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High Hydrostatic Pressure Inhibits the Biosynthesis of Eukaryotic Elongation Factor-2

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Abstract High continuous hydrostatic pressure is known to inhibit the total cellular protein synthesis. In this study, our goal was to identify pressure-regulated proteins by using two dimensional gel electrophoresis and mass spectrometry. This analysis showed that under 30 MPa continuous hydrostatic pressure the biosynthesis of eukaryotic elongation factor-2 (eEF-2) was inhibited both in HeLa carcinoma and T/C28a4 chondrocytic cell lines. Western blot analysis of HeLa cells revealed that the cellular protein level of eEF-2 decreased by 40%–50% within 12 h of the pressure treatment. However, the steady-state mRNA level of eEF-2 was not affected by the pressure. Cycloheximide addition after 4 h-pressure treatment suggested that the half-life of eEF-2 protein was shorter in pressurized cells. eEF-2 is responsible for the translocation of ribosome along the specific mRNA during translation, and its phosphorylation prevents the ribosomal translocation. Therefore, increased phosphorylation of eEF-2 was considered as one mechanism that could explain the reduced level of protein synthesis in pressurized HeLa cell cultures. However, Western blot analysis with an antibody recognizing the Thr56-phosphorylated form of eEF-2 showed that phosphorylation of eEF-2 was not elevated in pressurized samples. In conclusion, the inhibition of protein synthesis under high pressure occurs independent of the phosphorylation of eEF-2. However, this inhibition may result from the decrease of cellular eEF-2 protein. J. Cell. Biochem. 94: 497–507, 2005.

Key words: hydrostatic pressure; protein synthesis, eukaryotic elongation factor-2; two-dimensional gel electrophoresis; mass spectrometry

The cells of our body are exposed to various forms of mechanical forces, such as hydrostatic pressure, compression, and shear stress [Lammi et al., 2001]. Especially the musculoskeletal system faces high amplitudes of forces during locomotion. Within synovial joints, the articular cartilage covers the ends of the long bones to distribute the compressive and shear forces evenly, and protects the underlying bone from excessive loading [Urban, 1994; Muir, 1995]. Although overloading may be harmful for the cartilage, it is known that adequate mechanical stimulation is required for the maintenance of cartilage matrix integrity and normal composition [Buckwalter, 1995; Jortikka et al., 1997].

In vitro studies have shown that hydrostatic pressure applied on chondrocytes affects the gene expression of extracellular matrix molecules [Hall et al., 1991; Parkkinen et al., 1993b; Urban, 1994; Ishihara et al., 1996; Smith et al., 1996, 2000; Karjalainen et al., 2003]. High continuous hydrostatic pressure suppresses macromolecule biosynthesis and secretion [Hall et al., 1991; Lammi et al., 1994], decreases steady-state level of aggrecan mRNA [Lammi et al., 1994], condenses the Golgi apparatus [Parkkinen et al., 1993b], and disturbs cytoskeletal organization [Parkkinen et al., 1995;

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Crenshaw et al., 1996]. Further, it influences the expression of cytokines, growth factors [Takahashi et al., 1997, 1998], proteins participating in the cell cycle control, and those generally induced after DNA damage [Sironen et al., 2002a].

Heat shock protein 70 (Hsp70) is considered to be the major cellular stress protein, and its expression is increased after various environmental stresses [Kregel, 2002]. High continuous hydrostatic pressure causes a stress response which is characterized by increased Hsp70 expression in various primary and continuous cells lines [Takahashi et al., 1997; Kaarniranta et al., 1998, 2001]. However, pressurized primary chondrocytes lack the stress response [Kaarniranta et al., 2001], suggesting that previous loading history may help the cells to adapt to stressful pressure conditions. The accumulation of the Hsp70 expression under 30 MPa continuous hydrostatic pressure involves hsp70 mRNA stabilization without a detectable transcriptional activation of the gene [Kaarniranta et al., 1998, 2000, 2001]. Interestingly, high hydrostatic pressure appears to decrease histone mRNA stability [Symington et al., 1991]. Therefore, high pressure experiments may be a useful tool to solve the mechanisms that are important for cellular stabilization of mRNAs in general.

Continuous high hydrostatic pressure inhibits protein synthesis in bacteria and eukaryotes [Hardon and Albright, 1974; Scheck and Landau, 1982a]. In addition to gene expression profiling on mRNA level [Sironen et al., 2000, 2002a,b], we have had a proteomic approach to investigate the cellular responses to high pressure. In a chondrocytic cell line T/C28a4 [Goldring et al., 1994], we identified tryptic peptides from various stress proteins by two-dimensional gel electrophoresis/mass spectrometry (2-DE/MS), and observed increases of Hsp70 and Hsp90 biosynthesis in metabolically labeled pressurized cells [Elo et al., 2000]. On the basis of this analysis, Hsp90β, instead of Hsp90α, responds to pressure treatment, and we could confirm this finding also in HeLa cells [Elo et al., 2003]. Hsp90 is an abundant cytosolic protein that has been shown to be associated with various cellular proteins, but its role in the regulation of specific cellular targets is not well understood [Nollen and Morimoto, 2002]. It is known to modulate the activity of the steroid hormone receptors, various kinases, and also Hsp70 expression [Richter and Buchner, 2001; Nollen and Morimoto, 2002]. Hsp90 has also been shown to immunoprecipitate with elongation factor-2 kinase (EF-2 kinase) [Palmquist et al., 1994; Yang et al., 2001]. EF-2 kinase phosphorylates eukaryotic EF-2 (eEF-2), a protein crucial for protein synthesis by mediating the translocation step of ribosome relative to mRNA [Browne and Proud, 2002]. The phosphorylation of eEF-2 decreases cellular protein synthesis by inhibiting the activity of eEF-2 [Ryazanov and Davydova, 1989; Redpath et al., 1993]. In this study, our principal goal was to screen for proteins whose biosynthesis is affected by high hydrostatic pressure. By mass spectrometric analysis we could identify eEF-2 from one protein spot whose biosynthesis was inhibited by high hydrostatic pressure. Since the general protein synthesis is known to be inhibited by the pressure treatment we investigated further the behavior of eEF-2 in pressurized cells.

MATERIALS AND METHODS

Cell Cultures

HeLa cervical carcinoma cells and T/C28a4 cells (SV40 immortalized human chondrocytic cell line) [Goldring et al., 1994] 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 (PAA, Linz, Austria), penicillin (50 U/ml, PAA), streptomycin sulfate (50 U/ml, PAA), and 2 mM glutamine (PAA). The cells were used for pressure treatments when they had reached 80%–90% confluency (24 h after the passage).

Pressure and Stress Treatments

Hydrostatic pressure was applied to cell cultures using the device described in detail previously [Parkkinen et al., 1993a]. Briefly, the system consists of cylindrical pressure chamber made of acid-resistant steel (inner diameter 8.5 cm) which is filled with prewarmed deionized water (37°C) just before the onset of hydrostatic pressure. The pressure is created by hydraulic system driven by computer-controlled hydraulic valves.

Before exposure to hydrostatic pressure, the medium described above was changed and 15 mM HEPES (pH 7.3, Gibco) was added. For metabolic labeling experiments with Tran^{35}S-label (30 μCi/ml, ICN Biochemicals, Irvine, CA),

the medium was prepared by mixing one part of DMEM containing methionine and cysteine and nine parts of DMEM without methionine and cysteine (Sigma, St. Louis, MO). Medium was supplemented with 4 mM glutamine, 10% FCS and penicillin/streptomycin. The culture dishes were filled with the medium described above, and sealed with a covering plastic membrane. Continuous 30 MPa hydrostatic pressure for up to 12 h was applied to the cultures in absence and presence of Hsp90 inhibitor geldanamycin (0.5 μM, Sigma). Ionophore A23187 (1 μM, Sigma), thapsigargin (25 nM, Sigma), and nocodazole (10 μM, Sigma) were used to study eEF-2 phosphorylation in comparison with hydrostatic pressurization. All experiments were repeated two to three times.

Metabolic Labeling and Two-Dimensional Gel Electrophoresis

The metabolic labeling was performed by injecting Tran^{35}S-label (ICN Biochemicals) to the culture medium before the onset of pressurization. After 12 h labeling period, the medium was removed and the cells were washed twice with cold PBS. The cells were lysed with a solution containing 9.8 M urea, 2% (v/v) Nonidet P-40, and 100 mM dithiotreitol. Viscosity caused by DNA was diminished with a brief sonication while keeping the lysate on ice before centrifugation at 20,000*g* for 30 min. The amount of incorporated label was determined from trichloroacetic acid (TCA) precipitates, and a volume containing 5×10^5 cpm of TCA-precipitable radioactive material was loaded to gel. The isoelectric focusing was performed according to the manufacturer's instructions using 13 cm long 3–10 non-linear IPG strips (Amersham Pharmacia Biotech, Uppsala, Sweden) manufactured for Multiphor™ II system (Amersham Pharmacia Biotech). The separation program was 150 V for 30 min, 300 V for 1 h, 1,500 V for 1 h, and 3,000 V for 5 h. After isoelectric focusing, the gels were equilibrated and proteins were further separated in 10% SDS–polyacrylamide gels in Protean™ II xi system (Bio-Rad, Hercules, CA). The gels were dried, and the radioactivity signal was analyzed using Storm PhosphorImager™ (Molecular Dynamics, Sunnyvale, CA). For MS analysis, approximately 5×10^5 cells were lysed and the proteins separated with 2-DE as described above. The gels were stained with PlusOne™ Silver Staining kit (Amersham Pharmacia Biotech).

In-Gel Digestion of Proteins

Silver stained protein spots of interest were cut out of gels and digested in-gel as previously described [Shevchenko et al., 1996]. Proteins were reduced and alkylated with iodoacetamide before overnight digestion with trypsin (Sequencing Grade Modified Trypsin, Promega, Madison, WI) at 37°C. The peptides were extracted once with 25 mM ammoniumbicarbonate and twice with 5% formic acid, and the extracts were pooled. Before MALDI-TOF MS analysis, the peptide mixture was desalted using Millipore ZipTip™ μ-C18 pipette tips (Millipore, Bedford, MA).

Mass Spectrometry

Mass mapping of the peptides generated by trypsin-treament was performed with a Biflex™ MALDI-TOF mass spectrometer (Bruker-Daltonics, Bremen, Germany) in a positive ion reflector mode using α-cyano-4-hydroxycinnamic acid as the matrix [Poutanen et al., 2001; Vasiljeva et al., 2001]. The MALDI spectra were internally calibrated with the standard peptides, angiotensin II and adrenocorticotropin-18-39 (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany). In the LC-MS/MS analysis, the peptides were first separated by micropore reversed-phase HPLC on an 0.075×150 mm PepMap column (LC packings, Amsterdam, The Netherlands) by elution with a linear gradient of acetonitrile in 0.1% formic acid. Chromatography was performed at a flow rate of 0.25 μl/min, and the eluent was directly injected into a Q-TOF mass spectrometer (Micromass, Manchester, UK) equipped with an electrospray ionization source. MS/MS spectra were acquired by colliding the doubly charged precursor ions with argon collision gas accelerated with voltages of 30–45 V. Database searches were carried out using programs ProFound (http://prowl.rockefeller.edu/cgi-bin/ProFound) or Mascot MS/MS ion search (http://www.matrixscience.com/).

Western Blot Analyses

Western blot analysis of eEF-2 was performed from the whole cell extracts as previously described [Mosser et al., 1988]. To analyze the relative amount of phosphorylated eEF-2 the cellular proteins were extracted into RIPA buffer (1× 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), and centrifuged at 10,000*g* for 10 min at 4°C before electrophoresis. The protein extracts (15 μg per lane) were electrophoresed in 10% SDS–polyacrylamide gels, and proteins transferred to a nitrocellulose membrane. The proteins were stained with Ponceau S to confirm an equal protein loading and transfer on the membrane. An antibody raised in rabbit against the Thr56-phosphorylated eEF-2 [McLeod et al., 2001] was used to study the major physiological phosphorylation site of eEF-2. Polyclonal antibody recognizing eEF-2 (C-14, Santa Cruz Biotechnology, San Francisco, CA) and Hsp90-α (N-17, Santa Cruz Biotechnology), and peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology) were used for the Western blots. The membranes were developed with an enhanced chemiluminescence kit (Pierce, Rockford, IL) or with 3-amino,9-ethyl-carbazole chromogen (Zymed Laboratories, San Francisco, CA).

To study the half-life of eEF-2 protein the cell cultures were exposed to 30 MPa continuous hydrostatic pressure for 4 h, then cellular proteins were extracted into RIPA buffer immediately after the pressurization, as well as 3, 6, and 9 h after the addition of 10 μM cycloheximidine (Sigma) into the culture medium. The immunoblotting for eEF-2 and Hsp90α was performed as described above.

Northern Blot Analysis

Total RNA samples (20 μg) isolated with Trizol™ reagent (Gibco) were separated on a 1% agarose/formaldehyde gel, transferred to a nylon membrane (Hybond-N, Amersham Pharmacia Biotech), and hybridized with [α-^{32}P]dCTP-labeled plasmids specific for human eEF-2, hsp70 [Wu et al., 1985] and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) [Fort et al., 1985]. Human eEF-2 specific probe (1,303 bp) was made with PCR reaction using primers 5′-GGA GAC ACG CTT CAC TGA TAC C-3′ and 5′-GGT GAT GGT GCC CGT CTT C-3′ that were designed based on the previously published cDNA sequence (Genbank access NM_001961) [Rapp et al., 1989]. The amplified PCR product was cloned into pCR®II-TOPO vector (Invitrogen, Carlsbad, CA), and verified by DNA sequencing. The hybridization was made in ULTRAhyb™ hybridization solution (Ambion, Austin, TX) according to the manufacturer's instructions. The autoradiography signals were quantitated using the Storm PhosphorImager™ (Molecular Dynamics), and the values obtained were normalized against GAPDH signals.

Image Analysis

All the image analyses to estimate the protein band or spot intensities were performed using ImageJ image analysis software (Wayne Rasband, NIH, http://rsb.info.nih.gov/ij/).

RESULTS

High Hydrostatic Pressure Decreases eEF-2 Protein Synthesis

The effects of 30 MPa hydrostatic pressure on cellular protein synthesis in HeLa cervical carcinoma and immortalized human chondrocytic cell line was investigated with 2-DE/MS. An equal amount of incorporated radioactivity was loaded on the gels to compensate for the general inhibition of total protein synthesis observed in the pressurized cultures (72% decrease in HeLa cells). The relative intensity of several protein spots were affected by the pressurization. In immortalized chondrocytic cell line T/C28a4, a number of stress proteins, such as Hsp70, constitutive Hsp70 (Hsc70), Grp78/BiP and Grp94, were previously identified with mass spectrometry [Elo et al., 2000]. Quantitation of Hsp70 and Hsc70 protein spots (Fig. 1, spots 1 and 2, respectively) showed that ratio of radioactively labeled Hsp70 to Hsc70 increased by 30%–40% in the pressurized T/C28a4 and HeLa cells.

In this study, our major goal was to identify additional proteins that were similarly affected by high pressure treatment in the two independent cell lines used for this study. The spot marked with an arrow in Figure 1 was noticed to have lower relative radioactivity in both cell line cultures exposed to hydrostatic pressure, therefore, it was chosen for further identification. In MALDI-TOF mass mapping, twelve tryptic peptides from this spot were found to match into eEF-2 tryptic peptides with less than 0.1 Da mass accuracy. The peptides covered 14% of the whole eEF-2 amino acid sequence. The identification was further confirmed by LC-MS/MS analysis in which seven tryptic peptides could be sequenced giving sequences and molecular masses identical to eEF-2 tryptic peptides (Table I). In this spot, no other peptides referring to any other protein could be identified in

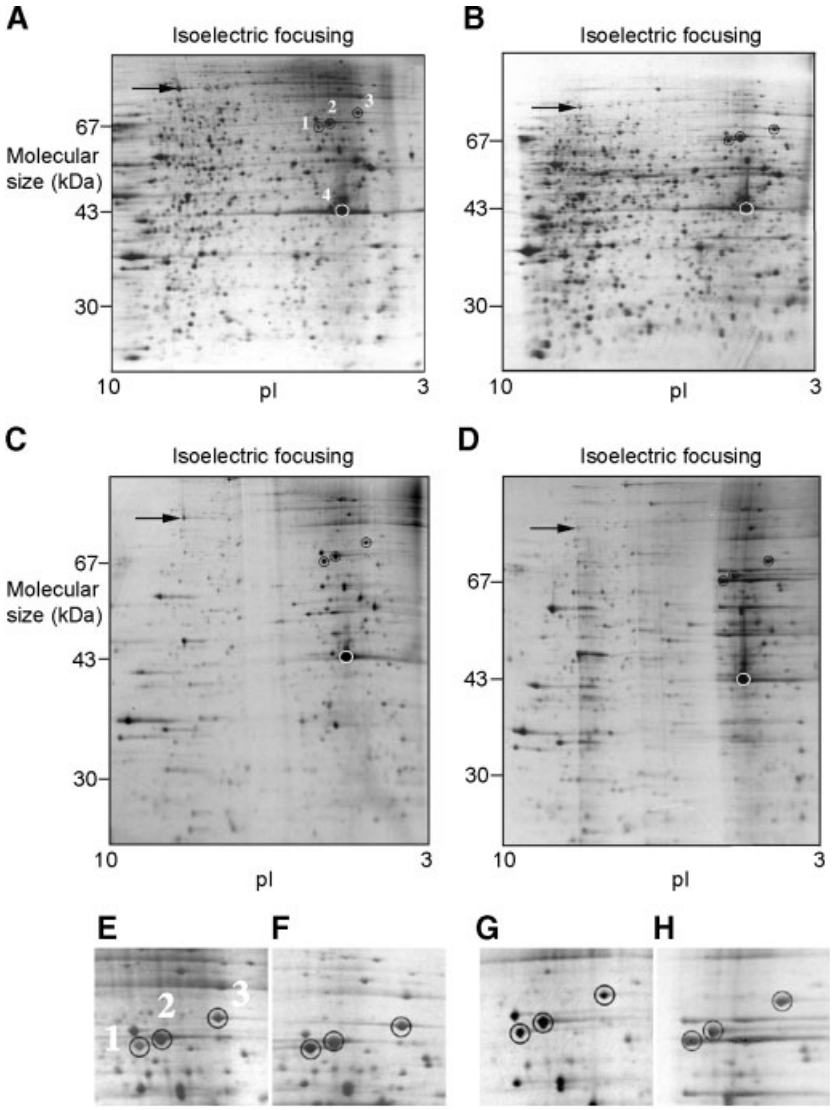

Fig. 1. Two-dimensional gel electrophoresis of metabolically labeled samples. The autoradiography signals of non-pressurized (**A**, **E**) and pressurized (**B**, **F**) T/C28a4 cells, and non-pressurized (**C**, **G**) and pressurized (**D**, **H**) HeLa cells were compared after Phosphorimager scanning analysis of the dried gels. The areas in E–H are enlarged and adjusted to show the effect of pressure on Hsp70. The spots indicating the previously identified proteins are encircled and numbered: 1, Hsp70; 2, Hsc70; 3, Grp78; and 4, actin. The spot marked with arrows was identified as eukaryotic elongation factor-2 (eEF-2) with mass spectrometry.

this sample, with the exception of few peptides matching with the expected keratin tryptic fragments. Therefore, 30 MPa continuous hydrostatic pressure was concluded to decrease the biosynthesis of eEF-2. Since hydrostatic pressure had a stronger influence on protein synthesis in HeLa cells than in the chondrocytic cells, the following protein and mRNA analyses were performed in HeLa cells.

TABLE I. Peptide Sequences of eEF-2 Identified by LC-MS/MS

Peptide sequence	Calculated mass	Experimental mass
ETVSEESNVLCLSK	1593.76	1593.98
EGALCEENNMR	1207.50	1207.68
YEWDVAEAR	1137.51	1137.68
STLTDSLVCK	1122.56	1122.74
VFSGLVSTGLK	1106.63	1106.82
VNFTVDQIR	1090.58	1090.76
FSVSPVVR	889.50	889.66

Amount of eEF-2 Protein Is Decreased in Pressurized Cells

Western blot analysis was used to estimate the effect of 30 MPa hydrostatic pressure on the

amount of cellular eEF-2 protein. Samples collected at 6 and 12 h after the beginning of pressure treatment were analyzed. According to the analysis, eEF-2 protein level was decreased by 50%–55% in the pressurized cells within 12 h of pressurization (Fig. 2A). Hsp90 inhibitor geldanamycin, which has been shown to decrease the amount of EF-2 kinase in glioma cells [Yang et al., 2001], caused a full 53%–57% inhibition of eEF-2 protein level in pressurized cultures within 6 h, while it took 12 h to reach this level by hydrostatic pressure alone (Fig. 2A). Hsp70 level was elevated in pressurized samples within the 12 h treatment (Fig. 2A). Geldanamycin increased the amount of Hsp70 both in the control and the pressurized cells (Fig. 2A).

The steady-state level of eEF-2 mRNA was analyzed from controls and samples pressurized for 3, 6, and 12 h. As expected hsp70 mRNA level was clearly increased due to the pressurization. However, mRNA level for eEF-2 remained at the constant level in comparison to GAPDH (Fig. 2B).

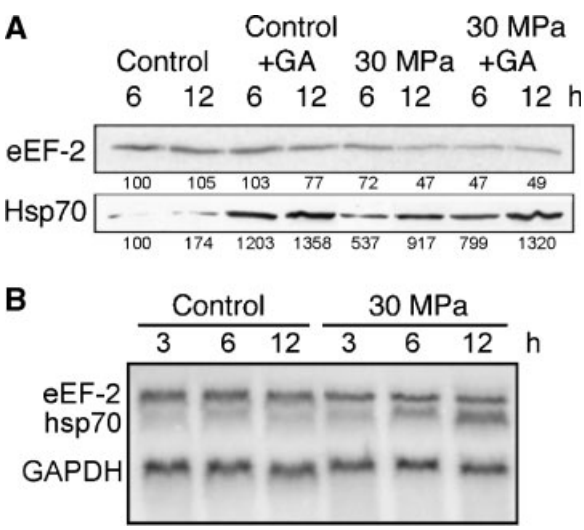

Fig. 2. Western blot analysis of pressurized HeLa cells (30 MPa continuous hydrostatic pressure). The blots show the difference in eEF-2 and Hsp70 contents after exposure to hydrostatic pressure in the absence and presence 0.5 μM geldanamycin (GA). GA was added on the control and pressurized cultures at the onset of 30 MPa hydrostatic pressure, and the samples were collected 6 and 12 h afterwards. **A**: The values below the blots show the intensity of the bands related to 6 h control sample. Northern blot analysis (**B**) of pressurized HeLa cells (30 MPa continuous hydrostatic pressure). Steady-state levels of eEF-2, hsp70, and GAPDH were analyzed.

High Hydrostatic Pressure Does not Increase eEF-2 Phosphorylation

Phosphorylation of eEF-2 inhibits the protein synthesis by preventing the translocation of ribosome during peptide elongation. It is also well known that high pressure markedly inhibits cellular protein synthesis. Thus, the possibility that phosphorylation level eEF-2 would be increased in pressurized cells was investigated at 3 and 6 h after the onset of 30 MPa continuous hydrostatic pressure. Total eEF-2 content was adjusted to approximately the same level in all of the lanes to make the comparisons between the total and the phosphorylated form easier. As a result, it was observed that high hydrostatic pressure did not increase the phosphorylation of eEF-2 in comparison with the total eEF-2 content (Fig. 3A), and thus cannot explain the inhibition of general protein synthesis due to high hydrostatic pressure. Hsp90α, a protein not affected by the pressure, was immunodetected from the same membrane as total eEF-2, and the difference in relative band intensity was determined from densitographs (Fig. 3A). As expected, hydrostatic pressure decreased the ratio of eEF-2 to Hsp90α within 6 h.

Because the phosphorylation state of eEF-2 remained steady in pressurized cell cultures we analyzed phosphorylation state of eEF-2 under conditions that are likely to affect protein synthesis and eEF-2 phosphorylation. In cell

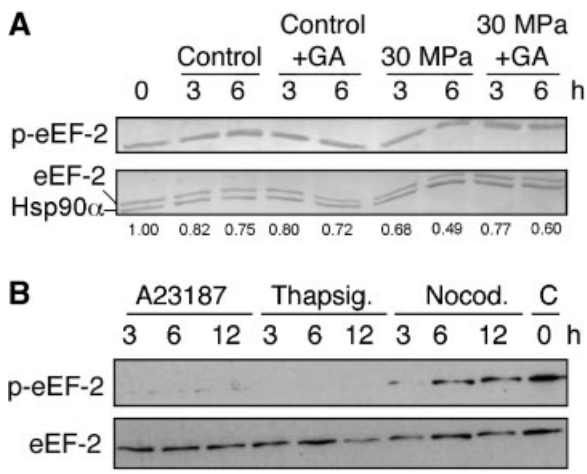

Fig. 3. The analysis of eEF-2 phosphorylation status (**A**). Hsp90α was analyzed from the same membrane as eEF-2, and the band intensity ratios of eEF-2/Hs90α have been included in the figure (A). Effects of ionophore A23187 (1 μM), thapsigargin (25 nM), and nocodazole (10 μM) on the phosphorylation status of eEF-2 (**B**).

cultures treated with drugs that increase intracellular calcium concentration (ionophore A23187 and thapsigargin), the total eEF-2 protein level remained at a constant level during 12 h treatment (Fig. 3B), whereas the phosphorylation of eEF-2 was decreased due to these treatments. Nocodazole caused dephosphorylation of eEF-2 within 3 h, while rephosphorylation occurred 6 h after the onset of treatment (Fig. 3B).

Half-Life of eEF-2 Is Shorter in Pressurized Cell Cultures

Analysis of steady-state level of eEF-2 mRNA did not give explanation to the observed decrease in the cellular eEF-2 protein. Therefore, we investigated whether eEF-2 protein is degraded faster in the pressurized cells. We assumed that pressure-induced mechanisms possibly affecting the protein stability would be turned on relatively soon after the onset of pressure. HeLa cells were pressurized for 4 h before adding cycloheximide into the cultures to prevent protein synthesis. We analyzed both total eEF-2 and Hsp90 α with specific antibodies (Fig. 4A), and used their ratio as an estimate of the relative stability of the proteins. It could be observed that relative to Hsp90α the content of eEF-2 was actually decreased in the pressurized cell cultures (Fig. 4B).

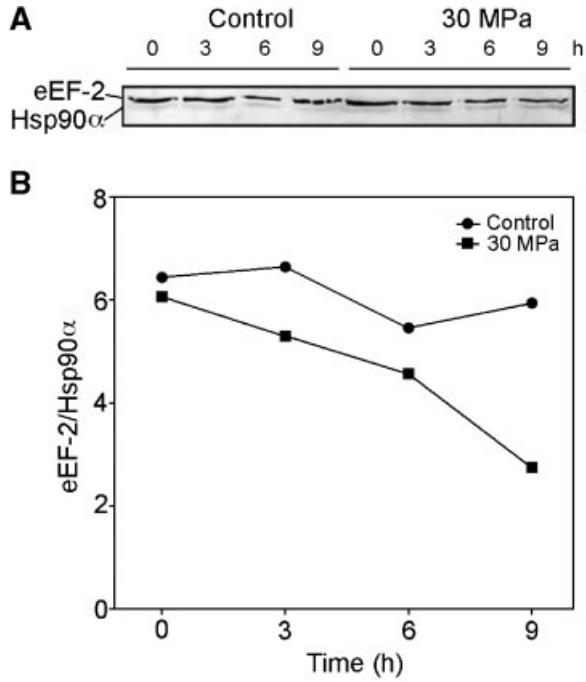

Fig. 4. Half-life analysis of eEF-2 and Hsp90α. Immunodetection of eEF-2 and Hsp90α was performed simultaneously for controls and samples pressurized at 30 MPa continuous hydrostatic pressure for 4 h (**A**). Cycloheximide (10 μg/ml) was added to block protein synthesis, and samples were collected immediately after pressurization (0 h), and 3, 6, and 9 h after addition of cycloheximide. Ratio of eEF-2 to Hsp90α staining intensity (**B**) analyzed from the densitograph shown in (A).

DISCUSSION

Various cellular stresses often lead to inhibition of protein synthesis. For instance, heat stress at 41–42°C inhibits a fraction of active polysomal ribosomes, which recover their activity after removal of the stress [Duncan and Hershey, 1989]. Numerous chemical stressors can decrease protein synthesis, and they apparently mediate their actions through many different translation initiation factors [Duncan and Hershey, 1987]. Therefore, the machinery responsible for initiation stage of translation has attracted a lot of scientific interest [Gingras et al., 1999; Scheper and Proud, 2002]. Cellular stresses modulate also the phosphorylation states of other translation factors, including eEF-2, and regulate their contribution to the rate of protein synthesis [Patel et al., 2002].

Early studies in bacteria [Landau, 1966, 1967; Pope et al., 1975a,b] and eukaryotic cells [Scheck and Landau, 1982a,b] have shown that high hydrostatic pressure inhibits the protein synthesis. The activity of rat ribosomes decreased with the increase of the applied pressure, so that total inhibition was reached at 240 MPa [Lu et al., 1997]. However, the mechanism of inhibition is not exactly known. In this study, the proteomic analysis clearly revealed that biosynthesis of one key player involved in protein translation, eEF-2, was partly inhibited by high continuous hydrostatic pressure. Western blot analysis confirmed a decreased amount of eEF-2 also at total protein level. However, increased phosphorylation of eEF-2 could not be detected in the pressurized cells. Thus, if eEF-2 participates in the regulation of translation rate it is likely due to the decrease in its total content. The decrease of eEF-2 in the pressurized cells appeared to involve a shorter half-life of the protein.

In addition to inhibition of general protein synthesis, stressing conditions cause accumulation of a number of specific proteins in the cells. Heat shock protein family is a major stress-responsive group of proteins, which normally

are regulated by heat shock transcription factors (HSFs) [Pirkkala et al., 2001]. Hsp70 is normally strongly induced during cellular stress and it acts mainly as a chaperone, while Hsp90 is involved in several cellular signaling pathways also under normal conditions. In HeLa cells, 30 MPa continuous hydrostatic pressure increases the relative contents of both Hsp70 and Hsp90β [Kaarniranta et al., 2000; Elo et al., 2003]. Notably, EF-2 kinase (also known as Ca^{2+}/calmodulin-dependent protein kinase III) interacts with Hsp90 [Palmquist et al., 1994]. Disruption of this interaction by geldanamycin was previously shown to decrease the amount of EF-2 kinase in glioblastoma cells [Yang et al., 2001] which effectively may affect the phosphorylation state of eEF-2. A strong increase of Hsp70 in geldanamycin-treated cells showed that the geldanamycin concentration used here was effective, however, it did not affect much the level of eEF-2 in HeLa cells within 12 h of treatment. In the pressurized cell cultures, geldanamycin did not have an additional effect on the decline in eEF-2 content. Therefore, it appears that Hsp90 interactions with cellular proteins, such as EF-2 kinase, are not involved with the decrease of relative eEF-2 protein level under high hydrostatic pressure.

The regulation of EF-2 kinase activity is rather complicated. A number of kinases, such as cAMP-dependent protein kinase [Redpath and Proud, 1993], stress-activated protein kinase SAPK4/p38 [Knebel et al., 2001], p90 RSK1 and p70 S6 kinase [Wang et al., 2001], are known to modulate its activity. Slight changes in the intracellular pH which occur under stressful conditions, such as hypoxia and ischemia, can also regulate EF-2 kinase activity [Dorovkov et al., 2002]. EF-2 kinase was initially shown to be calcium-dependent [Nairn et al., 1985; Ryazanov, 1987], thus, intracellular calcium fluctuations may change the phosphorylation of eEF-2. However, recent data question whether calcium plays a role in the regulation of protein synthesis by eEF-2 phosphorylation [Laitusis et al., 1998].

Calcium signaling during hydrostatic loading is an interesting topic, since mechanical stimuli can induce transient calcium impulses within the cells [Hung et al., 1996; Wright et al., 1996; Guilak et al., 1999; D'Andrea et al., 2000; Roberts et al., 2001; Valhmu and Raia, 2002]. However, it is less clear whether changes in intracellular calcium concentration occur during high continuous hydrostatic pressurization. In mouse fibroblast cell line, no changes in cytosolic Ca^{2+} concentrations were detected at any level of continuous hydrostatic pressure, or at the release of pressure [Crenshaw and Salmon, 1996]. Similarly, 90 MPa continuous pressure did not affect cellular calcium concentration in bovine chromaffin cells, although it was suggested that pressure most likely acted directly on the channel proteins and/or their modulating reactions [MacDonald, 1997]. In immortalized chondrocytic cell line, proteomic analysis indicated that continuous 30 MPa hydrostatic pressure induced a different pattern of changes than chemicals that affect cellular Ca^{2+} homeostasis [Elo et al., 2000]. In this study, an opposite response of eEF-2 phosphorylation in pressurized versus thapsigargin-treated cells was observed suggesting that calcium signaling is not likely involved with the regulation of cellular protein synthesis in cell cultures submitted to 30 MPa continuous hydrostatic pressure.

CONCLUDING REMARKS

This study reveals eEF-2 as a possible regulator of protein synthesis during high pressure conditions. Although pressure-induced inhibition of eEF-2 biosynthesis may be sufficient to depress the rate of translation to the level observed in pressurized cells, other mechanisms may also be involved, such as phosphorylation status of the initiation factors. However, in this study, inhibition of protein synthesis took place independent of the phosphorylation of eEF-2. Hsp90 appeared not to be involved in the regulation of eEF-2, however, it may participate in other pathways affected by high hydrostatic pressure. This study is one of the few studies so far that investigates the molecular mechanisms behind the inhibition of protein synthesis caused by high hydrostatic pressure.

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REFERENCES

Browne GJ, Proud CG. 2002. Regulation of peptide-chain elongation in mammalian cells. Eur J Biochem 269: 5360–5368.

Buckwalter JA. 1995. Osteoarthritis and articular cartilage use, disuse, and abuse: Experimental studies. J Rheumatol (Suppl) 43:13–15.

Crenshaw HC, Salmon ED. 1996. Hydrostatic pressure to 400 atm does not induce changes in the cytosolic concentration of Ca^{2+} in mouse fibroblasts: Measurements using fura-2 fluorescence. Exp Cell Res 227:277–284.

Crenshaw HC, Allen JA, Skeen V, Harris A, Salmon ED. 1996. Hydrostatic pressure has different effects on the assembly of tubulin, actin, myosin II, vinculin, talin, vimentin, and cytokeratin in mammalian tissue cells. Exp Cell Res 227:285–297.

D'Andrea P, Calabrese A, Capozzi I, Grandolfo M, Tonon R, Vittur F. 2000. Intercellular Ca^{2+} waves in mechanically stimulated articular chondrocytes. Biorheology 37:75–83.

Dorovkov MV, Pavur KS, Petrov AN, Ryazanov AG. 2002. Regulation of elongation factor-2 kinase by pH. Biochemistry 41:13444–13450.

Duncan RF, Hershey JW. 1987. Translational repression by chemical inducers of the stress response occurs by different pathways. Arch Biochem Biophys 256:651–661.

Duncan RF, Hershey JW. 1989. Protein synthesis and protein phosphorylation during heat stress, recovery, and adaptation. J Cell Biol 109:1467–1481.

Elo MA, Sironen RK, Kaarniranta K, Auriola S, Helminen HJ, Lammi MJ. 2000. Differential regulation of stress proteins by high hydrostatic pressure, heat shock, and unbalanced calcium homeostasis in chondrocytic cells. J Cell Biochem 79:610–619.

Elo MA, Sironen RK, Karjalainen HM, Kaarniranta K, Helminen HJ, Lammi MJ. 2003. Specific induction of heat shock protein 90beta by high hydrostatic pressure. Biorheology 40:141–146.

Fort P, Marty L, Piechaczyk M, el Sabrouty S, Dani C, Jeanteur P, Blanchard JM. 1985. Various rat adult tissues express only one major mRNA species from the glyceraldehyde-3-phosphate-dehydrogenase multigenic family. Nucl Acid Res 13:1431–1442.

Gingras AC, Raught B, Sonenberg N. 1999. eIF4 initiation factors: Effectors of mRNA recruitment to ribosomes and regulators of translation. Annu Rev Biochem 68:913–963.

Goldring MB, Birkhead JR, Suen LF, Yamin R, Mizuno S, Glowacki J, Arbiser JL, Apperley JF. 1994. Interleukin-1 beta-modulated gene expression in immortalized human chondrocytes. J Clin Invest 94:2307–2316.

Guilak F, Zell RA, Erickson GR, Grande DA, Rubin CT, McLeod KJ, Donahue HJ. 1999. Mechanically induced calcium waves in articular chondrocytes are inhibited by gadolinium and amiloride. J Orthop Res 17:421–429.

Hall AC, Urban JP, Gehl KA. 1991. The effects of hydrostatic pressure on matrix synthesis in articular cartilage. J Orthop Res 9:1–10.

Hardon MJ, Albright LJ. 1974. Hydrostatic pressure effects on several stages of protein synthesis in *Escherichia coli*. Can J Microbiol 20:359–365.

Hung CT, Allen FD, Pollack SR, Brighton CT. 1996. Intracellular Ca^{2+} stores and extracellular Ca^{2+} are required in the real-time Ca^{2+} response of bone cells experiencing fluid flow. J Biomech 29:1411–1417.

Ishihara H, McNally DS, Urban JP, Hall AC. 1996. Effects of hydrostatic pressure on matrix synthesis in different regions of the intervertebral disk. J Appl Physiol 80:839–846.

Jortikka MO, Inkinen RI, Tammi MI, Parkkinen JJ, Haapala J, Kiviranta I, Helminen HJ, Lammi MJ. 1997. Immobilisation causes longlasting matrix changes both in the immobilised and contralateral joint cartilage. Ann Rheum Dis 56:255–261.

Kaarniranta K, Elo M, Sironen R, Lammi MJ, Goldring MB, Eriksson JE, Sistonen L, Helminen HJ. 1998. Hsp70 accumulation in chondrocytic cells exposed to high continuous hydrostatic pressure coincides with mRNA stabilization rather than transcriptional activation. Proc Natl Acad Sci USA 95:2319–2324.

Kaarniranta K, Holmberg CI, Helminen HJ, Eriksson JE, Sistonen L, Lammi MJ. 2000. Protein synthesis is required for stabilization of hsp70 mRNA upon exposure to both hydrostatic pressurization and elevated temperature. FEBS Lett 475:283–286.

Kaarniranta K, Holmberg CI, Lammi MJ, Eriksson JE, Sistonen L, Helminen HJ. 2001. Primary chondrocytes resist hydrostatic pressure-induced stress while primary synovial cells and fibroblasts show modified Hsp70 response. Osteoarthritis Cartilage 9:7–13.

Karjalainen HM, Sironen RK, Elo MA, Kaarniranta K, Takigawa M, Helminen HJ, Lammi MJ. 2003. Gene expression profiles in chondrosarcoma cells subjected to cyclic stretching and hydrostatic pressure. A cDNA array study. Biorheology 40:93–100.

Knebel A, Morrice N, Cohen P. 2001. A novel method to identify protein kinase substrates: eEF2 kinase is phosphorylated and inhibited by SAPK4/p38delta. EMBO J 20:4360–4369.

Kregel KC. 2002. Heat shock proteins: Modifying factors in physiological stress responses and acquired thermotolerance. J Appl Physiol 92:2177–2186.

Laitusis AL, Brostrom CO, Ryazanov AG, Brostrom MA. 1998. An examination of the role of increased cytosolic free Ca^{2+} concentrations in the inhibition of mRNA translation. Arch Biochem Biophys 354:270–280.

Lammi MJ, Inkinen R, Parkkinen JJ, Häkkinen T, Jortikka M, Nelimarkka LO, Järveläinen HT, Tammi MI. 1994. Expression of reduced amounts of structurally altered aggrecan in articular cartilage chondrocytes exposed to high hydrostatic pressure. Biochem J 304:723–730.

Lammi MJ, Sironen RK, Elo MA, Oksala N, Kaarniranta K, Karjalainen HM, Helminen HJ. 2001. Responses of mammalian cells to mechanical forces. In: Pandalai SG, editor. Recent research developments in biophysics and biochemistry. Trivandrum, India: Research Signpost. pp 77–98.

Landau JV. 1966. Protein and nucleic acid synthesis in *Escherichia coli*: Pressure and temperature effects. Science 153:1273–1274.

Landau JV. 1967. Induction, transcription and translation in *Escherichia coli*: A hydrostatic pressure study. Biochim Biophys Acta 149:506–512.

Lu B, Li Q, Liu WY, Ruan KC. 1997. Effects of hydrostatic pressure on the activity of rat ribosome and cell-free translation system. Biochem Mol Biol Int 43:499–506.

MacDonald AG. 1997. Effect of high hydrostatic pressure on the BK channel in bovine chromaffin cells. Biophys J 73:1866–1873.

McLeod LE, Wang L, Proud CG. 2001. b-Adrenergic agonists increase phosphorylation of elongation factor 2 in cardiomyocytes without eliciting calcium-independent eEF2 kinase activity. FEBS Lett 489:225–228.

Mosser DD, Theodorakis NG, Morimoto RI. 1988. Coordinate changes in heat shock element-binding activity and HSP70 gene transcription rates in human cells. Mol Cell Biol 8:4736–4744.

Muir H. 1995. The chondrocyte, architect of cartilage. Biomechanics, structure, function and molecular biology of cartilage matrix macromolecules. Bioessays 17:1039–1048.

Nairn AC, Bhagat B, Palfrey HC. 1985. Identification of calmodulin-dependent protein kinase III and its major Mr 100,000 substrate in mammalian tissues. Proc Natl Acad Sci USA 82:7939–7943.

Nollen EA, Morimoto RI. 2002. Chaperoning signaling pathways: Molecular chaperones as stress-sensing 'heat shock' proteins. J Cell Sci 115:2809–2816.

Palmquist K, Riis B, Nilsson A, Nygård O. 1994. Interaction of the calcium and calmodulin regulated eEF-2 kinase with heat shock protein 90. FEBS Lett 349:239–242.

Parkkinen JJ, Ikonen J, Lammi MJ, Laakkonen J, Tammi M, Helminen HJ. 1993a. Effects of cyclic hydrostatic pressure on proteoglycan synthesis in cultured chondrocytes and articular cartilage explants. Arch Biochem Biophys 300:458–465.

Parkkinen JJ, Lammi MJ, Pelttari A, Helminen HJ, Tammi M, Virtanen I. 1993b. Altered Golgi apparatus in hydrostatically loaded articular cartilage chondrocytes. Ann Rheum Dis 52:192–198.

Parkkinen JJ, Lammi MJ, Inkinen R, Jortikka M, Tammi M, Virtanen I, Helminen HJ. 1995. Influence of short-term hydrostatic pressure on organization of stress fibers in cultured chondrocytes. J Orthop Res 13:495–502.

Patel J, McLeod LE, Vries RGJ, Flynn A, Wang X, Proud CG. 2002. Cellular stress profoundly inhibit protein synthesis and modulate the states of phosphorylation of multiple translation factors. Eur J Biochem 269:3076–3085.

Pirkkala L, Nykänen P, Sistonen L. 2001. Roles of the heat shock transcription factors in regulation of the heat shock response and beyond. FASEB J 15:1118–1131.

Pope DH, Connors NT, Landau JV. 1975a. Stability of *Escherichia coli* polysomes at high hydrostatic pressure. J Bacteriol 121:753–758.

Pope DH, Smith WP, Swartz RW, Landau JV. 1975b. Role of bacterial ribosomes in barotolerance. J Bacteriol 121: 664–669.

Poutanen M, Salusjärvi L, Ruohonen L, Penttilä M, Kalkkinen N. 2001. Use of matrix-assisted laser desorption/ionization time-of-flight mass mapping and nanospray liquid chromatography/electrospray ionization tandem mass spectrometry sequence tag analysis for high sensitivity identification of yeast proteins separated by two-dimensional gel electrophoresis. Rapid Commun Mass Spectrom 15:1685–1692.

Rapp G, Klaudiny J, Hagendorff G, Luck MR, Scheit KH. 1989. Complete sequence of the coding region of human elongation factor 2 (EF-2) by enzymatic amplification of cDNA from human ovarian granulosa cells. Biol Chem Hoppe Seyler 370:1071–1075.

Redpath NT, Proud CG. 1993. Cyclic AMP-dependent protein kinase phosphorylates rabbit reticulocyte elongation factor-2 kinase and induces calcium-independent activity. Biochem J 293:31–34.

Redpath NT, Price NT, Severinov KV, Proud CG. 1993. Regulation of elongation factor-2 by multisite phosphorylation. Eur J Biochem 213:689–699.

Richter K, Buchner J. 2001. Hsp90: Chaperoning signal transduction. J Cell Physiol 188:281–290.

Roberts SR, Knight MM, Lee DA, Bader DL. 2001. Mechanical compression influences intracellular Ca^{2+} signaling in chondrocytes seeded in agarose constructs. J Appl Physiol 90:1385–1391.

Ryazanov AG. 1987. Ca^{2+}/calmodulin-dependent phosphorylation of elongation factor 2. FEBS Lett 214:331–334.

Ryazanov AG, Davydova EK. 1989. Mechanism of elongation factor 2 (EF-2) inactivation upon phosphorylation. Phosphorylated EF-2 is unable to catalyze translocation. FEBS Lett 251:187–190.

Scheck AC, Landau JV. 1982a. The effect of high hydrostatic pressure on eukaryotic protein synthesis. Biochim Biophys Acta 698:149–157.

Scheck AC, Landau JV. 1982b. The stability of rabbit reticulocyte polysomes at high hydrostatic pressure. Biochim Biophys Acta 699:226–231.

Scheper GC, Proud CG. 2002. Does phosphorylation of the cap-binding protein eIF4E play a role in translation initiation? Eur J Biochem 269:5350–5359.

Shevchenko A, Wilm M, Vorm O, Mann M. 1996. Mass spectrometric sequencing of proteins from silver-stained polyacrylamide gels. Anal Chem 68:850–858.

Sironen R, Elo M, Kaarniranta K, Helminen HJ, Lammi MJ. 2000. Transcriptional activation in chondrocytes submitted to hydrostatic pressure. Biorheology 37: 85–93.

Sironen RK, Karjalainen HM, Elo MA, Kaarniranta K, Törrönen K, Takigawa M, Helminen HJ, Lammi MJ. 2002a. cDNA array reveals mechanosensitive genes in chondrocytic cells under hydrostatic pressure. Biochim Biophys Acta 1591:45–54.

Sironen RK, Karjalainen HM, Törrönen K, Elo MA, Kaarniranta K, Takigawa M, Helminen HJ, Lammi MJ. 2002b. High pressure effects on cellular mRNA stability. A cDNA array analysis. Biorheology 39:111–117.

Smith RL, Rusk SF, Ellison BE, Wessells P, Tsuchiya K, Carter DR, Caler WE, Sandell LJ, Schurman DJ. 1996. In vitro stimulation of articular chondrocyte mRNA and extracellular matrix synthesis by hydrostatic pressure. J Orthop Res 14:53–60.

Smith RL, Lin J, Trindade MC, Shida J, Kajiyama G, Vu T, Hoffman AR, van der Meulen MC, Goodman SB, Schurman DJ, Carter DR. 2000. Time-dependent effects of intermittent hydrostatic pressure on articular chondrocyte type II collagen and aggrecan mRNA expression. J Rehabil Res Dev 37:153–161.

Symington AL, Zimmerman S, Stein J, Stein G, Zimmerman AM. 1991. Hydrostatic pressure influences histone mRNA. J Cell Sci 98:123–129.

Takahashi K, Kubo T, Kobayashi K, Imanishi J, Takigawa M, Arai Y, Hirasawa Y. 1997. Hydrostatic pressure influences mRNA expression of transforming growth factor-beta 1 and heat shock protein 70 in chondrocyte-like cell line. J Orthop Res 15:150–158.

Takahashi K, Kubo T, Arai Y, Kitajima I, Takigawa M, Imanishi J, Hirasawa Y. 1998. Hydrostatic pressure induces expression of interleukin 6 and tumour necrosis factor alpha mRNAs in a chondrocyte-like cell line. Ann Rheum Dis 57:231–236.

Urban JP. 1994. The chondrocyte: A cell under pressure. Br J Rheumatol 33:901–908.

Valhmu WB, Raia FJ. 2002. myo-Inositol 1,4,5-trisphosphate and Ca(2+)/calmodulin-dependent factors mediate transduction of compression-induced signals in bovine articular chondrocytes. Biochem J 361:689–696.

Vasiljeva L, Valmu L, Kääriäinen L, Merits A. 2001. Site-specific protease activity of the carboxyl-terminal domain of Semliki Forest virus replicase protein nsP2. J Biol Chem 276:30786–30793.

Wang X, Li W, Williams M, Terada N, Alessi DR, Proud CG. 2001. Regulation of elongation factor 2 kinase by p90(RSK1) and p70 S6 kinase. 20:4370–4379.

Wright M, Jobanputra P, Bavington C, Salter DM, Nuki G. 1996. Effects of intermittent pressure-induced strain on the electrophysiology of cultured human chondrocytes: Evidence for the presence of stretch-activated membrane ion channels. Clin Sci 90:61–71.

Wu B, Hunt C, Morimoto R. 1985. Structure and expression of the human gene encoding major heat shock protein Hsp70. Mol Cell Biol 5:330–341.

Yang J, Yang JM, Iannone M, Shih WJ, Lin Y, Hait WN. 2001. Disruption of the EF-2 kinase/Hsp90 protein complex: A possible mechanism to inhibit glioblastoma by geldanamycin. Cancer Res 61:4010–4016.