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HIGH HYDROSTATIC PRESSURE INDUCES ERK AND PI3 KINASE PHOSPHORYLATION IN HUMAN HCS-2/8 CHONDROSARCOMA CELLS

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Abstract - High continuous hydrostatic pressure has been shown to affect many cellular functions within the pressurised cells, for instance, accumulation of heat shock protein 70 occurs during pressurisation. Various signal transduction pathways are likely to mediate these changes, however, at the present time our knowledge of the pathways involved is rather limited. The aim of this study was to investigate whether some of the well known transduction pathways are activated by the exposure of human chondrosarcoma cells to 15-30 MPa hydrostatic pressure. The results showed an increased presence of the active, phosphorylated forms of extracellular signal-related kinase (ERK) and phosphoinositide 3-kinase (PI3K) in cells exposed to 15 and 30 MPa continuous hydrostatic pressure, while 0.5 Hz cyclic loading had weaker effects. Inhibition of ERK-pathway with UO126 did not prevent the accumulation of heat shock protein 70. No activation of c-Jun N-terminal protein kinase (JNK) or p38 could be noticed in pressurised cells. In conclusion, we could identify at least two different signal transduction pathways that are activated under high continuous hydrostatic pressure. Accumulation of heat shock protein 70 was independent of ERK-activation.

Key words: High hydrostatic pressure, signal transduction, chondrosarcoma, heat shock protein 70

INTRODUCTION

Mechanical loading is a well known modifier of cellular metabolism, and a number of signal transduction pathways are activated to transmit the information of extracellularly generated forces (19). Although practically all the cells within our body experience some level of loading, it is the cells within bone, cartilage and intervertebral discs that in particular have to withstand transient high forces (37). In cartilage, the mechanical forces are present in various forms, such as compression, hydrostatic pressure, fluid flow and streaming potentials (17,28). Low and moderate hydrostatic pressure appears to increase the production of extracellular matrix (9,24,29,34,38). Hydrostatic pressure at 5 MPa level changed the expression level of numerous genes in human chondrosarcoma cells, and differential gene expression profiles were observed between continuous and intermittent hydrostatic loading (16). High hydrostatic pressure has many effects on living cells (25,32,33), and in chondrocytes it is known to inhibit cellular protein and proteoglycan synthesis (9,18,29).

Accumulation of heat shock protein 70 (Hsp70) occurs in cell cultures exposed to high hydrostatic pressure (12,35). The mechanism behind the accumulation is rather specific, as no activation of the hsp70 gene could be observed (12). A longer half-live of hsp70 in pressurised cells is the explanation for increased Hsp70 biosynthesis (12). The phenomenon is rather general, and occurs in most of the cell types so far examined (13-15). Primary chondrocytes (14) and Neuro-2a cells (15) were the only cell types that did not show this stress response.

Hydrostatic pressure obviously mediates its actions on the cells via several possible signal transduction pathways. However, our present knowledge of the mechanotransduction mechanisms activated by hydrostatic pressure is limited. Therefore, in this study we have investigated whether some of the best known signaling cascades are activated in the human chondrosarcoma cells exposed to high hydrostatic pressure.

Abbreviations: **Erk**: extracellular signal-related kinase; **Hsp70**: heat shock protein 70; **JNK**: c-Jun N-terminal protein kinase; **p38**: p38 mitogen-activated protein kinase; **PI3K**: phosphoinositide 3-kinase

MATERIALS AND METHODS

Antibodies

The antibodies recognizing the phosphorylated forms of extracellular signal-related kinase (p-ERK, Zymed, San Francisco, CA, USA), p38 mitogen-activated protein kinase (p-p38, Promega, Madison, WI), c-Jun N-terminal protein kinase (p-JNK, Santa Cruz Biotechnology, San Francisco, CA, USA), phosphoinositide 3-kinase (p-PI3K, Santa Cruz Biotechnology) and phospho-serine (p-Ser, Zymed) were used. The antibody recognizing Hsp70 was from StressGen Biotechnologies (Victoria, BC, Canada). The secondary antibodies were from Santa Cruz Biotechnology.

Cell culture

Human chondrosarcoma HCS-2/8 cells (36) were cultured in a humidified 5% CO_2/95% air atmosphere at 37°C in DMEM (Gibco, Paisley, UK) supplemented with 10% fetal calf serum, penicillin (50 units/ml), streptomycin sulphate (50 units/ml) and 2 mM glutamine (all from PAA, Linz, Austria).

Hydrostatic pressure treatment

Before exposure to hydrostatic pressure, the medium was changed and 15 mM HEPES (pH 7.3, Gibco) was added. Medium was supplemented with 2 mM glutamine, 10% fetal calf serum and penicillin/streptomycin. The culture dishes were filled with the medium described above and sealed with a covering plastic membrane, as previously described in detail (29). Continuous 30 MPa hydrostatic pressure for up to 8 hr was used for the experiments.

Western blotting

After pressurisation, the cells were detached from the culture plate and centrifuged for 5 min at 1,000 g, and the cell pellets were stored at –70°C until used for further analysis. The samples were then resuspended in RIPA buffer (1 x PBS, 1% Nonidet P 40, 0.5% sodium deoxycholate, 0.1% SDS, 0.1 mg/ml PMSF, 30 ml/ml aprotinin, 1 mM sodium orthovanadate). The protein concentration of the samples were measured using Bradford´s assay (1). Cellular samples (15 or 30 µg of protein) were mixed with the electrophoresis sample buffer, boiled for 5 min, and the proteins were separated in 10% sodium dodecyl sulphate polyacrylamide gel. The proteins were then transferred onto nitrocellulose membrane (Protran, Schleicher & Schuell, Dassel, Germany). Ponceau S solution (Sigma-Aldrich, St. Louis, MO) was used to check for the presence of equal amounts of transferred proteins. The blotted membranes were placed in blocking buffer (Tris-buffered saline, pH 8.0, supplemented with 0.1% Tween 20 and 1% non-fat dry milk for p-JNK, p-p38 and p-Ser; and Tris-buffered saline, pH 8.0, supplemented with 0.3% Tween 20 and 5% non-fat dry milk for p-PI3K, Hsp70 and p-ERK) for 1 hr. Overnight incubation with primary antibodies diluted with Tris-buffered saline, pH 8.0, supplemented with 0.1% Tween 20 and 1% non-fat dry milk (0.3% Tween for Hsp70 and p-ERK) was then performed. Following dilutions were used: p-ERK 1:1000, p-p38 1:625, p-JNK 1:200, p-PI3K 1:250, p-Ser 1:1000 and Hsp70 1:10000. Erk-inhibitor UO126 (10 µM, Calbiochem, San Diego, CA, USA) was used to block the phosphorylation of Erk-1/2 during some of the pressure treatments. Secondary antibodies were diluted 1:1000 (p-Ser), 1:5000 (p-p38, p-JNK and p-PI3K) in Tris-buffered saline (pH 8.0) containing 0.1% Tween 20 and 1% non-fat dry milk, or 1:10000 (Hsp70 and p-ERK) in Tris-buffered saline (pH 8.0) containing 0.3% Tween 20 and 3% non-fat dry milk. The membranes were incubated for 1 hr. The membranes were developed with the Supersignal West Pico Chemiluminescent Substrate detection kit (Pierce, Rockford, IL, USA).

RESULTS

Many of the responses initiated by application of high continuous hydrostatic pressure on cell cultures require several hours to take place. However, because the signaling events within the cells normally occur within minutes or few hours, we focused mainly on the first 6 hr after the onset of hydrostatic loading.

A gradual increase in the content of p-ERK (5.3-fold increase at 6 hr) was observed in human chondrosarcoma cells pressurized for 6 hr with continuous 30 MPa hydrostatic pressure (Fig. 1A), and a slightly weaker response (2.7-fold increase) was observed for p-PI3K (Fig. 1A). Two other common signaling kinases, JNK and p38, were not activated as shown by the lack of increased content of their phosphorylated forms (Fig. 1A). Continuous 15 MPa elicited a weaker response in p-ERK (at maximum 1.7-fold increase), while the content of p-PI3K reached at maximum a 8-fold increase compared with the control level (Fig. 1B). The response in p-PI3K occurred rapidly after the onset of pressure and started to fall already after 1 h of the pressure treatment. Cyclic 0.5

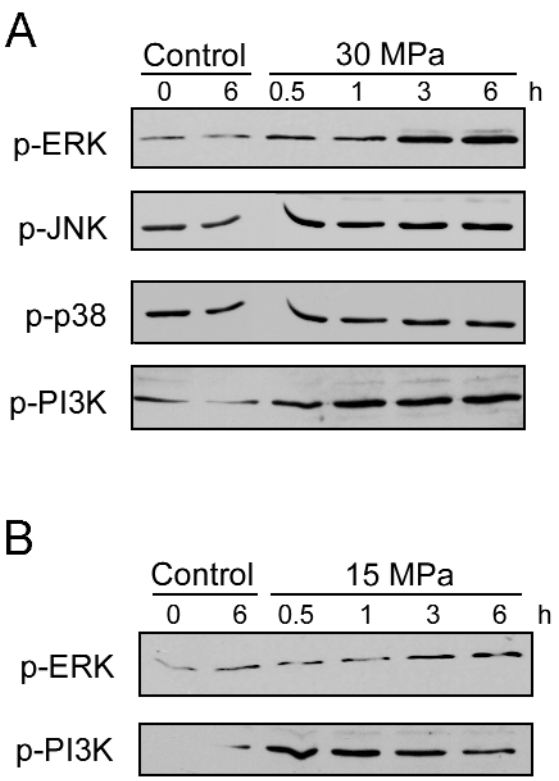

Fig. 1 Analysis of phosphorylated forms of ERK, JNK, p38 and PI3K in chondrosarcoma cells exposed to 30 (**A**) and 15 (**B**) MPa continuous hydrostatic pressure. p-ERK and p-PI3K were increased during the both pressure treatments.

Hz loading at 30 MPa increased also slightly the level of p-ERK (1.4-fold increase), with no changes observed in p-JNK or p-p38 (Fig. 2).

Heat shock protein 70 is known to accumulate in several cell lines when exposed to continuous 30 MPa hydrostatic pressure. This response obviously involves cellular signaling, therefore, we investigated whether inhibition of ERK-pathway would prevent the accumulation of Hsp70 in the pressurised cells. A clear accumulation of Hsp70 (1.8-fold increase) could be observed in HCS-2/8 chondrosarcoma cells within 6 hr of the application of hydrostatic pressure (Fig. 3A). The phosphorylation of ERK reached the maximum within 3 hr of pressurisation, remaining at approximately the same level at 6 hr (Fig. 3A). Inhibition of ERK phosphorylation by UO126 blocked the phosphorylation of ERK, however, accumulation of Hsp70 could be observed even in the presence of UO126 (Fig. 3B). Therefore, the accumulation of Hsp70 under continuous 30 MPa hydrostatic appears to be independent of ERK activation.

We also screened for the possible other changes in the contents of serine-phosphorylated proteins in the samples exposed to continuous 30 MPa hydrostatic pressure. The sensitivity of the immunoblot revealed approximately ten bands of different sizes. Four major bands were detected, of which 80-85 and 70-75 kDa bands were down-regulated (Fig. 4). The other bands were rather faint, and no consistent changes could be observed. This result shows that in addition to increased kinase activity, some specific phosphatase activity could also be triggered by the presence of high hydrostatic pressure.

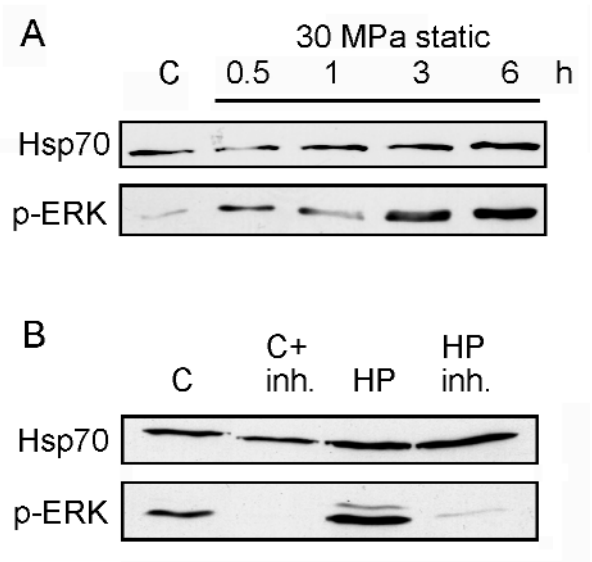

Fig. 3 Hsp70 and p-ERK levels in control cell cultures and those exposed to 30 MPa continuous hydrostatic pressure for up to 6 hr (**A**). Simultaneous increase in Hsp70 and p-ERK could be noticed. To test the involvement of ERK phosphorylation in the accumulation of Hsp70 during hydrostatic loading, the phosphorylation was inhibited by addition of UO126 (inh.) in control cell and those exposed to 30 MPa continuous hydrostatic pressure (HP) for 8 hr (**B**). Inhibitor blocked the phosphorylation of ERK almost completely, however, its peresence did not prevent the accumulation of Hsp70 in the pressurised cells.

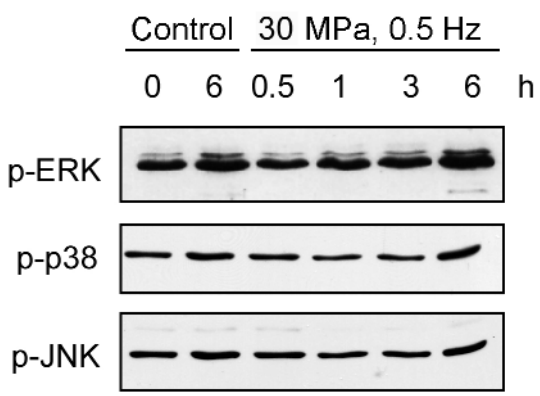

Fig. 2 Analysis of phosphorylated forms of ERK, p38 and JNK in chondrosarcoma cells pressurised at 30 MPa intermittent (0.5 Hz) hydrostatic pressure. p-ERK was slightly increased during intermittent loading.

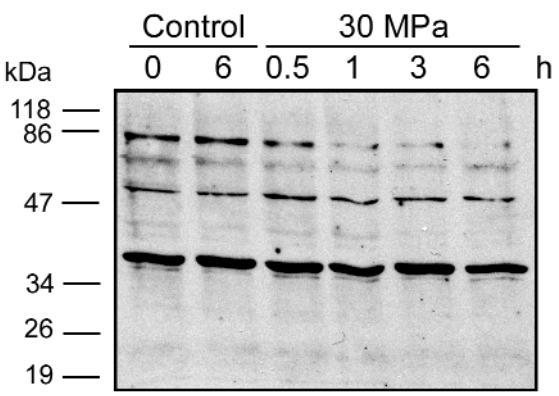

Fig. 4 Changes in proteins phosphorylated at serine residues. Approximately ten phosphorylated proteins were detected, of which 80-85 and 70-75 kDa proteins had decreased content of phosphorylation in cells exposed to 30 MPa hydrostatic pressure.

DISCUSSION

Various mechanical forces are known to regulate the cellular functions of chondrocytes (8). One of the best characterised mechanotransduction mechanisms in chondrocytes involves signaling during cyclic pressure-induced strain. Studies with human chondrocytes have revealed that integrins α_5 and β_1 can function as a mechanoreceptor (40). In another report, this integrin combination was noticed to promote chondrocyte survival in chondrocytes cultured on fibronectin-coated culture plates (30). When this receptor is stimulated, a signal cascade is activated that appears to involve stretch-activated ion channels (39), the cellular actin cytoskeleton (41), phosphorylation of β-catenin and focal adhesion proteins (21), and secretion of interleukin-4 (27). Osteoarthitic chondrocytes respond differently compared with normal chondrocytes (31). A number of reports suggest that nitric oxide also plays a role in response of articular chondrocytes to dynamic compression, shear stress and intermittent hydrostatic pressure (2,3,20,22). Surprisingly, a recent report revealed that substance P, an agent generally considered as neuropeptide, is expressed in human articular cartilage and is also involved in chondrocyte mechanotransduction (26).

This study shows that especially ERK-2 was activated in response to exposure to continuous high level of hydrostatic pressure. Interestingly, the same pathway was triggered by static mechanical compression of cartilage explants in a way that mechanical compression first induced mainly ERK-1 phosphorylation within 10 min, while ERK-2 activation occurred later lasting for up to 24 hr (6). In contrast to mechanical compression, high continuous hydrostatic pressure did not activate p38 or JNK, while both of these pathways were activated by compression of cartilage explants (6). Dynamic compression also has been shown to activate ERK-pathway (23). Mitogen-activated protein kinase signalling in articular chondrocytes is apparently very sensitive to mechanical loading, since it is activated also by fluid flow (10). ERK-activation was linked to a simultaneous decrease in aggrecan promoter activity (10).

The signalling cascades associated with PI3K are known to control cell growth, proliferation and survival (42). One of the best-characterized PI3K-dependent pathways is the activation of Akt/protein kinase B (7). Akt is known to promote cell survival signals through the PI3K pathway, which lead to inactivation of a number of pro-apoptotic proteins (4). The target of PI3K signalling pathway triggered by high hydrostatic pressure is not known, especially because the 15 MPa hydrostatic pressure induced clearly a faster response than 30 MPa pressure. It is obvious that continuous 30 MPa hydrostatic pressure is a stressful condition, yet, the chondrocytes survive quite well this stress maintaining a high level of proteoglycan synthesis even at 48-68 hr after release of the pressure (11). A cDNA array analysis revealed neither any particular increase in the expression level of apoptotic proteins (32). Therefore, activation of PI3K pathway may be involved in the promotion of cellular survival under exposure to high pressure.

Continuous high hydrostatic pressure initiates a stress response in a number of cell types in contrast to intermittent loading (12-15). However, in this study the cyclic 30 MPa hydrostatic loading induced the same signalling pathways as continuous 30 MPa hydrostatic pressure, although the response was not always as strong as with continuous pressure.

We have shown previously that hsp90 β mRNA was specifically induced by 30 MPa hydrostatic pressure, while hsp90 α mRNA remained at the control level in HeLa cells (5). Since Hsp90 proteins participate in the mitogen-activated protein kinase signalling pathway, the activation of this route was considered as a possible transduction pathway leading to accumulation of hsp70 mRNA in cell cultures exposed to 30 MPa continuous hydrostatic pressure. Interestingly, in HeLa cells geldanamycin (an inhibitor of Raf in the mitogen-activated protein kinase pathway via its binding to Hsp90) activated the hsp70 gene without an increased phosphorylation of ERK, while Hsp70 accumulated in the cells exposed to 30 MPa continuous hydrostatic pressure with a simultaneous phosphorylation of ERK in the absence and presence of geldanamycin (unpublished results). However, in this study the experiments utilizing a specific inhibitor of ERK phosphorylation indicated that Hsp70 protein accumulation during hydrostatic loading occured independent of ERK-signalling in chondrosarcoma cells. This suggests that despite the phosphorylation of ERK by high hydrostatic pressure, the pressure does not activate Raf, the upstream kinase of mitogen-activated kinase kinase.

In conclusion, this study shows that at least two different signalling pathways were activated by high continuous hydrostatic pressure, therefore, the results must be interpeted with caution. Nevertheless, cellular signalling via ERK pathway appears to be a rather common response to mechanical loading, while activation of PI3K has not been previously reported to be associated with mechanotransduction. Accumulation of Hsp70 protein was independent of ERK-phosphorylation.

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