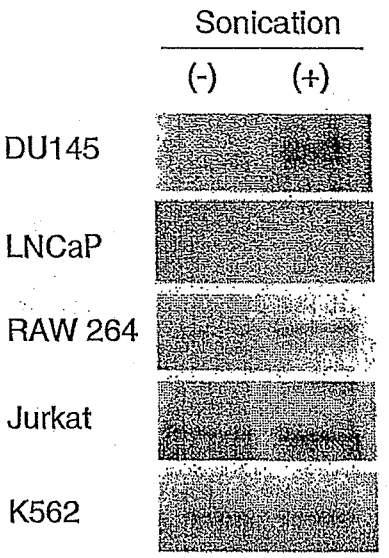

Fig. 4. Western blot analysis of HO-1 in five cell lines. The cells were sonicated for 60 s with 1.0-MHz continuous wave at 1.1 W/cm² and harvested 7 h after sonication

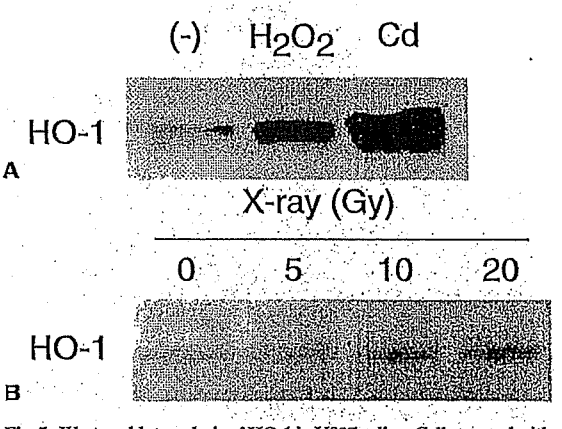

Fig. 5. Western blot analysis of HO-1 in U937 cells. a Cells treated with H_2O_2 (600 μM) or with $CdCl_2$ (5 μM). The cells were treated with H_2O_2 or $CdCl_2$ for 6 h and then harvested for Western blotting. b Cells exposed to X-rays at 5, 10, and 20 Gy. After X-irradiation, the cells were incubated for 6 h and harvested for Western blotting

lation, and *PCTK1-V2* was identified as another upregulated gene. Two other genes, *LIFR* and *CXCL10*, were found to be downregulated. In addition, the data suggest that O_2^- secondarily generated in damaged mitochondria resulting from sonomechanical effects gives rise to intracellular oxidative stress to induce *HO-1* expression.

HO is an enzyme-catalyzing heme, which coordinately bonds to oxygen in the living body as a prosthetic group of hemoglobin or cytochrome p-450, Fe^{2+}, carbon monoxide and biliverdin. Biliverdin is subsequently converted to bilirubin in the cytosol by biliverdin reductase. HO is considered a rate-limiting enzyme in the process of heme catabolism.[19] HO consists of three isozymes: HO-1, HO-2 and HO-3.[20] HO-1 has been extensively studied. It is an isozyme induced by metal ions, such as cadmium[21,22] and cobalt,[23] and by physical and chemical stresses such as treatment with 12-o-tetradecanoylphorbol 13-acetate,[24] ultraviolet light,[25] hydrogen peroxide[25] and donors of nitric oxide.[26] In addition, oxidative stress caused by ischemia-reperfusion injury is known to induce HO-1.[27] It has been reported that HO-1-deficient embryonic fibroblasts are hypersensitive to both hemin and hydrogen peroxide cytotoxicity, as supported by genetic evidence that upregulation of HO-1 serves as an adaptive mechanism to protect cells from oxidative damage during stress.[28] However, the mechanism by which HO-1 is induced by ultrasound, a form of mechanical stress, has not yet been elucidated.

Bioeffects of ultrasound occur because of thermal and nonthermal effects. The latter are further classified into cavitational and noncavitational effects. Cavitation is known to lead to both mechanical shear stress and/or shock waves (mechanical effects) and free radical formation (an example of chemical effects) arising from the collapse of oscillating bubbles. Because upregulation of HO-1 was observed at intensities greater than 0.8 W/cm² in the present study, where a significant decrease in cell survival rate and apparent free radical production were observed, upregulation of HO-1 is likely to be cavitation-related. The next question is whether the upregulation is caused by the mechanical effects or the chemical effects associated with cavitation.

To answer this question, we sonicated cells with or without NAC, which is a potent antioxidant, and measured HO-1 expression by using real-time quantitative PCR. As shown in the results, even when NAC was added immediately after sonication, HO-1 induction was significantly suppressed, suggesting that HO-1 induction is most likely due to intracellularly generated ROS after sonication because the addition of culture medium sonicated at 1.1 W/cm² even for 5 min did not induce HO-1 in this cell line (the mean (±SD) fold induction was 1.2 ± 0.8 ($n = 4$)). Interestingly, our previous data have shown that the addition of NAC after sonication also increased intracellular glutathione concentration and suppressed apoptosis, but did not affect intracellular superoxide formation or mitochondrial transmembrane potential.[9] Restored intracellular glutathione appears to scavenge a variety of peroxides to reduce the intracellular oxidative stress and suppress HO-1 induction and apoptosis without affecting superoxide formation and mitochondrial function. Data on HO-1 induction in a variety of cell lines suggest that HO-1 induction by ultrasound appears to be a cell line-specific event. Ultrasound induced HO-1 in the U937 and Jurkat human leukemia cell lines, but not in the K562 cell line. Not only ultrasound, but also other oxidative stresses, including H_2O_2, $CdCl_2$ and X-irradiation, were able to induce HO-1 in U937 cells. We have shown that these stresses also induce apoptosis in this cell line.[29–31]

Recently, the enhancement of ischemia-reperfusion-induced lung apoptosis by inhibition of HO-1 using a

siRNA technique has been reported.[32] We have already reported apoptosis induced by ultrasound in human lymphoma cell lines, and discovered that the apoptosis is due to an increase in intracellular oxidative stress and intracellular free calcium ions.[9] Therefore, the elucidation of intracellular oxidative stress-mediated interactions between apoptosis and HO-1 induced by ultrasound remains a subject for future studies.

Conclusion

We used a cDNA microarray system to confirm the identity of known genes and discover new genes that respond to ultrasonic cavitation. We confirmed upregulation of the *HO-1* gene and further identified one upregulated and two downregulated genes. In addition, we suggest that increased intracellular oxidative stress secondary to sonomechanical effects arising from ultrasonic cavitation is a potential mechanism of enhancement of HO-1 expression.

Acknowledgment This study was supported by the Research and Development Committee Program of the Japan Society of Ultrasonics in Medicine.

References

1. Feril LB Jr, Kondo T, Umemura S, et al. Sound waves and antineoplastic drugs: The possibility of an enhanced combined anticancer. J Med Ultrasonics 2000;29:173–87.
2. Feril LB Jr, Kondo T. Biological effects of low intensity ultrasound: The mechanism involved, and its implications on therapy and on biosafety of ultrasound. J Radiat Res 2004;45:479–89.
3. Henglein A. Sonochemistry: Historical developments and modern aspects. Ultrasonics 1987;25:6–16.
4. Suslick KS. Sonochemistry. Science 1990;247:1439–45.
5. Riesz P, Kondo T. Free radical formation induced by ultrasound and its biological implications. Free Radic Biol Med 1992;13: 247–70.
6. Ashush H, Rozenszajn LA, Blass M, et al. Apoptosis induction of human myeloid leukemic cells by ultrasound exposure. Cancer Res 2000;60:1014–20.
7. Honda H, Zhao QL, Kondo T. Effects of dissolved gases and an echo contrast agent on apoptosis induced by ultrasound and its mechanism via the mitochondria-caspase pathway. Ultrasound Med Biol 2002;28:673–82.
8. Feril LB Jr, Kondo T, Zhao Q-L, et al. Enhancement of ultrasound apoptosis and cell lysis by echo-contrast agents. Ultrasound Med Biol 2003;29:331–7.
9. Honda H, Kondo T, Zhao QL, et al. Role of intracellular calcium ions and reactive oxygen species in apoptosis induced by ultrasound. Ultrasound Med Biol 2004;30:683–92.
10. Feril LB Jr, Kondo T, Takaya K, et al. Enhanced ultrasound-induced apoptosis and cell lysis by a hypotonic medium. Int J Radiat Biol 2004;80:165–75.
11. Feril LB Jr, Kondo T, Cui Z-G, et al. Apoptosis induced by the sonomechanical effects of low intensity pulsed ultrasound in a human leukemia cell line. Cancer Lett 2005;221:145–52.
12. Cheung VG, Morley M, Aguilar F, et al. Making and reading microarrays. Nat Genet 1999;21(Suppl 1):15–19.
13. Schena M, Shalon D, Davis RW, et al. Quantitative monitoring of gene expression patterns with a complementary DNA microarray. Science 1995;270:467–70.
14. Tabuchi Y, Kondo T. cDNA microarray analysis reveals chop-10 plays a key role in Sertoli cell death induced by bisphenol A. Biochem Biophys Res Commun 2003;305:54–61.
15. Hirano H, Tabuchi Y, Kondo T, et al. Analysis of gene expression in apoptosis of human lymphoma U937 cells induced by heat shock and the effects of α-phenyl N-tert-butylnitrone (PBN) and its derivatives. Apoptosis 2005;10:331–40. 15
16. Tabuchi Y, Kondo T, Suzuki Y, et al. Genes involved in nonpermissive temperature-induced cell differentiation in Sertoli TTE3 cells bearing temperature-sensitive simian virus 40 large T-antigen. Biochem Biophys Res Commun 2005;329:947–56.
17. Tabuchi Y, Kondo T, Ogawa R, et al. DNA microarray analysis of genes elicited by ultrasound in human U937 cells. Biochem Biophys Res Commun 2002;290:498–503.
18. Kondo T, Kano E. Effect of free radicals induced by ultrasonic cavitation on cell killing. Int J Radiat Biol 1988;54:475–86. 16 17
19. Maines MD. ■■. In: ■■ (eds) Heme oxygenase: clinical applications and function. Boca Raton: CRC Press; 1992. p. 145–201.
20. Maines MD. The heme oxygenase system: a regulator of second messenger gases. Annu Rev Pharmacol Toxicol 1997;37:517–54.
21. Alam J, Shibahara S, Smith A. Transcriptional activation of the heme oxygenase gene by heme and cadmium in mouse hepatoma cells. J Biol Chem 1989;264:6371–5.
22. Alam J, Wicks C, Stewart D, et al. Mechanism of heme oxygenase-1 gene activation by cadmium in MCF-7 mammary epithelial cells. Role of p38 kinase and Nrf2 transcription factor. J Biol Chem 2000;275:27694–702.
23. Gong P, Hu B, Stewart D, et al. Cobalt induces heme oxygenase-1 expression by a hypoxia-inducible factor-independent mechanism in Chinese hamster ovary cells: regulation by Nrf2 and MafG transcription factors. J Biol Chem 2001;276:27018–25.
24. Muraosa Y, Shibahara S. Identification of a cis-regulatory element and putative trans-acting factors responsible for 12-O-tetradecanoylphorbol-13-acetate (TPA)-mediated induction of heme oxygenase expression in myelomonocytic cell lines. Mol Cell Biol 1993;13:7881–91.
25. Keyse SM, Tyrrell RM. Heme oxygenase is the major 32-kDa stress protein induced in human skin fibroblasts by UVA radiation, hydrogen peroxide, and sodium arsenite. Proc Natl Acad Sci USA 1989;86:99–103.
26. Immenschuh S, Tan M, Ramadori G. Nitric oxide mediates the lipopolysaccharide dependent upregulation of the heme oxygenase-1 gene expression in cultured rat Kupffer cells. J Hepatol 1999;30:61–9.
27. Vulapalli SR, Chen Z, Chua BH, et al. Cardioselective overexpression of HO-1 prevents I/R-induced cardiac dysfunction and apoptosis. Am J Physiol Heart Circ Physiol 2002;283:H688–94.
28. Poss KD, Tonegawa S. Reduced stress defense in heme oxygenase 1-deficient cells. Proc Natl Acad Sci USA 1997;94:10925–30.
29. Lin M, Kondo T, Zhao QL, et al. Apoptosis induced by cadmium in human lymphoma U937 cells through Ca^{2+}-calpain and caspase-mitochondria-dependent pathways. J Biol Chem 2000;275:39702–9.
30. Cui ZG, Kondo T, Ogawa R, et al. Enhancement of radiation-induced apoptosis by 6-formylpterin. Free Radical Res 2004;38: 363–73.
31. Cui ZG, Kondo T, Feril LB Jr, et al. Effects of antioxidants on X-ray- or hyperthermia-induced apoptosis in human lymphoma U937 cells. Apoptosis 2004;9:757–63.
32. Zhang X, Shan P, Jiang D, et al. Small interfering RNA targeting heme oxygenase-1 enhances ischemia-reperfusion-induced lung apoptosis. J Biol Chem 2004;279:10677–84.

Available online at www.sciencedirect.com
SCIENCE DIRECT
ELSEVIER
Cancer Letters xx (2006) 1–8
CANCER Letters
www.elsevier.com/locate/canlet

Identification of genes responsive to low intensity pulsed ultrasound in a human leukemia cell line Molt-4

Yoshiaki Tabuchi [a,*], Hidetaka Ando [c], Ichiro Takasaki [a], Loreto B. Feril Jr [b,e], Qing-Li Zhao [b], Ryohei Ogawa [b], Nobuki Kudo [d], Katsuro Tachibana [e], Takashi Kondo [b]

[a] *Division of Molecular Genetics, Life Science Research Center, University of Toyama, 2630 Sugitani, Toyama 930-0194, Japan*
[b] *Department of Radiological Sciences, Faculty of Medicine, University of Toyama, Toyama 930-0194, Japan*
[c] *First Department of Surgery, Faculty of Medicine, University of Toyama, Toyama 930-0194, Japan*
[d] *Laboratory of Biomedical Instrumentation and Measurement, Graduate School of Information Science and Technology, Hokkaido University, Sapporo 060-0814, Japan*
[e] *Department of Anatomy, Fukuoka University School of Medicine, Fukuoka 814-0180, Japan*

Received 20 December 2005; received in revised form 15 February 2006; accepted 15 February 2006

Abstract

We examined the gene expression of human leukemia Molt-4 cells treated with non-thermal low intensity pulsed ultrasound. Six hours after 0.3 W/cm^2 pulsed ultrasound treatment, apoptosis (24±3.3%, mean±SD) with minimal cell lysis was observed. Of approximately 16,600 genes analyzed, BCL2-associated athanogene 3 (*BAG3*), DnaJ (Hsp40) homolog, subfamily B, member 1 (*DNAJB1*), heat shock 70 kDa protein 1B (*HSPA1B*), and heat shock 70 kDa protein 6 (*HSPA6*) showed increased levels of expression while isopentenyl-diphosphate delta isomerase (*IDI1*) and 3-hydroxy-3-methylglutaryl–coenzyme A synthase 1 (*HMGCS1*) showed decreased levels in the cells 3 h after the ultrasound treatment. The expression levels of these six genes were confirmed by a real-time quantitative polymerase chain reaction. To our knowledge, this is the first report of DNA microarray analysis of genes that are differentially expressed in response to apoptosis induced by non-thermal low intensity pulsed ultrasound in human leukemia cells. The present results will provide a basis for further understanding of the molecular mechanisms of effects of not only low intensity pulsed ultrasound but also that of mechanical shear stress in the cells.

Keywords: DNA microarray; Gene expression; Low intensity pulsed ultrasound

1. Introduction

In many medical fields, ultrasound (US) has been widely used for diagnosis and therapy. Biophysical actions of US are divided into three modes, thermal, cavitational and non-thermal non-cavitational effects. Cavitation is known to lead to both mechanical shear stress and free radical formation arising from the oscillation and collapse of cavitation bubbles [1–3]. In general, these two effects (mechanical shear stress and free radicals) of cavitation have been inferred to act simultaneously on all of biological materials. It is well known that fairly intense US induces cell killing, cell lysis, loss of viability and loss of clonogenicity [2]. Currently, of particular interest is its ability to induce apoptosis in human leukemia cell lines [4–12]. Different factors that can influence apoptosis were cited. Among them were, sonochemical mechanism [12], cavitation-mediated enhancement of intracellular calcium ion concentration, intracellular free radical

* Corresponding author. Tel.: +81 76 434 7187; fax: +81 76 434 5176.
E-mail address: ytabu@cts.u-toyama.ac.jp (Y. Tabuchi).

0304-3835/$ - see front matter © 2006 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.canlet.2006.02.011

formation due to mitochondrial membrane damage and free radical formation [10,11], and sonomechanical mechanism [9].

It has been indicated that DNA microarrays are one of the most powerful technologies for functional genomics as it can simultaneously analyze the expression levels of many 100s or many 1000s of genes [13,14]. Recently, we applied DNA microarray technologies to analyze gene expression in cellular differentiation [15] and in a variety of biological responses to physical and chemical stresses, such as sodium butyrate [16], bisphenol A [17,18] and hyperthermia [19]. In our previous study, using UniGEMV Ver2.0 human gene expression microarrays to detect approximately 9200 genes, five up-regulated genes and two down-regulated genes were identified in the human lymphoma U937 cells at 6 h after exposure of fairly intense continuous waves (1 MHz, 4.9 W/cm^2, for 1 min), where not only free radical formation, but also cell lysis and apoptosis were significantly observed [20]. Moreover, under nearly same condition in U937 cells, another microarray system and real-time quantitative polymerase chain reaction (PCR) confirmed up-regulation of heme oxygenase-1 (HO-1) and revealed that HO-1 is the most sensitive gene for US in U937 cells [21]. More recently, following a report by Lagneaux et al. [12], we also reported that non-thermal low intensity pulsed US treatment (1 MHz, 0.3 W/cm^2, 1 min) induced apoptosis in human leukemia cell lines such as U937, Jurkat and Molt-4, and it was revealed that the up-regulation of HO-1 was observed above apoptosis-inducing intensities [9,22]. However, the knowledge of the details of molecular signaling in response to mechanical pressure or pressure waves such as US, particularly low intensity pulsed waves, remains elusive.

In the present study, the gene expression of human leukemia Molt-4 cells treated with low intensity pulsed US were examined by using high-density IntelliGene HS human Expression microarrays to detect approximately 16,600 genes.

2. Materials and methods

2.1. Cell culture

Molt-4 human leukemia cells were obtained from Japanese Cancer Research Resource Bank (Tokyo, Japan). The cells were grown in RPMI 1640 medium (Invitrogen Co., Tokyo, Japan) supplemented with 10% fetal bovine serum (Invitrogen Co.) at 37 °C in humidified air with 5% CO_2.

2.2. US apparatus and intensity measurement

The ultrasonic apparatus (Sonicmaster ES-2, OG Giken Co. Ltd, Okayama, Japan) with a resonant frequency of 1 MHz with 100 Hz pulse repetition frequency (PRF) was used in all the sonication experiments. This device is equipped with a built-in digital timer, intensity regulator, and duty factor (DF) controller. For the sonication procedure, the transducer with a diameter of 5 cm was fixed with a clamp attached to a metal stand to keep the transducer facing directly upward (Fig. 1). We used such near acoustic field produced by the liquid–air interface and did not reduce the standing waves because of the effective occurrence of cavitation.

The spatial-average–temporal-average intensities (ISATA) at 10% DF corresponding to the reading output intensity were measured using an ultrasonic power meter (PM-DT-10E, Ohmic instrument Co., Easton, MD). The peak acoustic amplitude in degassed water was also measured at the distance of 5 cm from the transducer with a calibrated poly-(vinyledene difluoride–trifluoroethylene) needle-type hydrophone, 0.5 mm in diameter (Toray techno Co., Ltd, Shiga, Japan) connected to a PC/AT compatible computer and a digitizing oscilloscope (TDS3034, Tektronix Japan, Ltd, Tokyo, Japan). The ISATA and the peak acoustic pressures corresponding to the reading output of 0.1, 0.2, 0.3, 0.4 and 0.5 W/cm^2 (device-indicated) were 0.048, 0.072, 0.081, 0.092 and 0.105 W/cm^2, and 0.061, 0.105, 0.132, 0.144 and 0.146 MPa, respectively. In this paper we used device-indicated intensities to refer to these values.

For most of the experiments, 0.3 W/cm^2 was used. During such an ultrasonic exposure experiment the change in absorbance of 2 ml of aqueous air-saturated ferrous (Fricke) dosimeter solution after 5 min exposure at 304 nm was 0.0082 ± 0.0015 (mean$\pm$SD, $n=4$) in a 1 cm path length quartz cell. This dosimeter monitors the extent of the cavitational activity induced by ultrasound by measuring the sum of H_2O_2, OH radicals, and H atoms available to react with ferrous ions.

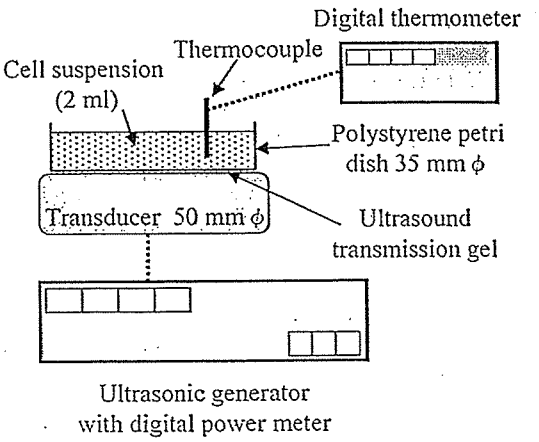

Fig. 1. A set-up for ultrasonic exposure.

2.3. Sonication procedure

The cell concentration used in the experiments was about 2.0×10^6 cells/ml. The cells were gently mixed to homogenize and 1 ml/sample of cell suspension was transferred to a 3 cm polyethylene culture dish. Just before sonication, 1 ml culture medium was added to the cell suspension after gentle shaking. After sonication, the cells were incubated in humidified air with 5% CO_2 at 37 °C for an indicated period before evaluation of DNA fragmentation and other parameters.

2.4. Measurement of cell viability

Trypan blue dye exclusion test was performed. In short, cell suspension was mixed with an equal amount of 0.3% Trypan blue solution (Sigma–Aldrich Co., St Louis, MO) in phosphate-buffered saline. After 5 min incubation at room temperature, the number of cells excluding Trypan blue (unstained) was counted using a Burker Turk hemocytometer to estimate the number of intact viable cells and the number of non-viable cells.

2.5. DNA fragmentation assay

The amount of DNA extracted from cells that had undergone DNA fragmentation was assayed using the method of Sellins and Cohen [23] with a few modifications. Briefly, cells were lysed in lysis buffer (1 mM EDTA, 0.2% Triton X-100 and 10 mM Tris–HCl, pH 7.5) and centrifuged at 13,000 g for 10 min. Subsequently, each DNA sample in the supernatant and the resulting pellet were precipitated in 12.5% trichloroacetic acid (TCA) at 4 °C, and quantified using the diphenylamine reagent after hydrolysis in 5% TCA at 90 °C for 20 min. The percentage of fragmented DNA for each sample was calculated as the amount of DNA in the supernatant divided by the total DNA for that sample (supernatant plus pellet).

2.6. Electron paramagnetic resonance (EPR)-spin trapping for the detection of free radical formation

In this study, we used EPR-spin trapping with 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) to detect the US-induced hydroxyl radical formation [2,24]. The cells suspended in culture medium with 10 mM of DMPO were sonicated at 0–0.5 W/cm^2 of 1 MHz 10% DF for 1 min. At 9.425 GHz with a field modulation of 0.1 mT amplitude using a microwave power of 4 mW, EPR spectra of the sonicated solution in a capillary tube were recorded with EPR spectrometer (RFR-30, Radical Research Inc., Tokyo, Japan) at room temperature. The yields of spin adducts were determined using a stable nitroxide radical, TEMPOL (4-hydroxy-2,2,6,6-tetramethyl-1-piperidinyloxy) as a standard. A calibration curve was determined by plotting the product of the peak-to-peak derivative amplitude and the square of the width at maximum slope of the signal versus the different concentrations of the standard nitroxide radical. One unit of the *Y*-axis labeled 'DMPO-OH adduct' is calculated to correspond to about 19.0 μM of TEMPOL.

2.7. Separation of total RNA and mRNA

Total RNA was extracted from the cells using an RNeasy Total RNA Extraction Kit (Qiagen K.K., Tokyo, Japan). Then, RNA samples were treated with RNase-free DNase (Qiagen K.K.) for 30 min at room temperature. mRNAs were extracted from the DNase-treated samples using an Oligotex-dT30 mRNA Purification Kit (Takara Bio Inc., Shiga, Japan).

2.8. cDNA microarray analysis

cDNA microarray analysis was performed by IntelliGene HS human Expression glass microarrays (Takara Bio Inc.), which were spotted with approximately 16,600 cDNA fragments of human genes. A list of these genes is available at Takara's Web site (http://www.takara-bio.co.jp/). Antisense RNA (aRNA) labeled with Cy3 (control group) or Cy5 (treated group) from mRNAs from cells at 3 h after US treatment (for 1 min at 0.3 W/cm^2) by using an RNA Transcript SureLABEL Core Kit (Takara Bio Inc.). In some experiments, control sample was labeled with Cy5; and in others, it was labeled with Cy3, with essentially identical results. Hybridization and washing of the microarray were carried out according to the manufacturer's instructions. In brief, aRNA probe solutions containing both Cy3- and Cy5-labeled aRNA probes were applied to the microarrays, and the microarrays were covered with a spaced glass cover slip (Takara Bio Inc.) and placed in a humidified chamber at 70 °C for 16 h with gentle shaking. Then, the microarrays were sequentially washed in 2× SSC (150 mM NaCl and 15 mM sodium citrate) containing 0.2% SDS for 10 min three times at 65 °C and in 0.05× SSC for 1 min once at room temperature. The microarrays were scanned in both Cy3 and Cy5 channels with a ScanArray Lite (Packard BioChip Technologies, Billerica, MA, USA). QuantArray software (Packard BioChip Technologies) was used for image analysis. Genes were considered to be positive-expressed if the signal/background ratio was >2.0. The average of RPS27A (ribosomal protein S27a, an internal control gene) Cy3 and Cy5 signal (12 spots each) gives a ratio that was used to balance or normalize the signals.

2.9. Real-time quantitative PCR assay

Real-time quantitative PCR was performed on a Real-Time PCR system (Mx3000P, Stratagene Japan K.K., Tokyo, Japan) using Brilliant SYBR Green qPCR Master Mix (Stratagene Japan K.K.) according to the manufacturer's protocol. Reverse transcriptase reaction (Omniscript Reverse Transcriptase, Qiagen K.K.) was carried out with DNase-treated total RNA by using an oligo d(T)$_6$ primer. Real-time

Table 1
Nucleotide sequences of primers for target genes

Genes	Orientation	Nucleotide sequence (position)	GenBank accession no.
HSPA1B	Sense	5′-AGGTGCAGGTGAGCTACAAG-3′ (509–528)	NM_005346
	Antisense	5′-ATGATCCGCAGCACGTTGAG-3′ (722–703)	
BAG3	Sense	5′-CGACCAGGCTACATTCCCAT-3′ (574–593)	NM_004281
	Antisense	5′-TCTGGCTGAGTGGTTTCTGG-3′ (749–730)	
HSPA6	Sense	5′-GGCCATGACCAAGGACAACA-3′ (1760–1779)	NM_002155
	Antisense	5′-AACCATCCTCTCCACCTCCT-3′ (1976–1957)	
DNAJB1	Sense	5′-ACCCGGACAAGAACAAGGAG-3′ (135–154)	NM_006145
	Antisense	5′-GCCACCGAAGAACTCAGCAA-3′ (364–345)	
IDI1	Sense	5′-CACTAACCACCTCGACAAGC-3′ (304–323)	NM_004508
	Antisense	5′-CTTCTTGGTCTCAGCTCC-3′ (400–383)	
HMGCS1	Sense	5′-ACGGTATGCCCTGGTAGTTG-3′ (564–583)	NM_002130
	Antisense	5′-GCGGTCTAATGCACTGAGGT-3′ (807–788)	
RPS27A	Sense	5′-TTACGGGGAAGACCATCACC-3′ (61–80)	NM_002954
	Antisense	5′-CCACCACGAAGTCTCAACAC-3′ (265–246)	

BAG3, BCL2-associated athanogene 3; *DNAJB1*, DnaJ (Hsp40) homolog, subfmaily B, member 1; *HMGCS1*, 3-hydroxy-3-methylglutaryl–coenzyme A synthase 1; *HSPA1B*, heat shock 70 kDa protein 1B; *HSPA6*, heat shock 70 kDa protein 6; *IDI1*, isopentenyl-diphosphate delta isomerase; *RPS27A*, ribosomal protein S27a

quantitative PCR was performed by using the specific primers listed in Table 1. Temperature cycling conditions for each primer consisted of 10 min at 95 °C followed by 40 cycles for 30 s at 95 °C, 1 min at 60 °C and 1 min at 72 °C. The dissociation analysis was carried out over the range from 55 to 95 °C by monitoring SYBR Green fluorescence and PCR-specific products were determined as a single peak at the melting curves more than 80 °C. In addition, the specificity of primers was confirmed as a single band with the correctly amplified fragment size through an agarose gel electrophoresis of the real-time quantitative PCR products. Each mRNA expression level was normalized with respect to the mRNA expression of RPS27A.

2.10. Statistical analysis

Data are presented as means ± SD. Statistical analysis was carried out using Student's *t*-test and *P* values less than 0.05 were regarded as significant.

3. Results

3.1. Effects of ultrasonic intensity on cell viability and apoptosis in human leukemia Molt-4 cells

As shown in Fig. 2(A), the cell viability detected by trypan blue dye exclusion test 6 h after the low intensity pulsed US treatment (1 min) was significantly decreased at intensities more than 0.2 W/cm^2, with the cell viability of 88.4 ± 9.3 (%), 79.8 ± 8.5 and 44.2 ± 6.2 at intensities of 0.3, 0.4 and 0.5 W/cm^2, respectively. On the other hand, the apoptosis detected by observing the DNA fragmentation assay 6 h after the low intensity pulsed US treatment (1 min) was significantly increased at intensities more than 0.2 W/cm^2, 24.0 ± 3.3 (%), 26.0 ± 4.6, and 26.5 ± 2.4 at intensities of 0.3, 0.4 and 0.5 W/cm^2, respectively (Fig. 2(B)). Next, the relationship between ultrasonic intensity and free radical formation was studied using EPR-spin trapping with DMPO. The EPR signal of DMPO-OH adducts 1 min after the low intensity pulsed US treatment (1 min) was not detected up to 0.2 W/cm^2, and increased with increasing intensity up to 0.5 W/cm^2. The values of intensities of 0.3, 0.4 and 0.5 W/cm^2 were 0.015 ± 0.01 (relative units), 0.044 ± 0.023 and 0.059 ± 0.022, respectively (Fig. 2(C)). During the sonication procedure, low intensity pulsed US at 0.3 W/cm^2 for 1 min slightly increased the temperature of medium 0.30 ± 0.10 °C as measure by a digital thermometer (model 7563, Yokogawa Electric Co., Tokyo, Japan). Thus, we considered that thermal effect could be neglected in this US condition. Considering the effects of US on the basis of these data, we chose intensity of 0.3 W/cm^2 for the remainder of our studies.

3.2. Identification of genes responsive to low intensity pulsed ultrasound

To identify genes responsive to non-thermal low intensity pulsed US in the cells, we carried out DNA microarray analysis of cells cultured at 3 h after US treatment (for 1 min at 0.3 W/cm^2). Genes were considered up- or down-regulated, if each value and the average fold change were 1.5 or greater in two different experiments. Of approximately 16,600 genes

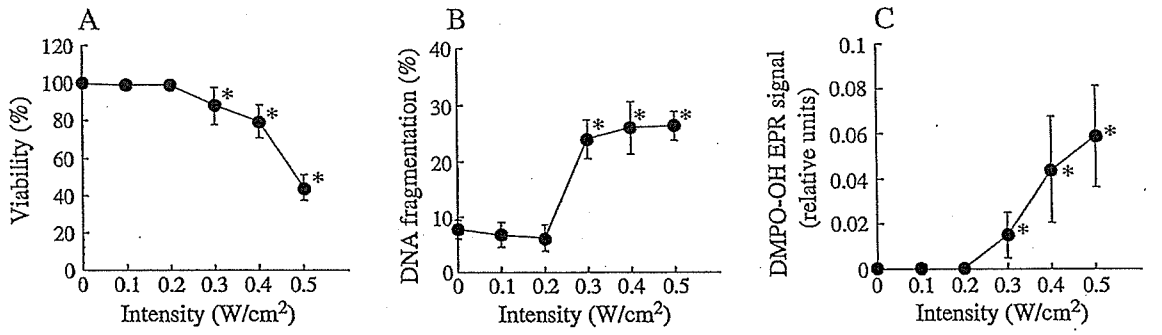

Fig. 2. The effects of ultrasonic intensity on cell viability (A), DNA fragmentation (B) and free radical formation induced by US (C). The cells were sonicated for 1 min at 0–0.5 W/cm^2, 1 MHz pulsed (10% DF, 100 Hz) and cultured at 37 °C. (A) After 6 h culture, the cell viability was evaluated by trypan blue dye exclusion test. (B) After 6 h culture, DNA fragmentation was assayed. (C) After 1 min culture, EPR-spin trapping with DMPO was employed to detect US generated free radicals. One unit of the *Y*-axis is calculated to correspond to about 19.0 μM of TEMPOL. Data indicate means ± SD for four different experiments. *$P<0.05$ vs. control (intensity: 0 W/cm^2) (Student's *t*-test).

examined, four up-regulated genes, BCL2-associated athanogene 3 (*BAG3*), DnaJ (Hsp40) homolog, subfamily B, member 1 (*DNAJB1*), heat shock 70 kDa protein 1B (*HSPA1B*), and heat shock 70 kDa protein 6 (*HSPA6*), and two down-regulated genes, isopentenyl-diphosphate delta isomerase 1 (*IDI1*) and 3-hydroxy-3-methylglutaryl–coenzyme A synthase 1 (*HMGCS1*) were identified (Table 2). To verify the results of the microarray experiments, real-time quantitative PCR was performed. The results are summarized in Fig. 3. Although the expression levels of these genes were not completely comparable to that found by microarray analysis, the expression levels of *BAG3*, *DNAJB1*, *HSPA1B* and *HSPA6* were significantly increased, with expression levels being 6.3-, 3.3-, 7.3- and 311-fold, respectively. In contrast, the expression levels of *IDI1* and *HMGCS1* were significantly decreased, with the expression levels being 0.45 and 0.34-fold, respectively.

4. Discussion

US have been widely used for diagnosis and therapy in many medical fields. More recently, following a report by Lagneaux et al. [12], we also reported that non-thermal low intensity pulsed US treatment (1 MHz, 0.3 W/cm^2, 1 min) induced apoptosis in human leukemia cell lines such as U937, Jurkat and Molt-4 [9,22]. In addition, low intensity pulsed US have been shown to promote cell proliferation in human skin fibroblasts [25] and to stimulate cell proliferation and differentiation in human periosteal cells [26]. In the present study, non-thermal low intensity pulsed US induced apoptosis with minimal cell lysis and free radical formation was observed in human leukemia Molt-4 cells in the cases of our previous studies [9,22]. In addition, DNA microarray and real-time quantitative PCR analyses suggested that six genes showed changed levels of expression in US-treated Molt-4 cells. To our best knowledge, this is the first report of DNA microarray analysis of genes that are differentially expressed in response to apoptosis induced by non-thermal low intensity pulsed US in human leukemia cells. Of the approximately 16,600 genes analyzed, four genes including *BAG3*, *DNAJB1*, *HSPA1B* and *HSPA6* showed increased levels of expression while two genes including *IDI1* and *HMGCS1* showed decreased levels in Molt-4 cells 3 h after the US treatment. Of the four up-regulated genes, three heat shock proteins (HSPs), HSPA1B, HSPA6 and DNAJB1, were involved. HSPs are important modifying factors in cellular responses to a variety of physiological relevant conditions such as hyperthermia, oxidative stress, exercise and so on, and play important physiological roles for both normal cellular functions and survival after a stress [27]. HSPA1B and HSPA6 or DNAJB1 are isoforms of inducible Hsp70 or Hsp40, respectively. Inducible

Table 2
Up- and down-regulated genes after sonication in Molt-4 cells

Gene	Fold change			Genbank accession no.
	Exp. 1	Exp. 2	Average	
Up-regulated				
HSPA1B	1.9	1.5	1.7	NM_005346
BAG3	1.5	2.1	1.8	NM_004281
HSPA6	1.7	2.6	2.1	NM_002155
DNAJB1	2.2	2.9	2.6	NM_006145
Down-regulated				
IDI1	0.58	0.67	0.62	NM_004508
HMGCS1	0.52	0.66	0.59	NM_002130

Microarray analysis was performed. Details of experimental conditions described in Section 2.

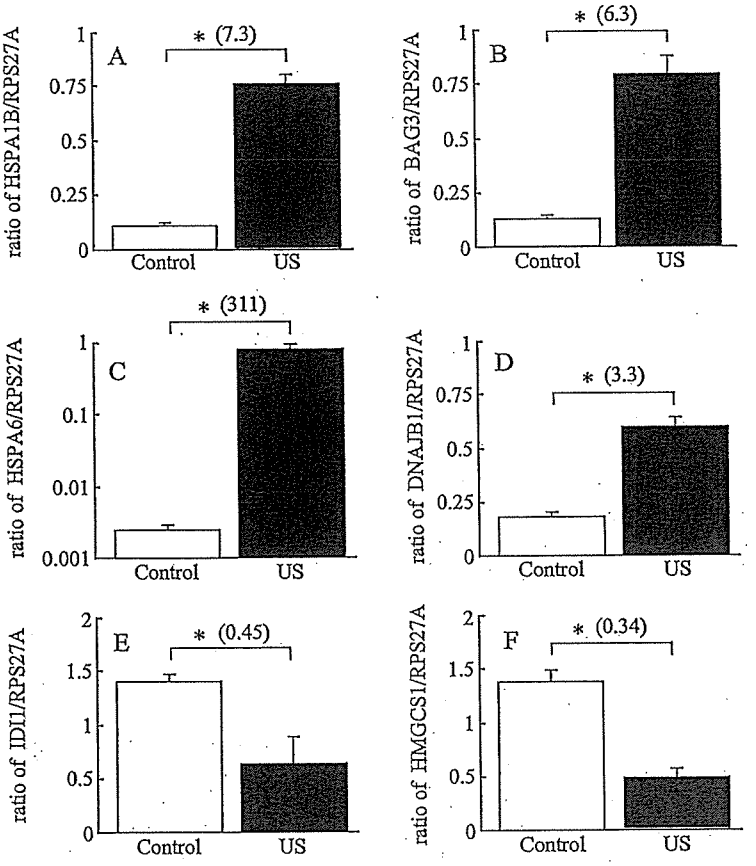

Fig. 3. Verification of the DNA microarray results with real-time quantitative PCR. The cells were exposed to US for 1 min at 0.3 W/cm^2, 1 MHz pulsed (10% DF, 100 Hz) and cultured for 3 h at 37 °C. Reverse transcriptase reaction was carried out with total RNA. Real-time quantitative PCR was performed according to the manufacture's instructions. mRNA level was normalized by *RPS27A*. A, *HSPA1B*; B, *BAG3*; C, *HSPA6*; D, *DNAJB1*; E, *IDI1* and F, *HMGCS1*. Data indicate means±SD for four different experiments. *$P<0.05$ vs. control (Student's *t* test).

Hsp70 and Hsp40 have been shown to protect cells from necrosis [28] and apoptosis [29,30] and co-operate with Hsp70 [31], respectively. It has been demonstrated that the anti-apoptotic activities of BAG-family proteins including BAG3 may be dependent on their interactions with Hsp70 and/or binding to Bcl-2, an anti-apoptotic protein [32,33]. Interestingly, in the present study, the expression of *BAG3* was significantly elevated in US-treated cells. We therefore speculate that *BAG3* and *Hsps*, *DNAJB1*, *HSPA1B* and *HSPA6*, may be up-regulated to protect cells from apoptosis. IntelliGene HS human Expression microarray used here is spotted with genes for 42 HSPs containing 11 Hsp70 and 21 Hsp40 proteins and four BAG-family proteins. However, only four genes out of 46 genes were increased in this experiment. The mechanisms by which low intensity pulsed US up-regulates these four genes and their physiological roles in US-treated cells remain a subject of further study.

In the present study, genes including IDI1 and HMGCS1 showed decreased levels of expression in the cells treated with US. Both HMGCS1 and IDI1 are enzymes in cholesterol biosynthesis, and the former drives the condensation of acetyl-CoA with acetoacetyl-CoA to form HMG-CoA and the latter catalyzes the rearrangement of isopentenyl diphosphate to its highly electrophilic isomer, dimethylallyl diphosphate [34,35]. It seems likely that the cholesterol biosynthesis pathway is down-regulated in apoptotic cells induced by US. Further studies will be necessary to clarify functions of these two genes in the apoptotic cells induced by low intensity pulsed US.

By using UniGEMV Ver2.0 human gene expression DNA microarrays to detect approximately 9200 genes, we previously demonstrated that five up-regulated genes (ferritin, heavy polypeptide 1, AU RNA-binding protein/enoyl–Coenzyme A hydratase, v-jun avian sarcoma virus 17 oncogene homolog, expressed

sequence tag (GenBank Accession number: N35555) and HO-1) and two down-regulated genes (v-myb avian myeloblastosis viral oncogene homolog and cathepsin G) were identified in the human lymphoma U937 cells at 6 h after exposure to fairly intense continuous waves (1 MHz, 4.9 W/cm^2, for 1 min) which induced significant cell lysis and free radicals [20]. Moreover, under nearly experimental condition, DNA microarray analysis indicated that PCTAIRE protein kinase 1 transcript variant 2 and HO-1 were up-regulated while leukemia inhibitory factor and chemokine ligand 10 were down-regulated by using IntelliGene II Human CHIP 1 DNA microarrays to detect approximately 3900 genes [21]. These microarray and real-time quantitative PCR results suggested that HO-1 is the most sensitive gene in U937 cells treated with low intensity continuous US [20,21]. However, all of these differentially expressed genes containing HO-1 were not identified in our present experiments using low intensity pulsed US. This discrepancy may be due to different experimental conditions such as the type of microarray, intensity of US and origin of the cell.

In conclusion, the present results will provide a basis for further understanding of the molecular mechanisms of biological effects of low intensity pulsed US. Here, we used the standing wave field induced by millisecond pulsed-ultrasound, and the detection of change of gene expression in the progressive wave field remains for further experiment.

Acknowledgements

The authors express their appreciation to Drs Shinichiro Umemura and Hideki Yoshikawa, Central Research Laboratory, Hitachi Co. Ltd, Tokyo, Japan for their support on measurement of peak pressure amplitude of the ultrasonic apparatus. This study is supported in part by the Grant in Aid for Scientific Research on Priority Areas (12217049) from the Ministry of Education, Culture, Sports, Sciences and Technology; in part by the 21st Century COE Program of the Toyama Medical and Pharmaceutical University, Japan; and also in part by Research and Development Committee Program of the Japan Society of Ultrasonics in Medicine.

References

[1] K.S. Suslick, Sonochemistry, Science 247 (1990) 1439–1445.
[2] P. Riesz, T. Kondo, Free radical formation induced by ultrasound and its biological implications, Free Radic. Biol. Med. 13 (1992) 247–270.
[3] L.B. Feril Jr., T. Kondo, Biological effects of low intensity ultrasound: the mechanism involved, and its implications on therapy and on biosafety of ultrasound, J. Radiat. Res. 45 (2004) 479–489.
[4] A. Abdollahi, S. Domhan, J.W. Jenne, M. Hallaj, G. Dell'Aqua, M. Mueckenthaler, et al., Apoptosis signals in lymphoblasts induced by focused ultrasound, Fed. Am. Soc. Exp. Biol. J. 18 (2004) 1413–1414.
[5] H. Ashush, L.A. Rozenszajn, M. Blass, M. Barda-Saad, D. Azimov, J. Radnay, et al., Apoptosis induction of human myeloid leukemic cells by ultrasound exposure, Cancer Res. 60 (2000) 1014–1020.
[6] L.B. Feril Jr., T. Kondo, Major factors involved in the inhibition of ultrasound-induced free radical production and cell killing by pre-sonication incubation or by high cell density, Ultrason. Sonochem. 12 (2005) 353–357.
[7] L.B. Feril Jr., T. Kondo, Q.L. Zhao, R. Ogawa, K. Tachibana, N. Kudo, et al., Enhancement of ultrasound-induced apoptosis and cell lysis by echo-contrast agents, Ultrasound Med. Biol. (2003) 331–337.
[8] L.B. Feril Jr., T. Kondo, K. Takaya, P. Riesz, Enhanced ultrasound-induced apoptosis and cell lysis by a hypotonic medium, Int. J. Radiat. Biol. 80 (2004) 165–175.
[9] L.B. Feril Jr., T. Kondo, Z.G. Cui, Y. Tabuchi, Q.L. Zhao, H. Ando, et al., Apoptosis induced by the sonomechanical effects of low intensity pulsed ultrasound in a human leukemia cell line, Cancer Lett. 221 (2005) 145–152.
[10] H. Honda, Q.L. Zhao, T. Kondo, Effects of dissolved gases and an echo contrast agent on apoptosis induced by ultrasound and its mechanism via the mitochondria-caspase pathway, Ultrasound Med. Biol. 28 (2002) 673–682.
[11] H. Honda, T. Kondo, Q.L. Zhao, L.B. Feril Jr., H. Kitagawa, Role of intracellular calcium ions and reactive oxygen species in apoptosis induced by ultrasound, Ultrasound Med. Biol. 30 (2004) 683–692.
[12] L. Lagneaux, E.C. de Meulenaer, A. Delforge, M. Dejeneffe, M. Massy, C. Moerman, et al., Ultrasonic low-energy treatment: a novel approach to induce apoptosis in human leukemic cells, Exp Hematol. 30 (2002) 1293–1301.
[13] A. Butte, The use and analysis of microarray data, Nat. Rev. Drug Discov. 1 (2002) 951–960.
[14] J. DeRisi, L. Penland, P.O. Brown, M.L. Bittner, P.S. Meltzer, M. Ray, et al., Use of a cDNA microarray to analyse gene expression patterns in human cancer, Nat. Genet. 14 (1996) 457–460.
[15] Y. Tabuchi, T. Kondo, M. Suzuki, M. Obinata, Genes involved in nonpermissive temperature-induced cell differentiation in Sertoli TTE3 cells bearing temperature-sensitive simian virus 40 large T-antigen, Biochem. Biophys. Res. Commun. 329 (2005) 947–956.
[16] Y. Tabuchi, Y. Arai, T. Kondo, N. Takeguchi, S. Asano, Identification of genes responsive to sodium butyrate in colonic epithelial cells, Biochem. Biophys. Res. Commun. 293 (2002) 1287–1294.
[17] Y. Tabuchi, T. Kondo, cDNA microarray analysis reveals chop-10 plays a key role in Sertoli cell injury induced by bisphenol A, Biochem. Biophys. Res. Commun. 305 (2003) 54–61.
[18] Y. Tabuchi, Q.L. Zhao, T. Kondo, DNA microarray analysis of differentially expressed genes responsive to bisphenol A, an alkylphenol derivative, in an in vitro mouse Sertoli cell model, Jpn. J. Pharmacol. 89 (2002) 413–416.

[19] H. Hirano, Y. Tabuchi, T. Kondo, Q.L. Zhao, R. Ogawa, Z.G. Cui, et al., Analysis of gene expression in apoptosis of human lymphoma U937 cells induced by heat shock and the effects of alpha-phenyl *N*-*tert*-butylnitrone (PBN) and its derivatives, Apoptosis 10 (2005) 331–340.

[20] Y. Tabuchi, T. Kondo, R. Ogawa, H. Mori, DNA microarray analyses of genes elicited by ultrasound in human U937 cells, Biochem. Biophys. Res. Commun. 290 (2002) 498–503.

[21] G. Kagiya, Y. Tabuchi, L.B. Feril, Jr., R. Ogawa, Q.L. Zhao, N. Kudo, et al., Confirmation of enhanced expression of heme oxygenase-1 gene induced by ultrasound and its mechanism: analysis by cDNA microarray system, real-time quantitative PCR, and western blotting, J. Med. Ultrasonics (in press).

[22] H. Ando, L.B. Feril, Jr., T. Kondo, Y. Tabuchi, R. Ogawa, Q.L. Zhao, et al., An echo-contrast agent, Levovist lowers the ultrasound intensity required to induce apoptosis of human leukemia cells, Cancer Lett., in press.

[23] K.S. Sellins, J.J. Cohen, Nuclear changes in the cytotoxic T lymphocyte-induced model of apoptosis, Immunol. Rev. 146 (1995) 241–266.

[24] P. Riesz, T. Kondo, C.M. Krishna, Free radical formation by ultrasound in aqueous solutions. A spin trapping study, Free Radic. Res. Commun. 10 (1990) 27–35.

[25] S. Zhou, A. Schmelz, T. Seufferlein, Y. Li, J. Zhao, M.G. Bachem, Molecular mechanisms of low intensity pulsed ultrasound in human skin fibroblasts, J. Biol. Chem. 279 (2004) 54463–54469.

[26] K.S. Leung, W.H. Cheung, C. Zhang, K.M. Lee, H.K. Lo, Low intensity pulsed ultrasound stimulates osteogenic activity of human periosteal cells, Clin. Orthop. Relat. Res. 418 (2004) 253–259.

[27] K.C. Kregel, Heat shock proteins: modifying factors in physiological stress responses and acquired thermotolerance, J. Appl. Physiol. 92 (2002) 2177–2186.

[28] J.A. Yaglom, D. Ekhterae, V.L. Gabai, M.Y. Sherman, Regulation of necrosis of H9c2 myogenic cells upon transient energy deprivation. Rapid deenergization of mitochondria precedes necrosis and is controlled by reactive oxygen species, stress kinase JNK, HSP72 and ARC, J. Biol. Chem. 278 (2003) 50483–50496.

[29] A. Saleh, S.M. Srinivasula, L. Balkir, P.D. Robbins, E.S. Alnemri, Negative regulation of the Apaf-1 apoptosome by Hsp70, Nat. Cell Biol. 2 (2000) 476–483.

[30] R. Steel, J.P. Doherty, K. Buzzard, N. Clemons, C.J. Hawkins, R.L. Anderson, Hsp72 inhibits apoptosis upstream of the mitochondria and not through interactions with Apaf-1, J. Biol. Chem. 279 (2004) 51490–51499.

[31] R. Kaneko, Y. Hayashi, I. Tohnai, M. Ueda, K. Ohtsuka, Hsp40 a possible indicator for thermotolerance of murine tumour in vivo, Int. J. Hyperthermia 13 (1997) 507–516.

[32] H. Doong, A. Vrailas, E.C. Kohn, What's in the 'BAG'?–A functional domain analysis of the BAG-family proteins, Cancer Lett. 188 (2002) 25–32.

[33] S. Takayama, Z. Xie, J.C. Reed, An evolutionarily conserved family of Hsp70/Hsc70 molecular chaperone regulators, J. Biol. Chem. 274 (1999) 781–786.

[34] F.M. Hahn, J.W. Xuan, A.F. Chambers, C.D. Poulter, Human isopentenyl diphosphate: dimethylallyl diphosphate isomerase: overproduction, purification, and characterization, Arch. Biochem. Biophys. 332 (1996) 30–34.

[35] D.J. Wilkin, S.Y. Kutsunai, P.A. Edwards, Isolation and sequence of the human farnesyl pyrophosphate synthetase cDNA. Coordinate regulation of the mRNAs for farnesyl pyrophosphate synthetase, 3-hydroxy-3-methylglutaryl coenzyme A reductase, and 3-hydroxy-3-methylglutaryl coenzyme A synthase by phorbol ester, J. Biol. Chem. 265 (1990) 4607–4614.