KUOPION YLIOPISTON JULKAISUJA D. LÄÄKETIEDE 395
KUOPIO UNIVERSITY PUBLICATIONS D. MEDICAL SCIENCES 395

MIKA ELO

Stress-Related Protein Synthesis in Mammalian Cells Exposed to Hydrostatic Pressure

Doctoral dissertation

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Institute of Biomedicine
Department of Anatomy
University of Kuopio

KUOPION YLIOPISTO
KUOPIO 2006

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ISBN 951-27-0575-3
ISBN 951-27-0592-3 (PDF)
ISSN 1235-0303

Kopijyvä
Kuopio 2006
Finland

Elo, Mika. Stress-Related Protein Synthesis in Mammalian Cells Exposed to Hydrostatic Pressure. Kuopio University Publications D. Medical Sciences 395. 2006. 74 p.
ISBN 951-27-0575-3
ISBN 951-27-0592-3 (PDF)
ISSN 1235-0303

ABSTRACT

In many cases, adequate mechanical stress is required by cells and tissues to maintain their normal functional structure and composition. On the other hand, mechanical forces of unphysiological mode or magnitude may damage or even kill their target cells. This work was designed to study and compare the stress response in two mammalian continuous cell lines after exposures to hydrostatic pressure (HP), to elevated temperature, or to various chemicals.

SV40 -immortalized T/C28a4 human chondrocytic cells and human HeLa cervical carcinoma cells were pressurized in a custom-made pressurization chamber. The results were compared to those observed when the cell were exposed to elevated temperature (HS) or chemicals known to interfere with intracellular Ca^{2+} homeostasis. When the changes in protein synthesis were analyzed with two-dimensional electrophoresis and mass spectrometry, it was found that the synthesis of certain stress proteins had been changed. Of the different pressure treatments, only continuous 30 MPa HP resulted in a marked down-regulation of total protein synthesis, and simultaneous up-regulation of heat shock protein 70 (Hsp70) and the Hsp90β isoform. This up-regulation of stress proteins differed from that seen after HS or treatments with chemical agents, suggesting that disturbed Ca^{2+} homeostasis was not responsible for the stress response seen after high continuous HP. Expression of the Hsp90α isoform remained at a constant level, possibly indicating functional differences between these two isoforms during HP loading.

Hsp90 is known to influence the Hsp70 gene expression, e.g., by repressing the activity of HSF1 transcription factor. The DNA-binding activity of HSF1 was increased in Hsp90 inhibitor geldanamycin (GA) -treated cells but in line with previous studies it remained unchanged in HP-treated cells. However, simultaneous treatment with GA and HP decreased the HSF1 DNA-binding activity. When Hsp70 mRNA and protein levels were evaluated, the effect of GA alone or together with HP loading was stronger compared with that seen in cultures treated only with HP. The increased expression of Hsp70 was not only due to transcriptional activation of the Hsp70 gene, but also to the fact that Hsp70 mRNA was stabilized during HP as well as GA treatments.

We further investigated how HP could change the syntheses of proteins unlikely to be major stress proteins. A protein whose synthesis was decreased in both of the cell lines during continuous 30 MPa HP was identified as eukaryotic elongation factor-2 (eEF-2). The reduced total levels of eEF-2 protein may be related to a decrease in protein synthesis since there was no change in the phosphorylation-regulated activity of this elongation factor.

National Library of Medicine Classification: QU 55.6, QU 58.5, QU 325, QU 350, QU 475

Medical Subject Headings: Cellular Structures; DNA-Binding Proteins; Gene Expression Regulation; Electrophoresis, Gel, Two-Dimensional; Enzyme Inhibitors; HSP70 Heat-Shock Proteins; HSP90 Heat-Shock Proteins; Hydrostatic Pressure; Peptide Elongation Factor 2; Phosphorylation; Spectrum Analysis, Mass; Protein Biosynthesis

ACKNOWLEDGEMENTS

This thesis work was carried out in the Institute of Biomedicine, Department of Anatomy, University of Kuopio during the years 1995-2006.

I owe my deepest thanks to my supervisor and Head of the Department of Anatomy, Professor Heikki J. Helminen, M.D., Ph.D. His enthusiasm and support have been invaluable during the years. I am deeply grateful to my second supervisor, Docent Mikko J. Lammi, Ph.D. His support, expertise in cell biology and patience especially during the past couple of years have been crucial.

I am greatly indebted to emeritus Professor Pekkä Mäenpää, M.D., Ph.D., and Docent Tuula Nyman, Ph.D., for their thorough reviewing of my thesis and constructive comments. I am also grateful to Ewen MacDonald, Ph.D., for revising the language of my thesis.

I thank my closest co-workers and colleagues Kai Kaarniranta, M.D., Ph.D., M.Sc., and Reijo Sironen, M.D., Ph.D., M.Sc., for their friendship, advice and support during the many years that we were "in the same boat". I wish to thank Hannu Karjalainen, M.Sc., Kari Törrönen, M.Sc., Mika Hyttinen, M.D., Teemu Långsjö, M.D., for their friendship, help and support; especially I would like to thank Kari Törrönen for his guidance in the world of computers. I wish to express my gratitude to Professor Markku Tammi, M.D., Ph.D., Docent Raija Tammi, M.D., Ph.D., and the entire hyaluronan research group for their advice and help with the laboratory work, as well as in creating such a pleasant environment in the Department of Anatomy. I wish to express my sincere thanks to all the other researchers, laboratory personnel as well as office staff in the Department of Anatomy for their help during the years, especially I would like to thank Ms. Elma Sorsa for her excellant technical assistance.

I am grateful to Seppo Auriola, Ph.D., Docent Leena Valmu, Ph.D., and Nisse Kalkkinen, Ph.D., for their expertise in mass spectrometry.

I wish to thank the head physicians in the health centers of Ilomantsi and Liperi for providing me with the opportunity to finish my thesis while I was doing clinical work.

My loving thanks belong to my sister and her family, my mother Eira and father Docent Heikki Elo, Ph.D., for their love and support. Finally, I wish to thank Satu for her patience and support during the last year.

For their financial support I want to thank the Academy of Finland, the Ministry of Education of Finland, the University of Kuopio, the North Savo Fund of the Finnish Cultural Foundation, the Sigrid Juselius Foundation, the Duodecim Foundation and TULES Graduate School.

Joensuu, July 2006

Mika Elo

ABBREVIATIONS

ActD	actinomycin D
ARE	AU-rich element
BiP	immunoglobulin heavy chain-binding protein
CAMKII	calcium/calmodulin-dependent protein kinase II
CBB	Coomassie Brilliant Blue
cDNA	complementary DNA
CID	collision-induced dissociation
DMSO	dimethyl sulfoxide
ECM	extracellular matrix
eEF	eukaryotic elongation factor
eIF	eukaryotic initiation factor
ER	endoplasmic reticulum
ERK	extracellular signal-regulated kinase
ESI	eletrospray ionization
GA	geldanamycin
GADD	growth arrest and DNA damage
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GRP	glucose-regulated protein
HP	hydrostatic pressure
HPLC	high performance liquid chromatography
HS	heat stress
Hsc	heat shock cognate
HSE	heat shock element
HSF	heat shock factor
Hsp	heat shock protein
IEF	isoelectric focusing
IL	interleukin
IP_3	inositol triphosphate
JNK	c-Jun N-terminal kinase
MAPK	mitogen-activated protein kinase
MEK	extracellular signal-regulated kinase kinase
MMP	matrix metalloproteinase
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PI3K	phosphoinositide 3-kinase
pkr	double-stranded RNA-dependent protein kinase
RTK	receptor thyrosine kinase
SDS	sodium dodecyl sulfate
SHR	steroid hormone receptor
STAT	signal transducer and activator of transcription
TCA	trichloroacetic acid
TG	thapsigargin
TIMP	tissue inhibitor of metalloproteinases
Trap1	tumor necrosis factor-associated protein 1
TPR	tetratricopeptide
2-DE	two-dimensional polyacrylamide gel electrophoresis

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by the Roman numerals I-IV.

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

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

III Elo MA, Kaarniranta K, Helminen HJ, Lammi MJ: Hsp90 inhibitor geldanamycin increases hsp70 mRNA stabilisation but fails to activate HSF1 in cells exposed to hydrostatic pressure. Biochim. Biophys. Acta 1743, 115-119, 2005

IV Elo MA, Karjalainen HM, Sironen RK, Valmu L, Redpath NT, Browne GJ, Kalkkinen N, Helminen HJ, Lammi MJ: High hydrostatic pressure inhibits the biosynthesis of eukaryotic elongation factor-2. J. Cell. Biochem. 94, 497-507, 2005

TABLE OF CONTENT

1. INTRODUCTION

Numerous cells and tissues in our bodies are continuously subjected to various kinds of mechanical stresses such as compression, shear stress, hydrostatic pressure, and osmotic pressure. In many cases, mechanical stress is required by the cells and tissues to maintain their normal functional structure and composition. For example, in articular cartilage, the chondrocytes are able to modify the amount and composition of the extracellular matrix when the joint is subjected to adequate loading (Kiviranta et al., 1988). On the other hand, immobilization of the joint disturbs the formation of the extracellular matrix and leads to cartilage softening (Jurvelin et al., 1986b) a process which is likely to impair the mechanical properties of tissue.

One of the mechanical forces encountered in the articular cartilage is hydrostatic pressure (HP), and studies made with isolated articular chondrocytes or intact articular cartilage plugs have shown that cells respond to HP in a pressure-dependent manner. Most of the earlier studies have been focused on the extracellular matrix, and thus have reported how its formation and composition changes when HP is applied. Recently also the intracellular responses to HP have received attention as they precede the extracellular responses. When cells are exposed to various chemical and physical stresses, the expression of heat shock proteins (Hsps) becomes induced (Wegele et al., 2004). Many, but not all, cell types experience high HP as a stress, and up-regulate Hsp70 synthesis (Kaarniranta et al., 1998; Kaarniranta et al., 2001; Takahashi et al., 1997). This protein functions as a chaperone and prevents protein aggregation and helps to refold misfolded proteins. Hsps have also numerous other functions under normal conditions. This thesis focuses on the stress response in pressure-sensitive mammalian cells when they are exposed to high HP.

2. REVIEW OF THE LITERATURE

2.1. Hydrostatic pressure as a physical factor

Hydrostatic pressure describes the pressure in water when it is compressed. In biological systems, HP acts strongly between two different phases, such as fluid and membranes. In the human body, HP is experienced by a variety of cells and tissues, with the highest forces being placed on the musculoskeletal system, especially the articular cartilage. Articular cartilage is an avascular tissue covering the diarthropodial joints, and articular cartilage chondrocytes occupy only 10% of the total tissue volume (Stockwell 1979). Due to its avascularity, the oxygen tension inside cartilage is only about 6%, which directs energy metabolism towards anaerobic glycolysis where glucose is metabolised into lactic acid (Lee et al., 2002; Marcus, 1973). The extracellular matrix (ECM) accounts for up to 90% of the dry weight of the tissue (Hardingham and Fosang, 1992) and is composed of a variety of collagens, polyanionic proteoglycans such as aggrecan, leucine-rich glycoproteins, and other small proteins (Buckwalter 1999). Based on their expression, collagen types II, IX, X and XI are said to be cartilage-specific (Cremer et al., 1998) whereas types VI and XII are present also in many types of non-cartilaginous tissues. Collagen type II fibrils form a mesh that entraps proteoglycans, which in turn bind cations. The osmotic pressure difference absorbs fluid into the tissue creating HP because the swelling is resisted by collagen fibrils. It is estimated that, at rest, HP within articular cartilage is approximately 0.2 MPa (2 atm) (Maroudas and Bannon, 1981). When articular cartilage is loaded, HP rises within milliseconds, after which the interstitial fluid is expelled out of the tissue and the extracellular osmolality becomes increased while pH is reduced (Urban, 1994). During normal walking, the

pressure in hip joint is approximately 3-4 MPa (30-40 atm), but may momentarily increase up to 20 MPa (200 atm) during some physical activities (Hodge et al., 1986). However, these pressures were measured from a 73-year-old female i.e. intra-articular pressures in a young and physically active individual may be even higher.

When load is applied to articular cartilage, the chondrocytes are subjected to a change in their physico-chemical environment. In addition to HP, the cells experience deformation, strain, and streaming potentials caused by fluid flow, as well as changes in tissue osmolality. Because of these concomitantly existing variables, *in vivo* animal models and *in vitro* models utilizing mechanical compression are unable to determine the importance of pure HP on cartilage. Nevertheless, *in vivo* animal models have shown that joint loading both thickens the cartilage and increases the proteoglycan concentration (Kiviranta et al., 1988; Tammi et al., 1983), resulting in the formation of mechanically stronger tissue (Jurvelin et al., 1986a). In addition, the chondrocytes in load-bearing areas are larger, and have a greater volume of intracellular organelles (Eggli et al., 1988). Reduced loading or immobilization reduces the concentration of proteoglycan and hyaluronan (Haapala et al., 1996; Kiviranta et al., 1987), which leads to softening of the cartilage (Jurvelin et al., 1986b).

2.2. Hydrostatic pressure and the cell

2.2.1. Hydrostatic pressure -induced changes in the extracellular space

A correctly balanced ECM is a crucial part of the normal function of cartilaginous tissue and HP is a potential factor that regulates its maintenance. Several *in vitro* studies have been performed with isolated HP and articular cartilage, isolated

articular cartilage chondrocytes or continuous cell lines that express a chondrocytic phenotype.

To understand the effect of HP on the ECM over the past two decades it has become evident that HP is an important factor regulating the physical environment of the chondrocytes. The cells' response to HP is clearly dependent on the magnitude of the pressure, and also on the loading protocol (continuous or cyclic pressure). Most of the studies have utilised pressures claimed to be in the physiological range, i.e., less than 20 MPa. In articular cartilage slices, continuous HP of 2.1-15 MPa was shown to stimulate proteoglycan synthesis, whereas lower HP has an adverse effect (Hall et al., 1991; Lippiello et al., 1985). In isolated primary articular cartilage chondrocytes, pressures over 10 MPa inhibit proteoglycan synthesis, whereas lower pressure either increases synthesis or has no effect (Jortikka et al., 2000; Lammi et al., 1994; Parkkinen et al., 1993; Smith et al., 1996). Extracellular osmolality appears to be an important factor in addition to applied HP, since elevated extracellular Na^+ has been shown to have an additive effect on proteoglycan synthesis in bovine chondrocytes when they are subjected to 5 MPa pressure (Browning et al., 2004). Continuous HP at a physiological level also induces collagen type II mRNA expression (Hall et al., 1991), though different cell culture conditions during HP treatment can abolish this phenomenon or even decrease mRNA levels (Smith et al., 1996).

Cyclic HP at physiological level increases proteoglycan synthesis in cartilage explants and isolated chondrocytes, although the duration and frequency of the HP treatment significantly modifies the response (Ikenoue et al., 2003; Lammi et al., 1994; Parkkinen et al., 1993; Smith et al., 1996). Cyclic HP up to 10 MPa increases collagen type II mRNA levels, but also in this case, different cell culture conditions during HP have been shown to cause even decreased expression (Ikenoue et al., 2003; Smith et al.,

2000; Smith et al., 1996). Type I collagen mRNA levels are down-regulated by cyclic HP (Wong et al., 2003).

Articular cartilage responds to mechanical loading by changing the synthesis rate of ECM constituents, and also the ECM degrading enzymes and their inhibitors. Cyclic HP decreases matrix metalloproteinase-2 (MMP-2) levels in human osteoarthritic chondrocytes (Trindade et al., 2004) and collagenase-3 mRNA (MMP-13) levels in chondrocytes seeded in alginate (Wong et al., 2003). MMP-2 degrades collagens I, X, XI, and XIV, whereas MMP-13 is known to have an important role in collagen type II metabolism (Cawston et al., 1999). Cyclic HP can elevate the mRNA levels of tissue inhibitor of metalloproteinase 1 (TIMP-1) (Wong et al., 2003), but it has also been reported that TIMP-1 release into extracellular space is not affected in osteoarthritic chondrocytes (Trindade et al., 2004). Continuous HP up to 50 MPa does not change the mRNA levels for stromelysin (MMP-3) or TIMP-1 (Takahashi et al., 1998). This suggests that the decrease in proteoglycan concentration observed after high HP is more likely to be a result of low synthesis rather than increased breakdown since stromelysin has a crucial role in proteoglycan metabolism (Cawston et al., 1999).

2.2.2. Hydrostatic pressure -induced changes in the intracellular space

In addition to affecting the ECM production, HP has been reported to evoke a variety of changes in the intracellular space. Low continuous HP of 0.016 MPa caused cell membrane depolarization in isolated chondrocytes and fibroblasts, whereas cyclic pressure resulted in frequency-dependent hyperpolarization or depolarization of these cells, changes that involved both Na^+ channels and Ca^{2+} -dependent K^+ channels (Wright et al., 1992). In addition, increased extracellular osmolality in the form of an elevated

extracellular Na^+ concentration further increased the HP-induced rise in intracellular Na^+ concentration (Browning et al., 2004). Calcium is an important regulator of many cellular functions, and it is also thought to participate in mechanotransduction. Intracellular Ca^{2+} concentrations were elevated after mechanical compression in primary chondrocytes seeded in agarose (Roberts et al., 2001), and also in primary chondrocytes and immortalized chondrocytes treated with isolated HP (Browning et al., 2004; Mizuno, 2005). One recent study conducted with immortalized chondrocytes suggested that high continuous HP initially results in the generation of inositol triphosphate (IP_3) which then mobilises Ca^{2+} from the intracellular stores (Browning et al., 2004). Later, the elevated cytosolic levels of Ca^{2+} become sustained because of a capacitative influx of Ca^{2+} through cell membrane channels. However, there may be cell-type specific regulation mechanisms involved in this Ca^{2+} signalling since, in mouse fibroblasts, high continuous HP failed to alter the intracellular calcium homeostasis (Crenshaw and Salmon, 1996). High continuous HP also inhibits glucose intake by chondrocytes hence disturbing glycolysis and ATP production (Lee et al., 2002). This may lead to a decrease in ECM production being seen during the same pressure conditions since sulphate incorporation into proteoglycans requires ATP.

Various cellular stresses often lead to inhibition of protein synthesis. For instance, heat stress at 41-42°C inhibits the functions of one fraction of active polysomal ribosomes, which recover their activity after removal of the stress (Duncan and Hershey, 1989). Early studies in bacteria (Landau 1966 and 1967; Pope et al., 1975) and eukaryotic cells (Scheck and Landau, 1982) have shown that also high hydrostatic pressure can inhibit protein synthesis. In cell-free *E. coli* preparations, a progressive inhibition of protein synthesis was observed, starting at approximately 20 MPa that was

instantaneously reversible after release of the pressure (Schwarz and Landau, 1972b). Studies on whole-cell preparations of *E. coli* have shown that 67 MPa HP totally inhibited protein synthesis at the elongation stage (Schwarz and Landau, 1972a). In eukaryotes, the activity of rat ribosomes decreased as the applied pressure was increased, and total inhibition was reached at 240 MPa (Lu et al., 1997). The mechanism of protein synthesis inhibition is still not exactly understood. The response of bacterial protein synthesis to HP has been associated with the structure of the 30S ribosomal subunit (Landau et al., 1977; Smith et al., 1975), while it is also suggested that the binding of aminoacyl-tRNA into the ribosome-mRNA complex is disturbed under HP (Schwarz and Landau, 1972b). HP did not, however, affect amino acid permeability, amino acid activation, aminoacyl-tRNA formation (Schwarz and Landau, 1972a), or peptide bond formation (Schwarz and Landau, 1972b). There have been conflicting results concerning the effect of HP on ribosomal stability. Earlier investigations suggested that a decrease in protein synthesis occurs without any simultaneous destabilization of the ribosomes (Pope et al., 1975; Schwarz and Landau, 1972a). However, it has been later demonstrated that ribosome dissociation does take place under high HP in prokaryotes (Gross and Jaenicke, 1990; Gross et al., 1993; Niven et al., 1999), and the activity of eukaryotic ribosomes is also decreased under HP loading (Lu et al., 1997).

In primary chondrocytes, continuous HP over 15 MPa alters the structure of the Golgi apparatus, and disrupts the organization of stress fibers (Parkkinen et al., 1993; Parkkinen et al., 1995). Since proteoglycan modifications take place in the Golgi apparatus, this observation made under continuous HP may partly explain the decrease in proteoglycan synthesis. The microtubules in chondrocytes appear to be rather resistant to high continuous HP and the use of specific inhibitors known to affect microtubule

stability have indicated that the inhibition of proteoglycan synthesis was not linked to microtubules (Jortikka et al., 2000). However, the increase in proteoglycan secretion seen after cyclic HP is likely to be dependent on vesicle traffic associated with microtubules (Jortikka et al., 2000). Intact stress fibers are needed in the mechanical stimuli-induced change in membrane potential since this phenomenon was not apparent in chondrocytes or bone cells when the actin cytoskeleton was disrupted with cytochalacin B or D (Salter et al., 1997; Wright et al., 1992). In contrast to continuous HP, cyclic HP of up to 30 MPa had only a minor effect on the organelle or cytoskeletal structures. Continuous HP induces cytoskeletal changes also in non-chondrocytic cells such as HeLa cells, where pressures over 20 MPa can disrupt the structures of the intermediate filaments, stress fibers and focal contact sites (Crenshaw et al., 1996). In focal contact sites, the actin cytoskeleton is linked via multiprotein complexes to integrins that mediate cell attachment to the ECM components (Wozniak et al., 2004). Integrins are known to be involved in mechanotransduction in chondrocytes (Wright et al., 1997) and bone cells (Salter et al., 1997). In chondrocytes, hyperpolarisation caused by intermittent pressure-induced strain involves integrins $\alpha 5$ and $\beta 1$ (Wright et al., 1997), and, in another report, this integrin combination was noted to promote chondrocyte survival in chondrocytes cultured on fibronectin-coated culture plates (Pulai et al., 2002).

cDNA array data has revealed that cyclic and static 5 MPa pressures have partially differential effects in chondrosarcoma cells (Karjalainen et al., 2003). There were several similarly down-regulated genes, for example certain genes involved in cell adhesion such as integrin α_E and β_8. The use of the same cell line in another cDNA array study further revealed that continuous HP of 30 MPa up-regulated certain interesting genes e.g. those involved in heat-shock response or growth arrest, but down-regulated

genes that are involved in cellular differentiation (Sironen et al., 2002). The heat-shock response (see next Chapter) was characterized by up-regulation of heat-shock proteins 27, 40 and 70 (Hsp27, Hsp40 and Hsp70, respectively) and occurred after high continuous but not cyclic HP in chondrosarcoma cells and immortalized chondrocytes (Kaarniranta et al., 1998; Sironen et al., 2002; Takahashi et al., 1997). The mechanism by which Hsp70 mRNA and protein accumulate in HP is rather unique because it is due to mRNA stabilization rather than to transcriptional activation (Kaarniranta et al., 1998). The heat-shock response after HP appears to be a rather cell line-independent phenomenon (Kaarniranta et al., 2000; Kaarniranta et al., 2001) with the exception of primary articular cartilage chondrocytes which exhibit only negligible Hsp70 induction (Kaarniranta et al., 2001).

High continuous HP is known to increase the expression of interleukin-6 (IL-6) and tumor necrosis factor-α mRNAs, and to decrease transforming growth factor-β mRNA levels in chondrosarcoma cells (Takahashi et al., 1997; Takahashi et al., 1998), whereas cyclic HP of 10 MPa decreased IL-6 secretion in chondrocytes isolated from osteoarthrotic cartilage (Trindade et al., 2004). IL-4 has been shown to be released from chondrocytes subjected to pressure-induced strain, and an autocrine or paracrine action of IL-4 was claimed to be crucial to the membrane hyperpolarization occurring during loading (Millward-Sadler et al., 1999).

Since the above changes involve gene expression, it is likely that the HP-induced effects on the cellular function are transmitted through activation of specific signal transduction cascades. The intracellular signaling of β1 integrin in chondrocytes involves the Ras-mitogen-activated protein kinase (Ras-MAPK) signaling pathway (Shakibaei et al., 1999). The MAPK system has three well-characterized subfamilies of kinases, namely extracellular signal-regulated kinases (e.g. ERK-1 and ERK-2), c-Jun

N-terminal or stress-activated protein kinases (JNK), and the p38 kinases (Johnson and Lapadat, 2002). The activation of the ERK pathway is linked to cell proliferation, differentiation, and survival, whereas p38 and JNK are usually activated under stress conditions by inflammatory cytokines and sometimes by growth factors. In chondrosarcoma cells, ERK-1 and especially ERK-2 were activated after continuous HP higher than 15 MPa (Kopakkala-Tani et al., 2004), and the same pathway was triggered by static and dynamic mechanical compression of cartilage explants (Fanning et al., 2003; Li et al., 2003). It appears that different modes of mechanical stress may activate different signal transduction pathways, because HP failed to activate p38 and JNK while both of these kinases were activated by static compression (Fanning et al., 2003). In addition to direct mechanical loading, ERK is also activated by fluid flow in articular chondrocytes, and in this model, ERK -activation was linked to a simultaneous decrease in aggrecan promoter activity (Hung et al., 2000).

High continuous HP also resulted in the activation of PI3K pathway (Kopakkala-Tani et al., 2004) which is known to control cell growth, proliferation, metabolism, and survival, as well as the arrangement of cytoskeletal structures (Wymann et al., 2003). The target of the PI3K signaling pathway triggered by HP remains unknown. Activation of the pathway may promote cellular survival because one of the best-characterized PI3K-dependent pathways is the activation of Akt/protein kinase B (Wymann et al., 2003). Akt is known to enhance cell survival since it can inactivate a number of pro-apoptotic proteins. Interestingly, the viability of chondrosarcoma cells was not affected by continuous 50 MPa HP (Takahashi et al., 1998), and a cDNA array analysis revealed no increase in the expression levels of apoptotic genes after HP of 30 MPa (Sironen et al., 2002).

2.3. The heat shock response

2.3.1. The heat shock proteins

Cells and tissues frequently encounter environmental stress factors such as elevated temperature, heavy metals, ischemia-reperfusion, oxygen radicals, and also mechanical loading. This may lead to changes in protein structure and function, and should protein aggregation occur, this can endanger the survival of the cells. To prevent this phenomenon, cells increase the expression of the evolutionarily highly conserved Hsps (Morimoto, 1998), which act as chaperones and refold partly denatured proteins (Wegele et al., 2004). In addition to their role in the stress response, these proteins are known to participate in numerous tasks also under normal environmental conditions. For example they assist in folding of newly synthesized proteins, guide protein translocation across membranes, and control the activity of various regulatory proteins such as transcription factors and kinases (Wegele et al., 2004). The Hsps are divided into classes according to their molecular size or function (Table 1.): (I) the glucose-regulated protein 170 (Grp170); (II) the Hsp105/Hsp110 protein family; (III) the Hsp90 family; (IV) the Hsp70 family; (V) the Hsp60 family; the (VI) The Hsp40 family, and (VII) the small Hsps. Grp170 is located in the rough endoplasmic reticulum (ER). Its physiological role is poorly characterized, but it is known that Grp170 binds to secreted proteins in the ER (Easton et al., 2000). The Hsp105/Hsp110 family has two members, Hsp105α and Hsp105β. Hsp105 regulates the activity of the heat shock cognate protein 70 (Hsc70) chaperone system (Yamagishi et al., 2000). The Hsp90 family has five members, glucose-regulated protein 94 (Grp94), Hsp90α, Hsp90β, tumor necrosis factor receptor-associated protein 1 (Trap1 or Hsp75) and the recently discovered and so far poorly

characterized Hsp90N (Felts et al., 2000; Grammatikakis et al., 2002; Hickey et al., 1989; Lee, 2001; Wegele et al., 2004). Grp94 is located in the endoplasmic reticulum and cell membrane, and is up-regulated in several situations, for example after glucose starvation, hypoxia, low pH, or disturbed Ca^{2+} homeostasis (Lee, 2001). It functions as a chaperone, and is also a Ca^{2+} binding protein and it can protect the cell against apoptosis (Lee, 2001). Hsp90 is an abundantly expressed protein (1-2% of total soluble proteins in the cell) with multiple functions and is found widely distributed inside the cell, and recent studies suggest that it also acts in the extracellular space (Eustace et al., 2004). Trap1 is located in mitochondria and though its functions have not been yet fully established, it may protect the cells against apoptosis (Masuda et al., 2004).

The three members of the Hsp70 family are Hsp70, Hsc70 and Grp78. Hsp70 is induced by stress and is a widely distributed chaperone with additional regulatory functions, while Hsc70 is a constitutively expressed chaperone. Grp78, also called immunoglobin heavy chain-binding protein (BiP), is located in the endoplasmic reticulum, and its gene regulation and function resembles Grp94 (Lee, 2001).

The smaller Hsps usually act as general chaperones (Hsp60 in mitochondria) but some are targeted towards specific substrates. Hsp47 belongs to the Hsp40 family and is a collagen-specific chaperone located in the endoplasmic reticulum (Nagata, 2003). It is required in collagen synthesis and triple helix assembly in connective tissues. The smallest Hsps vary in size from 15 to 30 kDa. This family includes, for example, Hsp27 which is known to protect cell against apoptosis and to stabilize the actin cytoskeleton (Concannon et al., 2003).

Table 1. Heat shock protein classes and the functions of stress proteins

Class	Proteins	Functions
I	Grp170	regulates protein secretion from the ER ?
II	Hsp105α, Hsp105β	prevent protein aggregation, regulation of the Hsc70 chaperone system
III	Grp94	protein folding, regulation of apoptosis
	Hsp90α, Hsp90β	protein folding and degradation, regulation of cellular signalling
	Trap1/Hsp75	regulation of apoptosis ?
	Hsp90N	regulation of neoplastic transformation ?
IV	Hsp70, Hsc70	Regulation of protein folding, transport and aggregation. Regulation of apoptosis and cellular signaling
	Grp78	protein folding, regulation of apoptosis
V	Hsp60	protein folding
VI	e.g. Hsp47	regulation of collagen maturation
VII	e.g. Hsp27	regulation of apoptosis, stabilization of actin filaments

2.3.2. Hsp70 structure, regulation and function

In mammals, heat shock protein 70 has two isoforms; a 73-kDa form that is constitutively expressed (Hsc70) and a 72-kDa, stress-inducible form (Hsp70) (Milner and Campbell, 1990). Hsp70 is encoded by two different genes, hsp70-1 and 2. Hsc70 is encoded by hsp70-Hom that shares 90% homology with Hsp70-1 gene (Milner and Campbell, 1990). Both hsc70-Hom and hsp70 genes have promotor regions with basal transcriptional regulatory sequences (Lis and Wu, 1993; Wilke et al., 2000; Williams et al., 1989), as well as heat shock elements (HSE). In the case of hsp70 genes, these regulatory sites are occupied by heat shock factor-1 (HSF-1) following environmental stress such as elevated heat (Sarge et al., 1993). Before HSF1 can acquire its transcriptional activity, it must undergo oligomerization from a monomer into a trimer, and bind to the HSE-region, where it is ultimately subjected to hyperphosphorylation (Pirkkala et al., 2001). In conjunction with the basal transcription factors, this results in induction of hsp70 transcription (Lis and Wu, 1993). In addition to transcriptional activation the hsp70 mRNA levels can be regulated by mRNA stabilization. hsp70 mRNAs contain AU-rich elements (ARE) in their 3' untranslated region, which, together with various regulatory proteins, are known to associate with a shorter half-life of the mRNA molecule (Bevilacqua et al., 2003). Following heat shock treatment, the hsp70 mRNA becomes stabilized (Theodorakis and Morimoto, 1987) by a mechanism that is not fully established. Hsp70 protein may control its own mRNA stability (Bevilacqua et al., 2003) along with additional regulators, and a recent study has suggested that a double-stranded RNA-dependent protein kinase (*pkr*) is crucial for heat-induced hsp70 mRNA stabilization (Zhao et al., 2002). Interestingly, the Hsp90 chaperone complex was found to be an important regulator of the *pkr* activity (Donze et

al., 2001).

Hsp70 proteins consist of a 44-kDa amino terminal ATPase domain, a 18-kDa peptide binding domain, and a 10-kDa carboxy-terminal domain of partly unknown function (Wegele et al., 2004). The carboxy-terminal domain is involved at least in the cofactor interaction because it contains an EEVD motif that is known to bind the tetratricopeptide (TPR) domain of various cofactors. TPR is a structural motif present in a wide range of proteins with different functions, and it mediates protein-protein interactions and the assembly of multiprotein complexes (D'Andrea and Regan, 2003). Hsp70 exists in close proximity to various co-chaperones, of which Hip and bag1 are known to affect its ATPase activity (Nollen et al., 2001). This results in coordinated regulation of Hsp70 chaperone activity because, after substrate peptide binding, ATP hydrolysis can stabilize the substrate protein – Hsp70 interaction (Greene et al., 1995).

When cells are subjected to a stress treatment, Hsp70 chaperones together with cofactors are known to prevent protein aggregation and to refold misfolded protein, whereas in normal conditions they assist in the folding of newly synthesized proteins (Hartl and Hayer-Hartl, 2002), help to translocate proteins across membranes (Zimmermann, 1998), uncoat clathrin-coated vesicles (Chappell et al., 1986), and are involved in protein degradation in proteasomes (Shin et al., 2005). Hsp70 controls the activity of a variety of regulatory proteins. For example, together with Hsp90 and co-chaperones it regulates cell growth via the steroid hormone receptors and the MAPK pathway (Nollen and Morimoto, 2002). Hsp70 is also an antiapoptotic chaperone since it can inhibit caspase activity (Beere and Green, 2001)

2.3.3. Hsp90 structure, regulation and function

In mammalian cells, hsp90α and hsp90β are the two genes that encode Hsp90 proteins. They partly differ in their transcriptional regulation because their promotor regions are different. However, both genes are activated after heat shock treatment. The first exon of both genes is untranslated, and the translation is started at the beginning of the second exon. In hsp90α, the HSE targeted to HSF1 is located in the promotor region from -96 to -60 (Zhang et al., 1999). Together with this region, the upstream enhancers are crucial in the highly inducible expression of Hsp90α. In the hsp90β gene it is the HSE in the first intron that is primarily needed for both constitutional and inducible expression of hsp90β (Shen et al., 1997). However, the 5' flanking region contains additional HSE sequences partly overlapping with binding elements to the signal transducer and activator of transcription 1 and 3 (STAT1 and 3), and nuclear factor interleukin-6 (NF-IL6). HSF1 has a weaker binding affinity to these HSE sequences than to the sequence in the first intron (Shen et al., 1997). The other binding sites are occupied with structurally related transcription factors, for example, after IL-6 and interferon-γ treatments (Stephanou et al., 1998; Stephanou et al., 1999). An additional regulator of hsp90β was recently discovered: its promotor region contains a binding site for tumor suppressor p53, a protein known to take part in cell cycle control, DNA damage repair, and apoptosis (Zhang et al., 2004).

At the protein level, Hsp90α and Hsp90β isoforms share 85% sequence identity (Hickey et al., 1989). The Hsp90 protein has an amino terminal ATPase domain and a carboxy terminal dimerization domain, separated by a middle domain (Maruya 1999; Prodromou et al., 1997). Ansamycin antibiotics such as geldanamycin (GA) have been widely used to study the function of Hsp90. The mechanism of GA is attributed to the

fact that this chemical is a specific Hsp90 inhibitor that has a high affinity for the ATPase site (Roe et al., 1999). ATP hydrolysis is considered to be crucial for Hsp90 function and thus when GA disturbs the normal chaperone function of Hsp90, the dissociation of Hsp90-substrate protein complex is seen. This results in a misfolded substrate protein that is degraded by the proteasome (Schulte et al., 1997). The carboxy-terminal domain is recruited to Hsp90 dimerization permitting the formation of homodimers (α–α, β–β) and heterodimers (α–β) *in vivo* (Minami et al., 1991; Nemoto et al., 1995). However, it has also been suggested that, under non-stressful conditions, the isoforms join into larger oligomers in the cytosol (Nemoto and Sato, 1998). At the carboxy-terminus one can find the pentapeptide MEEVD that is needed for the interaction with co-chaperones such as Hop via its TPR domain (Ramsey et al., 2000). Although the function of the middle domain is still obscure, it has been suggested to be involved in substrate binding (Muller et al., 2004) or in the ATPase cycle (Wegele et al., 2003).

In various normal and pathological cellular processes, the expression of hsp90 gene is induced, and it is involved in cell growth, differentiation, apoptosis, and embryonic development. Hsp90α gene expression in known to be induced during certain stages of the cell cycle (Jerome et al., 1993), and by growth factors (Jerome et al., 1991). In acute leukemia, hsp90α mRNA levels are markedly elevated when compared with hsp90β mRNA levels (Yufu et al., 1992), and in breast cancer elevated hsp90α levels are associated with poorer prognosis (Jameel et al., 1992). On the other hand, in gastric cancer, hsp90β mRNA transcripts are up-regulated especially in poorly differentiated cancer types (Liu et al., 1999), and increased expression of hsp90β has been shown to be associated with multidrug resistance of the transformed cells (Bertram et al., 1996; Liu et al., 1999). Hsp90 is also up-regulated in certain autoinflammatory diseases. In

systemic lupus erythematosus, the Hsp90 protein level is increased in peripheral blood mononuclear cells (Dhillon et al., 1993) due to activation of the hsp90β gene (Twomey et al., 1993). In rheumatoid arthritis, the patients express circulatory antibodies to Hsp90 (Hayem et al., 1999). During embryogenesis, the two Hsp90 isoforms are differently expressed (Vanmuylder et al., 2002), and are believed to have specific functions, since homozygotic knock-out mice harboring the mutated hsp90β gene die because they fail to develop the placental labyrinth (Voss et al., 2000).

Hsp90 together with its co-chaperones and accessory proteins, is an essential regulator of many crucial cellular functions, e.g. cytoskeletal organization, protein folding and degradation, gene transcription and kinase activity (Wegele et al., 2004). Hsp90 is associated with microtubules (Redmond et al., 1989), and is known to regulate the polymerization of tubulin (Garnier et al., 1998). Hsp90 co-localizes also with intermediate filaments (Czar et al., 1996) and an *in vitro* study has suggested that Hsp90 may crosslink actin filaments (Koyasu et al., 1986).

During the protein folding process, Hsp90 forms a protein complex with co-chaperones Hsp70 and Hsp40 together with accessory proteins Hop, p23, and immunophilins such as peptidylprolyl isomerase FKBP52 (Hernandez et al., 2002; Morishima et al., 2003). The steroid hormone receptors (SHRs) are among the most extensively studied substrate proteins for this complex. Hsp90 is responsible for maturation of those receptors into the ligand-binding state (Whitesell and Cook, 1996; Morishima et al., 2000). In addition, dynein-mediated nuclear translocation of glucocorticoid receptors and p53 along the microtubules requires the Hsp90-binding immunophilins (Galigniana et al., 2004; Harrell et al., 2004). In proteasomes, Hsp90 is involved in protein degradation (Whittier et al., 2004). Hsp90 inhibitors have been reported to express antitumor activity as they can disrupt the normal chaperone-substrate

protein relationship (Kamal et al., 2004). Consequently, the substrate proteins such as Raf-1 kinase are directed towards the proteasomes for protein degradation (Schulte et al., 1997).

At present, Hsp90 is known to regulate the activity of various transcription factors and kinases controlling the cell cycle, growth and apoptosis (Wegele et al., 2004). As stated earlier, SHRs require Hsp90 assistance before they can acquire their DNA binding activity. There are other Hsp90 -regulated transcription factors e,g., tumor suppressor p53 (Muller et al., 2004) and HSF1(Bharadwaj et al., 1999). p53 is known to be activated in response to DNA damage and oncogenic transformation, and it also controls the cell cycle (Hofseth et al., 2004). Hsp90 is recruited to maintain p53 in its active form. HSF1 regulates the Hsp70 gene transcription and is repressed by Hsp90 (Zou et al., 1998) (Fig. 1). Hsp90 participates in the Ras-Raf-1-MEK-MAPK pathway that transmits mitogenic stimuli from the plasma membrane to the nucleus as it binds and regulates various key regulatory kinases such as Raf-1 (Schulte et al., 1995; Schulte et al., 1996) and its downstream kinases ERK 1/2 (Setalo et al., 2002) (Fig. 1). Finally, it is also involved in the PI3K pathway via interactions with 3-phosphoinositide-dependent kinase 1 (PDK-1) (Fujita et al., 2002) and its substrate Akt/protein kinase B (Basso et al., 2002; Storz and Toker, 2002). This signal transduction cascade is involved in cellular survival, glucose transport and metabolism, tumor progression as well as participating in protein translation (Storz and Toker, 2002).

2.4. The proteomics

The genome describes the whole information of an organism that is encoded in the DNA, while the term proteome is used to describe the entire complement of proteins

in some biological system. Proteomics refers to the large scale study of proteins, and includes their separation, identification and characterization.

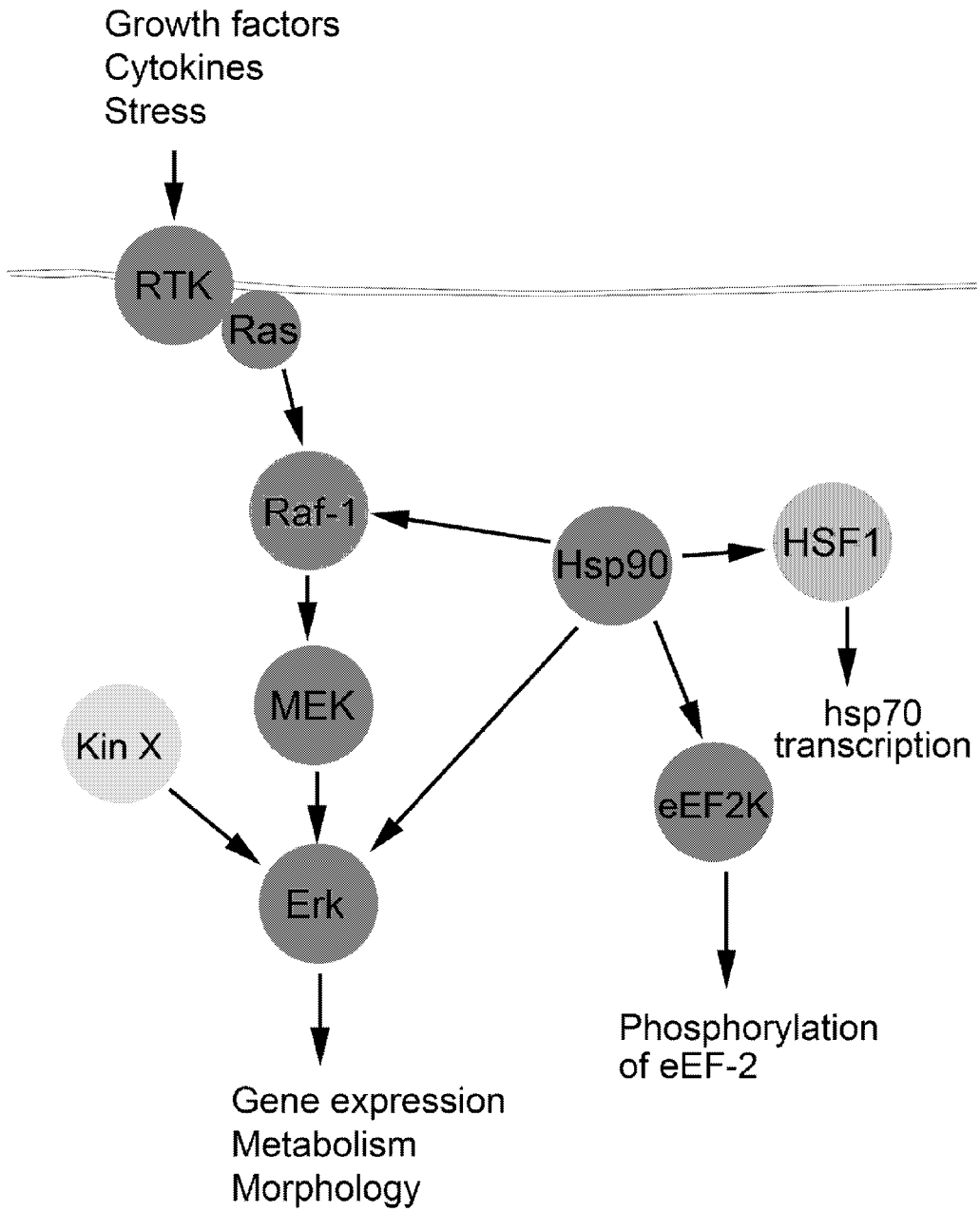

Figure 1. Schematic illustration of some of the many regulatory functions of Hsp90. Hsp90 participates in the Ras-Raf-MEK-MAPK pathway tha transmits mitogenic stimuli from the plasma membrane to the nucleus. During high continuous HP, activation of ERK is, however, at least partly dependent on yet unknown kinase (Kin X, kinase X) (Kopakkala-Tani et al., 2004) eEF-2 specific kinase (eEF2K) is known to interact with Hsp90, hence by regulating the phosphorylation status of eEF-2, Hsp90 influences the rate of protein synthesis. Hsp90 negatively regulates the activity of HSF1, and dissociation of this complex is needed before HSF1 can acquire its transcriptional activity.

3. AIMS OF THE STUDY

The mammalian cells are constantly subjected to environmental stresses such as mechanical loading. Stress of an unphysiological magnitude is known to elicit a stress response characterized by up-regulation of Hsps. The highest mechanical forces in the form of HP are attributed to the musculoskeletal system, and especially articular cartilage and mechanical loading is known to affect the function of the chondrocytes. The cellular responses to HP are both amplitude- and frequency-dependent, as well as being cell type-dependent. Interestingly, although non-physiologically high HP usually induces a stress response in cells by some unknown mechanism, primary chondrocytes have been shown to be pressure-resistant. The aims of the study were:

- To study the changes in protein expression in pressurized cells known to be sensitive to high HP, and to compare the HP-induced stress response to those induced by disturbed intracellular calcium homeostasis and elevated temperature (**I**)

- To study the effect of HP on Hsp90α and Hsp90β expression (**II**)

- To study whether Hsp90 is involved in the regulation of HSF1 activity and Hsp70 expression during HP (**III**)

- To study the expression and phosphorylation-regulated activity of eukaryotic elongation factor 2 (eEF-2) during HP (**IV**)

4. MATERIALS AND METHODS

4.1. Cell culture and treatments

SV40 -immortalized T/C28a4 human chondrocytic cells provided by Dr. M.B. Goldring (Goldring et al., 1994) and human HeLa cervical carcinoma cells (American Type Culture Collection, ATCC) were cultured in a humidified 5% CO_2/95% air atmosphere at 37°C in DMEM with 10% fetal calf serum, penicillin (50 units/ml), streptomycin (50 units/ml) and 2 mM glutamine. The cells were grown to a subconfluent state before the experiments. Before HP treatments, the medium was changed and 15 mM Hepes (pH 7.3) was added. To expose the cells to pressure, the culture dishes were filled with the medium described above and sealed with a plastic membrane. The apparatus for hydrostatic pressurization of the cells has been previously described in detail (Parkkinen et al., 1993). Cells were exposed either to continuous or cyclic (0.5 Hz, 1 second on / 1 second off) HP of 4 or 30 MPa. Control dishes were prepared similarly and kept in an incubator. In the same experiments, the cell were allowed to recover after the HP treatment, during this period the dishes were kept in an incubator.

For heat shock treatment, the culture plates were filled with medium, sealed with a plastic membrane and submerged in a 43°C water bath. An intracellular calcium imbalance was produced by adding thapsigargin (TG) (25 μM stock solution in dimethyl sulfoxide (DMSO), final concentration 25 nM) or calcium ionophore A23187 (1 mM stock solution in DMSO, final concentration 1 μM) into the cultures. TG is a highly specific inhibitor of sarco-endoplasmic reticulum Ca^{2+} ATPases and it increases the cytosolic Ca^{2+} concentration via passive leakage of Ca^{2+} ions from the endoplasmic reticulum, while the Ca^{2+} ionophore, A23187, increases intracellular Ca^{2+} concentration

by transporting cations across the cell membrane. Hsp90 was inhibited with geldanamycin (2 mM stock solution in DMSO, final concentration 0.5-10 μM) and microtubular structures were disassembled with nocodazole (1 mM stock solution in DMSO, final concentration 10 μM). The stability of mRNA was studied after pressurization by adding a fresh medium supplemented with actinomycin D (ActD) to the cultures at a final concentration of 3 μg/ml. The stability of proteins after HP treatment was studied by introducing 10 μM cycloheximidine (Sigma) into the fresh culture medium.

4.2. *In vitro* metabolic labeling and two-dimensional electrophoresis (2-DE)

To study the protein synthesis immediately after different stress exposures (I), the cells were washed with phosphate-buffered saline (PBS) after treatments, and the medium was replaced with methionine- and cysteine-free DMEM supplemented with antibiotics, 10% fetal calf serum and 4 mM glutamine. The labeling was performed with 50 μCi/ml Tran ^{35}S label (ICN) for 1 h in a cell incubator. For conventional SDS-PAGE, whole cell extracts were prepared as previously described (Mosser et al., 1988). For 2-DE, the cells were washed twice with PBS after labeling, and lysed with a solution containing 9.8 M urea, 2% (v/v) Nonidet P-40, and 100 mM dithiotreitol after which the samples were briefly sonicated and centrifuged. The amount of incorporated label was determined with trichloroacetic acid (TCA) precipitation, and a volume containing 1x10^6 TCA-precipitable counts per minute (c.p.m) was loaded to either conventional SDS-PAGE or to 2-DE.

For 2-DE (I) isoelectric focusing (IEF) was performed with self-casted polyacrylamide gels containing 9.3 M urea, 2.7% (v/v) ampholyte pH 3–10, 2.7% (v/v)

ampholyte pH 5–7, and 2% (v/v) Nonidet P-40. After IEF, the gels were equilibrated in a solution containing 0.06 M Tris-HCl pH 6.8, 2% SDS, 100 mM dithiotreitol, and 10% (v/v) glycerol. Then proteins were further separated using 10% SDS-polyacrylamide gel. For metabolically labeled samples, the gels were dried and the autoradiography signal was detected by using a PhosphorImager (Molecular Dynamics). Autoradiograms were analyzed visually without any computer-assisted image analysis. The unlabelled samples used for mass spectrometric (MS) analysis were separated with 2-DE as described above. The gels were stained with 0.05% (w/v) Coomassie Brilliant Blue (CBB) dissolved in 50% (v/v) methanol and 10% (v/v) acetic acid.

To study the protein synthesis during HP exposure (IV), the metabolic labeling was performed by injecting Tran^{35}S-label (30 μCi/ml) with syringe and hypodermic needle through the plastic membrane to the culture medium before the onset of pressurization. To avoid harmful amino acid deprivation during longterm pressurization experiments, the labeling medium contained 10% of standard DMEM and 90% of methionine- and cysteine-free DMEM. The medium was supplemented with antibiotics, 10% fetal calf serum and 4 mM glutamine. After labeling, the samples were prepared as previously described and a volume containing 5x10^5 TCA-precipitable cpm was loaded onto the gel.

For 2-DE (IV), the IEF was performed according to the manufacturer's instructions using 13 cm long 3-10 non-linear IPG strips (Amersham Pharmacia Biotech, presently GE Healthcare) manufactured for the Multiphor™ II system (Amersham Pharmacia Biotech, presently GE Healthcare). After IEF, the gels were equilibrated, and proteins were further separated using 10% SDS-polyacrylamide gels in the Protean™ II xi system (Bio-Rad). The gels were dried, and the radioactivity signal was analysed by Storm PhosphorImager™(Molecular Dynamics). Autoradiograms were analyzed

visually without any computer-assisted image analysis. Unlabelled samples were used for mass spectrometric analysis where approximately $5x10^5$ 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).

4.3. In-gel digestion of the proteins

The CBB-stained (I) protein spots of interest were excised from the gel and digested with modified trypsin solution (Bio-Rad). As a control sample, standard Hsp90 protein (1 μg and 5 μg) isolated from bovine brain (Sigma) was digested under the same conditions. Silver-stained protein spots of interest were cut out of gels (IV) and in-gel digested as previously described (Shevchenko et al., 1996). The proteins were reduced and alkylated with iodoacetamide before overnight digestion with trypsin (Sequencing Grade Modified Trypsin, Promega) at 37°C. The peptides were extracted once with 25 mM ammonium bicarbonate and twice with 5% formic acid, and the extracts were pooled. Finally the peptide mixture was desalted using Millipore ZipTip™ μ-C18 pipette tips (Millipore).

4.4. Protein identification by mass spectrometry (MS)

Positive ion mass spectra were acquired with on-line HPLC-electrospray ionisation/mass spectrometry (HPLC-ESI/MS) (I). 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 peptides obtained after tryptic digestion from

CBB-stained protein spots (20 μl) were injected onto a reversed-phase column (Syncropak RP-8, 5 μM, 100 x 2.1 mm I.D., Lafayette), and the peptides were separated using a linear gradient from 2-100 % B 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 effluent flow of 0.2 ml/min was directed to the ESI source. 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 - 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 data dependent scan mode of the instrument. Full scan MS data were collected until the base peak ion exceeded a preset intensity threshold ($2x10^4$). This treshold triggered the acquisition of a CID MS/MS product ion spectrum of the ion. The collision energy was 35% and the isolation width was two mass units. The scan range was dependent on the parent mass ion. Database searches with the obtained MS/MS data were carried out using the program MS-Tag (http://prospector.ucsf.edu/) against Swiss-Prot-database (http://ca.expasy.org/sprot/).

Mass mapping of the peptides resulting after tryptic digestion from silver-stained protein spots was performed with a Biflex™ MALDI-TOF mass spectrometer (Bruker-Daltonics) in the positive ion reflector mode using α-cyano-4-hydroxycinnamic acid as the matrix (IV). The MALDI spectra were internally calibrated with the standard peptides, angiotensin II and adrenocorticotropin-18-39 (Sigma-Aldrich Chemie). Alternatively, peptides were analysed using LC-MS/MS analysis, where the peptides were first separated by microbore reversed-phase HPLC using a 75 μm x 150 mm

PepMap™ column (LC packings) and elution with a linear gradient of acetonitrile (0-50% in 50 min) in 0.1% formic acid. Chromatography was performed at a flow rate of 250 nl/min, and the eluent was directly injected into a Q-TOF mass spectrometer (Micromass) equipped with an ESI source. MS/MS spectra were acquired by colliding the doubly charged precursor ions with an argon collision gas accelerated with voltages of 30-45 V. Database searches were carried out using two programs, ProFound (current address: http://prowl.rockefeller.edu/profound_bin/WebProFound.exe) or Mascot MS/MS ion search (current address: http://www.matrixscience.com/).

4.5. Western blotting

For Western blot analysis, whole cell extracts were prepared as previously described (Mosser et al., 1988). To analyze the half-life of eEF-2 and the relative amount of phosphorylated eEF-2, the cellular proteins were extracted into RIPA buffer (1 x PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 0.1 mg/ml PMSF, 30 μl/ml aprotinin, 1 mM sodium orthovanadate). Protein extracts (15 μg per lane) were separated on 10% SDS-polyacrylamide gels and transferred to a nitrocellulose membrane (Millipore or Schleicher & Schuell). The transferred proteins were visualized with Ponceau S stain to ensure equal protein loading and transfer. Monoclonal antibody recognizing Hsp70 (SPA-810) (StressGen) and polyclonal antibodies recognizing Grp78 (N-20), Hsp90α (N-17), Hsp90β (D-19) and eEF-2 (C-14) (Santa Cruz Biotechnology) were used, as well as an antibody raised in rabbit against the Thr56-phosphorylated eEF-2 (McLeod et al., 2001). Peroxidase-conjugated secondary antibodies (Zymed or Santa Cruz Biotechnology) were used. The membranes were developed with an enhanced chemiluminescence method (Santa Cruz Biotechnology or Pierce) or with 3-amino-9-

ethyl-carbazole chromogen (Zymed).

4.6. Gel mobility shift assay

Whole cell extracts for the electrophoretic mobility shift assay were prepared as previously described (Mosser et al., 1988). The double-stranded oligonucleotide probe corresponding to the proximal heat shock element (HSE) of the human hsp70 promoter was labeled with [γ-^{32}P]dCTP using T4 polynucleotide kinase (Promega). After probing, PhosphorImager™ was used to detect the autoradiography signal.

4.7. Northern blotting

RNA (20 μg), isolated with Trizol (Gibco) or EUROzol (Euroclone) reagents, was separated on a 1% agarose/formaldehyde gel, transferred to a nylon membrane (Hybond-N, Amersham) and hybridized with [α–^{32}P]dCTP-labeled plasmids specific for human hsp70 (Wu and Hunt, et al., 1985), hsp90α (Hickey et al., 1989), hsp90β (Hickey et al., 1989), eEF-2, and rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Fort et al., 1985). The human eEF-2 specific probe (1303 bp) was made with the 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), and verified by DNA sequencing. PhosphorImager™ (Molecular Dynamics) was used for quantification of the levels of the specific mRNAs.

5. RESULTS

5.1. Differential regulation of major stress proteins by high HP, heat shock, and unbalanced calcium homeostasis

Eukaryotic cells up-regulate the synthesis of certain proteins involved in the stress response when exposed to HP, elevated temperature or disturbed Ca^{2+} homeostasis. Possible differences in protein synthesis could be evidence that these treatments activate different kinds of signal transduction cascades. Therefore, we used SV40 -immortalized T/C28a4 chondrocytic cells to investigate the changes in protein synthesis after various treatments known to induce a stress response. Cells were stressed either with HP, heat shock, or with chemical agents that interfere with calcium homeostasis (TG or A23187). After the stress experiments, the cells were lysed with 2-DE sample solution, and an equal volume from each experiment was mixed with TCA. Protein precipitation with TCA followed by scintigraphy was used to estimate the effect of various stressors on total protein synthesis. Continuous and cyclic 30 MPa HP for 12 h decreased total protein synthesis by about two-thirds and one-third, respectively. Heat shock (HS) treatment (43°C for 5 h) was the most potent inhibitor of protein synthesis, while 25 nM TG treatment for 12 h had only a minor effect. The protein patterns from differentially treated cells were then analysed using 1- and 2-DE. After SDS-PAGE from HP - and HS -treated samples, we observed an increased synthesis of 67 and 82 kDa class of proteins in HS - and continuous 30 MPa HP - treated samples (**I**, Fig. 1). Continuous HP for 4 MPa or cyclic HP from 4 to 30 MPa failed to induce any marked changes in protein synthesis.

2-DE revealed that continuous 30 MPa HP for 12 h did not cause any major changes in the general pattern of the proteins. Visual inspection, however, suggested that the intensities of certain spots had changed. The use of an image analysis program confirmed that continuous high HP induced by approximately twofold the synthesis of a 67 kDa protein (pI 5.4), and the synthesis of an 82 kDa protein (pI 4.9 –5.0) was also increased by 15–20% (**I**, Fig. 2B). The same spots were induced by heat shock at 43°C for 5 h (**I** Fig. 2D). These proteins were identified as Hsp70 and Hsp90, respectively, using MS and database searches. A 70 kDa protein (pI 5.3) was also slightly increased with both of the stress treatments. The size and pI value suggested that this protein would be Hsc70, which was later confirmed by its identification by MS. In addition, the syntheses of a group of proteins ranging from 89 kDa to 92 kDa (pI 5.1–5.3) were downregulated after high continuous HP (**I**, Fig. 2B). Cyclic 30 MPa HP showed no clear changes in protein synthesis (**I**, Fig. 2C). Compared with controls or other treatments, TG and A23187 (**I**, Fig. 2E&F) resulted in a clearly different pattern of newly-synthesized proteins. They induced the synthesis of two proteins, molecular weights 74 kDa and 89 kDa (pI 5.0 and 4.9, respectively), identified as Grp78 and Grp94. Synthesis of Hsp70 and Hsp90 was not up-regulated, rather the production of Hsp90 tended to be down-regulated. The syntheses of a 74 kDa and a 106 kDa proteins (pI 5.2 and 5.3, respectively) were also increased after chemical treatments.

A total of six spots of interest (**I**, Fig. 3) were excised from the 2-DE gel of the sample separated after continuous HP treatment. After successful MS analysis of the peptides, we were able to identify all of our proteins of interest (**I**, Table I). Interestingly, Hsp90α and Hsp90β are both found from two separate spots (**I**, Fig. 3, spots 2 and 3), and the different ratios of these proteins after HP suggested that especially Hsp90β had been up-regulated (data not shown).

Finally, the protein levels of four selected Hsps were analyzed by conventional SDS-PAGE, Western blotting and densitometric analysis (**I**, Fig. 5A&B). Static HP clearly increased Hsp70 and slightly Grp78 levels, but did not have any effect on the levels of Hsp90α and Hps90β (**I**, Fig. 5A). Cyclic HP caused a minor increase only in Hsp70 levels. After HS treatment, Hsp70, Hsp90α, and Grp78 levels were increased, whereas the Hps90β level remained rather constant. A different kind of stress protein profile was observed after TG treatment, i.e. Grp78 levels were increased, while Hsp70, Hsp90α, and Hsp90β levels were decreased. Table 2. summarizes the effects of different stress treatments on stress protein synthesis.

Table 2. The effects of different stress treatments on stress protein synthesis

	Continuous HP	Cyclic HP	TG/ A23187	HS
Hsp70	⇑	⇔/⇑	⇓	⇑
Hsc70	⇔	⇔	⇔	⇔
Hsp90α	⇔	⇔	⇓	⇑
Hsp90β	⇑*	⇔	⇓	⇔
Grp78	⇑	⇔	⇑	⇑
Grp94	⇔	⇔	⇑	⇔

⇑ = increase, ⇔ = no effect, ⇓ = decrease, ⇔/⇑ = no marked increase, ⇑* = see next chapter

5.2. Specific induction of Hsp90β by high hydrostatic pressure

2-DE and MS analysis showed that the expression Hsp90β was up-regulated in chondrocytic cells by high continuous HP, although this was not confirmed by Western blot analysis. However, Northern blot analysis made from these cells after similar HP treatment gave results indicating that hsp90β mRNA levels had indeed been up-regulated whereas the hsp90α levels remained rather constant (data not shown). Since high continuous HP has been previously shown to cause a strong stress response in extensively characterized HeLa cervical carcinoma cells, we used this cell line to further study the effect of 30 MPa continuous HP on Hsp90 expression.

An induction of hsp90β mRNA level could be observed already after 3 h of the pressure treatment, and the level increased up to 12 h (**II**, Fig. 1A). The levels of hsp90α mRNA did not change markedly in response to continuous pressure. The recovery of the hsp90β mRNA level back to normal required a rather long time, since at 12 h after the release of the pressure its level was still higher than that occurring in control samples (**II**, Fig. 1B). At the protein level, Western blotting and densitographs showed that continuous 30 MPa HP changed the ratio of Hsp90β to Hsp90α already at 3 h after the beginning of the experiment, and the difference was strongest 12 h after the pressurization (**II**, Fig. 2A). The ratio returned nearly back to normal within 12 h after the release of pressure (**II**, Fig. 2B).

5.3. Hsp90 inhibition with GA increases hsp70 mRNA stabilisation but fails to activate HSF1 in cells exposed to HP

Hsp90 is known to change Hsp70 gene expression for example, by regulating the activity of the HSF1 transcription factor. We used HeLa cells and the Hsp90 inhibitor GA to investigate whether Hsp90 is involved in the regulation of Hsp70 expression also under 30 MPa continuous HP. First, under normal cell culture conditions, we determined the effect of different GA concentrations on Hsp70 protein expression and HSF1 activation. Hsp70 protein levels were up-regulated at 3 h after the onset of GA treatments in a concentration range from 0.5 μM to 10 μM (**III**, Fig. 1A). There were no marked differences in either the intensity or the kinetics of Hsp70 up-regulation in response to the different GA concentrations. The DNA-binding activity of HSF1 was also increased at 3 h after GA treatment (**III**, Fig. 1B). Interestingly, the HSF1 activation lasted for the entire GA treatment period up to 12 h. Since there were no concentration-dependent differences in the Hsp70 expression levels or HSF1 activation, we used 0.5 μM GA in the following pressurization experiments.

When cells were subjected to HP treatment, elevated Hsp70 protein levels were observed at 12 h after the onset of pressure (**III**, Fig. 2A). The effect of GA alone or together with HP loading was stronger compared with the cultures treated only with HP. The hsp70 mRNA levels were also increased in response to HP, and again the increase was stronger when GA was used alone or together with HP (**III**, Fig. 2B). However, the kinetics of hsp70 mRNA up-regulation were different in HP and/or GA treated cells. In HP-treated cells, the hsp70 mRNA levels were increased for up to 12 h, whereas in the cultures treated with GA alone, the levels reached their maximum at 6 h (**III**, Fig. 2B).

The DNA-binding activity of HSF1 was increased in GA-treated cells but, in line

with previous studies, it remained unchanged in HP-treated cells (**III**, Fig. 2C). Notably, the simultaneous treatment with GA and HP decreased the DNA-binding activity of HSF1 (**III**, Fig. 2C). To examine whether HP inhibits trimerization of HSF1 and thus affects the DNA-binding activity, the cells were exposed for 1 h to the elevated temperature (43°C) and HP treatment. This treatment evoked clear HSF1 activation, which excluded the possibility of impaired ability of HSF1 to form a trimer under HP loading (**III**, Fig. 3).

Finally, hsp70 mRNA stabilization during HP and/or GA treatments was studied by inhibiting RNA synthesis with ActD. The cells were first exposed for 3 h to HP and/or GA, before the medium was changed and ActD was introduced (**III**, Fig. 4A). The dishes were placed in an incubator and the treatment was continued for up to 6 h. In comparison with GAPDH mRNAs, the hsp70 mRNAs accumulated in the GA-treated and in the pressurised cell cultures (**III**, Fig. 4B). In the pressurised cultures treated with GA, the hsp70 mRNAs also had longer half-lives than the controls. However, the stabilisation effect was not additive when compared with HP or GA treatments alone.

5.4. High hydrostatic pressure inhibits the biosynthesis of eEF-2

In addition to up-regulating the synthesis of some stress proteins, high continuous HP is known to widely affect the normal structure and function of cells. Thus, we investigated in HeLa cells and T/C28a4 immortalized human chondrocytic cells whether HP could change the syntheses of proteins unlikely to be major stress proteins. We were especially interested in those proteins which responded similarly to HP in both cell lines.

When metabolically labeled proteins were separated by 2-DE, we observed two marked differences in protein synthesis that were identically present in both cell lines. A clear increase after HP treatment was obvious in the synthesis of a 65 kDa protein (pI 4.8). The same protein was induced by high continuous HP also in human chondrosarcoma cell line HCS 2/8 (data not shown). Due to the low expression level even after HP treatment, we were unable to acquire sufficient material for MS identification. Instead, the protein that showed lower radioactivity in the pressurized samples in both cell lines was selected for mass spectrometric identification (**IV**, Fig. 1).

Twelve tryptic peptides were found in MALDI-TOF mass mapping to match eEF-2 tryptic peptides with a discrimination of less than 0.1 Da mass units. The identification was further confirmed by LC-MS/MS analysis in which 7 tryptic peptides could be sequenced, providing amino acid sequences and molecular masses identical to eEF-2 tryptic peptides. Since HP had a stronger influence on total protein synthesis in HeLa cells than in the chondrocytic cells, the subsequent protein and mRNA analyses were performed mainly using HeLa cells.

According to Western blot analysis, eEF-2 protein level was decreased by 40-50% in the pressurized cells within 12 h of pressurization (**IV**, Fig. 2A), and similar results were obtained from T/C28a4 cells (data not shown). However, in addition to the reduced levels of total of eEF-2, the inhibition of protein synthesis may be explained by reduced activity of eEF-2. Previous studies have demonstrated that phosphorylation of eEF-2 results in inhibition of protein synthesis by preventing the translocation of ribosome during peptide elongation (Ryazanov et al., 1988). Hsp90 has been shown to be associated with the EF-2 kinase (Palmquist et al., 1994), which is the only kinase known to regulate the activity of eEF-2 (Ryazanov et al., 1988). When the Hsp90-EF-2 kinase complex was disrupted by GA, the amount of EF-2 kinase was decreased in glioma cells

(Yang et al., 2001) and, recently, hsp90 was found to protect EF-2 kinase from degradation by the ubiquitin-proteasome pathway (Arora et al., 2005). However, 30 MPa continuous HP did not increase the phosphorylation of eEF-2 in comparison with the total eEF-2 content (**IV**, Fig. 3A). The total eEF-2 content was adjusted to approximately the same level in all of the lanes to ease the comparisons between the total and the phosphorylated form. Hsp90α was considered to be a constantly expressed internal standard, and thus immunodetected from the same membrane as the total eEF-2. The difference in relative band intensity was determined from the densitographs (**IV**, Fig. 3A) and as expected, HP decreased the ratio of eEF-2 to Hsp90α within 6 h.

As expected, the introduction of 0.5 μM GA to cells increased the amount of Hsp70 protein levels present in both the unpressurized and pressurized cells (**IV**, Fig. 2A). GA with or without HP, however, did not affect to any major extent the total level of eEF-2 in HeLa cells within 12 h of treatment. HP treatment also increased the steady-state level of hsp70 mRNA but eEF-2 mRNA levels remained at a constant level when compared to GAPDH (**IV**, Fig. 2B).

Since the phosphorylation state of eEF-2 remained steady in pressurized cell cultures, we analyzed the phosphorylation state of eEF-2 under conditions that are likely to affect protein synthesis and eEF-2 phosphorylation. These experiments were also made so that we could test the function of our antibody. EF-2 kinase is known to be calcium-dependent (Nairn et al., 1985; Ryazanov, 1987). In cell cultures treated with drugs that increase the intracellular calcium concentration (A23187 and TG), the total eEF-2 protein level remained at a constant level during the 12-h treatment (**IV**, Fig. 3B), whereas the phosphorylation of eEF-2 was decreased due to these treatments. Destabilization of microtubules with nocodazole caused dephosphorylation of eEF-2

within 3 h, while rephosphorylation occurred at 6 h after the onset of treatment (**IV**, Fig. 3B).

Analysis of steady-state level of eEF-2 mRNA did not provide an explanation for the observed decrease in the cellular eEF-2 protein. Therefore, we investigated whether eEF-2 protein was being 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 addition of cycloheximide into the cultures to prevent protein synthesis. We analyzed both total eEF-2 and Hsp90α with specific antibodies (**IV**, 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 clearly decreased in the pressurized cell cultures (**IV**, Fig. 4B), indicative of its faster degradation in pressurized cells.

6. DISCUSSION

6.1. High hydrostatic pressure up-regulates the synthesis of Hsps

HP is experienced in the human body by a variety of cells and tissues, but this is especially the case in cartilage, and therefore chondrocytes are regularly subjected to mechanical stress. *In vitro* studies have shown that HP may be necessary for the cartilage matrix to maintain its composition (Lammi et al., 1994; Parkkinen et al., 1993; Wong et al., 2003). However, excessive physical forces are detrimental to chondrocytes as they are to other cells, and usually some kind of stress response is evoked. High continuous HP has been shown to induce a stress response for example in the SV40 immortalized chondrocytic cell line T/C-28a4 and the human chondrosarcoma cell line HCS-2/8, whereas the primary articular cartilage cells appear to be pressure-resistant (Kaarniranta et al., 1998; Kaarniranta et al., 2001; Sironen et al., 2002). Although these continuous cell lines have been shown to be capable of producing cartilage-specific ECM molecules such as aggrecan and type II collagen (Goldring et al., 1994; Takigawa et al., 1989), it is evident that they still differ from the native chondrocyte *in vivo*. If we want to understand the function of a chondrocyte for example how it is affected by mechanical stress, we need to characterize the HP-induced changes in the intracellular events on chondrocytes as well as chondrocytic and non-chondrocytic cells.

Proteomics is the large scale study of proteins, including their separation, identification and characterization. During the past decade the technical advances in 2-DE and MS techniques, and the creation of publicly available protein databases have made it possible to conduct proteome studies. For example, screening with cDNA arrays has revealed that HP changes the transcriptional activity of many genes, and in this

study proteomics was used to study the possible HP -induced changes at the protein level.

We investigated the stress response after various stress factors, and found marked differences in the pattern of newly-synthesized stress proteins. Earlier studies have reported that the HP-induced stress response markedly differs from that induced by HS but the reason for this difference is a mystery (Kaarniranta et al., 1998; Kaarniranta et al., 2000; Kaarniranta et al., 2001). Ca^{2+} may be involved in the response since HP affects intracellular Ca^{2+} homeostasis by a still largely obscure mechanism. However, it has also been suggested that high continuous HP does not influence intracellular Ca^{2+} concentrations at least in mouse fibroblasts (Crenshaw and Salmon, 1996). Treatment with certain chemical agents e.g. TG and A23187 has resulted in up-regulation of Grp78 and 94 while Hsp70 and Hsp90β were down-regulated. These results support the previous studies where A23187 and thapsigargin were found to induce Grp78 and Grp94 expression (Li et al., 1993; Watowich and Morimoto, 1988). In addition, A23187 has decreased Hsp70 synthesis in HeLa cells (Watowich and Morimoto, 1988) but up-regulated the synthesis of αB-crystallin, a small Hsp, in chondrocytes (Bennardini et al., 1995). Somewhat different results were obtained with primary bovine chondrocytes where TG and A23187 increased the levels for Hsp60, 70 and 90 as well as Grp78 and 94 (Cheng and Benton, 1994). It remains unclear whether the immortalization of human chondrocytes changes their response to Ca^{2+} inhibitors.

HS treatment induced the well-known up-regulation of Hsp70 and Hsp90 synthesis. No markedly increased synthesis of Grps was observed although heat shock disturbs and increases the intracellular Ca^{2+} concentration (Stevenson et al., 1986). *In vitro* Ca^{2+} has been shown to regulate the activity of HSF1 (Mosser et al., 1990) and recently it was suggested that the transcriptional activation of HSF1 is strongly

dependent on phosphorylation by calcium/calmodulin-dependent protein kinase II (Holmberg et al., 2001). However, other regulatory mechanisms for HSF1 activation are likely to exist because in immortalized chondrocytic cells, high continuous HP induced an elevation in the intracellular Ca^{2+} concentration (Browning et al., 2004) but failed to activate HSF1 (Kaarniranta et al., 1998).

According to 2-DE and Western blot analysis, high continuous HP induced a slight up-regulation of Grp78, perhaps due to disturbed Ca^{2+} homeostasis. Interestingly, in HCS 2/8 chondrosarcoma cells and the T/C28a4 immortalized chondrocyte cell line, the same type of HP loading strongly up-regulates the mRNA levels of e.g. growth arrest and DNA-damage-inducible proteins 45 and 153 (GADD45 and GADD153, respectively) (Sironen et al., 2002). The levels of GADD45 are induced by several stress factors such as irradiation, genotoxic drugs and withdrawal of growth factors (Sanchez and Elledge, 1995), and simultaneous up-regulation of Grp78 and GADDs has been reported to occur when cells are exposed to other stress factors such as glutamine deprivation (Abcouwer et al., 1999), A23187, and hypoxia (Price and Calderwood, 1992). It is possible that HP can disturb the function of the amino acid transporters in the cell membrane, resulting in amino acid deprivation and a stress response. It is unlikely that the membrane-sealed and pressurized cells experience hypoxia, as demonstrated previously with primary chondrocytes (Parkkinen et al., 1993). In addition, the metabolically more active control cells are unable to up-regulate the synthesis of the previously mentioned proteins.

In addition to Hsp70, also the Hsp90β isoform was found to be up-regulated by continuous HP. According to 2-DE and MS analysis, the Hsp90α and Hsp90β isoforms were located in two different spots. However, a Western blot analysis made after 2-DE (data not shown) identified these proteins from two or more well-defined spots. Their

slightly different pI values may reflect post-translational modifications such as phosphorylation. However, it has to be taken into account that the use of self-made gels in isoelectric focusing reduces the reproducibility of protein separation. For example, the lengths of the individual IEF gels may vary slightly each time when the gels are cast inside the hollow glass rod. Also, the pH gradient may differ from gel-to-gel since the ampholytes are not bound to the gel matrix. At the protein level, the ratio of Hsp90β to Hsp90α was increased already after 3 h of pressure treatment. Such a rather rapid change suggests that the differences in protein turnover or distribution are attributable to the high HP. In fibrosarcoma and breast cancer cell cultures, Hsp90α level is specifically elevated in the extracellular space, where it modulates the activity of MMP-2 (Eustace et al., 2004). Elevated MMP-2 levels are also present in osteoarthritic cartilage (Tetlow et al., 2001), whereas cyclic HP loading reduces the MMP-2 levels in isolated osteoarthritic chondrocytes (Trindade et al., 2004). At present it is not known whether continuous high HP increases either the level or the activity of MMP-2, resulting in accelerated turnover of collagen molecules. Hsp90β, in turn, has been reported to be structurally related to human microtubule-interacting protein (Mip-90) (Cambiazo et al., 1999). Therefore, one possible mechanism is that there is a specific interaction between Hsp90β and the cytoskeleton.

However, two isoforms of Hsp90 often function interchangeably and up-regulation of the Hsp90β isoform may be related to any of a number of regulatory functions of Hsp90. The role of Hsp90 during HP loading still needs to be studied since, for example, the high HP-induced ERK activation is not dependent on this chaperone protein (Kopakkala-Tani et al., 2004).

6.2. Hsp90 regulates Hsp70 gene expression during hydrostatic pressure

In this study, we investigated whether the up-regulated Hsp90 has a role in the hsp70 gene regulation that appears in response to continuous HP (Elo et al., 2000; Kaarniranta et al., 1998; Kaarniranta et al., 2001). In general, potentially harmful environmental factors lead to the induction of the heat shock genes that are regulated by the activation of HSF1 transcription factor (Pirkkala et al., 2001). In an inactive state, the HSF1 monomer exists as a multiprotein complex which contains the Hsp70 and Hsp90 proteins (Guo et al., 2001; Zou et al., 1998). In addition to Hsp70 and Hsp90, HSF1 activation is attenuated by Hsp40 (Hdj-1) and heat shock factor binding protein 1 (HSBP1) (Morimoto, 1998). In response to stress, Hsp90 and Hsp70 are released from the complex, HSF1 forms a trimer, binds to the heat shock gene promoter, and undergoes phosphorylation (Abravaya et al., 1992; Holmberg et al., 2001; Sarge et al., 1993; Zou et al., 1998). The important role of Hsp90 in HSF1 regulation was also demonstrated in our study when this chaperone was inhibited by GA. In HeLa cells cultivated under regular cell culture conditions, a long-lasting HSF1 activation was seen with up-regulation of Hsp70 expression. Our previous study revealed that in primary fibroblasts, HP *per se* does not prevent the DNA-binding activity of HSF1 upon simultaneous heat shock treatment (Kaarniranta et al., 2001). The results were similar also in HeLa cells where HSF1 was able to trimerise under HP loading, if an appropriate additive stress stimulus such as HS was present. However, HP effectively attenuated GA-induced HSF1 gene activation. Only a slight activation of HSF1 was detected on the gel mobility shift assay after 3 h of simultaneous HP and GA treatments. This is most likely due to the experimental procedure where GA is supplemented 10–15 min prior to HP exposure. Thus, the increased DNA-binding activity of HSF1 is clearly a response to GA treatment. The attenuation of HSF1 by HP seems to mediate influences on both

Hsp70 mRNA and protein levels under the influence of simultaneous GA treatment.

Gene expression in eukaryotes involves also posttranscriptional regulation when the stability of mRNA is controlled. The half-lives of various mRNA species are regulated by their stabilisation or destabilisation by many different proteins and mechanisms (Bevilacqua et al., 2003). Many short-lived mRNA species have AREs in their 3'-untranslated regions and there are several ARE-binding proteins, such as AUF1, which have high affinity for this sites (Bevilacqua et al., 2003). Hsp70 mRNA contains AREs but remains stabilised when cells are exposed to HS treatment (Laroia et al., 1999), hypertonicity (Alfieri et al., 2002), or to high continuous HP (Kaarniranta et al., 1998; Kaarniranta et al., 2001). During HP loading, protein synthesis is needed for hsp70 mRNA stabilization (Kaarniranta et al., 2000). A Hsp90-regulated kinase, *pkr*, is known to be involved in the stabilization of hsp70 mRNA (Zhao et al., 2002). In addition, the activated *pkr* also decreases total protein synthesis by inhibiting translational initiation (Yan et al., 2002). A recent study revealed that constant stretching can induce *pkr* activation in fibroblasts, after which a *pkr* downstream effector, eukaryotic initiator factor-2α (eIF-2α), becomes phosphorylated, possibly explaining the decrease in protein synthesis (Wang et al., 2005). When GA and HP treatments were tested on their own, they increased hsp70 mRNA stability, and simultaneous treatment with GA and HP did not have any additive influence on the stabilisation. The increased Hsp70 protein level may control the rate of its own mRNA decay in both of these treatments, as suggested previously (DiDomenico et al., 1982). On the other hand, simultaneous treatments with GA and HP may evoke opposite effects on hsp70 mRNA stabilisation or perhaps the stabilisation represents maximal effects after HP or GA treatment.

6.3. Hydrostatic pressure inhibits the synthesis of eEF-2

In eukaryotes, protein translation is a complicated process that demands the tightly co-ordinated interplay of dozens of different molecules (Kapp and Lorsch, 2004). Together with the ribosomal 40S subunit, at least 12 eukaryotic initiation factors (eIFs) participate in the formation of the mRNA recognition and preinitiation complex that scans the mRNA to find a correct initiation codon. Once the initiation site is found, the preinitiation factors will dissociate from the small subunit of ribosome. The ribosomal 60S subunit then joins the complex, forming a functional ribosome and the translation proceeds to the elongation stage. During elongation, eukaryotic elongation factors (eEFs) are recruited for peptide chain elongation until a stop codon is encountered and the termination stage ends the translation (Kapp and Lorsch, 2004).

Phosphorylation events of various eIFs are involved in the initiation stage. For example, the phosphorylation of eIF-2α frequently occurs under stressful conditions, reducing the translational rates (Clemens, 2001). The activity of eIF-2B is altered in response to viral infection, hormones, nutrients, growth factors, and stresses such as elevated temperature, and it may be regulated directly by its own phosphorylation (Duncan and Hershey, 1984; Webb and Proud, 1997). The activity of eEFs has also been shown to depend on phosphorylation status, for example eEF-2 is inactivated by its phosphorylation, such as occurring during oxidative stress (Patel et al., 2002; Redpath et al., 1993; Ryazanov and Davydova, 1989).

We were able to demonstrate that the biosynthesis of one key player involved in protein translation, eEF-2, was partly inhibited by high continuous hydrostatic pressure. This protein has a role in protein translation acting to facilitate the movement of peptidyl-tRNA from ribosomal A site to the P site (Kapp and Lorsch, 2004). Western

blot analysis confirmed the decreased amount of eEF-2. However, no sign of increased phosphorylation of eEF-2 could be detected in the pressurised cells. This observation supports the proposal that there is no correlation between the protein synthesis and eEF-2 phosphorylation status (Laitusis et al., 1998; Garcia et al., 2004). Thus, if eEF-2 participates in the regulation of the translation rate during HP, this it is likely due to a decrease in its amount. The inhibition of protein synthesis by cycloheximide indicated that the decrease of eEF-2 in the pressurized cells involved a shorter half-life of this protein. However, it is possible that the HP-induced changes in protein synthesis can be partly explained by altered activity of other proteins, such as eIFs.

A eEF-2 -specific kinase, the EF-2 kinase (also known as Ca^{2+}/calmodulin-dependent protein kinase III), is known to interact with Hsp90 (Palmquist et al., 1994). Disruption of this interaction by GA was previously shown to decrease the amount of the eEF-2 kinase in glioblastoma cells (Yang et al., 2001), and was likely to affect the phosphorylation status of eEF-2. We concluded that, at ambient pressure, GA it did not affect to any significant extent the level of eEF-2 in HeLa cells and that, in HP-treated cell cultures, GA did not have any additional effect on the decline in the eEF-2 content. Therefore, it appears that Hsp90 interactions with cellular proteins, such as the EF-2 kinase, are not involved in the decrease of relative eEF-2 protein level that occurs under high hydrostatic pressure.

Chemical agents that disturb the Ca^{2+} homeostasis also change the phosphorylation status of eEF-2 (Laitusis et al., 1998). An opposite response of eEF-2 phosphorylation in pressurised vs. thapsigargin-treated cells was observed, suggesting that calcium signalling is not likely to be involved in the regulation of cellular protein synthesis in cell cultures subjected to 30 MPa continuous hydrostatic pressure.

7. SUMMARY AND CONCLUSIONS

Cells and tissues are constantly exposed to mechanical stress such as HP, with the highest forces being experienced in the musculoskeletal system, especially the articular cartilage. In a number of *in vivo* and *in vitro* studies, the effect of various loading methods has been investigated on articular cartilage or isolated chondrocytes, and it has been concluded that relevant loading is crucial for proper cartilage structure and function. Non-physiologically high HP applied in a constant mode disturbs the normal function of articular cartilage chondrocytes. However, unlike the situation in most other cells, HP does not up-regulate the level of the major stress protein, Hsp70.

In an immortalized human chondrocytic cell line (T/C28a4), high continuous, but not cyclic, HP induces Hsp70 and Hsp90β expression, while Grp78 is only marginally up-regulated and Hsp90α expression is totally unaffected. These changes in stress protein synthesis are markedly different from those seen after HS treatment or when cellular Ca^{2+} homeostasis is chemically disturbed. Based on these observations, we conclude that, although intracellular Ca^{2+} homeostasis is disturbed by high continuous HP in these same cells, the stress response is not related to this phenomenon. Interestingly, in T/C28a4 as well as in HeLa cells, high continuous HP up-regulated specifically the Hsp90β isoform, possible evidence that there are differences in the functions of the two isoforms.

Hsp90 is known to be a regulator of dozens of proteins some of which are transcription factors such as HSF1. Hsp90 inhibition by GA resulted in HSF1 activation and Hsp70 up-regulation, but simultaneous HP treatment effectively attenuated HSF1 - mediated gene activation. In addition, both GA and HP treatments increased hsp70 mRNA stability, while simultaneous treatment with GA and HP did not have any

additive influence on the extent of the stabilisation. The results indicate that HP regulates the transcriptional activity of HSF1 by some yet unknown mechanism, and that during pressurization, Hsp70 levels are preferentially elevated by mRNA stabilization rather than transcriptional activation.

High continuous HP decreases total protein synthesis in the cell. The mechanism behind this effect is currently not known, although it is most likely that HP disturbs the translational machinery at multiple sites. We were able to show that the level of eEF-2 protein was decreased in HP-loaded cells, and that the degradation of eEF-2 was accelerated. Phosphorylation regulates the activity of eEF-2, but no HP -induced change occurred in the phosphorylation-status of this elongation factor.

In conclusion, the results presented in this thesis provide new information about the rather exceptional Hsp gene expression and stress response occurring in HP -treated cells.

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