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Primary chondrocytes resist hydrostatic pressure-induced stress while primary synovial cells and fibroblasts show modified Hsp70 response

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Summary

Objective: During joint loading, chondrocytes in the articular cartilage are subjected to gradients of high compressive hydrostatic pressure (HP). In response to diverse chemical or physical stresses, heat shock genes are induced to express heat shock proteins (Hsps). This study sought to examine the role of Hsps in baroresistance in primary bovine chondrocytes and synovial cells, as well as in primary human fibroblasts.

Methods: Northern blotting was used to analyze the steady-state levels of hsp70 mRNA in the primary cells exposed to HP or heat stress. Hsp70 protein accumulation was analyzed by Western blotting, and the DNA-binding activity was examined by gel mobility shift assay.

Results: Primary bovine chondrocytes which have been adapted to live under pressurized conditions showed negligible Hsp70 response upon HP loading, whereas primary bovine synovial cells and human fibroblasts accumulated hsp70 mRNA and protein when subjected to HP. The response was initiated without activation of the heat shock transcription factor 1. Interestingly, pre-conditioning of the barosensitive fibroblasts with HP or heat shock reduced the Hsp70 response, indicating induction of baroresistance.

Conclusion: This study suggests that Hsp70 can play an important role in the early stages of adaptation of cells to HP. Thus, the Hsp70 gene expression upon HP loading may serve as one indicator of the chondrocytic phenotype of the cells. This can be of use in the treatment of cartilage lesions. © 2001 OsteoArthritis Research Society International

Key words: Chondrocyte, Gene regulation, Heat shock protein, Hydrostatic pressure.

Introduction

The magnitude of hydrostatic pressure (HP) in the biosphere ranges from 0.1 MPa to 120 MPa, and various organisms show differences in their sensitivity to pressure.[1] The ability of cells to tolerate high HP often occurs via adaptation at the biochemical level: enzymes exhibit altered functional properties,[2,3] membranes have fluidities adapted to high HP,[4] and proteins may acquire an enhanced structural stability,[5] partly due to expression of pressure-tolerant proteins.[6] Furthermore, energy metabolism varies in different pressure conditions.[7]

High HP has been shown to alter cell morphology and cytoskeletal organization in a cell-type dependent manner, when cultured primary chondrocytes and continuously dividing transformed cells have been exposed to HP.[8–10] Moreover, strenuous HP loading has been shown to reduce proteoglycan synthesis in explants of articular cartilage and intervertebral disc, as well as in cultured chondrocytes.[11–13] Chondrocytes are highly differentiated cells originating from mesenchymal stem cells, and their properties differ remarkably from many other cell types. They produce and maintain highly anionic extracellular matrix of cartilage in conditions where their energy metabolism occurs mainly through anaerobic glycolysis.[14,15] The main functions of articular cartilage are related to load bearing; thus, cartilage is commonly subjected to severe stresses and during normal locomotor activity articular pressures may rise to 20 MPa.[16] Hence, it has been proposed that HP is one of the mediators of joint loading effects on chondrocytes.[14] Today, it is widely accepted that certain amount of joint loading is required for the maintenance of healthy articular cartilage.[17–21] Moderate exercise efficiently maintains the functional properties of cartilage,[22] whereas strenuous exercise may eventually cause adverse reactions in the joint.[23] Degenerative changes in chondrocytes and in extracellular matrix are integral parts of the clinical syndrome of osteoarthrosis [osteoarthritis (OA), degenerative joint disease]. Although the molecular basis of this syndrome is still unknown, it is obvious that excessive dynamic joint loading or prolonged static loading causes degradation of the matrix and, eventually, degeneration of the joint.[21]

In response to diverse chemical or physical stresses the expression of heat shock genes is induced, resulting in elevated synthesis of the heat shock proteins (Hsps) that are highly conserved throughout the evolution.[24] Hsps have been shown to function as molecular chaperones in normal and stressful conditions.[25] The elevated synthesis

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of Hsps in response to severe stress ensures the survival of many organisms and Hsps have been associated with the induction of thermotolerance in cells.[26–29] Heat shock gene expression is mainly regulated at transcriptional level. The transcriptional regulation is mediated by the stress-responsive heat shock transcription factor 1 (HSF1), which is present in non-stressed cells in an inactive state and becomes activated in response to stress stimuli. When HSF1 is completely activated, it is converted from a monomer to a trimer, translocated into the nucleus to bind to the promoter of heat shock genes, and is then hyperphosphorylated.[30,31] Numerous studies have shown that the transcription of heat shock genes implies multiple regulatory processes, and the induction can vary in intensity and kinetics in a modulator- and cell-type specific manner.[32–39]

In this study, we analyzed the effect of high HP on the heat shock gene expression in primary bovine chondrocytes, synovial cells, and primary human fibroblasts. We could demonstrate that in primary chondrocytes, which are cells normally adapted to live under pressure, hsp70 gene expression was unaffected by high continuous HP, whereas exposure of the cells to elevated temperature resulted in a classical heat shock response. Interestingly, high HP loading caused a significant Hsp70 response in primary synovial cells and fibroblasts. In addition, we show that pre-conditioning of the barosensitive human fibroblasts with HP or heat modified the Hsp70 response, indicating that Hsp70 can play an important role at the early stages of adaptation of cells to mechanical loading.

Materials and methods

CELL CULTURE AND TREATMENTS

Primary bovine chondrocyte and synovial cell cultures were established from 1–2-year-old animals (N=5 for chondrocytes, N=2 for synovial cells). The articular cartilage from the patellar surface of the femur and synovial tissue was cut into 1–2 mm^3 pieces, washed twice with PBS, and digested with hyaluronidase (0.5 mg/ml, Sigma), supplemented with gentamycin (0.1 mg/ml, PAA), fungizone (2.5 μg/ml, PAA), ascorbic acid (0.5 μg/ml, Sigma) in Dulbecco's MEM Nut MIX F-12 medium (Gibco) at 37°C for 30 min with continuous shaking. The medium was then exchanged to collagenase (3 mg/ml, Sigma) digestion solution, supplemented with 10% fetal calf serum (FCS), DNase (0.2 mg/ml, Sigma), 2 mM glutamine (PAA), fungizone 10 μl/ml, penicillin 50 units/ml (PAA), streptomycin 50 units/ml (PAA), ascorbic acid (0.5 μg/ml) in Dulbecco's MEM Nut MIX F-12-medium (Gibco), and kept at 37°C in a periodical magnetic stirring overnight. After extracellular matrix digestion, chondrocytes and synovial cells were cultured in Dulbecco's MEM Nut MIX F-12 medium including 10% FCS, penicillin, streptomycin, and glutamine. Primary human fibroblasts were isolated from the skin of a young man. The tissue explants were cultured in Dulbecco's MEM medium (Gibco) including 10% FCS, penicillin, streptomycin, and glutamine. The fibroblasts migrated out from the explants and attached to the bottom of dish. Passages III–V were used for the experiments. The human fibroblasts were cultured in Dulbecco's MEM medium (Gibco) with the same supplements as described above. The cells were grown to confluency on 60 mm plates in a humidified 5% CO_2/95% air atmosphere at 37°C. Before exposure to HP or elevated temperature, the medium was changed and 15 mM HEPES (pH 7.3) was added.

For heat shock treatment, the plates were sealed with Parafilm and immersed in a 43°C water bath. Before the cells were exposed to HP, the culture dishes were completely filled with the medium described above and sealed with a covering plastic membrane. The apparatus for hydrostatic pressurization of the cells has been described in detail previously.[40]

NORTHERN BLOTTING

Total cellular RNA was isolated with Trizol reagent (Gibco). Total RNA (20 μg or 25 μg) 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 hsp90α,[41] hsp70,[42] hsp27 (StressGen, SPD-910) and rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH).[43] The levels of hsp90, hsp70, hsp27 and GAPDH mRNA were quantitated with a PhosphorImager™ (Molecular Dynamics).

WESTERN BLOTTING

Whole cell extracts (20 μg) were electrophoresed on a 10% SDS-PAGE, and transferred to nitrocellulose membrane (Millipore) with a semi-dry transfer apparatus (Scleicher & Schuell). The analysis was performed using monoclonal antibodies that recognize the inducible form of Hsp70 (StressGen, SPA-810), the constitutive form of Hsc70 (Stress-Gen, SPA-815) and peroxidase-conjugated secondary antibodies (DAKO).

GEL MOBILITY SHIFT ASSAY

Preparation of whole cell extracts and gel mobility shift assay were performed as previously described.[44,45] As a probe [γ-^{32}P]dCTP-labeled oligonucleotide corresponding to the proximal heat shock element (HSE) of the human hsp70 promoter was used. The synthetic oligonucleotide was [γ-^{32}P]dCTP-labeled with T4 polynucleotide kinase (Promega).

Results

PRIMARY CHONDROCYTES SHOW BARORESISTANCE WHEN EXPOSED TO HIGH CONTINUOUS HYDROSTATIC PRESSURE

Previous studies have shown that high continuous HP causes cell-type dependent alterations in the cytoskeleton of pressurized cells and modifies extracellular matrix synthesis of cartilage.[8,10–12] In addition, our recent findings revealed that high HP induced heat shock response in human immortalized T/C28a4 chondrocytic cells.[44] Therefore, we wanted to investigate whether the response to high static HP varied in different primary cell types, including primary chondrocytes, which in articular cartilage are adapted to live under pressure.[14] Primary bovine chondrocytes and synovial cells, as well as primary human fibroblasts, were exposed to 30 MPa continuous HP for up to 12 h, or to heat stress at 43°C for 1 h. Northern blotting was used to analyze the steady-state mRNA levels. In primary chondrocytes, the hsp70 mRNA levels were not affected by static 30 MPa HP loading, whereas an exposure to elevated temperature caused a marked increase in the amount of hsp70 mRNA, indicating that these cells are able to respond to heat stress [Fig. 1(a) and (b)]. Interestingly, when exposed to high HP or heat shock, the primary synovial cells and fibroblasts showed a significant increase

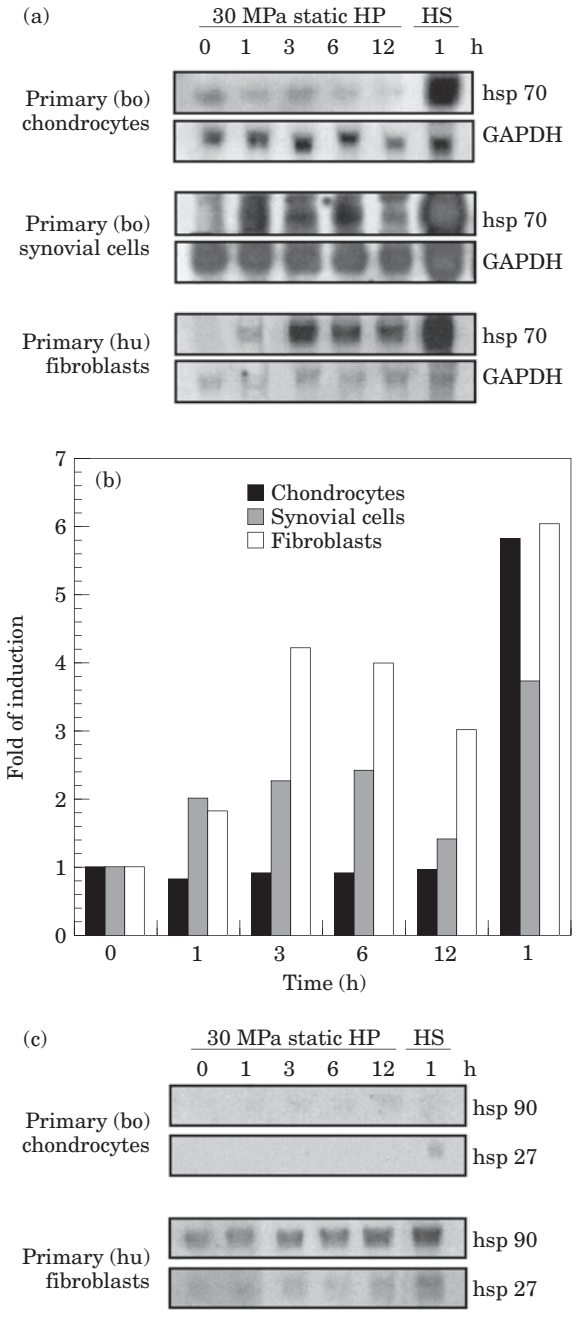

Fig. 1. Primary synovial cells and human fibroblasts but not primary chondrocytes show induction of hsp70 gene expression when exposed to HP loading. (a) The levels of hsp70 mRNA were examined by Northern blot hybridization of untreated (0 h) cells, cells exposed to static hydrostatic pressure (HP, 30 MPa, 1–12 h), or to heat shock (HS, 43°C, 1 h). The total RNA (20 μg) was run on 1% agarose gel, transferred to nylon membrane, and hybridized with [α-^{32}P]dCTP-labeled cDNA probes for hsp70 and GAPDH. (b) Quantification of the hsp70 mRNA was performed with a PhosphorImager. The hsp70 mRNA values were normalized against respective GAPDH values. (c) The levels of hsp90α and hsp27 mRNA were examined by Northern blot hybridization of untreated (0 h) cells, cells exposed to static hydrostatic pressure (HP, 30 MPa, 1–12 h), or to heat shock (HS, 43°C, 1 h). Abbreviations: (bo), bovine; (hu) human.

in steady-state levels of hsp70 mRNA [Fig. 1(a) and (b)]. HP caused a gradual induction of hsp70 mRNA, reaching the maximal 2.5-fold induction at 3–6 h in synovial cells and four-fold induction in fibroblasts [Fig. 1(b)]. In comparison, heat shock led to approximately 4–6-fold induction of the hsp70 mRNA levels in the cells [(Fig. 1(b)]. The amounts of hsp90α and hsp27 mRNA were investigated in the primary chondrocytes and fibroblasts, but they were not markedly affected by HP [Fig. 1(c)]. However, a minor increase in the hsp90α and hsp27 mRNA levels was detected in both primary cell types after heat shock treatment [Fig. 1(c)].

To assess whether the increased levels of hsp70 mRNA corresponded with Hsp70 protein accumulation, Western blot analyses with antibodies against Hsp70 and Hsc70 were performed for samples pressurized at 30 MPa continuous HP for up to 12 h or exposed to heat stress for 6 h at 43°C. In primary chondrocytes, the basal level of Hsp70 remained unaffected by HP loading, although accumulation of Hsp70 was readily detectable in the heat-shocked cells (Fig. 2). In contrast, elevated Hsp70 levels were observed in both pressurized and heat-shocked primary synovial cells and fibroblasts (Fig. 2). Hence, the protein levels of Hsp70 followed consistently the amount of hsp70 mRNA in both analyzed cell types (Fig. 2). As expected, no alterations were noticed in Hsc70 levels in any of the cell types (Fig. 2).

The effect of cyclic (0.5 Hz) 10 or 30 MPa, and 10 MPa static HP loading on Hsp70 expression was examined by Western blot analysis. No increase in the amount of Hsp70 or Hsc70 proteins could be detected in either primary chondrocytes or fibroblasts (data not shown) which is in accordance with our previous study on immortalized T/C28a4 chondrocytic cells.[44] Taken together, our observations indicate that primary chondrocytes, but not primary synovial cells or fibroblasts are resistant to high continuous HP, when Hsp70 expression was used as a stress marker.

HSF1 DNA-BINDING IS NOT INDUCED BY HIGH CONTINUOUS HYDROSTATIC PRESSURE

Since the stress response to high HP was different between the cell types (Figs 1 and 2), we examined the DNA-binding activity of HSF1 by gel mobility shift assay with a synthetic oligonucleotide containing the proximal HSE sequence of the human hsp70 promoter. As shown in Fig. 3(a), binding of HSF1 to HSE was not induced either in chondrocytes or fibroblasts exposed to high HP, whereas the binding was clearly detected in both of the heat-shocked cells [Fig. 3(a)].

High HP may induce conformational and catalytic changes in certain proteins.[46–49] To examine whether high continuous HP might cause conformational changes in the structure of HSF1, thereby inhibiting its DNA-binding capacity, we analyzed the HSF1 binding to HSE in untreated cells, cells exposed to 43°C for 1 h, and cells exposed both to 30 MPa continuous HP and heat shock at 43°C for 1 h. The analysis was restricted to the primary fibroblasts that showed most positive Hsp70 response in the absence of HSF1 binding to HSE when exposed to high HP. Simultaneous exposure to high HP and heat shock did not prevent the DNA-binding activity of HSF1 in the cells [Fig. 3(b)], excluding the possibility that during high HP the ability of HSF1 to trimerize and to bind to DNA would be impaired. In summary, our biochemical assays suggest that in contrast to heat-shock exposure, HSF1 DNA-binding activity is not required for the elevated expression of heat shock genes under 30 MPa continuous HP.

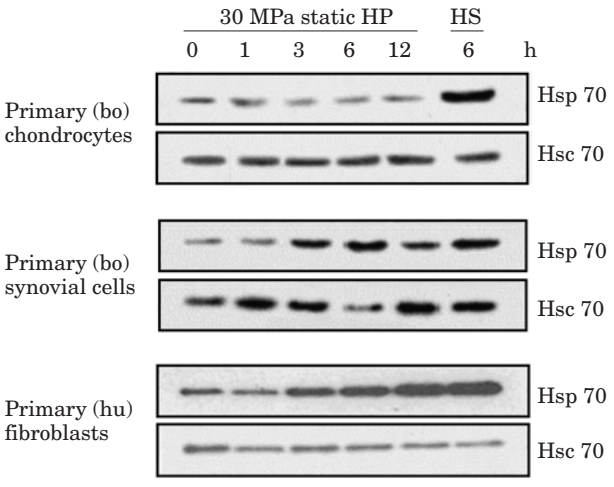

Fig. 2. Primary synovial cells and fibroblasts but not primary chondrocytes accumulate Hsp70 protein exposed to HP. Whole cell extracts of untreated (0 h) cells, cells exposed to HP (30 MPa, 1–12 h), or to HS (43°C, 6 h) were analyzed by Western blotting using antibodies against Hsp70 and Hsc70. Abbreviations: (bo), bovine; (hu) human.

ELEVATED HSP70 EXPRESSION OBTAINED BY PRE-CONDITIONING CONTRIBUTES TO MODIFIED HP RESPONSE

In general, the stress response is considered to represent a cellular defense mechanism against environmental disturbances. If cells are exposed to a mild, sublethal stress that is sufficient to upregulate the Hsps, they are often able to survive subsequent, otherwise lethal stress stimuli. This increased resistance is known as induced thermotolerance.[50] With regard to the finding that no heat shock gene other than hsp70 was induced upon HP (Fig. 1), it could be anticipated that Hsp70 protein has an essential role for triggering baroresistance in cells under HP. Therefore, Hsp70 levels were examined in primary fibroblasts—the cells adapted to live in subcutaneous connective tissue—that had been first exposed to 30 MPa continuous HP for 6 h or to 43°C heat treatment for 1.5 h, and then allowed to recover for 12 h. Interestingly, approximately two times higher accumulation levels of Hsp70 were detected in cells treated with heat shock than with HP [Fig. 4(a)]. Hsc70 was not affected by the stress stimuli.

To find out whether the pre-conditioning would affect the cellular response to HP, the fibroblast cultures were pre-treated as described above prior to the 30 MPa continuous pressure exposure for various time periods from 1 to 12 h, and the steady-state hsp70 mRNA levels were examined by Northern blotting. As shown in Figs 4(b) and (c), the induction of hsp70 mRNA was markedly reduced due to the pre-conditioning. However, in line with the protein data shown in Fig. 4(a), the heat pre-treatment caused a more pronounced baroresistance than HP pre-loading [Fig. 4(b) and (c)]. Taken together, our results indicate that in barosensitive cells, like fibroblasts, the response to HP can be modified in an Hsp70-dependent manner.

Discussion

This study demonstrates that acute HP stress causes cell-type specific induction of hsp70 gene expression. In barosensitive primary synovial cells and fibroblasts a significant HP-induced accumulation of Hsp70 occurred.

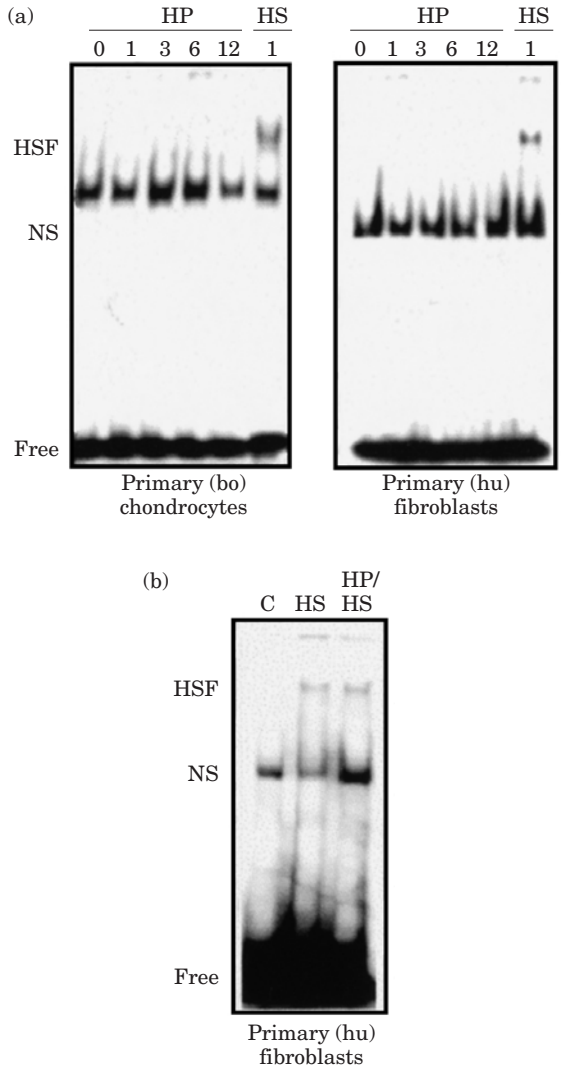

Fig. 3. The HP response occurs without activation of HSF1. (a) The HSF DNA-binding activity was analyzed by gel mobility shift assay in whole cell extracts from untreated cells (0 h), cells exposed to HP (30 MPa, 1–12 h) or to HS (43°C, 1 h). (b) Analysis of HSF DNA-binding activity in untreated cells (lane C), in cells exposed to HS (43°C, 1 h), and in cells exposed to both HS and HP (30 MPa and 43°C, 1 h). Abbreviations: (bo), bovine; (hu) human; NS, non-specific HSE-binding activity; Free, unbound labeled HSE oligonucleotide.

Interestingly, HP and heat shock pre-conditioning clearly reduced Hsp70 response upon subsequent HP loading. To our knowledge, this is the first time that a high static HP pre-conditioning treatment has been shown to modify the subsequent Hsp70 response to stresses, indicating acquired baroresistance. In contrast to the barosensitive cells, in primary bovine chondrocytes, cells that normally live under HP conditions,[14] no changes were observed in the low basal levels of Hsp70 under 30 MPa HP. Our results seem to indicate that elevated Hsp70 levels are required in response to acute HP stress in synovial cells and fibroblasts, and probably also during the early stages of cellular adaptation to regular mechanical loading. It can be speculated that once fully differentiated chondrocytes have adapted to live under pressurized conditions, they no

longer respond to strenuous mechanical loading by enhanced Hsp70 expression.

Hsps are prominently expressed in the hypertrophic chondrocytes of the growth plate,[51,52] where differentiating chondrocytes live under relatively high constant turgor pressure. The pressure is generated by proteoglycans and the high concentration of anionic hyaluronan entrapped between the plasma membrane of the chondrocyte and the lacunar wall.[53] We suggest that elevated Hsp70 levels are essential in the acute stage of cellular stresses. Primarily, the stress proteins might provide time for the cells to adapt and survive, while in cells that have acquired, e.g. baroresistance, high Hsp70 levels are no longer required for protection, as shown here in primary chondrocytes. In contrast to the load-bearing area of articular cartilage exposed to high pressures, the pressure in the synovial joint is even sub-atmospheric.[54] This probably explains the different stress response of synovial cells to HP compared with chondrocytes. It has previously been shown that Hsps have an essential role in maintaining the integrity of cytoskeletal structures, due to their function as molecular chaperones.[55–60] Since HP has multiple cell-type specific effects on the cytoskeleton,[8–10,61] the induction of Hsp70 under high HP might be an effort by the cell to repair damaged cytoskeleton, reform it, or to facilitate the assembly of newly synthesized cytoskeletal proteins in the barosensitive cells.

The development of osteoarthrosis is associated with aging and excessive local mechanical loading. The disease involves all the tissues that form the synovial joint, including articular cartilage, subchondral and metaphyseal bones, synovial tissue, joint capsule and muscles.[21] The disappearance of baroresistance in chondrocytes undergoing a pathophysiological process like OA or rheumatoid arthritis suggests an altered gene expression of the cartilage cells.[62–64] It remains to be shown if aging, too, can cause disappearance of baroresistance of chondrocytes. Despite the beneficial influences of moderate motion and loading on cartilage properties, the adult articular cartilage has only a weak capacity to repair structural damages.[21,22] However, mesenchymal cells that originate from the bone marrow possess the ability to differentiate into chondrocytes, and tentative experimental studies have revealed that mesenchymal cells implanted into a cartilage defect have the potential to adapt to the prevailing loading conditions and are often able to repair successfully the cellular organization of the tissue.[21] Our observation of acquired baroresistance upon acute stress in non-chondrocytic cells showing elevated Hsp70 levels, suggests that the Hsps could have an essential role in adaptation of cells to HP or mechanical loading. Thus, Hsp70 expression and metabolism may provide a new vision for molecular medicine when developing novel therapeutic strategies for cartilage repair.

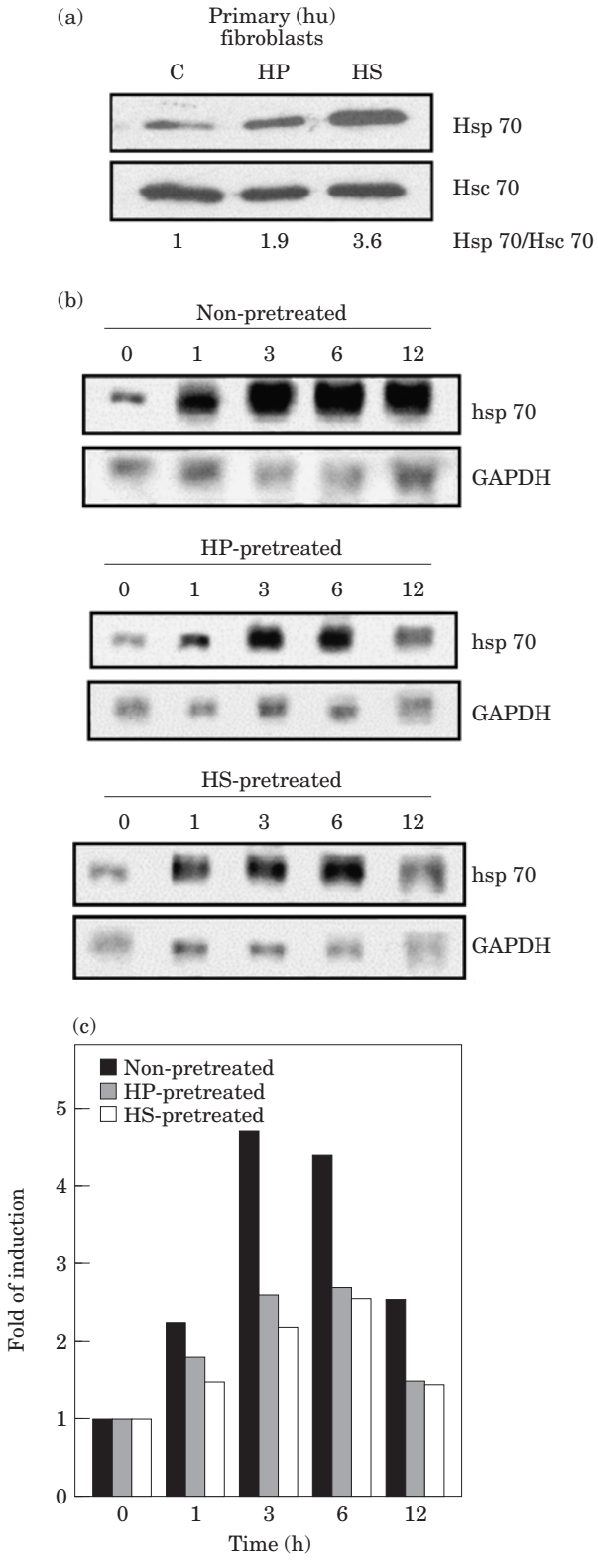

Fig. 4. Pre-treatment with HP or HS modifies the hsp70 response of primary fibroblasts to subsequent HP loading. (a) Hsp70 and Hsc70 protein levels were analyzed by Western blotting from whole cell extracts of untreated (lane C), pressurized (HP; 30 MPa, 6 h) and heat-shocked (HS; 43°C, 1.5 h) primary fibroblasts that had been allowed to recover for 12 h. The Hsp70 to Hsc70 ratio was determined densitometrically. (b) The non-pre-treated, HP-pre-treated, or HS-pre-treated primary fibroblasts were exposed to HP (30 MPa, 1–12 h) and the steady-state levels of hsp70 mRNA were analyzed by Northern blot hybridization. (c) Quantification of the hsp70 mRNA from Fig. 4(b) for primary fibroblasts was performed with a PhosphorImager. The hsp70 mRNA values were normalized against the respective GAPDH values. Abbreviations: (bo), bovine; (hu) human.

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