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DNA Microarray Analyses of Genes Elicited by Ultrasound in Human U937 Cells

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The gene expression of human histiocytic lymphoma cell line U937 at 6 h after 1 MHz ultrasound treatment in the presence of Ar or N_2O gas was examined by DNA microarrays. Of the 9,182 genes analyzed, only the keratin gene was identified as down-regulated in the cells exposed to ultrasound in the presence of N_2O where no internal cavitation was observed. In contrast, five up-regulated and two down-regulated genes were identified in the cells exposed to ultrasound in the presence of Ar where internal cavitation was apparently observed. Six changes of the gene expression were confirmed by the semiquantitative reverse transcriptase-polymerase chain reaction (RT-PCR). Gene expression of heme oxygenase was augmented by a factor of 6.6 in microarray and by 4.0 by RT-PCR. These results indicate that internal cavitation increased the expression of genes responsive to oxidative stress in sonicated cells but non-inertial cavitation had minimal effects on gene expression.

Key Words: **DNA microarray; gene expression; reverse transcriptase-polymerase chain reaction; ultrasound; internal cavitation; heme oxygenase.**

The study of acoustic cavitation in biological systems is of interest because of the widespread application of ultrasound not only in medical diagnosis and therapeutic application (1) but also in relation to the bioresearch application of ultrasound in the laboratory. Evidence has been presented for acoustic cavitation induced by pulsed ultrasound, which simulates diagnostic conditions (2, 3). Cavitation is known to lead to both mechanical shear stress and free radical formation arising from the oscillation and collapse of cavitation bubbles (4–6). Hydroxyl radicals and hydrogen atoms form as a result of thermal dissociation of water induced by very high temperatures (several thousand degree Kelvin) and pressures due to ultrasonic cavitation. In general it has been inferred that these two effects (free radicals and shearing stress) of cavitation act simultaneously on biological materials. However, the molecular mechanisms of biological damage elicited by cavitation have not been elucidated so far.

Triatomic N_2O and monoatomic Ar gas create different final temperatures of the cavitation bubbles, because of the different γ values (define as the ratio of specific heat at constant pressure to specific heat at constant volume) (7). It has been reported that N_2O suppresses free radical formation induced by ultrasound. On the other hand, monoatomic gases, such as Ar, in aqueous solutions promote free radical formation induced by ultrasound (8–11). The larger yield of free radicals in Ar- compared to N_2O-saturated solutions can be explained in terms of the higher final temperature of the collapsing cavitation bubbles calculated by Young to explain the sonoluninescence results (12).

The technology known as cDNA microarray expression profiling now offers tremendous potential for characterizing gene expression patterns during diseases and normal biological processes, as well as for the identification of specifically expressed genes induced by physical and chemical stress. Here, we examined a gene expression pattern in human histiocytic lymphoma U937 cultured cells exposed to 1 MHz ultrasound in Ar- or N_2O-saturated media by using UniGEMV Ver2.0 human cDNA microarrays.

MATERIALS AND METHODS

Cells and cell culture. The human histiocytic lymphoma cell line, U937, was obtained from the Japanese Cancer Research Resource Bank (Tokyo, Japan). The cells were grown in RPMI 1640 culture medium (Life Technologies, Inc., Rockville, MD) supplemented with 10% heat-inactivated fetal bovine serum (Life Technologies, Inc.) at 37°C in humidified air with 5% CO_2. The cells in log-phase (doubling time is 23.5 h) were used for experiments after confirmation that they were free of any mycoplasma contamination.

Preparation of erythrocytes suspensions and hemolysis measurements. Freshly drawn heparinized human blood from a healthy donor was washed three times with 10 mM phosphate buffered

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solution (pH 7.4) containing 150 mM NaCl following removal of the buffy coat and plasma by aspiration. After sonication of solutions containing erythrocytes (1.3% hematocrit) and centrifugation at 700*g* for 5 min, hemolysis was determined by measuring the absorbance at 415 nm due to hemoglobin in the diluted supernatant with a spectrophotometer. Absorbance was converted to percentage hemolysis by scaling to controls that had been 100% hemolyzed by adding water (11). Hemolysis induced by ultrasound was used as an indicator of inertial cavitation (12).

Ultrasonic exposure system. The ultrasonic apparatus consists of an acrylic tank and unfocused ceramic transducer (3 cm in diameter) driven by a 1.0 MHz ultrasonic generator (9, 10). Sonication was carried out using a disposable sterile culture tube (No. 25760; Corning Inc., Corning, NY) for which it was reported that the attenuation is about 35% with only the front half of the tube**.** When the sample solution was transferred into the tube, 3.5 ml of sample solution formed a column 3 cm high. The tube was positioned directly in front of the transducer face with the tube axis at 20 cm from the transducer, and the surrounding water in the acrylic tank was circulated by Coolnit (Model CL-80F, TAITEC, Tokyo, Japan) at 20.0 ± 0.05°C. During sonication, the tube was rotated at 30 rpm using a synchronous motor to improve mixing and to provide a more uniform exposure. The cells in the medium saturated with Ar or N_2O were sonicated at an intensity of 4.9 W/cm^2 (I_{SPTA} = Intensity of spatial peak and temporal average) (3.6 W/cm^2 (I_{SATA} = Intensity of spatial average and temporal average), where chemical and biological effects of ultrasound are the maximum in the rotation tube system (13). The detailed dosimetry and sonication procedures were described in previous reports (9, 10).

Electron spin resonance (ESR)-spin trapping. Aqueous solutions containing DMPO (5,5-dimethyl-1-pyrroline 1-oxide; Labotec Co., Tokyo, Japan) were saturated with various gases at a flow rate of 100 ml/min for more than 10 min and irradiated by 1 MHz ultrasound for 10 min. By 9.425 GHz field modulation with a 0.1 mT amplitude using a microwave power of 4 mW, ESR spectra of the sonicated solution in a quarts-flat cell were recorded with ESR (RFR-30; Radical Research Inc., Tokyo, Japan) at room temperature. The yields of spin adduct were determined using a stable nitroxide radical, 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 vs different concentrations of the standard nitroxide radical. The procedures in detail were described in previous report (8, 11, 13).

Measurement of fraction of intact cells and apoptosis. The remaining intact cells after sonication were counted with a Bruker Turk hemocytometer. For detection of apoptosis, U937 cells were stained simultaneously with Annexin V/FITC (Immunotech Co., Marseille, France) and propidium iodide and assessed with a flow cytometer (EPICS XL, Beckman-Coulter, Co., Tokyo, Japan). The method was described in detail in previous papers (14, 15).

Separation of mRNA. U937 cells were exposed to 1 MHz ultrasound at 20°C for 30 s in the presence of Ar or N_2O gas. mRNAs were extracted from the cells using a RNeasy Total RNA Extraction Kit (QIAGEN K.K., Tokyo, Japan) and an Oligotex-dT30 mRNA Purification Kit (Takara Shuzo Co., Kyoto, Japan) following the manufacturer's instructions.

Microarray analysis. Broad-scale expression profiling was performed by an UniGEMV Ver2.0 human gene expression microarray which is spotted with 9,182 cDNA fragments. A list of these genes is available at Kurabo's Web site (http://www.bio.kurabo.co.jp/data/HumanUniGene1.txt). Isolated mRNAs were reverse transcribed with 5′-Cy3- or -Cy5-labeled random 9-mer (Operon Technologies, Inc., Alameda, CA). Briefly, reaction mixtures were incubated for 2 h at 37°C with 200 ng mRNA, 200 units M-MLV reverse transcriptase (Life Technologies, Inc.), 4 mM DTT, 1 unit RNase Inhibitor (Ambion, Austin, TX), 0.5 mM dNTPs, and 2 μg labeled 9-mer in 25 μl volume with enzyme buffer supplied by the manufacturer. Labeled mRNAs were then purified with a TE-30 column (Clontech, Palo Alto, CA), and applied to the microarrays. After hybridization at 60°C for 6.5 h, the microarrays were washed three times consecutively at low ionic strength. The microarrays were scanned in both Cy3 and Cy5 channels with an Axon GenePix scanner (Foster City, CA). Incyte GEMtool software (Incyte Pharmaceuticals, Inc., Palo Alto, CA) was used for image analysis. The average of the resulting total Cy3 and Cy5 signal gave a ratio that was used to balance or normalize the signals.

Detection of mRNA expression by semiquantitative reverse transcriptase (RT)-polymerase chain reaction (PCR). Based on the previously published studies (16–21) and data base (GenBank Accession No. XM_004256 and AF240776), PCR primers were designed; glyceraldehyde 3-phosphate dehydrogenase (G3PDH) forward and reverse, 5′-AAGGCTGGGGCTCATTTGCA-3′ (379–398) and 5′-ATGACCTTGCCCACAGCCTT-3′ (722–703), respectively (16); v-myb avian myeloblastosis viral oncogene homolog (v-myb) forward and reverse, 5′-GAAGACCCCGGCACAGCATA-3′ (102–121) and 5′-TCCCCAGTCT- CTTGTGTGCC-3′ (584–565), respectively (GenBank Accession No. XM_004256); v-jun avian sarcoma virus 17 oncogene homolog (v-jun) forward and reverse, 5′-AGATGAGCAGGAGGGGTTCG-3′ (1286–1305) and 5′-ATCTCGGGCACTGTCTGAGG-3′ (1678–1659), respectively (17); heme oxygenase (decycling) 1 (HMOX1) forward and reverse, 5′-CTACACACCAGCCATGCAGC-3′ (398–417) and 5′-CACTTTGTTGCTGGCCCGCT-3′ (812–793), respectively (18); ferritin, heavy polypeptide 1 (FTH1) forward and reverse, 5′-GCGGGCTGAATGCAATGGAG-3′ (378–397) and 5′-TTCCGCCAAGCCAGATTCGG-3′ (595–576), respectively (19); AU RNA-binding protein/enoyl-Coenzyme A hydratase (AUH) forward and reverse, 5′-GGAGGAGGAGAACCGAGGAA-3′ (250–269) and 5′-CCACCACCTAAAGCGAGTCC-3′ (564–545), respectively (20); cathepsin G forward and reverse, 5′-TCCAGAGTCCAGCAGGTCAG-3′ (127–146) and 5′-GCCTATCCCTCTGCACTCTC-3′ (519–500), respectively (GenBank Accession No. AF240776); keratin, hair, acidic, 1 (KRTHA1), forward and reverse, 5′-GCTACCTGGAGAAAGTGCGT-3′ (259–278) and 5′-ACTTGCACAGGGTCAGCTCA-3′ (573–554), respectively (21). The PCR products for G3PDH, v-myb, v-jun, HMOX1, FTH1, AUH, cathepsin G and KRTHA1 were predicted to be 344, 483, 393, 415, 218, 315, 393, and 315 bp in length, respectively. RT-PCR was performed using a QIAGEN One-Step RT-PCR Kit (QIAGEN K.K.). Temperature cycling conditions for each primers consisted of 30 min at 50°C and 15 min at 95°C followed by 20–35 cycles for 1 min at 94°C, 1 min at 60°C and 1 min at 72°C, with a final extension for 10 min at 72°C. Preliminary experiments were performed to determine the number of amplification cycles required for the exponential phase of the PCR amplification of these genes. All reactions were performed in a Thermocycler (GeneAmp PCR System 9700; Applied Biosystems Japan K.K., Tokyo, Japan). PCR samples were electrophoresed through 2% agarose gels, and after staining with ethidium bromide, the product bands were visualized under ultraviolet light. The identity of PCR products was confirmed by sequencing. Bands of PCR products were quantified by densitometry using an ATTO Densitograph (ATTO Co., Tokyo, Japan). Fold induction was normalized by G3PDH.

RESULTS

Free radical formation and hemolysis induced by ultrasound in the presence of Ar or N_2O. The short-lived hydroxyl radicals and hydrogen atoms formed by sonolysis of aqueous DMPO solution reacted with the spin trap to form the following ESR observable radicals (DMPO + OH(H) → DMPO—OH(H)). Aqueous solutions of DMPO (2 mM) were sonicated in the presence of Ar or N_2O and their ESR spectra were obtained. The

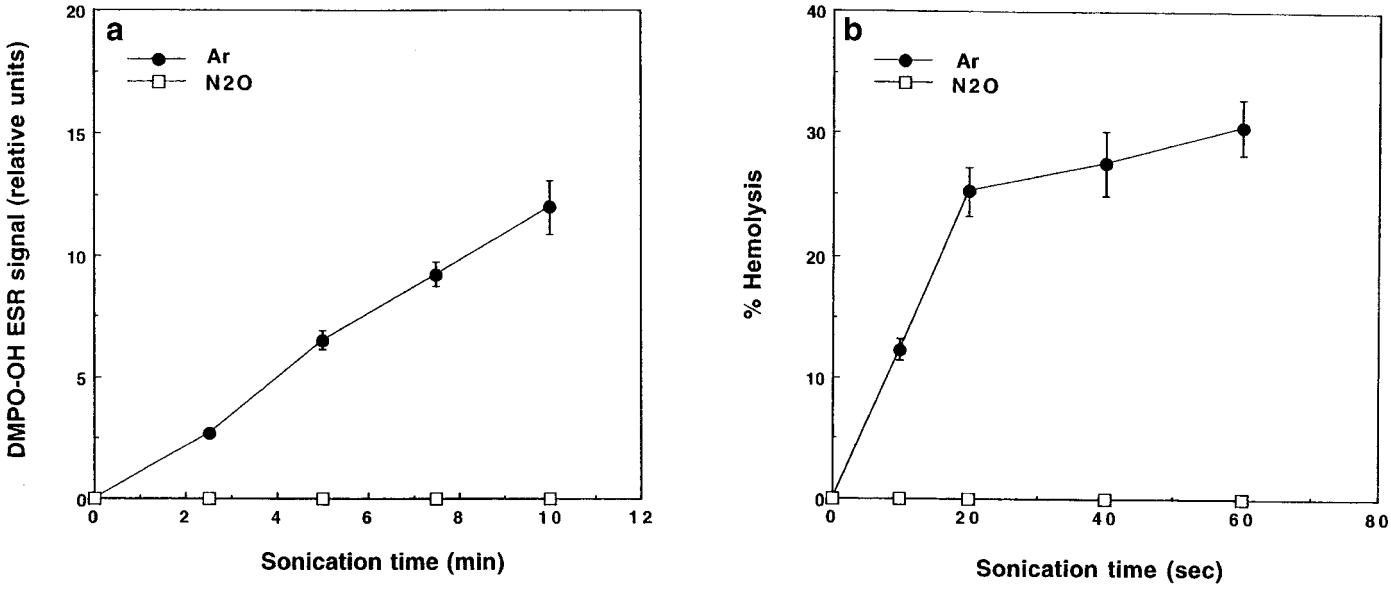

FIG. 1. (a) The effects of Ar and N_2O on hydroxyl radical formation induced by 1.0 MHz ultrasound. Hydroxyl radical formation was measured by the DMPO—OH ESR signal. One unit of the Y-axis was calculated to be 1×10^{-7} M of DMPO—OH in comparison with standard nitroxide. (b) The effects of Ar and N_2O on the hemolysis of erythrocytes exposed to 1.0 MHz ultrasound. Data indicate means ± SD from three independent experiments.

1:2:2:1 four line ESR spectrum has equal nitrogen and hydrogen hyperfine coupling constants ($a_N = a_H =$ 1.49 mT) and corresponds to the DMPO—OH spin adduct. Figure 1a shows the effect of sonication time on the formation of the DMPO—OH spin adducts in the presence of Ar or N_2O, respectively. The ESR signal intensity of the DMPO—OH adducts increased with the time of sonolysis when the solution was sonicated in the presence of Ar. On the other hand, when aqueous DMPO solution was sonicated in the presence of N_2O, the ESR signals of the DMPO—OH adduct were not observed. The effects of sonication time on hemolysis of erythrocytes in solutions bubbled with Ar or N_2O are shown in Fig. 1b. The percentage hemolysis increased with the time of sonolysis when the erythrocyte suspension was sonicated with Ar bubbling. However, no hemolysis was observed when the solution was sonicated with N_2O bubbling. These results indicate that inertial cavitation occur in the solution in the presence of monoatomic Ar but not in the presence of triatomic N_2O.

Fraction of intact cells and apoptosis. When U937 cells were sonicated at an intensity of 4.9 W/cm^2 (I_{SPTA}) for 1 min in the presence of Ar or N_2O, the fraction of intact cells measured immediately after sonication was 33.1 ± 9.2% (mean ± SD, $n = 3$) in the presence of Ar and 95.7 ± 8.2% (mean ± SD, $n = 3$) in the presence of N_2O. After incubation for 6 h, the fractions of early apoptosis (EA) and secondary necrosis (SN) estimated by flow cytometry were 8.7 ± 1.1 (EA) and 17.4 ± 4.0 (SN) (mean ± SD, $n = 5$) in the presence of Ar, and 1.8 ± 0.7 (EA) and 0.8 ± 0.3 (SN) (mean ± SD, $n = 5$) in the presence of N_2O, respectively. The fractions of EA and SN in nontreated cells were 2.2 ± 1.5 (EA) and 1.0 ± 0.8 (SA) (mean ± SD, $n = 10$). These results indicate that cell lysis, EA, and SN are produced by ultrasound significantly in the presence of monoatomic Ar but not in the presence of triatomic N_2O.

Microarray analysis. To identify genes responsive to physical and chemical stress due to ultrasound, we carried out microarray analyses of U937 cells at 6 h after 1 MHz ultrasound treatment in the presence of Ar or N_2O gas. Genes were considered up- or down-regulated if the fold change was at least 1.8 in individual experiments and the averaged fold change was 2.0 or greater in duplicate experiments (control experiment in the presence of N_2O gas: single experiment). Using three UniGEMV Ver2.0 human gene expression microarrays, we monitored the expression of 9,182 genes. Under the cells exposed to ultrasound in the presence of N_2O gas, KRTHA1 was the only gene identified as a down-regulated gene, with a level of 2.7-fold lower than that of the nontreated group (Table 1). On the other hand, five genes, FTH1, AUH, v-jun, EST (GenBank Accession No. N35555) and HMOX1, were up-regulated in the cells exposed to ultrasound in the presence of Ar gas, with levels 2.0-, 2.2-, 3.1-, 3.1-, and 6.6-fold higher than that of nontreated group. Under the same experimental conditions, two genes, cathep-

TABLE 1

Up- and Down-Regulated Genes after Sonication in U937 Cells

Gene	Fold change			GenBank Accession No.
	Exp. 1	Exp. 2	Average	
Under N_2O				
Down regulated				
Keratin, hair, acidic 1 (KRTHA1)	2.7		2.7	Y16787
Under Ar				
Up-regulated				
Ferritin, heavy polypeptide 1 (FTH1)	2.0	2.0	2.0	AA703944
AU RNA-binding protein/enoyl-coenzyme A hydratase (AUH)	2.4	1.9	2.2	NM001698
v-jun avian sarcoma virus 17 oncogene homolog (v-jun)	3.4	2.7	3.1	AW169889
EST	2.5	3.6	3.1	N35555
Heme oxygenase (decycling) 1 (HMOX1)	6.8	6.3	6.6	AU12980
Down-regulated				
v-myb avian myeloblastosis viral oncogene homolog (v-myb)	1.8	3.5	2.7	U22376
Cathepsin G	2.2	2.3	2.3	J04990

Note. U937 cells were exposed to ultrasound in the presence of N_2O gas or Ar gas. Microarray analysis was performed. Details of experimental conditions described under Materials and Methods.

sin G and v-myb, were down-regulated, with levels 2.3- and 2.7-fold lower than that of the nontreated group.

Semiquantitative RT-PCR analysis. To verify the results of microarrays, we performed semiquantitative RT-PCR analysis. PCR products with predicted sizes for AUH, FTH1, v-jun, HMOX1, cathepsin G, v-myb and G3PDH but not KRTHA1 were observed in control U937 cells (without ultrasound treatment in the presence of Ar or N_2O gas). In the presence of Ar gas, mRNA levels of v-jun (1.7-fold) and HMOX1 (4.0) were up-regulated, whereas mRNA levels of cathepsin G (1.5) and v-myb (3.1) were down-regulated (Fig. 2). These results confirm the findings found in microarray analysis. The mRNA levels of AUH and FTH1 did not change.

DISCUSSION

The present results showed that monoatomic Ar gas in sonicated aqueous solution produced inertial cavitation and triatomic N_2O gas eliminated inertial cavitation. Free radical formation and hemolysis were observed in the aqueous solution sonicated at an intensity of 4.9 W/cm^2 (0.6 MPa) in the presence of Ar but neither endpoint was observed in the presence of N_2O. Only KRTHA1 gene was identified as a down-regulated gene in the cell exposed to ultrasound in the presence of N_2O. However, practically no change of gene expression was estimated in the cells sonicated in the presence of N_2O, because KRTHA1 gene was not identified with RT-PCR analysis, and substantial free radical formation, cell lysis and apoptosis induced by ultrasound were not observed. These results suggest that the non-cavitational effects of ultrasound used in this study do not affect the gene expression and cause no cellular damage. In contrast, five up-regulated genes and two down-regulated genes were identified in the cells exposed to ultrasound in the presence of Ar. Six changes of the gene expression were confirmed by the method of semiquantitative RT-PCR. Gene expression of HMOX1 was augmented by a factor of 6.6 in microarray and by 4.0 by semiquantitative RT-PCR. These results indicate that inertial cavitation increased the expression of genes responsive to oxidative stress in sonicated cells but non-inertial cavitation had minimal effects on gene expression. The reason why in a population of cells by which a significant part is undergoing apoptosis, only few genes appear to change expression would be due to the fact that a large portion of the cell population maintained the normal expression pattern. Indeed, only about 25% of cells are destined to die as early apoptosis and secondary necrosis while about 75% of remained intact cells showed most likely no change in gene expression. On this point, we reported that structure and function of the membrane of remaining intact erythrocytes after sonication, regardless of free radical generation, have been kept normal (11). After certain time of sonication, most of unlysed intact cells appear to keep their integrity as normal cells do.

It is well known that a number of defense proteins and their activities are induced upon exposure to oxidative stresses. These proteins include HMOXs, superoxide dismutases (SOD), glutathione transferases (GTF), glutathione peroxidases (GPO), catalase, nitric oxide synthases (NOS) and heat shock proteins (HSP)

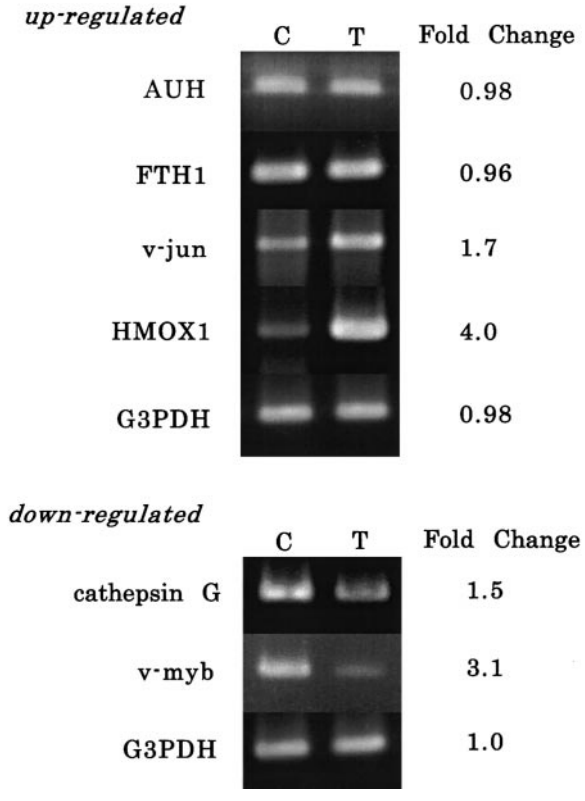

FIG. 2. Changes of transcript levels by semiquantitative RT-PCR. U937 cells were exposed to ultrasound in the presence of N_2O gas (control; C) or Ar gas (treatment; T). PCR products for AU RNA-binding protein/enoyl-Coenzyme A hydratase (AUH; 315 bp), ferritin, heavy polypeptide 1 (FTH1; 218 bp), v-jun avian sarcoma virus 17 oncogene homolog (v-jun; 393 bp), heme oxygenase (decycling) 1 (HMOX1; 415 bp), cathepsin G (393 bp), v-myb avian myeloblastosis viral oncogene homolog (v-myb; 483 bp), and glyceraldehyde 3-phosphate dehydrogenase (G3PDH; 344 bp) were detected in the cells. Fold induction was normalized by G3PDH. The experiments were done in duplicate. Typical results were shown.

(18, 22–28). The UniGEMV Ver2.0 human gene expression microarray used here is spotted with genes for HMOX1, SOD1, SOD2, GTFs (type 1, 2, and so on), GPO1, GPO2, GPO4, catalase, NOS1, NOS2A, NOS3, HSP40, HSP70, and HSP75. In this study, only HMOX1 gene was detected as up-regulated gene, indicating that HMOX1 may be the most important gene in these genes responsive to oxidative stress in the cells after ultrasound exposure in the presence of Ar. HMOX1 is predominantly induced under various oxidative stress conditions in many different cell types (18, 22–24). Since HMOX1-deficient embryonic fibroblasts are hypersensitive to the cytotoxicity of both hemin and hydrogen peroxide, genetic evidence that up-regulation of HMOX1 serves as an adaptive mechanism to protect cells from oxidative damage during stress has been reported (23). When the cells are sonicated in the presence of Ar, the hydroxyl radicals and hydrogen peroxide formed by the recombination of hydroxyl radicals create oxidative stresses. Therefore, enhancement of gene expression of HMOX1 in the cells sonicated in the presence of Ar appears to respond to these oxidative stresses due to inertial cavitation. The mechanism of enhancement of other gene expressions was thought to be complex because many genes, related to apoptosis, cell cycle, cell growth, etc. were induced in response to physical and chemical stresses due to inertial cavitation. The interaction between the cellular response to ultrasound and gene expression remains a subject of further study.

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