Asymmetrical Response of p38 Kinase Activation to Volume Changes in Primary Rat Astrocytes

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Activation of p38 kinase by osmotic stress has been documented in many cells; however, no report has distinguished the effects of cell volume on p38 activity from the effects of the altered osmotic condition *per se*. Here we report asymmetrical activation of astrocyte p38 mitogen-activated protein (MAP) kinase in response to volume increases and volume decreases. We separate effects of cell volume changes from the effects of osmotic exposure on p38 activation. Exposure to 400, 500, or 600 mOsm phosphate-buffered saline (PBS) caused cell shrinkage and an osmolality-dependent increase in p38 activity to 175%, 409%, or 518%, respectively, compared with cells maintained in control conditions (290 mOsm). Likewise, hyposmotic conditions ranging from 250 to 57 mOsm PBS caused the same activation of p38 (approximately 300% of the control value within 10 min). The activity in hyposmotic conditions did not diminish over 30 min despite cell volume recovery, indicating a dependence of extracellular osmolality or ionic strength rather than cell volume. Cells that were returned to isosmotic conditions following 30 min in 250, 150, or 57 mOsm PBS shrunk to 73%, 39%, or 26% of the control cell volume, respectively. In these cells, the activity of p38 increased further from approximately 300% of the control values in each hyposmotic condition to as much as 500% of the control activity as a function of the degree of cell shrinkage. Thus, p38 may be activated by cell shrinkage in hyperosmotic or in isoosmotic conditions, indicating reduced cell volume is a more important determinant of this enzyme activity than extracellular osmolality. Our results indicate distinct mechanisms of p38 activation in astrocytes exposed to hyperosmotic or hyposmotic PBS.

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Mitogen-activated protein (MAP) kinases are a spectrum of serine/threonine kinases that mediate cellular responses to extracellular mitogenic and stress signals (1–3). They are activated in response to various extracellular stimuli and they transmit signals to different intracellular effectors, mainly transcription factors in the nuclei. The three major subfamilies of MAP kinases are Erk-1/Erk-2, JNK, and p38 (4). JNKs and p38 mainly respond to stress signals such as anisosmotic exposure. The Erk-1/Erk-2 subfamily is mainly responsible for transmitting mitogenic signals from growth factors; however, a recent study demonstrated the Erk pathway also is activated by osmotic cell swelling in astrocytes (5). This finding raises the possibility that these MAP kinases also may be involved in volume regulatory responses of astrocytes. It has been reported that p38 is mainly activated by osmotic stress in most cell systems (1). Strong activation of p38 may lead to apoptosis (6, 7). Although p38 is activated in brain microglia after ischemia (8), little is known about the behavior and the role of this MAP kinase in brain astrocytes. p38 is homologous to yeast HOG-1 kinase, which is activated primarily by osmotic stress (1); however, it is not clear whether p38 activation is induced by exposure to anisosmotic conditions or by the resulting cellular volume change. Thus, this study was designed to distinguish osmotic induced- and volume-induced p38 activation in brain astrocytes.

Homeostasis of extracellular ions and neurotransmitters in the central nervous system (CNS) is largely regulated by glial cells via membrane transporters (9). As a result, these cells may be subjected to frequent changes in cell volume. Astrocytes in the CNS swell in many physiological and pathological conditions. For example, these cells swell as they buffer K^+ increases in extracellular space during normal neuronal activity (9, 10). Head trauma, ischemia, hypoxia, and Reye's syndrome also can cause cytotoxic swelling of astrocytes (9, 11, 12). Since the brain is constrained in the skull, swelling of the astrocytes must be regulated to avoid severe brain damage.

Exposure of cerebral astrocytes from primary culture to

hyperosmotic conditions causes cell shrinkage, whereas hyposmotic conditions elicits a swelling followed by a regulatory volume decrease (RVD) (10, 13–16). Astrocytes do not regulate volume following hyperosmotic shrinkage (10). RVD is mediated by loss of intracellular osmolytes, including the inorganic ions K^+ and Cl^- and organic osmolytes such as taurine and other amino acids (10, 13, 15, 16). The movement of osmolytes is mediated by volume sensitive K^+, Cl^-, and inorganic anion channels (17, 18). However, the intracellular signaling systems that respond to cell swelling and cell shrinkage are poorly understood.

The present study demonstrates an increase in p38 kinase activity in response to both hyperosmotic and hyposmotic conditions in rat brain astrocytes. Over a wide range of hyperosmolalities, the degree of p38 activation increases as medium osmolality increases and cell volume decreases. In addition, p38 activation increases by about 300% in response to moderate hyposmotic exposure, resulting in a 27% volume increase. There is no additional increase in p38 activity with further decreases in osmolality and cell swelling as great as 300% of the control cell volume. However, p38 activity could be increased further in hyposmoticly treated cells by shrinking them with a subsequent return to isosmotic conditions. These data suggest volume change rather than osmolality or ionic strength triggers p38 activation during cell shrinkage.

Materials and Methods

Primary cultures of rat brain astrocytes where prepared using methods previously described (19, 20). Briefly, cerebral cortices were removed from 2- to 3-day-old rat pups and were minced in Hank's balanced salt solution. The tissue was softened with a 15-min exposure to 0.125% trypsin, titrated with a 10-ml serum pipette, and filtered through an 80-μm nylon mesh. The resulting suspension of single cells was diluted with medium described previously (21) containing 20% newborn calf serum. Cells were plated into 35- or 100-mm plastic culture dishes at a density of 60,000 cell/cm^2. After 2 days and twice each week thereafter, medium was changed to a similar formulation except containing only 10% newborn calf serum. Cultures were used after 2 weeks when cells had become confluent. Previous studies from our laboratory have shown these cells are 95% astrocytes as determined by immunostaining for glial fibrillar acidic protein (19).

Growth medium was removed from cell cultures and was replaced with isosmotic, hyperosmotic, or hyposmotic phosphate-buffered saline (PBS). Isosmotic PBS contained 147 mM NaCl, 2.7 mM KCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.5 mM KH_2PO_4, 3.2 mM Na_2HPO_4, and 5.5 mM glucose, pH 7.3. Osmolality was adjusted to 290 mOsm by adding NaCl. Hyperosmotic PBS was made by adding sucrose to desired osmolalities, thus maintaining the same extracellular ionic strength. Hyposmotic PBS was made by reducing the concentration of NaCl to achieve the desired osmolalities. Isosmotic sucrose-PBS was made by replacing the NaCl with an equiosmotic quantity of sucrose, thus reducing the ionic strength while maintaining osmolality with a cell membrane-impermeant species. Isosmotic urea-PBS was made by replacing 50 mM of NaCl with an equiosmotic quantity of urea. Cells in this solution are subjected to reduced ionic strength in an isoosmotic solution containing a membrane permeable osmolyte. All osmolalities were verified using vapor pressure osmometry (Wescor, Logan, UT). Cells were incubated with PBS for various time periods before being harvested for measurements of cell volume, p38 activities, and protein levels.

p38 activities were measured by immune complex kinase assay with glutathione-sulfate-transferase (GST)-ATF-2 as the substrate (22). Cells were treated either for 10 min in isosmotic PBS (controls), for 10 min in hyperosmotic PBS, for 10 or 30 min in hyposmotic PBS, or for 30 min in hyposmotic PBS followed by 10 min in isosmotic PBS. Cells (2–4 mg of protein) were lysed in 1 ml of lysis buffer (20 mM Tris, pH 7.5, 137 mM NaCl, 1.5 mM, 2 mM EDTA, 10 mM sodium pyrophosphate, 25 mM β-glycerophosphate, 10% glycerol, 1% Triton X-100, 1 mM Na orthovanadate, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml leupeptin). Cell lysates were left on ice for 30 min and were then centrifuged at 13,000*g* for 30 min. p38 protein was immunoprecipitated with 1 μg rabbit polyclonal antibody against p38 (Santa Cruz Biotechnology, Santa Cruz, CA) and Protein A-Sepharose beads (Sigma, St. Louis, MO). Immune complexes were washed three times with lysis buffer and twice with kinase buffer (20 mM HEPES, pH 7.6, 20 mM $MgCl_2$, 25mM β-glycerophosphate, 100 mM sodium orthovanadate, and 2 mM dithiothreitol [DTT]) before being resuspended in 60 μl of kinase buffer. One microgram of GST-ATF-2 (Santa Cruz Biotechnology) was then added to a 30-μl aliquot of immune complex. The kinase reaction was initiated by adding 2 μl of ATP cocktail (20 μM ATP and 10 μCi [γ-^{32}P] ATP, 6000 Ci/mmol; Amersham, Chicago, IL) and was allowed to proceed at room temperature for 10 min before terminating by adding 30 μl of 2× Laemmli buffer. Phosphorylation of ATF-2 was visualized by autoradiography after SDS-PAGE (12%). ATF-2 phosphorylation levels were quantified by densitometry using Image Calc software by Chris van der Lest.

Levels of p38 protein were determined by Western blotting. Briefly, equal volumes of 2× Laemmli buffer were added to 20 μl of immune complex and were then boiled for 5 min. After resolution by 12% SDS-PAGE, proteins were transferred to PVDF membrane (Millipore, Bedford, MA) and probed with anti-p38 polyclonal antibodies. The membranes then were incubated with goat anti-rabbit IgG conjugated with alkaline phosphatase (Santa Cruz Biotechnology). Secondary antibodies were detected with PhototopeStar Western Blot Detection Kit (New England Biolabs, Beverly, MA). Based on measurements of blank samples containing no p38 activity, the mean value measured in isosmotic PBS was equivalent to the background levels.

Cell volumes were measured in parallel studies at the same time points used for p38 measurements. Because cell size may vary with time in culture (23), cell volume comparisons were performed only between cultures measured on the same day. Cell volume was defined as the intracellular 3-*O*-methylglucose (3-OMG) space. For volume determinations, culture dishes first were incubated in isosmotic, hyposmotic, or hyperosmotic PBS for 5 min. This PBS then was removed and exchanged with a similar PBS except with glucose replaced by 1.0 mM 3-OMG plus the radiotracers, 0.5 μCi/ml [^{3}H]-3-OMG and 0.5 μCi/ml [^{14}C]-sucrose. Cells remained in this solution for an additional 5 min, a time sufficient to equilibrate 3-OMG across the cell membrane (24). Other cultures were incubated in isosmotic or hyposmotic PBS for 25 min before changing to glucose-free osmotically matched PBS containing 1 mM 3-OMG and radiotracers for an additional 5 min. Still other cultures were incubated in isosmotic or hyposmotic PBS for 30 min, followed by 5 min in fresh isosmotic PBS and a final incubation for 5 min in glucose-free isosmotic PBS containing 1 mM 3-OMG and radiotracers. To obtain a measure of cell volume after only 3 min of exposure to various osmotic conditions, cells were incubated in glucose-free isosmotic PBS containing 1 mM 3-OMG and radiotracers for 2 min followed by 3 min in fresh glucose-free isosmotic or anisosmotic solution containing the same concentration of 3-OMG and radiotracers.

Following the incubation in radiolabeled PBS, dishes were rapidly rinsed three times with an osmotically matched sucrose solution containing 10 mM Tris buffer (pH 7.3), 0.5 mM $Ca(NO_2)_2$, and 1 mM phloretin. Cells then were fixed with 0.6 M $HClO_4$, scraped off the dish, and the resulting suspension was sonicated and then centrifuged at 10,000*g* for 1 min. Radioactivity and protein contents were determined in the supernatant and pellet, respectively. Tritium activity from residual extracellular fluid in the supernatant was corrected using the concentration of radiolabeled sucrose measured in the radioactive incubation PBS. Cell volumes are expressed as microliters per milligram of protein.

Cell volume and p38 activity were compared between control and experimental groups by analysis of variance (ANOVA) with *post hoc* Dunnett's test or Student's *t* test as appropriate. Statistical significance was indicated by $P < 0.05$.

Results

The effect of hyperosmolality on p38 activity in astrocytes was examined by treating cells with PBS containing sucrose to obtain osmolalities of 400, 500, or 600 mOsm (Fig. 1A). Activity in the control PBS (290 mOsm) was

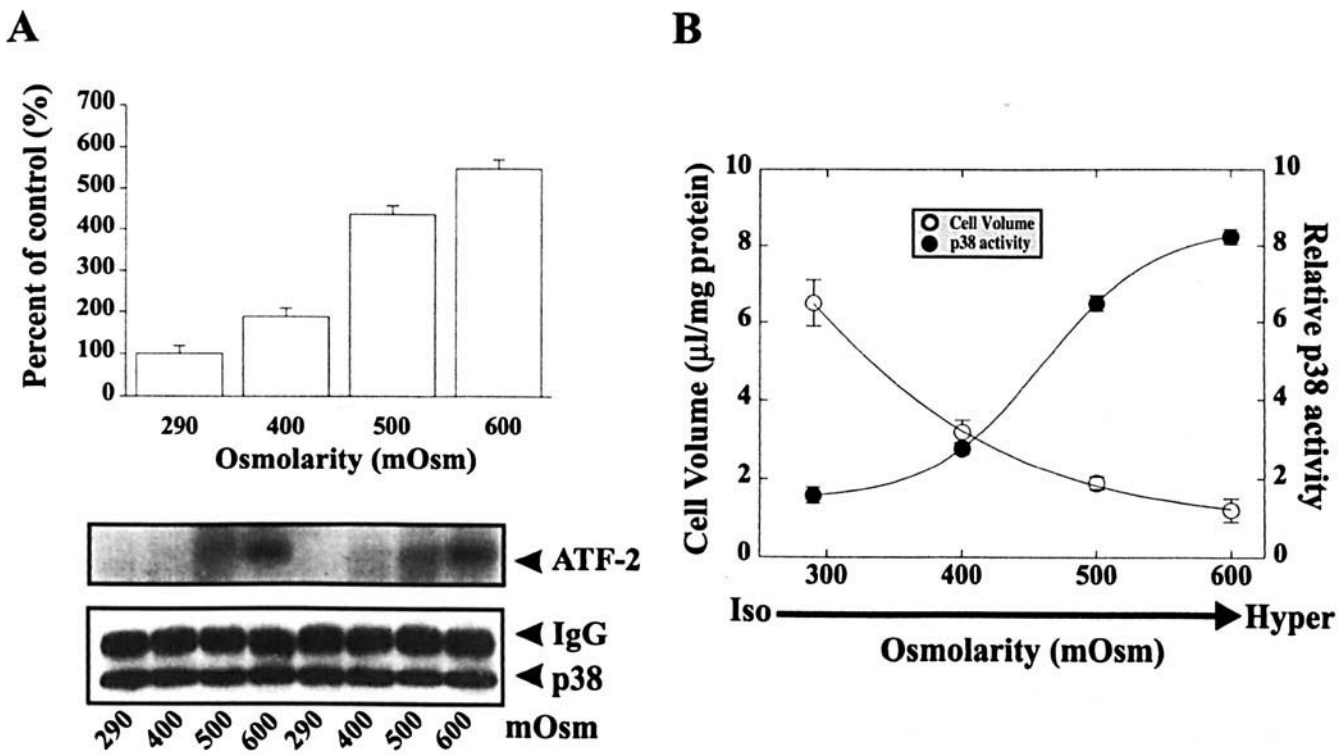

Figure 1. p38 kinase activity is increased by hyperosmotic exposure and cell shrinkage. (A) Effect of hyperosmotic stress on p38 activity in cultured rat astrocytes. Bar graph in upper panel shows increased p38 activity following 10 min of hyperosmotic exposure. Activity of p38 is expressed in arbitrary units reflecting the ratio of the density of the bands obtained after osmotic treatments and the density of the control band. Lower panel of the figure shows the results from of two representative kinase assays and measures of the concentration of p38 protein. (B) Comparison of p38 activity and cell volume following 10 min of hyperosmotic exposure. Cell volume decreased in response to hyperosmotic treatments. Data presented in A and B are plotted as mean values with SE bars from results of three independent experiments. An asterisk indicates mean values found to be significantly different from that measured in 290 mOsm conditions using Dunnett's test.

similar to that measured in blank samples. After a 10-min exposure to hyperosmotic PBS, p38 activity increased significantly. The activity of p38 was increased to 175%, 409%, and 518% of the control level as the extracellular osmolality was changed to 400, 500, and 600 mOsm, respectively. Although more than a 5-fold increase in p38 activity was observed at 600 mOsm, the biggest increase in p38 activity appeared between 400 and 500 mOsm. The content of p38 protein was unchanged by hyperosmotic exposure.

Hyperosmotic treatment caused a decrease in cell volume (Fig. 1B). The mean ± SEM volume of astrocytes decreased from 6.51 ± 0.63 μl/mg protein to 1.20 ± 0.32 μl/mg protein as the osmolality of the medium was increased from 290 to 600 mOsm. These data are plotted in Figure 1C together with corresponding measures of p38 activity for comparison. A good correlation appears between the cell volume decrease and p38 activity increase.

Increases in cell volume of 279%, 38%, and 27% relative to control cells in isosmotic PBS were observed after 10 min in PBS of 57, 150, and 250 mOsm (Fig. 2). After 30 min of exposure to these hyposmotic conditions, cells in 150 and 250 mOsm were only 21% and 8% larger than the control cell volume, and thus demonstrated volume regulation. No volume regulation was apparent for cells incubated in 57 mOsm PBS; however, some RVD may have already occurred by the 10-min time point in hyposmotic conditions when volume was measured (13, 15). Cell volume was reduced to approximately 74%, 38%, and 26% of the initial volume measured in isosmotic PBS when cells were returned to isosmotic PBS after a 30-min exposure to hyposmotic PBS of 250, 150, and 57 mOsm, respectively (Fig. 3B).

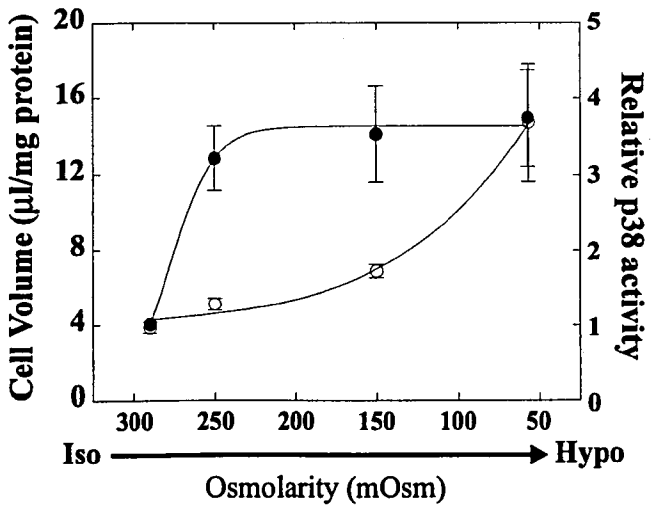

Figure 2. Effect of hyposmotic stress-induced cell volume increase on p38 activity. Data are from cells measured in isoosmotic PBS (290 mOsm) or after 10 min in hyposmotic PBS. p38 activity increased in moderate hyposmotic treatments and rapidly reached an apparent maximal value. Data are plotted as mean values with SE bars determined from results of three to six independent experiments. An asterisk indicates mean values found to be significantly different from that measured in 290 mOsm conditions using Dunnett's test.

Changes of cell volume following exposure to 10 min of hyposmotic conditions are shown as a function of extracellular osmolality in Figure 2. The activity of p38 increases dramatically to plateau at 306% to 361% of the control value after only a 27% increase in cell volume. Exposure of cells to the more extreme hyposmotic conditions of 57 mOsm caused additional swelling up to 400% of the initial cell volume measured in isosmotic PBS without further activation of p38 kinase activity (Fig. 2). Significant activation of p38 was measured in cells after 10 min of exposure to each of the three hyposmotic PBS (Figs. 2 and 3A). The magnitude of p38 activation was the same for cells in each hyposmotic PBS despite the differences in cell volumes measured at each osmolality (Fig. 3B). Furthermore, the activities of p38 were unchanged by an additional 20 min of exposure to hyposmotic PBS, while RVD was taking place (Fig. 3A).

Cells returned to isosmotic PBS for 10 min after 30 min in hyposmotic PBS shrunk to a volume smaller than their initial size measured in isosmotic PBS. The more severe hyposmotic treatments (57 and 150 mOsm) led to a greater decrease in cell volume than was observed following recovery from 250 mOsm PBS (Fig. 3A). Activity of p38 increased significantly to 490%, and 560% of that in control cells 10 min after cells were returned to isosmotic condition from 150 and 57 mOsm hyposmotic treatments, respectively (Fig. 3A).

To examine effects of cell volume changes in isosmotic conditions, astrocytes were incubated in isosmotic PBS (control), isosmotic sucrose-PBS, or isosmotic urea-PBS. The mean volume of cells after 3 min in isosmotic urea-PBS was slightly higher than that measured in control cells, but this volume change was not statistically significant. At the end of the 30-min observation period, there were no significant changes in cell volume in isosmotic PBS or isosmotic sucrose-PBS (data not shown), however, there was a volume decrease in cells treated with isosmotic urea-PBS (Fig. 4B). After 30 min in isosmotic urea-PBS, the solution was replaced with isosmotic PBS and a further reduction of cell volume was measured in these cells 10 min later. The increase in p38 activity of cells exposed to isosmotic urea-PBS was similar to that of cells swollen in hyposmotic PBS (compare Figs. 3 and 4A). After 30 min in isosmotic urea-PBS, activity of p38 was enhanced further by replacing isosmotic urea-PBS with isosmotic PBS for 10 min.

Discussion

It is very important to understand the intracellular signaling systems in astrocytes, which are activated by cell volume changes in response to hyposmotic and hyperosmotic conditions. During clinical brain edema, hyperosmotic treatment may be used to dehydrate the brain by reducing cell volume. However, following a prolonged period of edema, this procedure may cause net shrinkage of

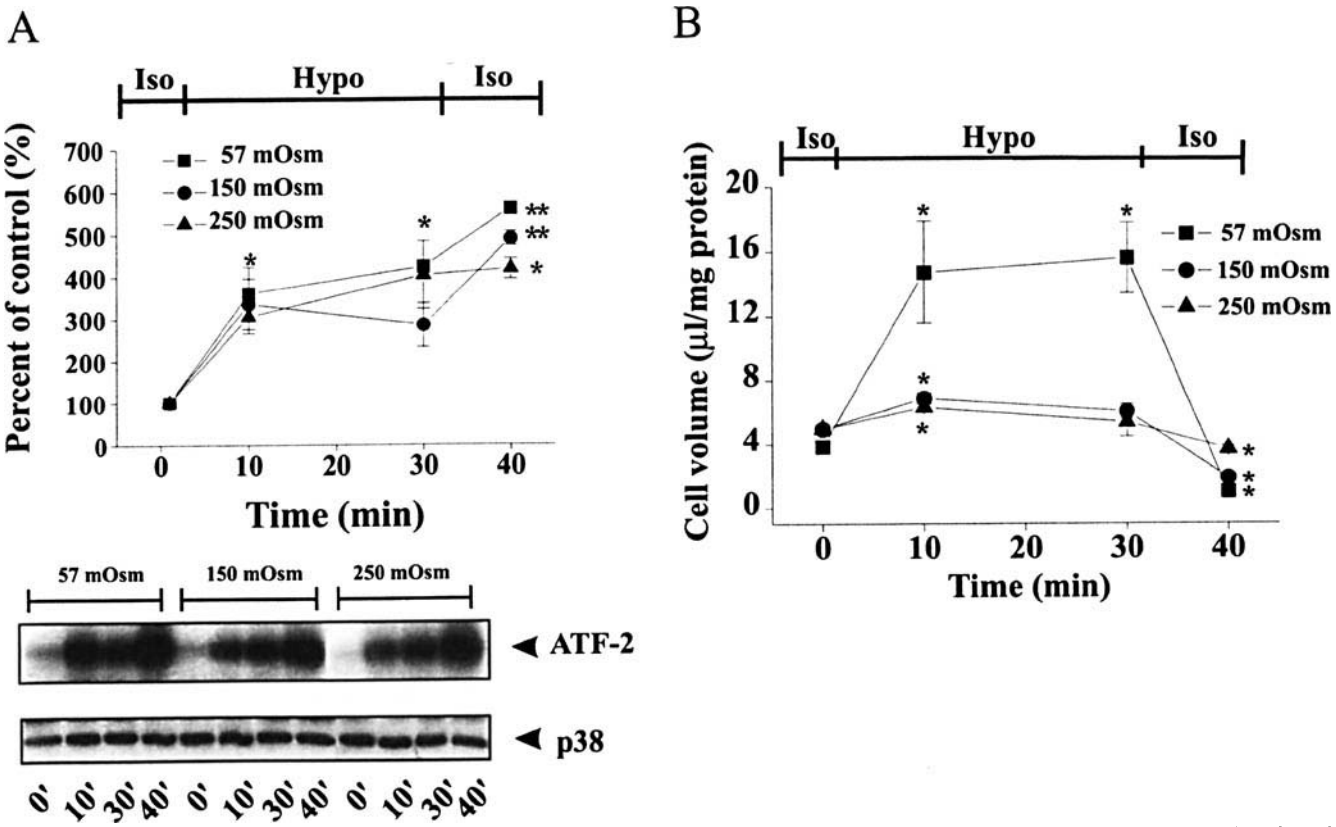

Figure 3. p38 kinase activity is increased by hyposmotic exposure and cell swelling. (A) Effect of hyposmotic stress on p38 activity in cultured rat brain astrocytes. Upper panel shows the time course of p38 kinase activation in response to hyposmotic exposure and after returning the cells to isosmotic conditions. Activity of p38 is expressed as a percentage of the activity of cells in isosmotic PBS (290 mOsm). The lower panel shows results of p38 activity after various times in hyposmotic conditions (57, 150, and 250 mOsm). The concentration of p38 protein measured at the same time points is unchanged as demonstrated in the bottom gel. (B) Cell volume changes during hyposmotic treatments and following return to isosmotic PBS. Cell volumes were measured in isosmotic PBS (290 mOsm) at 0 min or after 10 or 30 min in hyposmotic PBS (250, 150, or 57 mOsm). Other cells were exposed to hyposmotic PBS for 30 min starting at 0 min and were then changed back to isosmotic PBS for an additional 10 min. Cell volume increased in hyposmotic PBS, and then decreased below the initial value when cells were returned to isosmotic PBS. Data points are plotted as means with SE bars determined from results of three to six independent experiments. An asterisk indicates mean values found to be significantly different from that measured at 0 min in 290 mOsm conditions using Dunnett's test. Double asterisks indicate p38 activities that also are significantly different from the value measured at 30 min after returning the cells to isosmotic conditions for an additional 10 min (Student's *t* test).

brain cells, resulting in activation of intracellular signaling pathways that induce expression of some unusual genes. If gene expression is turned on by cell volume alterations, the long-term effects of the treatment and prognosis of brain edema will be altered. Our interest is to investigate characteristics of intracellular signaling pathways activated in response to anisosmotic exposure. One intracellular signaling pathway altered by response to osmotic stress and volume changes is the p38 MAP kinase pathway. Here we describe separate osmolality-dependent and cell volume-dependent p38 MAP kinase activation in primary rat astrocytes.

From our data, we conclude activation of p38 by hyperosmotic or hyposmotic exposure occurs by distinct mechanisms. p38 is activated by hyperosmotic exposure at constant ionic strength with resultant cell shrinkage. However, p38 also was activated during cell shrinkage in isoosmotic conditions after exposure to hyposmotic PBS or isosmotic PBS containing the cell permeant species, urea. Thus, reduced cell volume, rather than elevated extracellular osmolality, elevated ionic strength, or a rapid increase in extracellular osmolality is related to this enzymatic activity. Likewise, hyposmotic exposure with moderate cell swelling produced marked p38 activation within 10 min. Greater cellular swelling induced by a more severe hyposmotic challenge did not further elevate p38 activity. Furthermore, because p38 activity remained elevated for 30 min despite cell volume regulation, we conclude extracellular ionic strength or osmolality is a more important determinant of p38 activity than cell size during hyposmotic exposure.

Initial cell volume changes in response to osmotic stress reflect movement of water across the cell membrane and corresponding alterations in intracellular ionic strength. The cell volume changes we observed in anisosmotic conditions are consistent with previous studies on cultured rat brain astrocytes (13–15, 21, 25). The net shrinkage of cells returned to isosmotic conditions after hyposmotic exposure results from the net loss of potassium, chloride, amino acids, and potentially other osmolytes that occurs during the hyposmotic exposure (16, 17, 26). In addition to the volume regulation we observed between 10 and 30 min of hypos-

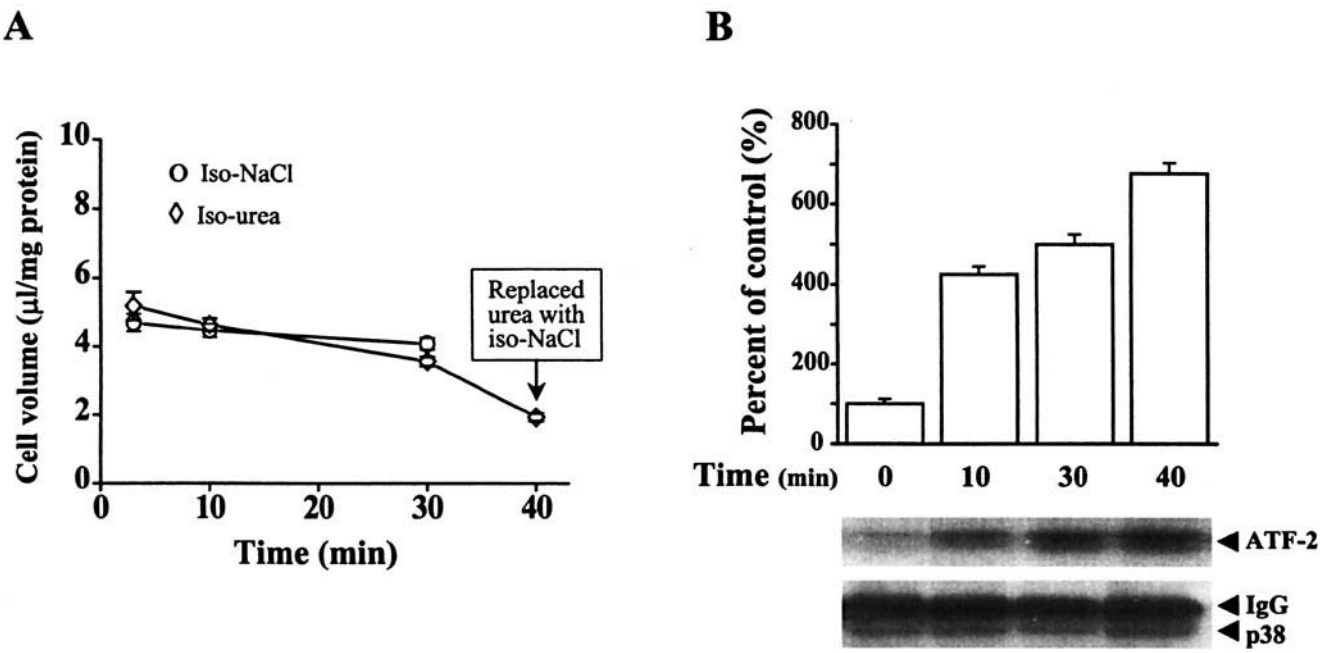

Figure 4. Effect of cell volume on p38 kinase activity in isosmotic urea-PBS. (A) p38 activity increased during a 30-min exposure to isosmotic urea-PBS yet increased further following a subsequent return to isosmotic PBS for 10 min. (B) Time course of cell volume change in isosmotic PBS and isosmotic urea-PBS. For cells exposed to isosmotic urea-PBS, the extracellular solution was replaced after 30 min with isosmotic PBS for an additional 10 min. An asterisk indicates mean values found to be significantly different from that measured at 0 min in 290 mOsm conditions using Dunnett's test. Double asterisks indicate values that also are significantly different from the value measured at 30 min after returning the cells to isosmotic conditions for an additional 10 min (Student's *t* test).

motic exposure, we anticipate some volume recovery occurred during the first 10 min of hyposmotic exposure (13, 27). Since cell shrinkage in isosmotic conditions occurs after recovery from prolonged hyposmotic exposure, we are able to distinguish effects of cell volume-dependent activation from effects of osmolality-dependent activation of this intracellular signaling pathway.

Activation of p38 MAP kinase has been reported in other cell types in response to hyperosmotic (1) and hyposmotic exposure (28, 29). However, the correlation of cell volume and p38 activity has not been well studied. The response to osmotic stress is believed to be mediated by transmembrane osmoreceptors in prokaryotes, plants, and yeast (30, 31). However, the osmosensor in mammalian systems has not been well defined. Recent evidence indicates hyperosmotic stress can activate receptors for growth factors and cytokines in mammalian cells (32, 33), suggesting a potential role as osmosensors of these receptors. In the present study, the degree of p38 activation corresponded to medium osmolality and reduced cell volume in hyperosmotic conditions in rat brain astrocytes. In contrast, both strong (57 mOsm) and weak (250 mOsm) hyposmotic conditions activated p38 equally. The activity of p38 was not diminished during subsequent volume regulation in hyposmotic PBS. Given the small cell volume change in 250 mOsm PBS (approximately 27%), these results suggest that the triggers for p38 activation in hyposmotic conditions have a low threshold and/or a high sensitivity.

We found p38 also can be activated by isosmotic cell shrinkage after recovery from hyposmotic exposure. Activation of p38 during cell shrinkage in isosmotic conditions suggests this enzyme is activated by reduced cell volume in hyperosmotic conditions rather than by exposure to the elevated osmolality or ionic strength *per se*. Similar activation of p38 by shrinking astrocytes in isosmotic PBS after an exposure to isosmotic PBS containing urea indicates a rapid increase in osmolality is not required for p38 activation. Comparing p38 kinase activity after cell swelling and cell shrinkage reveals asymmetrical effects of osmolality. Given the possible difference in mechanisms of p38 activation in hyperosmotic and hyposmotic conditions, the down-stream events of p38 also may be different in these conditions.

Swelling-induced activation of the Erk pathway has been reported to open anion channels that mediate osmolyte loss from cultured astrocytes (5). However, a larger increase in cell volume was needed to activate the kinases than was required to effect cell membrane anion conductance. It is unclear whether p38 activation in response to osmotic stress contributes to volume regulation through regulation of related gene expression in rat brain astrocytes. Although hyposmotic volume recovery was not addressed in the present study, the high sensitivity of p38 activity to increases in astrocyte volume suggest this pathway might mediate the adaptive response to cell swelling, including activation of an osmolyte efflux pathway. p38 MAP kinase could be involved in recovery from osmotic stress; however, it has been shown that p38 is not involved in activation of anion efflux in response to hyposmotic stress in intestine cells (28). Strong activation of p38 MAP kinase has been shown to induce apoptosis in many cell systems, including

PC-12 neuronal cells. If this mechanism also is present in rat brain astrocytes, p38 activation in response to clinical brain edema and treatment with osmotic agents may be a prognostic indicator in these conditions.

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