown in Figure 1. A Western blot of a typical experiment and the densitometric average of several experiments are depicted. Hyperosmolar NaCl significantly activated all MAPK members in a time dependent manner. Induction of ERK1/2 and JNK-p54/p46 occurred by 10 min and peaked at 30 min. Maximal fold induction were 2.9 ± 0.3, 3.4 ± 0.3, 2.3 ± 0.05, and 1.9 ± 0.08 at 30 min respectively. Induction of p38 activity was detected by 2 min (2.6 ± 0.3) and peaked at 30 min (3.1 ± 0.3). The degree of induction of p38 activity at 2 min was significantly higher compared with the other MAPK (2.6 ± 0.3 for p38 versus 1.1 ± 0.2 for ERK-1 and 1.5 ± 0.3 for JNK-p54, $P < 0.05$). Induction of MAPK activity in EC by hyperosmolar conditions was transient and returned to baseline within 120 min of exposure.

To evaluate the effect of exposure of EC to other osmotic agents on MAPK activity, we compared the induction of phosphorylation at 30 min that could be achieved by NaCl, contrast agent, glucose, mannitol, and urea. The results are summarized in Figure 2.

Induction of ERK-1/2 in EC was significant with NaCl (2.9 ± 0.2 and 2.7 ± 0.3) and with exposure to the contrast agent (2.4 ± 0.1 and 2.7 ± 0.1). ERK-1/2 activation by glucose (1.9 ± 0.1 and 1.8 ± 0.2) and by mannitol (1.9 ± 0.2 and 1.7 ± 0.1) was less robust but not significantly different compared with NaCl and contrast. Similarly, p38 was strongly activated by all agents except urea. Exposure of EC to NaCl

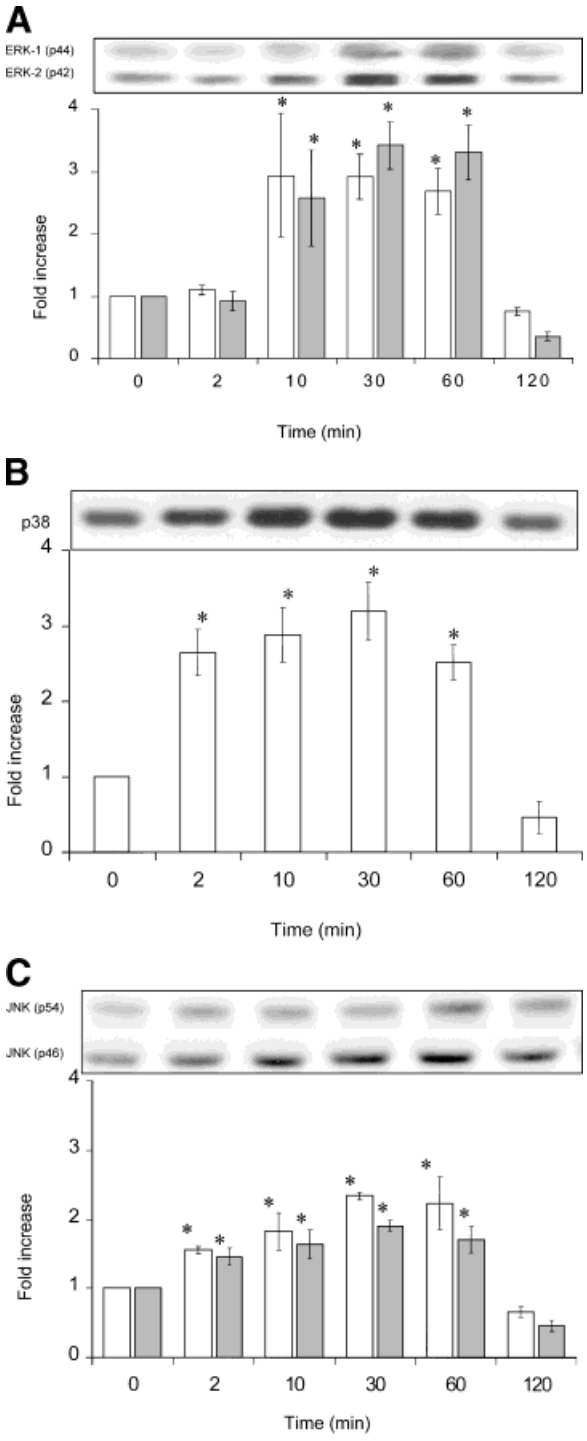

Fig. 1. Induction of mitogen-activated protein kinases (MAPK) activity by hyperosmolar NaCl. Bovine aortic ECs were exposed to 350 mOsm/kg H_2O of NaCl for up to 120 min. Detection of activated MAPK was performed by Western blotting with phospho-specific antibodies. A typical blot is shown, and the densitometric average normalized to control (300 mOsm/kg H_2O) of at least three separate experiments depicted below the blot. $^*P < 0.05$ compared with control. **A:** ERK1/2 activation. Open bars, ERK1 (44 kDa); shaded bars, ERK2 (42 kDa) (n = 4 experiments). **B:** p38 activation. Note the rapid increment of p38 phosphorylation at 2 min (n = 4 experiments). **C:** JNK activation. Open bar, 46 kDa; shaded bar, 54-kDa phosphorylated JNK proteins (n = 3 experiments).

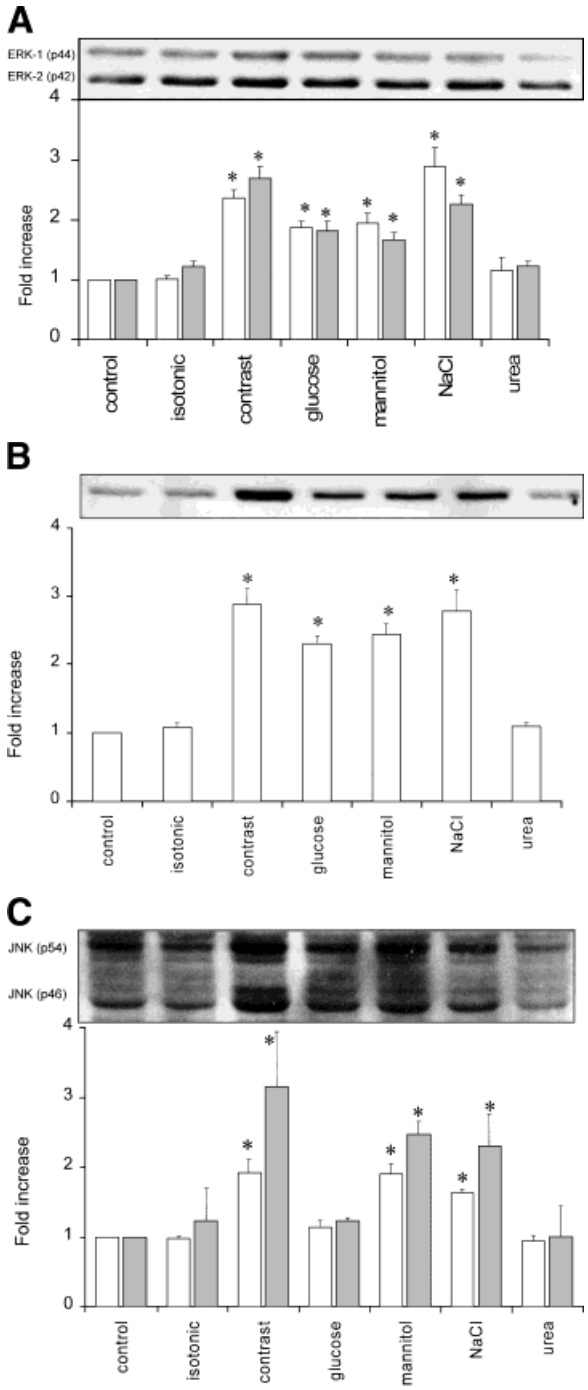

Fig. 2. Comparison of induction of MAPK activity by different hyperosmotic agents. Bovine aortic EC were exposed for 30 min to 350 mOsm/kg H_2O of either NaCl, glucose, mannitol, urea, or contrast agent. Since a small volume of highly concentrated osmotic agent was added to increase osmolality, the same amount of isotonic medium was added for the isotonic condition. MAPK activation was assessed by Western blotting with phospho-specific antibodies. A typical blot is shown and the densitometric average normalized to control (300 mOsm/kg H_2O) of at least three separate experiments depicted below the blot. $^*P < 0.05$ compared with control. **A:** ERK1/2 phosphorylation. Open bars, ERK1 (44 kDa); shaded bars, ERK2 (42 kDa) (n = 3 experiments). **B:** p38 activation. (n = 3 experiments). **C:** JNK activation. Open bar, 46 kDa; shaded bar, 54-kDa phosphorylated JNK proteins (n = 3 experiments)

(fold induction 2.3 ± 0.1), contrast agent (2.5 ± 0.1), glucose (2.3 ± 0.1), and mannitol (2.1 ± 0.1) resulted in a consistent increase in phosphorylation of p38. JNK 54 kDa/46-kDa isoforms were also activated after 30-min exposure to NaCl (1.6 ± 0.1 and 2.3 ± 0.4), contrast agent (1.9 ± 0.2 and 3.2 ± 0.5), or mannitol (1.9 ± 0.2 and 2.5 ± 0.3). However, exposure to glucose did not result in activation of JNK. Again, it should be emphasized that urea did not stimulate phosphorylation of any of the MAPK members.

DISCUSSION

Maintenance of homeostasis is a fundamental property of cells. Mammalian cells are often exposed to changing osmotic conditions that influence important cell functions. The ability to respond to a hyperosmotic challenge is an adaptive mechanism of cells to stress, which allows the cells to maintain viability and functional status [Fernandez et al., 1997].

Although hyperosmolar stimulation of MAPK has been reported in a variety of mammalian cells such as fibroblasts [Moriguchi et al., 1995], platelets [Nagata et al., 1997], and glial cells [Matsuda et al., 1995], it is unclear whether the activation of MAPK is a general biological phenomenon. For example, MAPK is not activated in human leukocytes exposed to hyperosmotic conditions [Krump et al., 1997]. Furthermore, differential activation of the various MAPK members occurs depending on cell type. Osmotic shock of Chinese hamster lung fibroblasts with 100 mOsm/kg H_2O sucrose activates ERK, but not p38 and JNK [Bianchini et al., 1997].

The present study demonstrates that vascular EC exposed to hyperosmotic conditions results in activation of the different MAPK subfamilies. However, this study clearly shows that not only do the different subfamilies of MAPK have a different temporal pattern and magnitude of phosphorylation, but also that different agents have varying abilities to upregulate MAPK expression. Unlike ERK and JNK, p38 induction by osmotic shock occurred as early as 2 min and this time point coincides with the activation of the volume regulatory proteins [O'Donnell et al., 1995; O'Neill and Klein, 1992]. This observation is consistent with the thesis that p38 activation is directly related to the initial cell response to osmotic shock. In this regard, HOG 1, the homologue of mammalian cell p38 in yeast, has been previously demonstrated to be a critical element of yeast adaptation to osmotic stress [Takekawa et al., 1997]. HOG 1 defective yeast do not proliferate under hyperosmotic condition [Brewster and Gustin, 1994]. The result of the present study indicates that although p38 was similarly activated by all osmotic agents except urea, the other MAPK members, ERK and JNK, were not uniformly activated. Taken together, these data suggest that p38 may play an important role in the osmotic shock response of EC.

None of the MAPK was activated in EC exposed to urea in our experiments. Although, urea increases the osmolality to the same degree as other agents, the cell membrane is permeable to urea and the urea molecules cross the cell membrane freely. When the urea concentration is increased, the osmotic charge is rapidly equilibrated in both side of the cell membrane and cell shrinkage does not occur [Kapus et al., 1999]. These results suggest that cell shrinkage may be a necessary component of osmotically-induced MAPK activation.

How cell deformation activates the MAPK cascade is not known. Obvious mechanisms include the involvement of focal adhesion plaques and/or the cytoskeleton. In this regard, osmotic shock at 900 mOsm/kg H_2O has been reported to activate focal adhesion kinase (FAK) and PECAM-1 phosphorylation [Kawasaki et al., 1996]. However, the 900-mOsm/kg H_2O condition is not relevant to normal physiology. In our preliminary experiments, NaCl and glucose at 350 mOsm/kg H_2O did not activate FAK or phosphorylate PECAM-1. Furthermore, MAPK was also strongly activated by glucose and NaCl at 350 mOsm/kg H_2O in PECAM-1-null ECV-304 cells (data not shown)[Kim et al., 1998]. Taken together, these data suggest that FAK and PECAM-1 are not necessary for osmotically induced MAPK phosphorylation. The role of cytoskeletal proteins in this process has not been studied and will require future experiments.

In conclusion, the results presented in this study clearly show that MAPK has a significant role in osmotic shock-induced signal transduction in endothelial cells. Further analysis of this pathway will be useful for understanding the stress response mechanism.

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