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Effect of osmotic stress on the expression of TRPV4 and BK_{Ca} channels and possible interaction with ERK1/2 and p38 in cultured equine chondrocytes

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Hdud IM, Mobasheri A, Loughna PT. Effect of osmotic stress on the expression of TRPV4 and BK_{Ca} channels and possible interaction with ERK1/2 and p38 in cultured equine chondrocytes. *Am J Physiol Cell Physiol* 306: C1050–C1057, 2014. First published March 26, 2014; doi:10.1152/ajpcell.00287.2013.—The metabolic activity of articular chondrocytes is influenced by osmotic alterations that occur in articular cartilage secondary to mechanical load. The mechanisms that sense and transduce mechanical signals from cell swelling and initiate volume regulation are poorly understood. The purpose of this study was to investigate how the expression of two putative osmolyte channels [transient receptor potential vanilloid 4 (TRPV4) and large-conductance Ca^{2+}-activated K^+ (BK_{Ca})] in chondrocytes is modulated in different osmotic conditions and to examine a potential role for MAPKs in this process. Isolated equine articular chondrocytes were subjected to anisosmotic conditions, and TRPV4 and BK_{Ca} channel expression and ERK1/2 and p38 MAPK protein phosphorylation were investigated using Western blotting. Results indicate that the TRPV4 channel contributes to the early stages of hypo-osmotic stress, while the BK_{Ca} channel is involved in responding to elevated intracellular Ca^{2+} and mediating regulatory volume decrease. ERK1/2 is phosphorylated by hypo-osmotic stress ($P < 0.001$), and p38 MAPK is phosphorylated by hyperosmotic stress ($P < 0.001$). In addition, this study demonstrates the importance of endogenous ERK1/2 phosphorylation in TRPV4 channel expression, where blocking ERK1/2 by a specific inhibitor (PD98059) prevented increased levels of the TRPV4 channel in cells exposed to hypo-osmotic stress and decreased TRPV4 channel expression to below control levels in iso-osmotic conditions ($P < 0.001$).

cartilage; chondrocyte; mitogen-activated protein kinase; osmotic; transient receptor potential vanilloid 4

ARTICULAR CARTILAGE covers the ends of bones in diarthrodial joints to provide protection from shearing and compressive forces generated secondary to joint articulation. Cartilage consists of extracellular matrix (ECM) and chondrocytes (3, 30). ECM is composed mainly of collagen type II and proteoglycan (PG), as well as other small protein and glycoprotein components. Chondrocytes are the only resident cells found in articular cartilage. Their metabolic activity is strongly influenced by environmental factors, including soluble mediators, ECM composition, and dynamic changes induced by mechanical loading (13, 46). Mechanical loading of articular cartilage induces fluid flow, mechanical membrane deformation, hydrostatic pressure, and osmotic stress (45).

The osmolarity of the tissue fluid that bathes chondrocytes in the cartilage ECM is different from that of most other tissues and typically exceeds 380 mosM (47). The presence of polyanionic PG molecules in the ECM attracts cations, such as Na^+, Ca^{2+}, and K^+, to neutralize the charge, which in turn increases cartilage osmotic pressure. An increase in interstitial osmolarity increases cartilage hydration (29). In addition, the osmotic pressure of the ECM is disturbed during physiological and pathological conditions. Osmolarity within cartilage has been reported to rise to 480 mosM under loading conditions (45). Osmotic pressure can also be altered during pathological conditions, where damage to the collagen network in the ECM permits PGs to attract water and increase tissue hydration (13).

Chondrocytes have been shown to initiate intracellular signaling cascades in response to acute volume change to prevent deleterious effects of osmotic alteration followed by regulatory volume pathways involving actin reorganization, as well as solute transport (9, 10, 22). Changes in extracellular osmolarity have been shown to elevate intracellular Ca^{2+} in human, bovine, and porcine articular chondrocytes (9, 55). This increase in intracellular Ca^{2+} could be initiated by extracellular influx and augmented by release from intracellular stores (9, 10). Recent studies suggest the transient receptor potential vanilloid (TRPV) 4 channel as a potential cellular osmosensor with possible involvement in mechanotransduction (17, 27, 54) and mediation of Ca^{2+} influx to regulate volume recovery following hypo-osmotic stress in porcine articular chondrocytes (33). The TRPV4 channel is a Ca^{2+}-permeable, nonselective cation channel (25, 26). Under physiological conditions, Ca^{2+} has priority in crossing the channel; however, in the absence of Ca^{2+}, the channel is permeable to Sr^{2+}, Ba^{2+}, and Mg^{2+} (37). The TRPV4 channel can be activated by hypotonicity, moderate heat (>27°C), 4α-phorbol 12,13-dideconate, and endogenous agonists such as arachidonic acid (14, 51, 53).

Cell swelling induced by exposure of cells to hypotonic stress is followed by initiation of a regulatory volume decrease (RVD) response to restore cell size. The process involves passive loss of Cl^- and K^+ via their corresponding channels and osmotically obligated water (4, 5). Investigations of several other cell types have shown that entry of extracellular Ca^{2+} and consequent activation of large-conductance Ca^{2+}-activated K^+ (BK_{Ca}) channels are essential for initiation of RVD. Expression of TRPV4 and BK_{Ca} channels varies be-

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tween tissues and cell types and was recently demonstrated in equine chondrocytes in vivo and in vitro (16). Expression of these channels at the mRNA and protein levels closely correlates with their activity (20, 49). Markedly altered levels of the functional proteins for these channels are associated with disease states (11, 32, 50). There is little information available, however, with regard to the regulation of expression of these channels in chondrocytes, in particular in response to environmental factors such as osmotic stimuli.

MAPKs have been implicated in chondrocyte biology (52). ERK1/2 and p38 pathways are activated by osmotic stress and induce activation of several subcellular signaling pathways (40, 44). Although the activity-related phosphorylation of ERK1/2 and p38 has been reported in response to osmotic stress, their role in chondrocyte volume regulation has not been elucidated.

In this study we examined the contribution of ERK1/2 and p38 MAPKs to the regulation of TRPV4 and BK_{Ca} channel expression in response to osmotic changes.

MATERIALS AND METHODS

Tissue Sources

Equine articular cartilage from load-bearing joints of the metacarpophalangeal joints of skeletally mature male and female animals (aged 9–22 yr) was obtained on the day of slaughter from a local abattoir (Nantwich, Cheshire, UK); these animals were euthanized for purposes other than research. All experiments were performed with local institutional ethical approval, in strict accordance with national guidelines.

Chondrocyte Isolation and Culture

Middle and superficial layers (but not full-depth) of equine articular cartilage were rinsed with PBS, and chondrocytes were isolated by overnight incubation with 0.1% type I collagenase from *Clostridium histolyticum* (Sigma-Aldrich, UK) in serum-free DMEM at 37°C. The filtered chondrocyte suspension was washed three times in PBS supplemented with 10% penicillin-streptomycin (Invitrogen, Paisley, UK), and the cells were cultivated in monolayer culture in DMEM supplemented with 10% FCS until ~80% confluent. All experiments were conducted on first-passage chondrocytes.

Induction of Osmotic Stress

Medium osmolarity was adjusted using a freezing-point osmometer (Advanced Micro Osmometer model 3300). Medium osmolarity of 380 mosM was used as the iso-osmotic point for chondrocytes (47). Hypo-osmotic medium (280 mosM) was prepared by addition of distilled water and hyperosmotic medium by addition of sucrose to the iso-osmotic medium (33, 38). Chondrocytes were seeded in six-well culture plates at 2×10^5 cells/well and maintained until 80% confluent. Before osmotic stress, the cells were adapted to serum-free medium by 1 h of exposure to iso-osmotic medium (380 mosM). Then the medium was changed to hypo-osmotic, mild hypo-osmotic, and hyperosmotic medium for 90 min, 3 h, and 6 h before chondrocytes were washed in ice using RIPA buffer (150 mM NaCl, 50 mM Tris·HCl, pH 7.5, 5 mM EGTA, 1% Triton, 0.5% sodium deoxycholate, and 0.1% SDS) supplemented with protease and phosphatase inhibitor cocktail (Roche Diagnostic, Mannheim, Germany). The whole cell protein lysate was collected, protein concentration was quantified using the Bradford assay, with BSA used as a standard (2), and the lysate was stored at −20°C until use. TRPV4 and BK_{Ca} channel expression and ERK1/2 and p38 MAPK phosphorylation were investigated. All cell culture was maintained at 37°C in 95% air-5% CO_2. Medium was changed every other day.

Pharmacological Inhibition Experiments

Influence of MAPK inhibitors on TRPV4 and BK_{Ca} channel expression during osmotic stress. Equine articular chondrocytes (EACs) were seeded at 2×10^5 cells/well and maintained until 80% confluent. Cells were adapted to serum-free medium for 1 h and subsequently incubated under the appropriate osmotic condition and supplemented with the specific pharmacological inhibitor. In p38 MAPK inhibitor experiments, chondrocytes were incubated with iso-osmotic or hyperosmotic medium in the presence or absence of the p38 inhibitor SB 203580 (10 μM; Invitrogen, UK) (34, 39); in ERK1/2 phosphorylation experiments, chondrocytes were incubated with iso-osmotic or hypo-osmotic medium in the presence or absence of the MEK1/2 inhibitor PD98059 (50 μM; Cell Signalling Technology, UK) for 90 min (34, 39). DMSO was added to the aqueous working medium without exceeding 0.1% (vol/vol) of DMSO in the medium. At the end of the incubation, chondrocytes were washed three times with sterile PBS, whole cell lysate was collected, and protein concentrations were quantified and used to investigate the influence of ERK1/2 and p38 inhibition on TRPV4 and BK_{Ca} channel expression.

Western Blotting

Total protein lysate was mixed with sample buffer (0.5 M Tris·HCl, pH 6.8, 100% glycerol, 20% SDS, 0.5% bromophenol blue, and 5% β-mercaptoethanol) and denatured at 90°C for 3 min. SDS-PAGE with 4–10% gels was used to separate 25 μg of whole cell lysate under denaturing conditions; then a semidry electroblotting apparatus (Bio-Rad, UK) was used to transfer the lysate to a polyvinylidene difluoride membrane (Invitrogen). The membranes were blocked in 5% (wt/vol) fat-free skimmed milk (Marvel) in TBS-0.1% Tween 20 for 1 h at room temperature and then probed with specific antibodies diluted in blocking reagent at 4°C overnight. After five washes in TBS-0.1% Tween 20, the membranes were incubated with goat anti-rabbit IgG conjugated with horseradish peroxidase (Dako, UK) secondary antibody for 1 h at room temperature. Finally, membranes were washed five times for 5 min each in TBS-0.1% Tween 20 and then developed using the Amersham ECL Western blot enhanced chemiluminescence kit (GE Healthcare, UK) and visualized by exposure to X-ray films (Fisher Scientific, UK).

Statistical Analysis

Values are means ± SE. Each experiment was performed in triplicate; relative expression represents the mean of a combination of three experiments. Differences between animals were analyzed utilizing Student's *t*-test. Statistical analysis was performed with ANOVA followed by Bonferroni's test. $P \leq 0.05$ was considered statistically significant.

RESULTS

Effect of Osmotic Stress on Expression of Ion Channels

BK_{Ca} channel. The expression level of the BK_{Ca} channel in EACs following exposure to hypo-osmotic, mild hypo-osmotic, and hyperosmotic stresses was monitored at different time points. Western blotting using a BK_{Ca} channel-specific antibody was used to examine the effect of osmotic stress on BK_{Ca} channel expression, as previously described (16). There were no significant changes in BK_{Ca} channel expression following hypo-osmotic and mild hypo-osmotic stress after 90 min and 3 h, whereas 6 h of incubation under hypo-osmotic conditions induced a significant (1.5-fold) increase in BK_{Ca} channel expression ($P < 0.01$; Fig. 1). In contrast, BK_{Ca} channel expression was significantly lower at the early stages (90 min) of hyperosmotic stress than during iso-osmotic stress

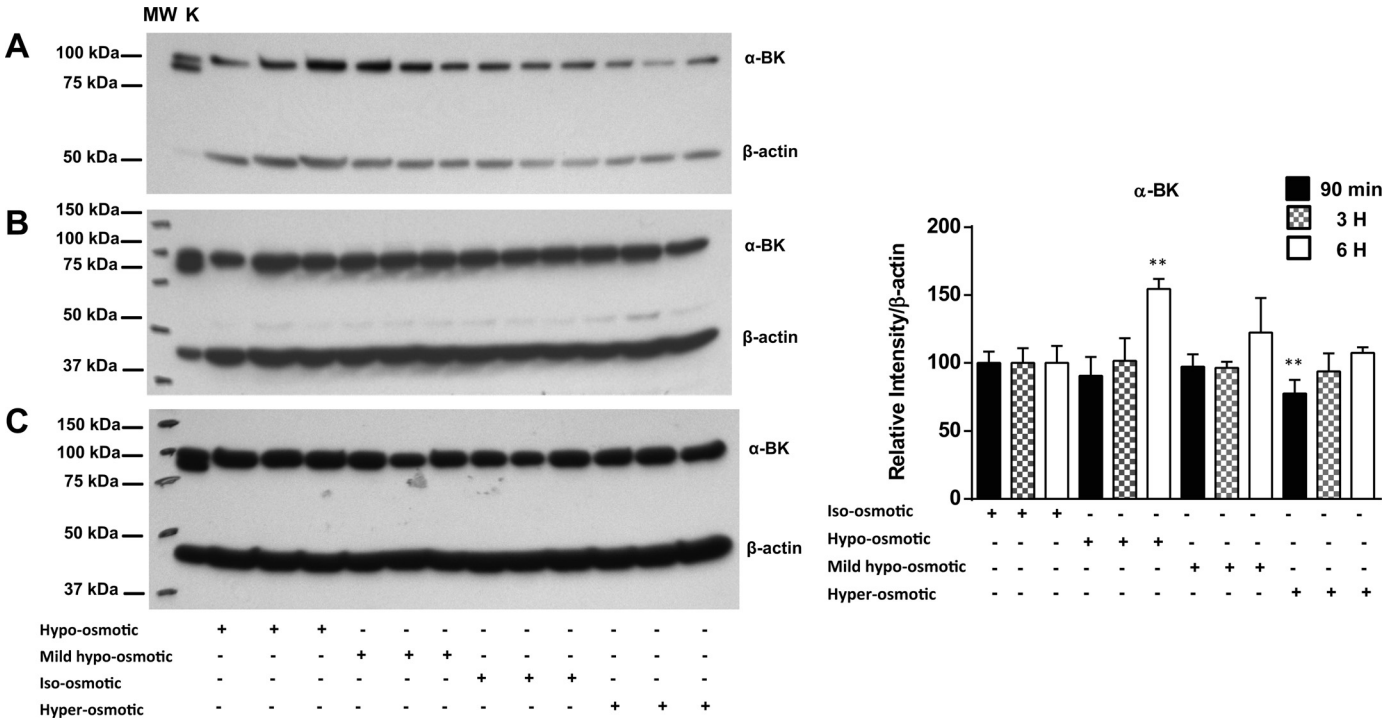


Fig. 1. Influence of hypo-osmotic (280 mosM), mild hypo-osmotic (320 mosM), iso-osmotic (380 mosM), and hyperosmotic (480 mosM) conditions on large-conductance Ca^{2+}-activated K^+ (α-BK_{Ca}) channel expression at 90 min (*A*), 3 h (*B*), and 6 h (*C*) of incubation. K, kidney. Expression relative to β-actin was determined by densitometric analysis of the Western blot. Values are means ± SE. $^{**}P < 0.01$ vs. iso-osmotic control.

($P < 0.01$). Extending the exposure to hyperosmotic stress for 3 and 6 h returned channel expression to the original level.

TRPV4 channel. A medium osmolarity of 380 mosM was used as the control condition. Western blotting using a TRPV4 channel-specific antibody, as described previously (16), was used to explore TRPV4 channel expression following hypo-osmotic, mild hypo-osmotic, and hyperosmotic stress. Exposure of chondrocytes to hypo-osmotic stress for 6 h ($P < 0.01$), 3 h ($P < 0.001$), and 90 min ($P < 0.001$) increased TRPV4 channel expression by >1.5-fold (Fig. 2). A mild hypo-osmotic environment induced an increase in TRPV4 channel expression at 90 min ($P < 0.001$) and 3 h ($P < 0.01$), but expression returned to control levels after 6 h (Fig. 2). In EACs exposed to hyperosmotic stress, TRPV4 channel expression was reduced by ~50% after 3 h ($P < 0.05$) and 90 min ($P < 0.01$) but returned to control levels by 6 h (Fig. 2).

Influence of Osmotic Stress on MAPK Phosphorylation

The influence of osmotic stress on activity-related phosphorylation of ERK1/2 and p38 MAPKs in chondrocytes was investigated at the protein level, as previously described (1, 39). Specific antibodies for the phosphorylated form of ERK1/2 and p38 MAPKs were used in Western blot experiments to investigate the phosphorylation of ERK1/2 and p38 MAPKs following exposure of EACs to osmotic stress for 90 min and 3 h. ERK phosphorylation was significantly (>2-fold) increased in response to hypo-osmotic stress at 90 min ($P < 0.001$) and was reduced at 3 h (Fig. 3). A significant decrease (~50%) was induced by exposure of chondrocytes to mild hypo-osmotic and hyperosmotic stress at 90 min and 3 h; however, the greatest decrease was observed in response to hyperosmotic stress at 90 min ($P < 0.001$).

In contrast, p38 MAPK phosphorylation was significantly increased by exposure of EACs to hyperosmotic stress for 90 min and 3 h. Phosphorylation was significantly increased (~7-fold) at 90 min ($P < 0.001$) but was reduced to ~1.5-fold at 3 h ($P < 0.001$; Fig. 4). No significant changes in phosphorylation were observed in chondrocytes exposed to hypo-osmotic stress at 90 min and 3 h. Phosphorylation of p38 MAPK was not changed by 90 min of mild hypo-osmotic stress, whereas it was downregulated at 3 h ($P < 0.001$).

Inhibition of ERK and p38 Activity During Osmotic Loading

ERK1/2- and p38 MAPK-specific pharmacological inhibitors were used to examine the influence of these MAPKs on TRPV4 and BK_{Ca} channel expression in EACs. Inhibition of ERK1/2 phosphorylation (by the MEK1/2 inhibitor PD98059) at iso-osmotic conditions for 90 min significantly decreased TRPV4 channel expression to below the endogenous levels ($P < 0.001$; Fig. 5). Moreover, inhibition of ERK1/2 phosphorylation under hypo-osmotic stress for 90 min significantly inhibited the elevation of TRPV4 channel expression induced by hypo-osmotic stress ($P < 0.001$; Fig. 5*B*). In contrast, BK_{Ca} channel expression was significantly elevated by inhibition of ERK1/2 phosphorylation under hypo-osmotic ($P < 0.001$) and iso-osmotic ($P < 0.01$) stress (Fig. 5*A*). The impact of p38 MAPK phosphorylation on TRPV4 and BK_{Ca} channel expression was investigated under hyperosmotic stress for 90 min. Inhibition of p38 MAPK (by the p38 inhibitor SB 203580) significantly elevated BK_{Ca} channel expression ($P < 0.001$) but did not influence TRPV4 channel expression (Fig. 6).

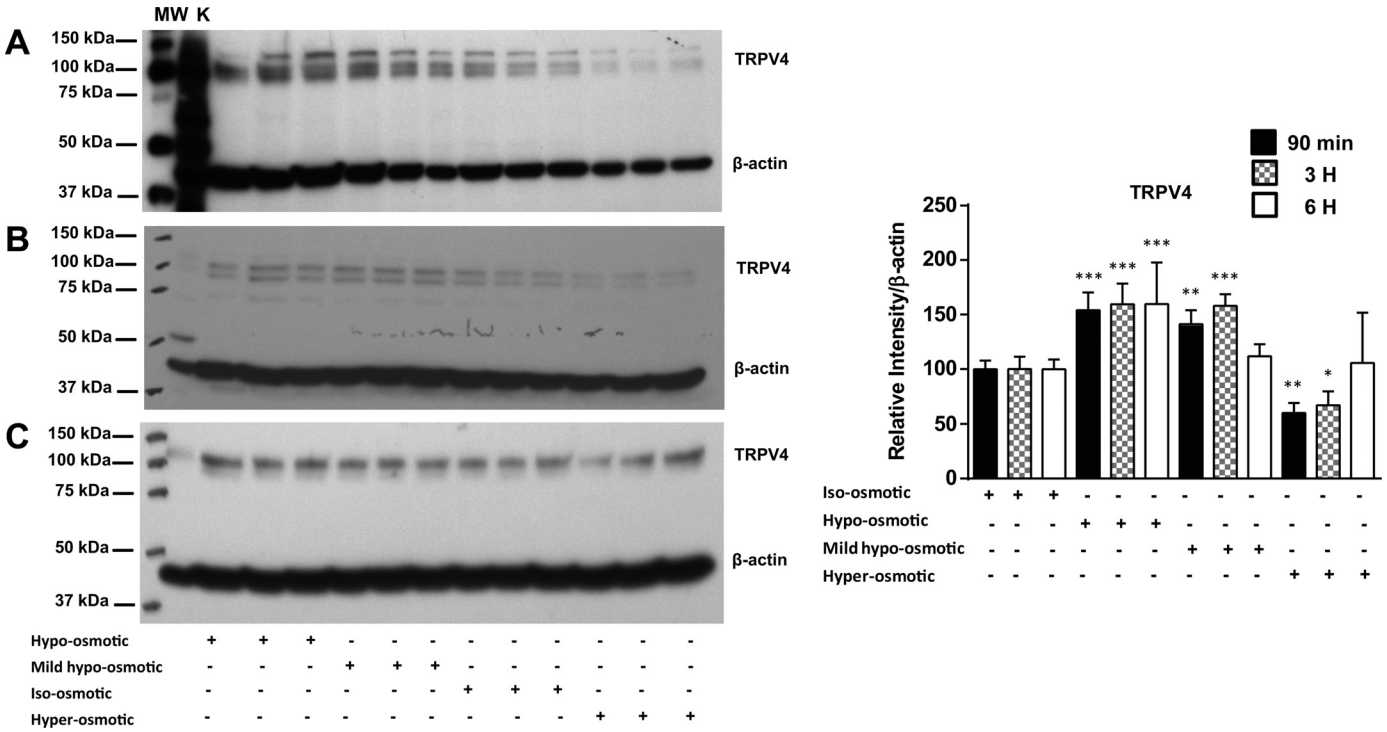


Fig. 2. Influence of hypo-osmotic (280 mosM), mild hypo-osmotic (320 mosM), iso-osmotic (380 mosM), and hyperosmotic (480 mosM) conditions on transient receptor potential vanilloid 4 (TRPV4) channel expression at 90 min (*A*), 3 h (*B*), and 6 h (*C*) of incubation. Expression relative to β-actin was determined by densitometric analysis of the Western blot. Values are means ± SE. $*P < 0.5$, $**P < 0.01$, $***P < 0.001$ vs. iso-osmotic control.

DISCUSSION

It has been shown in a number of cell types that the TRPV4 and BK_{Ca} ion channels play a role in the regulation of cell volume in altered osmotic environments. It is undoubtedly the case that changes in not only osmotic, but also mechanical and thermal, environments can lead to rapid and probably fluctuating changes in the activity of these channels. It is, however, reasonable to suggest that the overall capacity of these channels is dictated, at least in part, by their level of expression. Furthermore, the level of expression of these channels has been shown to differ in pathological cartilage, although whether this is causative or a result of the disease is unclear (24). In either case, altered expression could lead to progression of the disease and increased degeneration of the cartilage.

This study suggests that when chondrocytes are exposed to decreased osmolarity, TRPV4 channel protein expression increases rapidly (up to 6 h), whereas BK_{Ca} channel expression also increases, but only after 6 h. In contrast, increased osmolarity initially decreased expression of both channels, but expression levels were restored to the endogenous levels after 90 min for the BK_{Ca} channel and after 3 h for the TRPV4 channel. Regulation of cell volume following hypotonic swell-

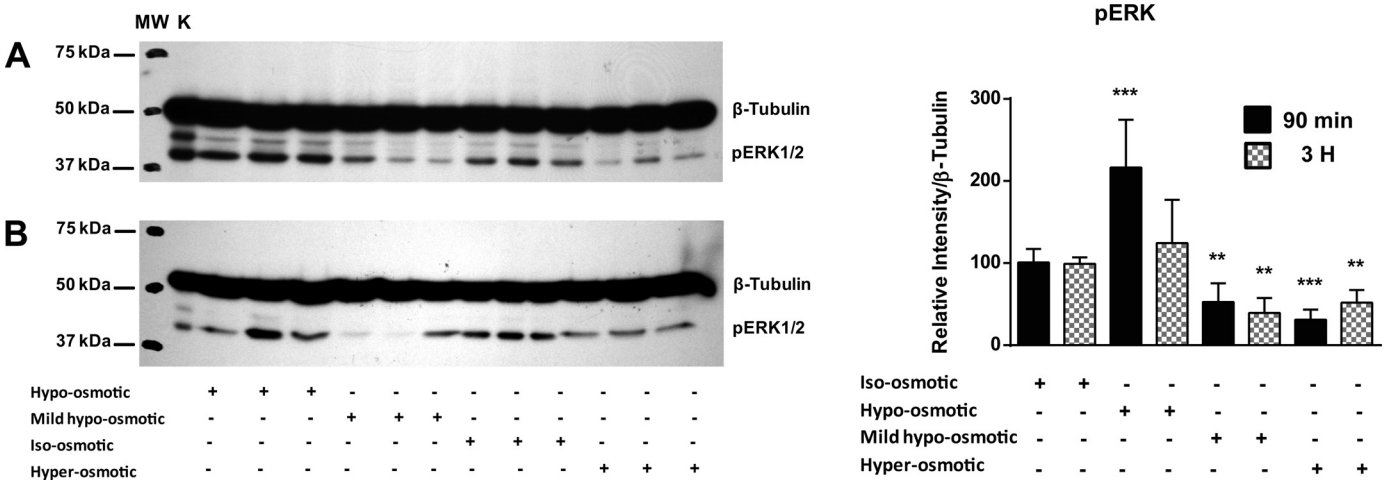


Fig. 3. Influence of hypo-osmotic (280 mosM), mild hypo-osmotic (320 mosM), iso-osmotic (380 mosM), and hyperosmotic (480 mosM) conditions on ERK1/2 phosphorylation at 90 min (*A*) and 3 h (*B*) of incubation. Expression relative to β-tubulin was determined by densitometric analysis of the Western blot. Values are means ± SE. $**P < 0.01$, $***P < 0.001$ vs. iso-osmotic control.

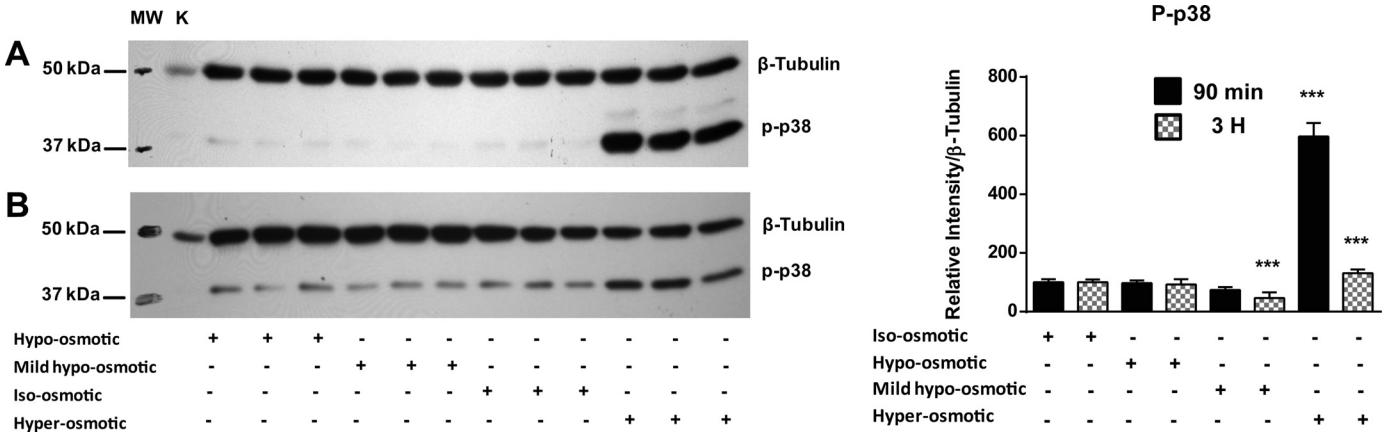
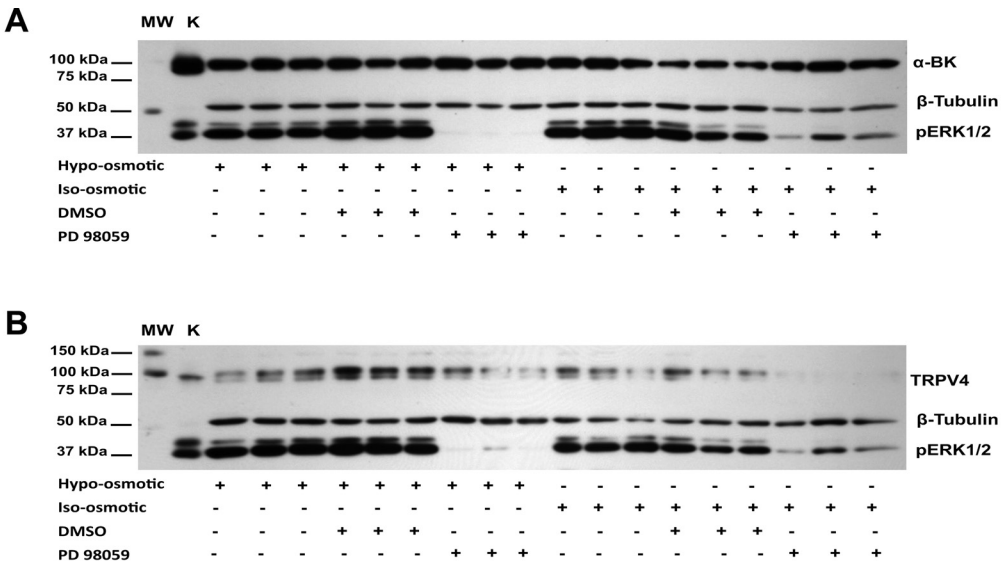


Fig. 4. Influence of hypo-osmotic (280 mosM), mild hypo-osmotic (320 mosM), iso-osmotic (380 mosM), and hyperosmotic (480 mosM) conditions on p38 MAPK phosphorylation at 90 min (*A*) and 3 h (*B*) of incubation. Expression relative to β-tubulin was determined by densitometric analysis of the Western blot. Values are means ± SE. ***$P < 0.001$ vs. iso-osmotic control.

ing is classically mediated by release of Cl^- and K^+ through activation of coordinated channels (18). In the majority of cell types, including chondrocytes, generation of an intracellular Ca^{2+} signal in response to hypotonic stress is followed by the RVD response, which allows cells to survive (18, 21, 55). This signal is initiated via Ca^{2+} entry from the extracellular space and augmented by Ca^{2+} release from intracellular stores (3). Elevation of intracellular Ca^{2+} induced by cell swelling acti-



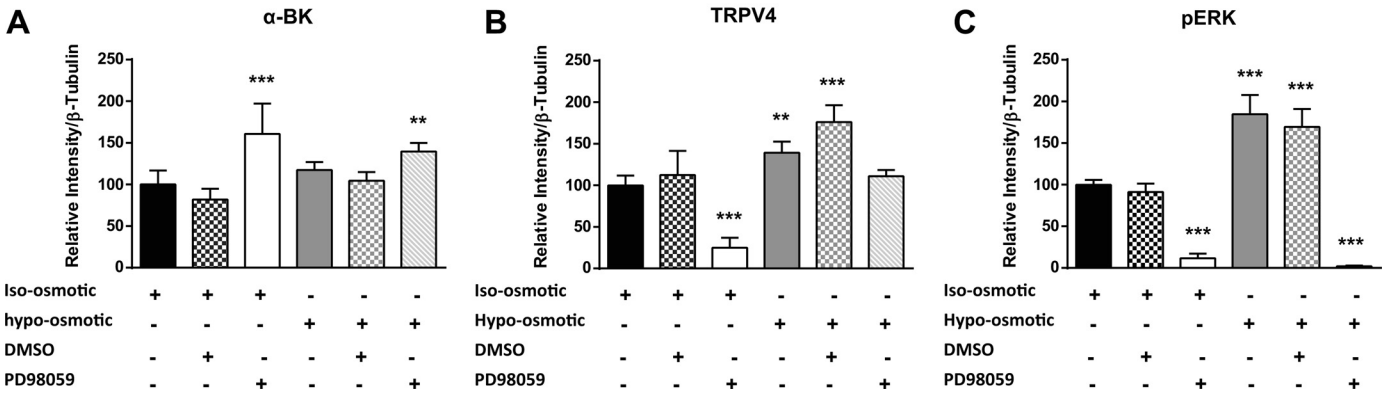


Fig. 5. Inhibitor sensitivity of ERK1/2 osmolarity-dependent activity. Western blot shows effect of hypo-osmotic (280 mosM) and iso-osmotic (380 mosM) conditions on ERK1/2 phosphorylation (pERK1/2), TRPV4 channel expression, and α-BK_{Ca} channel expression following 90 min of incubation in the absence (control) and presence of the pERK inhibitor PD98059 and vehicle (DMSO). Values are means ± SE. **$P < 0.01$, ***$P < 0.001$ vs. iso-osmotic control.

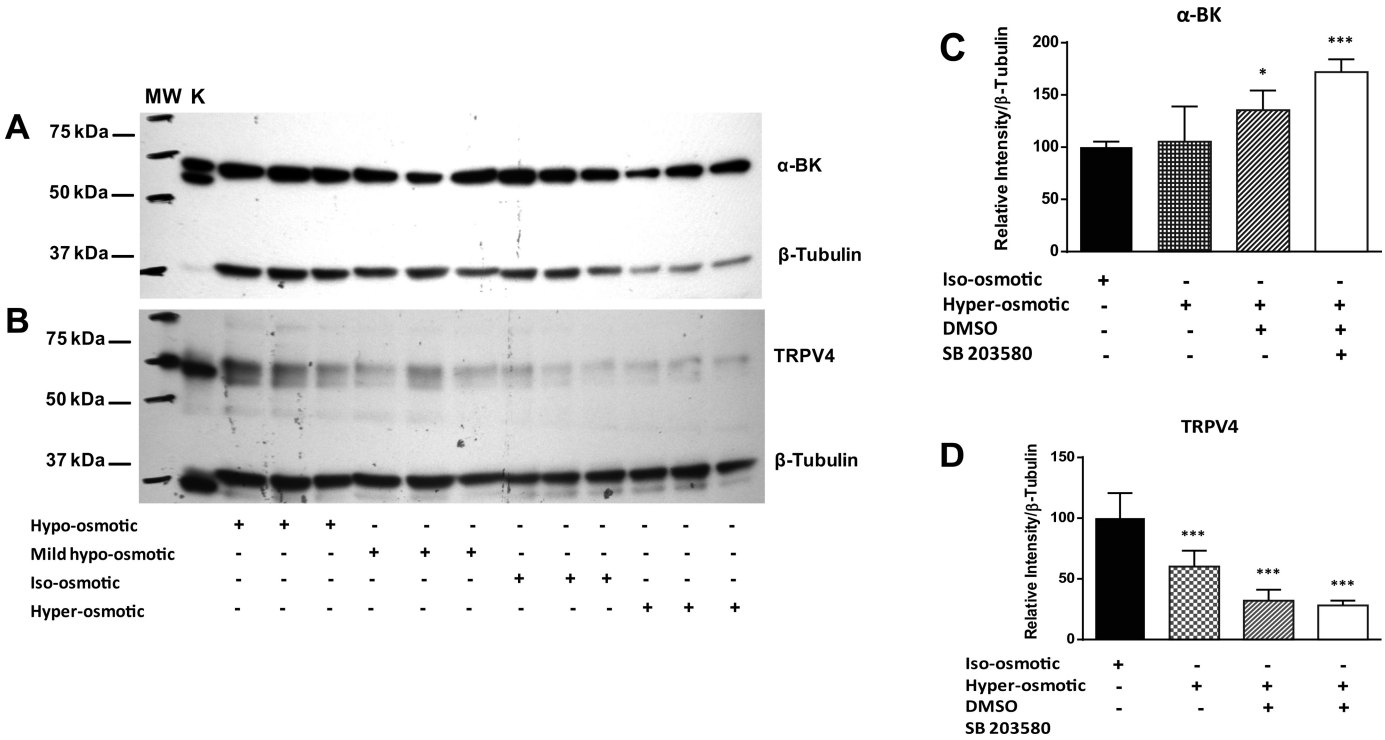


Fig. 6. Inhibitor sensitivity of p38 MAPK osmolarity-dependent activity. Western blot shows effect of the hyperosmotic (480 mosM) condition on TRPV4 and α-BK_{Ca} channel expression following 90 min of incubation in the absence (control) and presence of the p38 inhibitor SB 203580 and vehicle (DMSO). Values are means ± SE. *$P < 0.5$, ***$P < 0.001$ vs. iso-osmotic control.

vates Ca^{2+}-activated K^+ channels. Recently, the TRPV4 channel was identified as an osmosensor channel that mediates Ca^{2+} entry following cell swelling in response to hypo-osmotic challenge. The TRPV4 channel may interact with aquaporins to elicit the RVD response to facilitate rapid movement of water during hypotonic challenge (28). The current study showed an increase in TRPV4 channel expression at the protein level following hypo-osmotic challenge. This finding was in agreement with other reports in bronchial endothelial cells (12) and porcine articular chondrocytes (33). Several hypotheses have been proposed to implicate the BK_{Ca} channel in cell volume regulation. The BK_{Ca} channel may act as an osmolyte channel (15, 21), where elevation of intracellular Ca^{2+} induced by TRPV4 channel activation is sensed by the Ca^{2+} sensor in the BK_{Ca} channel, leading to its activation and release of K^+, subsequent decrease in intracellular osmotic potential, and cell volume regulation. The alternative hypothesis suggests that BK_{Ca} channel activation occurs by sensing membrane stretch, induced by cell swelling or interaction with other mechanoreceptors (31). Differentiating between these two hypotheses is rather difficult, as cell swelling is associated with membrane stretch. Previous studies reported a coupling between TRPV4 and BK_{Ca} channels in the vascular smooth muscle response to vasodilatory factors through the ryanodine receptor (RYR) (7), whereas in bronchial endothelial cells the RYR is not involved in the direct coupling between the two channels in response to hypotonic stress (12).

During the course of osmotic challenge in the current study, TRPV4 channel expression increased to allow Ca^{2+} entry at the early phases of challenge followed by increased BK_{Ca} channel expression to mediate K^+ efflux and facilitate volume regulation. As involvement of the RYR is not part of this study, the coupling between the two channels with or without involvement of the RYR in chondrocytes is possible.

The current study also showed that changes in cell volume induce MAPK cascades, leading to changes in phosphorylation of ERK1/2 and p38. Hypotonicity induced ERK1/2 phosphorylation, whereas hypertonicity provoked p38 phosphorylation during early phases of exposure. Previous studies showed changes in ERK1/2 phosphorylation during osmotic stress [i.e., increased phosphorylation in rat nucleus pulposus cells following increased osmolarity (44)]. In contrast, phosphorylation of ERK1/2 was increased by hypo-osmotic stress in intestinal 407 cells (48), astrocytes (6), and hepatoma cells (35). Taken together, ERK1/2 phosphorylation following osmotic stress seems to be cell-specific. The role of the ERK1/2 pathway in RVD has not been delineated; however, indirect activation of ERK1/2 via the Ras-Raf-MEK pathway has been suggested in hepatocytes (8). Other studies have linked activation of ERK1/2 to activation of the Cl^- channel in corneal epithelial cells (19) and astrocytes (6) and to activation of the K^+ channel in cervical cancer cells (36). Although Cl^- and K^+ play an important role in volume regulation following cell swelling in response to hypo-osmotic stress and elevation of intracellular Ca^{2+} (18), the current study suggests a link between ERK1/2 phosphorylation and TRPV4 channel expression, where ERK1/2 phosphorylation regulates endogenous TRPV4 channel expression.

Phosphorylation of p38 MAPK following hyperosmotic stress has been shown in several cell types, such as fibroblasts (23), human cervical cells (36), and human articular chondrocytes (41, 42). In agreement with these studies, we have shown

an elevation of p38 MAPK phosphorylation following hyperosmotic stress at early phases of the exposure. Therefore, activation of p38 could be implicated in the regulatory volume increase response to restore cell volume following hyperosmotic stress. Activation of p38 was strongly associated with upregulation of aggrecan gene expression (52). This was sustained by linking p38 pathway activation to elevation of tonicity-responsive enhanced binding protein, which in turn activates target genes such as aggrecan (43). Blocking phosphorylation of p38 MAPK did not change TRPV4 channel expression, whereas BK_{Ca} channel expression was upregulated.

In summary, we have shown that TRPV4 and BK_{Ca} channel expression in chondrocytes is sensitive to an altered osmotic environment. Furthermore, we have shown that some of these changes may involve activation of ERK and p38. The precise mechanism by which these signaling factors are involved in regulation of this expression is unclear, but further exploration is warranted to understand their role in normal chondrocyte function in healthy cartilage and their potential role in initiation and progression of pathological conditions such as osteoarthritis.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

I.M.H., A.M., and P.T.L. are responsible for conception and design of the research; I.M.H. performed the experiments; I.M.H. and P.T.L. analyzed the data; I.M.H. and P.T.L. interpreted the results of the experiments; I.M.H. prepared the figures; I.M.H. and P.T.L. drafted the manuscript; I.M.H., A.M., and P.T.L. edited and revised the manuscript; I.M.H. and P.T.L. approved the final version of the manuscript.

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