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# Differential Regulation of Stress Proteins by High Hydrostatic Pressure, Heat Shock, and Unbalanced Calcium Homeostasis in Chondrocytic Cells

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**Abstract** High hydrostatic pressure (HP) has recently been shown to increase cellular heat shock protein 70 (Hsp70) level in a specific way that does not involve transcriptional activation of the gene, but rather the stabilisation of the mRNA for Hsp70. In this study, we investigated whether there are other observable changes caused by HP stress, and compared them with those induced by certain other forms of stressors. A chondrocytic cell line T/C28a4 was exposed to 30 MPa continuous HP, heat shock at 43°C, and increased cytosolic calcium concentration by the addition of sarco-endoplasmic reticulum $Ca^{2+}$ ATPase inhibitor thapsigargin (25 nM) or calcium ionophore A23187 (1 μM) in the cultures. The protein synthesis was studied by in vitro metabolic labelling followed by one- and two-dimensional polyacrylamide gel electrophoresis, and mass spectrometry was utilized to confirm the identity of the protein spots on two-dimensional gels. Continuous 30 MPa HP increased remarkably the relative labelling of Hsp70. Labelling of Hsp90 was also increased by 15–20%, although no clear change was evident at the protein level in Western blots. Elevated intracellular $Ca^{2+}$ concentration induced by thapsigargin and calcium ionophore A23187 increased mainly the synthesis of glucose-regulated protein 78 (Grp78/BiP), whereas Hsp70 and Hsp90 were decreased by the treatment. Heat shock was the strongest inducer of Hsp70 and Hsp90. This study further confirmed the induction of Hsp70 in chondrocytic cells exposed to high HP, but it also showed that calcium-mediated responses are unlikely to cause the stress response observed in the hydrostatically pressurized cells. J. Cell. Biochem. 79:610–619, 2000. 

**Key words:** stress proteins; hydrostatic pressure; chondrocyte; heat shock; proteome analysis; mass spectrometry

Articular cartilage protects bone ends from excessive loading, and to fulfill this task it has to resist compression and shear stresses. The compressive forces within joint articular cartilage may rise in normal conditions up to 20 MPa on standing [Muir, 1995]. Nevertheless, joint loading is required for maintenance of the normal composition of articular cartilage [Kiviranta et al., 1987; Palmoski et al., 1979, 1980; Tammi et al., 1987], and moderate exercise preserves best the biological properties of cartilage [Jurvelin et al., 1990; Säämänen et al., 1989]. Strenuous exercise, on the other hand, may eventually start harmful reactions in the articular cartilage [Arokoski et al., 1993].

Previous in vitro studies on cartilage explants and cell cultures have shown that both the frequency and the amplitude of the forces applied on the cartilage affect the synthesis rate of cartilage-specific proteoglycans [Gray et al., 1988; Hall et al., 1991; Parkkinen et al., 1992, 1993b; Sah et al., 1989]. High continuous hydrostatic pressure (HP) inhibits macromolecule synthesis and secretion, reduces the steady-state level of aggrecan mRNA, alters the shape of the Golgi apparatus, and disturbs the stress fiber organization of microfilaments [Hall et al., 1991; Lammi et al., 1994; Parkkinen et al., 1993a, 1995; Symington et al., 1991]. Destabilization of microtubules with nocodazole inhibits proteoglycan secretion into the medium, but high HP has an additional effect on inhibition [Jortikka et al., 2000].

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Stress proteins, often referred to as heat shock proteins (Hsps), are generally induced by cellular insults such as hypertermia, ischaemia, and heavy metal intoxications. A number of Hsp families are known today. As molecular chaperones Hsps mediate the folding, assembly, and translocation of polypeptides across the intracellular membranes, and some of the essential components of the cytoplasmic ubiquitin-dependent degradative pathway are Hsps [Burel et al., 1992]. Induction of Hsps is normally regulated at both transcriptional and post-transcriptional levels [Lindquist and Craig, 1988; Morimoto, 1993], although transcriptional activation is not always involved [Kaarniranta et al., 1998]. The inducible Hsp70 is strongly involved in the chaperoning function [Macario, 1995], while the constitutively expressed hsc70 is important also for uncoating of clathrin-coated vesicles [Cheetham et al., 1996].

The Hsp90 family is a group of highly conserved stress proteins expressed in all eukaryotic cells [Parsell and Lindquist, 1993]. In human, there are two genes known to encode Hsp90; the gene products of Hsp90α and Hsp90β share 86% homology [Hickey et al., 1989; Rebbe et al., 1987]. Hsp90 is located in the cytoplasm at high concentration, as approximately 1–2% of the cytosolic proteins in unstressed eukaryotic cells consist of Hsp90 [Welch and Feramisco, 1982], and small amounts are present in the nucleus, too [Gasc et al., 1990]. Hsp90 assists in protein folding [Jacob and Buchner, 1994], and by binding to Raf-1, a down-stream kinase of Ras [Schulte et al., 1996], it participates in the signal transduction pathway that acts via receptor tyrosine kinases.

The so-called glucose-regulated proteins 78 (Grp78/BiP) and 94 (Grp94), the chaperones of the endoplasmic reticulum, are also members of Hsp families. Grp78 is a part of the translocation machinery and assists in the retrograde transport across the endoplasmic reticulum membrane of proteins that are to be degraded by the proteasome. Grp78 is also an abundant cellular protein, but its synthesis is markedly increased under conditions that lead to the accumulation of unfolded polypeptides in the endoplasmic reticulum [Gething, 1999]. Grp78 and Grp94 are also induced by disturbances in the intracellular $Ca^{2+}$ stores due to chemicals such as thapsigargin or calcium ionophores [Li et al., 1997].

The specific heat shock response due to high HP [Kaarniranta et al., 1998] led us to investigate whether some other indicators of stressful conditions would be observable at the protein synthesis in chondrocytic cells under high pressure. Two-dimensional gel electrophoresis technique combined with mass spectrometric identification of protein sequences was established in our laboratory as it was considered to be an effective tool to study the proteomes of the stressed cell cultures. The effects of two other forms of stress (heat shock and intracellular calcium imbalance) were used as reference for high HP.

## EXPERIMENTAL METHODS

### Cell Cultures

Human chondrocytic T/C28a4 cells, a cell line established by immortalizing juvenile costal chondrocytes with SV40 tumor antigen [Goldring et al., 1994], were cultured in a humidified 5% $CO_2$/95% air atmosphere at 37°C in DMEM supplemented with 10% fetal calf serum, penicillin (50 units/ml), streptomycin sulfate (50 units/ml), and 3 mM glutamine. Cells were grown to a density of $7.2–8.0 \times 10^4$ cells per $cm^2$ on 60-mm plates.

### Stress Conditions

Before exposure to HP or elevated temperature, the medium was changed and 15 mM HEPES (pH 7.3) was added. The culture dishes were filled with the above medium and sealed with a covering plastic membrane. The apparatus and protocol for hydrostatic pressurization of the cells has been described in detail previously [Parkkinen et al., 1993b]. The HP levels were selected to be either 4 or 30 MPa, and continuous or cyclic modes of HP were used. In the cyclic mode, the frequency of the pressure pulses was 0.5 Hz (1 s load/1 s rest). For heat shock, the plates were immersed in a water bath at 43°C. Intracellular calcium imbalance was produced by adding thapsigargin (25 μM stock solution in dimethyl sulfoxide, final concentration 25 nM) or calcium ionophore A23187 (1 mM stock solution in DMSO, final concentration 1 μM) into the cultures.

### Metabolic Labelling and Gel Electrophoresis

To study the protein synthesis after different exposures the cells were washed with PBS after treatments, and the medium was replaced with methionine- and cysteine-free DMEM supplemented with fetal calf serum, 3 mM glutamine, and antibiotics. The labelling was performed with 50 μCi/ml Tran$^{35}$S-label (ICN, Irvine, CA) for 1 h in a cell incubator. For 1D-PAGE, whole cell extracts were prepared as previously described [Mosser et al., 1988]. For 2D-PAGE, the cells were washed twice with PBS and directly lysed with a solution containing 9.8 M urea, 2% (v/v) Nonidet P-40 and 100 mM dithiotreitol. To reduce the interference caused by DNA during electrophoresis, the samples were briefly sonicated while on ice and centrifuged at 20,000*g* for 30 min. The amount of incorporated label was determined with trichloroacetic acid precipitation, and a volume containing $1 \times 10^6$ trichloroacetic acid-precipitable c.p.m was loaded to either 1D-PAGE or 2D-PAGE. For 2D-PAGE, isoelectric focusing was performed in polyacrylamide gels containing 9.3 M urea, 2.7% ampholyte pH 3–10, 2.7% ampholyte pH 5–7, and 2% (v/v) Nonidet P-40 at 667 V for 18 h at room temperature. After electrofocusing, the gels were equilibrated in a solution containing 0.06 Tris-HCl pH 6.8, 2% SDS, 100 mM dithiotreitol and 10% glycerol, then proteins were further separated in 12% SDS-polyacrylamide gel. For metabolically labelled samples, the gels were dried and autogradiography signal was detected by using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

### In-Gel Digestion of the Proteins

After 2D-PAGE, the gel was stained with Coomassie Brillian Blue, and the protein spots of interest were excised from the gel and digested with modified trypsin solution (Bio-Rad, Richmond, CA). As a control sample, standard Hsp90 protein (1 μg and 5 μg) isolated from bovine brain (Sigma, St. Louis, MO) was digested under the same conditions.

### HPLC-Electrospray Ionisation/Mass Spectrometry (ESI/MS)

Positive ion mass spectra were acquired with on-line HPLC-ESI/MS. The measurements were carried out using a LCQ quadrupole ion trap mass spectrometer equipped with an ESI source (Finnigan MATs), a Rheos 4000 pump (Flux Instruments), and a Rheodyne 7725 injector with a 20-μl-loop (Cotati). The tryptic digests (20 μl) were injected to reversed-phase column (Syncropak RP-8, 5 μM, 100 × 2.1 mm I.D., Lafayette), and the peptides formed during trypsin digestion were separated using gradient from 2–70% acetonitrile in 40 min, 23°C. Eluent A was 50 mM formic acid, and eluent B was 70% acetonitrile containing 50 mM formic acid. The total eluent flow of 0.2 ml/min was directed to the ESI source, which is designed to cope with flow rates up to 1 ml/min. The system was tuned by infusion of peptide MRFA from a T-split to the eluent flow. The spray needle potential was set to 5 kV. The spray was stabilized using a nitrogen sheath flow, with the value set to 85. The stainless steel inlet capillary was heated to 225°C. The capillary voltage was 20 V and the tube lens offset was 5 V. The full scan mass spectra from m/z 360 to m/z 2000 were measured using 500 ms for collection of the ions in the trap; two microscans were summed. Collision induced dissociation (CID) MS/MS spectra of the peptides were obtained using the dependent scan mode of the instrument. Full scan MS data were collected until the base peak ion exceeded a preset intensity threshold ($2 \times 10^4$). This triggers acquisition of a CID MS/MS product ion spectrum of the ion. The collision energy was 35% and isolation width was two mass units. Scan range was dependent on the parent mass ion. The protein mass spectrometry Internet pages of University of California San Francisco were utilized to compare the measured data with protein structures available in the data bank (http://prospector.ucsf.edu and http://prowl.rockefeller.edu).

### Western Blotting

For Western blot analysis, whole cell extracts were prepared as previously described [Mosser et al., 1988]. The protein extracts (15 μg per lane) were electrophoresed on 10% SDS-polyacrylamide gels, and transferred to nitrocellulose membrane. The transferred proteins were visualized with Ponceau stain to ensure equal protein loading and transfer. Monoclonal antibodies (SPA-810; StressGen, British Columbia, BC, Canada) recognizing Hsp70, and polyclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) recognizing Grp78 (N-20), Hsp90α (N-17), and Hsp90β

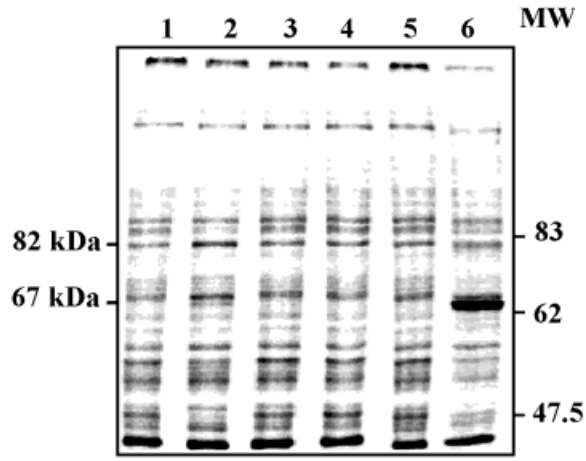


**Fig. 1.** Analysis of protein synthesis after HP and heat shock treatments studied by in vitro metabolic labelling and 1D-PAGE. After the cells had been exposed to different stress treatments, the newly-synthesised proteins were labelled with 50 μCi/ml of [$^{35}$S]methionine for 1 h in a cell culture incubator. The protein samples were prepared, and the proteins separated with 1D-PAGE. Autoradiography was performed with PhosphorImager. Lane 1: Control sample; lane 2–5: samples pressurized for 12 h with 30 MPa continuous (lane 2), 30 MPa cyclic (lane 3), 4 MPa continuous (lane 4), and 4 MPa cyclic HP (lane 5); lane 6, sample incubated for 5 h at 43°C. The 67 kDa and 86 kDa regions are indicated.

(D-19), as well as peroxidase-conjugated secondary antibodies (Zymed, San Francisco, CA and Santa Cruz Biotechnology) were used for the Western blots. The membranes were developed with an enhanced chemiluminescence method (Santa Cruz Biotechnology).

## RESULTS

### Protein Synthesis Analyzed With 1D-PAGE

Trichloroacetic acid precipitation was used to estimate the effect of various stressors on the protein synthesis of the cell cultures. Continuous 30 MPa HP for 12 h decreased total protein synthesis by 64%, heat shock by 83%, and thapsigargin by 7%, while cyclic 30 MPa HP inhibited protein synthesis only by 30%. Continuous or cyclic 4 MPa pressure did not have any significant effects on the amount of protein synthesis. In 1D-PAGE where equal amounts of radioactively labelled proteins from the different treatments were applied on the gel, continuous 30 MPa HP demonstrated the increased relative synthesis of 67 and 82 kDa class of proteins (Fig. 1, lane 2), whereas 30 MPa cyclic, and 4 MPa continuous or cyclic pressure caused no obvious changes in the relative intensity of the protein bands (Fig. 1, lanes 3, 4, and 5, respectively). As expected, heat shock treatment strongly induced the synthesis of a 67 kDa class of protein, and to a lesser extent a 82 kDa protein (Fig. 1, lane 6).

### 2D-PAGE Analysis

To further study the effect of high HP and other stressors on protein synthesis 2D-PAGE analyses were performed. It became evident that various stress conditions resulted in differential labelling patterns on 2D-PAGE. Continuous 30 MPa HP for 12 h did not cause very strong changes in the general pattern of proteins, however, it induced approximately two-fold the synthesis of 67 kDa protein (pI 5.4), and synthesis of an 82 kDa protein (pI 4.9–5.0) was also increased by 15–20% (Fig. 2B). The same spots were induced, although to a greater extent, by the heat shock at 43°C for 5 h (Fig. 2D), suggesting they were proteins Hsp70 and Hsp90, respectively. These findings are in line with the relative changes observed in 1D-PAGE (Fig. 1) at the same molecular weight range. A 70 kDa protein (pI 5.3) was also slightly increased in both of the shock treatments, the size and pI value suggesting it to represent Hsc70. On the other hand, syntheses for a group of yet unidentified proteins ranging from 89 kDa to 92 kDa, (pI 5.1–5.3) were down-regulated after high continuous HP (Fig. 2B). Cyclic 30 MPa HP showed no remarkable changes in protein synthesis (Fig. 2C).

Thapsigargin is a highly specific inhibitor of sarco-endoplasmic reticulum $Ca^{2+}$ ATPases and increases cytosolic $Ca^{2+}$ concentration via passive leakage of $Ca^{2+}$ ions from endoplasmic reticulum, while $Ca^{2+}$ ionophore A23187 increases intracellular $Ca^{2+}$ concentration by transporting cations across the cell membrane. Compared with gels from control, pressurized, and heat-shocked samples, thapsigargin (25 nM for 12 h) resulted in a clearly different pattern of newly-synthesized proteins visible on the gel. It induced syntheses for 74 kDa and 89 kDa proteins (pI 5.0 and 4.9, respectively), but not the likely representatives of Hsp70 and Hsp90 proteins, rather the spot obviously representing Hsp90 was down-regulated. The induced spots were expected to represent Grp78 and Grp94, based on their sizes and pI values and on the reported effects of thapsigargin on the synthesis of these proteins. Syntheses for two unknown 74 kDa and 106 kDa proteins (pI

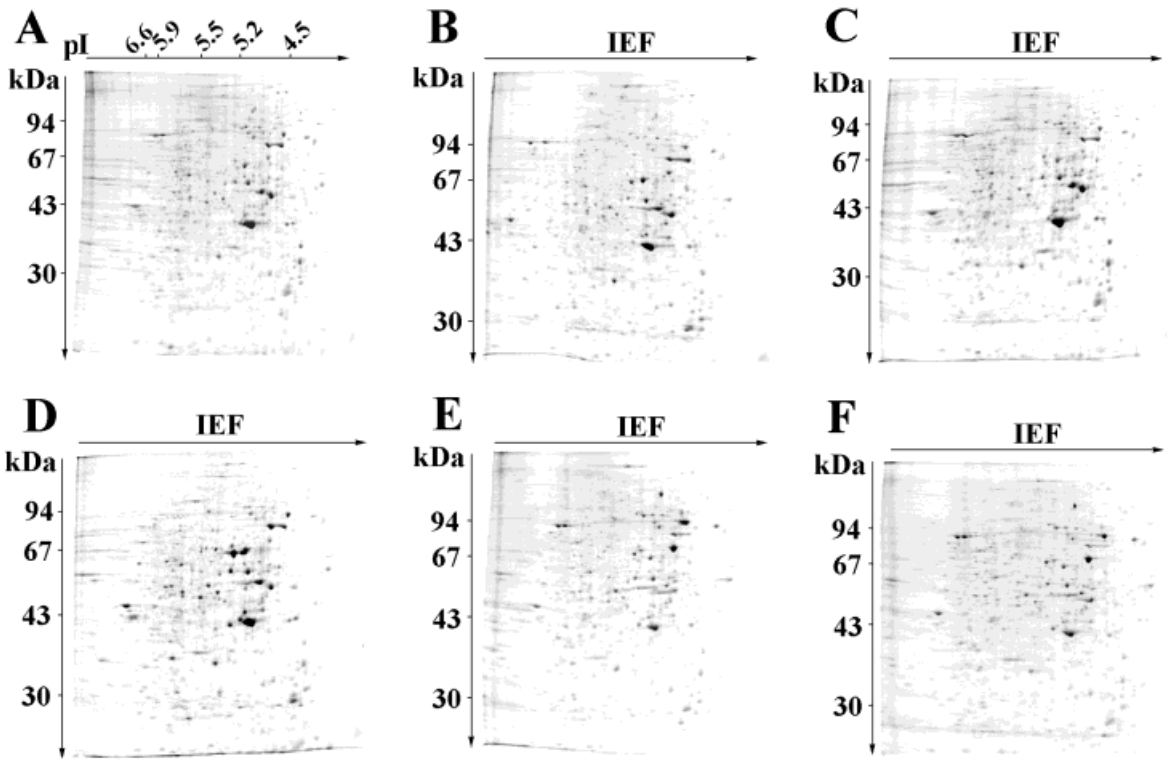


**Fig. 2.** Analysis of protein synthesis after various stress treatments studied by in vitro metabolic labelling and 2D-PAGE. After the cells had been exposed to different stress treatments, the newly-synthesised proteins were labelled with 50 μCi/ml of [$^{35}$S]methionine for 1 h. The protein samples were prepared, and the proteins were separated with 2D-PAGE. Autoradiography was performed with PhoshorImager. 2D-gels from (**A**) control culture, (**B**) cells exposed to 30 MPa continuous HP, (**C**) cells exposed to 30 MPa cyclic HP, (**D**) the sample incubated for 5 h at 43°C, (**E**) the one incubated at 25 nM thapsigargin, and (**F**) the one incubated at 1 μM calcium ionophore A23187.

5.2 and 5.3, respectively) were also increased after chemical treatment (Fig. 2E). Ionophore A23187 had a similar effect on the pattern of newly-synthesized protein as thapsigargin (Fig. 2F).

### Mass Spectrometric Analysis of Resolved Peptides

During the last years, identification of amino acid sequences of peptides present in tryptic digests has become possible by using the fragmentation mass spectra obtained from the separated peptides. In this study, we applied mass spectrometry for the identification of the proteins of interest isolated from the 2D-PAGE gels. Since the main goal in this study was to investigate the stress response, we chose spots that were likely to belong to the 70-kDa and 90-kDa classes of Hsps. That particular area of the gel is shown in higher magnification in Figure 3. Protein spots numbered 1–6 in Figure 3 were excised from the 2D-gel of the sample separated after treatment at 30 MPa static HP for 12 h, and analysed with HPLC-ESI/MS. The peptides identified from each of the spots by MS are presented in Table I. The proteins found in data bank search containing these particular peptide fragments and matching the size and pI values of the spots are also listed in Table I. Using this approach, we identified six different proteins based on the obtained mass spectrometric data.

Spots numbers 2 and 3, located at the 82 kDa range, were both noticed to consist of the two isoforms of Hsp90, therefore, we compared their mass spectra against that obtained from purified bovine Hsp90 sample to estimate the reliability of the mass analysis system in identification of the amino acid sequences present in the tryptic peptides. Figure 4 shows the full scan HPLC-MS base peak ion chromatogram of the tryptic digest from protein spot number 2. The peptides were identified by two methods. First, the data from tryptic digestion were com-

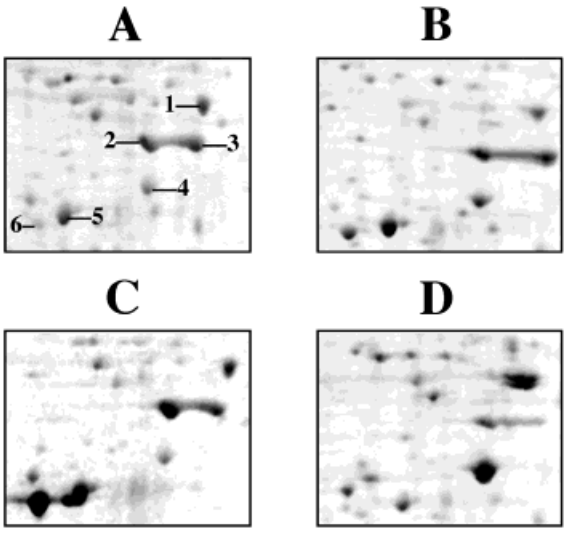


**Fig. 3.** 2D-PAGE-separated proteins selected for mass spectrometric identification. The six selected protein spots had their isoelectric points ranging from 4.9 to 5.4, and their molecular masses ranged from 67 kDa to 89 kDa. 2D-gels from (**A**) control culture, (**B**) cells exposed to 30 MPa continuous HP, (**C**) cells incubated for 5 h at 43°C, and (**D**) cells incubated with 25 nM thapsigargin. The code for each spot is given in Table I.

pared with the digest of the standard Hsp90 protein, a mixture of bovine Hsp90 isoforms Hsp90α and Hsp90β. Secondly, the molecular weights and fragmentation mass spectra of the identified peptides were compared with those of the theoretical peptides obtained from the protein digest. These peptides are identical in the human and bovine amino acid sequences.

The full scan HPLC-ESI mass spectra of the tryptic peptides show singly or doubly protonated molecules as base peaks (data not shown). In our protocol, the charge state of the peaks and the monoisotopic molecular weights were measured using 10 mass unit scan range (zoom scan function). The measured masses of the peaks found in the spot number 1 matched with the masses of the following expected tryptic peptides from Hsp90: GVVDSEDLPLNISR (measured monoisotopic mass 1512.4, calculated monoisotopic mass 1512.7), HLEINPDHSIIETLR (1785.6, 1785.94, this peptide is present only in Hsp90α, Fig. 4A,B), HLEINPDHPIVETLR (1781.7, 1781.8, present only in Hsp90β, Fig. 4C). The structures of these peptides were verified using CID of the $[M+H]^+$ ions, in case of GVVDSEDLPLNISR the $[M+2H]^{2+}$ ion at m/z 758. The fragment ions were labelled using the system developed by Biemann [1988]. The full scan MS-MS spectra of these peptides (Fig. 4) showed mainly y, B, and A type ions which are typical for low energy collision mass spectra. These results confirmed that the system was reliable and gave consistent information of amino acid sequences of tryptic peptides.

### Western Blot Analysis of Stress Proteins

Finally, protein levels of Hsp70, Grp78, Hsp90α, and Hps90β were analyzed with Western blotting. Static 30 MPa HP for 12 h increased clearly Hsp70, and slightly Grp78, levels, but did not have an effect on Hsp90α and Hps90β levels (Fig. 5A). Cyclic 30 MPa HP for 12 h caused a small increase in Hsp70 levels, but did not affect the protein levels of any other stress protein investigated. When cells were incubated at 43°C for 5 h, Hsp70, Hsp90α, and Grp78 levels were increased, while Hps90β level remained rather constant. A different kind of stress protein profile was observed when cells were treated with 25 nM thapsigargin for 12 h. Grp78 levels were then increased, while Hsp70, Hsp90α, and Hsp90β levels were decreased and level of Hps90β was unchanged. The relative changes are shown as densitographs in Figure 5B.

## DISCUSSION

Recently, we showed that high HP applied to immortalized human chondrocytic cell line causes an accumulation of Hsp70 in the pressurized cells [Kaarniranta et al., 1998]. Heat shock activates hsp gene transcription after trimerization and hyperphosphorylation of heat shock factor, however, hsp70 mRNA accumulation occurs via mRNA stabilization with no activation of transcription [Kaarniranta et al., 1998]. Since the mechanism of stress response to high HP appears rather unique, we used 2D-PAGE/HPLC/MS in this study to investigate whether we can observe some other indicators of stressful conditions. A number of Hsps were reliably identified from the 2D-PAGE gels, demonstrating that this technique is a valuable tool for the analysis of proteins of interest. The stress caused by 30 MPa continuous HP was compared with those induced by heat shock and calcium imbalance. Calcium effectors were chosen since previously it was proposed that increased intracellular $Ca^{2+}$-levels following the application of high HP may explain the observed reorganization of the cy-

**TABLE I. Peptide Sequences Determined by CID MS/MS***

| Spot no. | Peptide sequence Protein identified | Molecular determined by CID MS/MS | Mass (kDa) | pI |
|---|---|---|---|---|
| 1 | Grp94 | (K)EEASDYLELDTIK(N)<br>(R)FQSSHHPTDITSLDQYVER(M)<br>(K)GVVDSDDLPLNVSR(E) | 92.4 | 4.8 |
| 2, 3 | Hsp90α | (R)GVVDSEDLPLNISR(E)<br>(K)HLEINPDHSIIETLR(Q) | 84.5 | 4.9 |
| | Hsp90β | (K)HLEINPDHPIVETLR(Q) | 83.2 | 5.0 |
| 4 | Grp78 | (K)NQLTSNPENTVFDAK(R)<br>(R)ITPSYVAFTPEGER(L)<br>(R)ELEEIVQPIISK(L)<br>(K)SDIDEIVLVGGSTR(I) | 72.1 | 5.0 |
| 5 | Hsc70 | (K)STAGDTHLGGEDFDNR(M) | 70.8 | 5.4 |
| 6 | Hsp70 | (R)TTPSYVAFTDTER(L)<br>(K)NQVALNPQNTVFDAK(R)<br>(K)HWPFQVINDGDKPK(V) | 70.0 | 5.5 |

*Protein spots numbered 1–6 in Figure 4 were excised from the 2D-gel of the sample separated after treatment at 30 MPa static HP for 12 h, and analysed with HPLC-ESI/MS. The peptides identified from each of the spots by MS and the molecular mass for the whole protein obtained from the protein data bank are presented here.

toskeleton [Otter et al., 1987], and due to the well-known effects of calcium disturbances on Grp proteins, the endoplasmic reticulum chaperones.

We show here that Hsp70 induction occured at the protein synthesis level, irrespective of whether 1D- or 2D-PAGE was used for the analysis. Induction of Hsp70 was observed in rheumatoid synovial cells in the presence of proinflammatory cytokines interleukin-1 and interleukin-6, and shear stress [Schett et al., 1998], showing that pathologic processes may include Hsp activation in response to mechanical stress. The intensity of another protein band at 82 kDa (Hsp90) was also relatively increased in 1D-PAGE. In 2D-PAGE, only two major spots (both of them containing Hsp90α and Hsp90β isoforms) were observed and identified in that particular molecular weight area, suggesting that Hsp90α and Hsp90β were the proteins responsible for the increased labelling detected in 1D-PAGE. An approximately 15–20% increase in the synthesis for these two spots was obtained by Phosphorimager quantitation of 2D-PAGE gels. The fact that Hsp90 protein levels of the pressurized cell cultures were at the control level in immunoblots suggests that high HP increases their turnover rate.

New data seem to link mechanical factors with cytokines, Hsp90, and nitric oxide synthase. In cultured primary chondrocytes, fluid shear stress increased interleukin-6 both in mRNA and protein level [Mohtai et al., 1996], and a recent study showed that continuous HP elevated interleukin-6 mRNA levels in chondrosarcoma cells [Takahashi et al., 1998]. Interleukin-6 itself has been shown to induce Hsp90 expression [Stephanou et al., 1998]. Fluid shear stress in endothelial cells induces a binding of Hsp90 to endothelial nitric oxide synthase complex increasing also the enzyme activity [Garcia-Cardena et al., 1998]. The fact that geldanamycin inhibited the pathway showed that Hsp90 signalling is needed for production and release of NO in endothelial cells [Garcia-Cardena et al., 1998]. In chondrocytes, the proteoglycan synthesis is inhibited by increased production of NO [Taskiran et al., 1994] and by static loading [Gray et al., 1988; Hall et al., 1991; Lammi et al., 1994; Sah et al., 1989]. On the other hand, dynamic mechanical loading decreased cellular NO production in chondrocytes seeded in agarose [Lee et al., 1998], and generally induces proteoglycan synthesis [Gray et al., 1988; Lammi et al., 1994; Sah et al., 1989]. G-proteins appear to mediate the response to fluid shear stress and endothelial nitric oxide synthase activation [Das et al., 1997; Garcia-Cardena et al., 1998].

The three different stress treatments raised unique responses, heat shock showing the strongest response in the synthesis of Hsps. Thapsigargin had effects on a distinctive set of

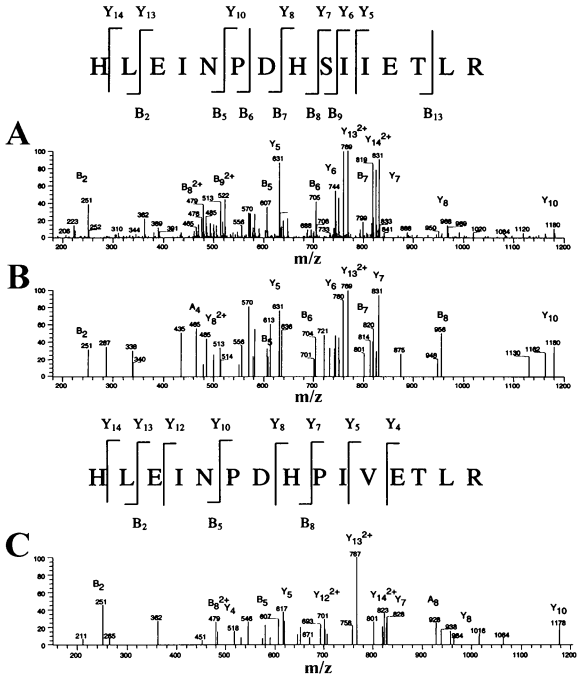


**Fig. 4.** Collision induced dissociation (CID) mass spectra from Hsp90 standard and tryptic peptide found in the selected protein spot. Peptides from the trypsinate of Hsp90 standard and the selected spot no. 2 from 2D-PAGE shown in Figure 3 were separated by reversed phase HPLC and analysed by electrospray ionization mass spectrometry. In both spectra, the triply protonated molecule at m/z 596.4 was selected for CID. The fragment ions are labelled according to Biemann [1988]. **A**: Tryptic peptide present only in Hsp90α, bovine Hsp90 standard, and (**B**) tryptic peptide from the protein spot number 2, pressurized cell culture, static 30 MPa, (**C**) tryptic peptide present only in Hsp90β, obtained from protein spot number 2, pressurized cell culture, static 30 MPa.

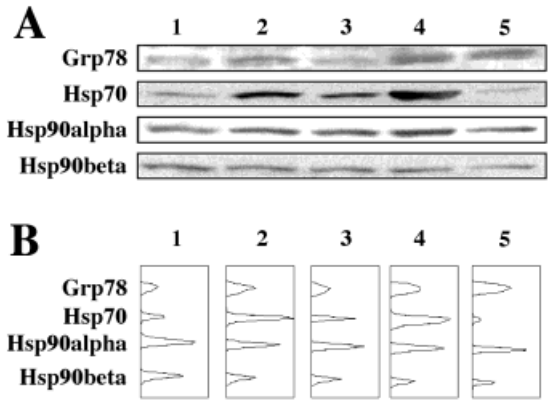


**Fig. 5.** Western blot analysis of protein levels after various stress treatments. **A**: After different treatments the whole cell extracts were prepared and the proteins separated with SDS-PAGE and blotted to a nitrocellulose membrane. Primary antibodies recognizing Hsp70, Grp78, Hsp90α, and Hsp90β were used. Control sample (lane 1), sample pressurized for 12 h with 30 MPa static HP (lane 2), sample pressurized for 12 h with 30 MPa cyclic HP (lane 3), sample incubated for 5 h at 43°C (lane 4) and sample incubated for 12 h with 25 nM thapsigargin (lane 5). **B**: The densitographs of the same samples show the relative changes of the proteins investigated.

proteins compared with heat shock and high HP. In bovine primary chondrocytes, high HP has been shown to result in the secretion of aggrecans that had retarded migration on agarose gels [Lammi et al., 1994]. Although a synthesis of aggrecans with a little longer and more densely substituted glycosaminoglycan chains on the core protein was the most likely explanation for the slower mobility on electrophoresis, a misfolded aggrecan core protein still remained one theoretical possibility for the phenomenon. The results of this study do not completely rule out the latter chance, since a minor increase of endoplasmic reticulum chaperone molecule Grp78 was visible in Western blot. However, the Grp78 response was small compared with the effect of thapsigargin and calcium ionophore A23187, as shown by the remarkably altered protein pattern on two-dimensional PAGE. An increased intracellular calcium level caused by thapsigargin or cyclopiazonic acid was previously shown to increase the levels of Hsp90α, Hsc70, Hsp70, and Hsp60 in bovine primary chondrocytes [Cheng and Benton, 1994], but in our experiments there was rather a relative decrease of Hsp70 and Hsp90β in thapsigargin-treated cells at the protein level. Whether the immortalization of human chondrocytes changes their response to the $Ca^{2+}$-pump inhibitors is not known.

Two-dimensional gel electrophoresis is a method that can resolve a large number of proteins in a quantitative way. However, we cannot ignore the specific features frequently associated with the technique [Abbott, 1999]. Firstly, cellular protein profile is constantly being influenced by numerous intra- and extracellular factors, therefore, the protein analysis made at a single time point cannot fully describe nor explain the effect of, e.g., mechanical stress. Secondly, although two-dimensional electrophoresis in currently considered as the most powerful method in protein separation it is also sensitive to errors and unable to detect some interesting cellular proteins such as membrane-bound or hydrophobic proteins. Thirdly, two-dimensional electrophoresis gives not only quantitative but also qualitative infor-

mation. This means that changes in a single protein spot may result from changes in absolute protein level or may reflect changes in protein modification, such as an altered phosphorylation state. For instance, Hsp90α and Hsp90β isoforms are both located on two or more separate spots on a two-dimensional gel, and in different ratios in two separate spots (data not shown), making the judgment of the true effects of the experiment more difficult.

In conclusion, high HP causes a stress response in cultured chondrocytic cells, however, it appears to be more inferior compared with heat shock and calcium imbalance. It changes cellular morphology and function, but it is not known which signal transduction cascade(s) high HP activates. Many G-protein coupled receptors activate the extracellular signal-regulated kinase/mitogen-activated protein kinase cascade [Gutkind, 1998; Lopez-Ilasaca, 1998], Hsp90 being involved in the upstream events of the cascade [Garcia-Cardena et al., 1998; Inanobe et al., 1994; Pratt, 1997]. Hydrostatic pressure affects at the border between two phases (for example at the plasma membrane), therefore, ion channels are among the potential targets. Whether we can learn more of the signal transduction pathways and gene regulation mechanisms involved in the fine-tuning of various stress responses requires further studies; in particular determining the role of Hsp90 in the cellular signaling cascades may uncover important new aspects of cellular regulatory control mechanisms.

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