Gene Expression Profiles of Cultured Rat Cardiomyocytes (H_9C_2 Cells) in Response to Arsenic Trioxide at Subcytotoxic Level and Oxidative Stress

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Epidemiologic studies have suggested a close correlation between arsenic exposure and cardiovascular disease. In mechanism studies of heart disease and arsenic exposure, blood vessels, vascular smooth muscle cells, and endothelial cells of the artery have long been thought to be the primary targets in arsenic exposure but there are only a few studies on cardiomyocytes. In this study, to predict more diverse responses of cardiomyocytes to arsenic exposure in the development of heart failure, gene expression profiles of cultured rat cardiomyocytes (H_9C_2 cell line) were evaluated using the rat whole-genome microarray when a subcytotoxic level of arsenic trioxide was treated. After 24 hr of arsenic 0.5 ppm exposure (As_2O_3 was used), 405 genes were up-regulated including heme oxygenase-1, and 499 genes were down-regulated including fibroblast growth factor. With the subcytotoxic dose of As_2O_3, oxidative stress was generated without cell death, and the transcription of stress-related genes such as heme oxygenase-1, glutathione S-transferase, metallothionein, and catalase were significantly increased. Direct measurement of reactive oxygen species using fluorescent dye showed that arsenic caused oxidative stress at the subcytotoxic level. Although no direct comparison was made among different types of cells in this study, it appears that arsenic can cause physiologic adverse reactions at a relatively low level in cardiomyocytes and that cardiomyocytes are also one of the vulnerable targets of heart failure by arsenic compounds.

Key words —— arsenic trioxide, cardiomyocytes, gene profiles, oxidative stress

INTRODUCTION

Arsenic is a widely occurring environmental pollutant in ambient air as well as drinking water.[1,2] It is classified as a human carcinogen and is likely to be related to the incidences of diabetes, peripheral disease such as blackfoot disease, and cardiovascular disease.[3–5] Cardiovascular effects of arsenic exposure include hypertension, atherosclerosis, and ischemic heart disease. Unfortunately, the exact induction mechanism of cardiovascular disease due to arsenic exposure has not been fully elucidated. Recent studies have shown that platelet aggregation may be one of the mechanisms of the arsenic-induced cardiovascular and/or peripheral disease. It is reported that the increased susceptibility of platelets to aggregation due to arsenic may induce thrombosis in the cardiovascular and/or peripheral vascular system.[6]

Regarding the arsenic toxicity to the heart, smooth muscle cells and endothelial cells of the artery have been major targets of arsenic exposure in toxic mechanism studies. In the previous report, arsenic inhibited acetylcholine-induced vascular relaxation *via* inhibition of nitric oxide synthase in vascular endothelial cells and showed increased vasoconstriction through calcium sensitization in vascular smooth muscle.[7] There have been many reports on the generation of reactive oxygen species (ROS) in vascular smooth muscle cells during arsenic metabolism. Proliferation of vascular smooth muscle cells by ROS may be implicated in the pathologic processes of atherosclerosis. There are other linking processes from the generation of ROS to inflammation of vascular cells to lead to atherosclerosis. Oxidative stress induced by arsenic increases various inflammation processes such as leukotriene bio-

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synthesis, prostaglandin biosynthesis, and cytokine productions. It also induces the transcription of heme oxygenase-1, monocyte chemoattractant protein-1, interleukin-6, and nuclear factor (NF)-kappa B, and causes DNA damage in vascular smooth muscle cells.[8,9] It is generally known that the dysfunction or loss of cardiomyocytes is as crucial as vascular smooth muscle cells or endothelial cells. Furthermore, it is known that the loss of cardiac myocytes through cell death from hypoxia or toxic chemicals leads to the progression of heart failure.[10] However, the effects of arsenic on cardiomyocytes, which are the constituent cells of heart, are not clear.

This study was designed to evaluate whether arsenic induces oxidative stress in cardiomyocytes at subcytotoxic level and to identify gene expression profiles to predict more diverse responses of cardiomyocytes to arsenic exposure in the development of heart failure.

MATERIALS AND METHODS

Cell Culture and Chemical Exposure —— Rat cardiomyocytes of the H_9C_2 cell line were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% heat-inactivated fetal bovine serum, penicillin 100 IU/ml, and streptomycin 10 μg/ml. Cells were grown and maintained in 28-cm^2 cell culture flasks at 37°C in a 5% CO_2 humidified incubator. As_2O_3 solution was prepared in phosphate-buffered saline (PBS) and diluted with the same culture medium with 10% fetal bovine serum when treated to the cells.

Cytotoxicity Test with the 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) Assay —— Cell viability was measured in the MTT assay. Cells were seeded on 96-well tissue culture plates with 5×10^3–2×10^4 cells per well and the medium was changed 24 hr after seeding. Cells were treated with 0.25, 0.5, 1, 2, 5, and 10 ppm of arsenic (As_2O_3 was used and the concentration represents that of inorganic As) for 24, 48, 72, and 96 hr, respectively. At the end of exposure, 40 μl of MTT solution (2 mg/ml) added and incubated for 4 hr at 37°C. Cells were treated with 150 μl of dimethyl sulfoxide (DMSO) and absorbance was quantified using a microplate reader.

Whole-Genome Microarray Analysis —— For the analysis of the gene expression profile in arsenic-treated cardiomyocytes, Applied Biosystems Rat Genome Survey Arrays (35 K) were used. Digoxigenin-11-UTP-labeled cRNA was generated and linearly amplified from 1 μg of total RNA purified from control and arsenic-treated groups, respectively, using Applied Biosystems Chemiluminescent RT-IVT Labeling Kit. Array hybridization, chemiluminescence detection, image acquisition, and analysis were performed using Applied Biosystems Chemiluminescence Detection Kit and Applied Biosystems 1700 Chemiluminescent Microarray Analyzer following the manufacturer's protocol. Briefly, each microarray was first prehybridized at 55°C for 1 hr in hybridization buffer with blocking reagent. Labeled cRNA targets (16 μg) were first fragmented into 100–400 bases by incubating with fragmentation buffer at 60°C for 30 min, mixed with an internal control target (ICT, 24-mer oligo labeled with LIZ fluorescent dye), and hybridized to each prehybridized microarray in a 1.5-ml volume at 55°C for 16 hr. After hybridization, the arrays were washed with hybridization wash buffer and chemiluminescence rinse buffer. Enhanced chemiluminescent signals were generated by first incubating arrays with antidigoxigenin-alkaline phosphatase, enhanced with chemiluminescence-enhancing solution, and finally adding chemiluminescence substrate. Each image was collected for each microarray using the 1700 analyzer equipped with a high-resolution, large-format CCD camera for gene expression analysis. Images were auto-gridded and the chemiluminescent signals were quantified, corrected for background and spot, and spatially normalized.

Reverse Transcriptase-Polymerase Chain Reaction —— For the preparation of total RNA, the RNAgent Total RNA Isolation System (Promega, Madison, WI, U.S.A.) was used according to the manufacturer's instructions. Reverse-transcription (RT)-PCR was performed using oligo deoxythymidine primer in 20-μl volumes at 42°C for 60 min. The RT-PCR reaction was done with 1 μg of total RNA, 1 μl of 20-μM oligo dT primer, and 18 μl of reaction mixture which was provided from *AccuPower* RT/PCR PreMix (Bioneer, Daejeon, Korea). Then PCR was performed in a 20-μl total mixture volume for 25 cycles at 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min. Amplified cDNA products were separated on 1.5% agarose gel by electrophoresis. The primer sequences of amplified genes are in Table 1.

Determination of ROS Production —— To measure ROS generation, a fluorometric assay using intracellular oxidation of 2,7-dichlorofluoroscein diacetate (DCF-DA) was performed. Cells grown to

Table 1. Primer Sequences for RT-PCR of Oxidative Stress-Related Genes

Gene name	GB no.	Primer sequence
Heme oxygenase -1	J02722.1	F : 5-GGTCCTGAAGAAGATTGCGCA-3
		R : 5-TCTGTGAGGGACTCTGGTCTT-3
Glutathione S-transferase	AF111160.1	F : 5-CTGCAGCTGGAGTAGAGTTT-3
		R : 5-TTGTATTTGGTGGCAATGTA-3
Metallothionein	AY341880.1	F : 5-TATTCACATGCTCGGTAGAA-3
		R : 5-TGCCCTCAGGTGTAAATAAT-3
Catalase	K01929.1	F : 5-TTAATCCATTTGACCTGACC-3
		R : 5-GTGTCTGGGTAAGCAAAAAG-3
Thioredoxin reductase	AF 108213.1	F : 5-GTAGATTAGCCAAGTCACCG-3
		R : 5-GTCAGATGGCTTAGTAGAGC-3

GB, Gene Bank.

confluence were pretreated with arsenic for 3, 6, 12, and 24 hr, washed with PBS, and then incubated with DCF-DA 40 μM for 30 min. At the end of DCF-DA incubation, cells were washed with PBS, the cells were lysed with NaOH, aliquots were transferred to the black well plate, and fluorescence was measured using the plate reader with excitation and emission wavelengths of 485 and 530 nm, respectively. For the visual image of ROS generation, the cells in a chamber slide were cultured in the presence or absence of arsenic, washed with PBS, and then loaded with DCF-DA 40 μM for 30 min. The fluorescence of DCF in cells was visualized using a fluorescent microscope.

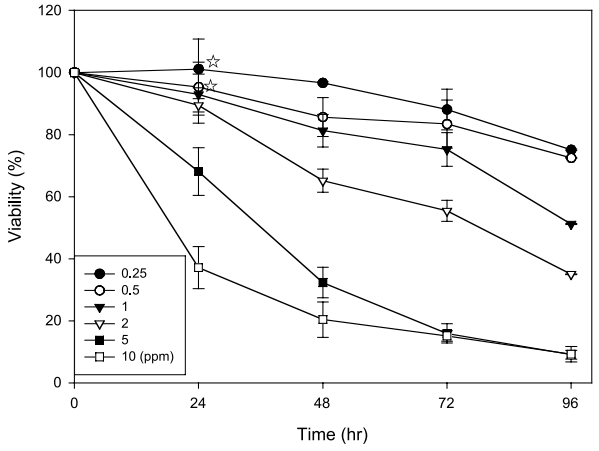

Fig. 1. Effects of Arsenic on Viability of H_9C_2 Cells *in Vitro*

Cells were treated with the indicated concentrations of arsenic for 24, 48, 72, and 96 hr. Cell viability was assessed in MTT assays. Data are presented as percentages of control. Cell viability was markedly reduced in a dose-dependent and time-dependent manner by arsenic trioxide. Results represent means of three independent experiments, and error bars represent the standard error of the mean. Stars (☆) indicate a statistically nonsignificant difference ($p > 0.05$) from control groups in Student's *t*-test. Points without asterisks are statistically different from control groups.

RESULTS AND DISCUSSION

Cytotoxicity of Arsenic in H_9C_2 Cells

To examine the toxic effects of arsenic, rat cardiomyocytes (H_9C_2 cells) were incubated with various concentrations of arsenite trioxide (0.25, 0.5, 1, 2, 5, 10 ppm of arsenic) and assayed for cell viability 24, 48, 72, and 96 hr after treatment, respectively. As shown in Fig. 1, inorganic arsenic showed strong cytotoxicity and the effect was dose and time dependent. Cytotoxicity was apparent in the 1, 2, 5, or 10 ppm arsenic-treated group after 24-hr and longer exposure. However, cell death was not significant at lower dose levels of 0.25 or 0.5 ppm after 24-hr exposure ($p > 0.05$, nonsignificant). Subcytotoxic of arsenic was defined in this study as the level of 0.5 ppm for 24 hr exposure.

Gene Expression Profiles at the Subcytotoxic Level of Arsenic

To identify gene expression profiles at the low concentration level exposure of arsenic which do not cause severe cytotoxicity, RNA was purified from cultured cells treated with arsenic 0.5 ppm for 24 hr. Hybridization was performed two times at the same concentration using independent total RNA preparations for reproducibility. Following normalization of the gene expression data, statistically significant genes that showed altered expression by more than 2-fold in each treatment group were identified. The total number of genes that were significantly changed by more than 2-fold in two separate hybridizations was 904. Among them, 405 transcripts (about 45% of total changed genes) were up-regulated and 499

Table 2. Partial List of Genes Significantly Up-Regulated in Rat Whole-Genome Microarray Analysis

Gene ID	GB no.	Gene Name	1st	2nd
rCG42048	AF380194.1	Trace amine receptor 6	4.3	50.0
rCG39800	U90888.1	Adenosine monophosphate deaminase 3	44.6	44.5
rCG26834	AJ278801.1	ProSAPiP1 protein	2.7	16.4
rCG39158	J02722.1	Heme oxygenase 1	17.5	14.4
rCG43530	AF111160.1	Glutathione S-transferase, alpha 1	13.9	13.4
rCG23561	M34097.1	Granzyme B	9.6	12.1
rCG39155	AY341880.1	Metallothionein	34.9	12.0
rCG60746	M33822.1	Gamma-glutamyl transpeptidase	8.4	10.5
rCG23807	AF254801.1	Brain-enriched SH3-domain protein Besh3	16.2	10.5
rCG51947	AF116896.1	PDZ domain-containing 1	5.5	8.3
rCG26882	AF486828.1	Prominin-related protein	5.8	7.4
rCG22494	AF110478.1	Aldehyde oxidase	2.2	7.2
rCG57104	BC060573.1	Ubiquitin carboxy-terminal hydrolase L1	8.6	6.8
rCG31944	U66322.2	Dithiolethione-inducible gene-1	3.8	5.9
rCG46099	AY219230.1	Transmembrane receptor FcgammaRIII-X	6.4	5.8
rCG23069	AF140347.1	Secreted frizzled-related protein 4	4.3	5.1
rCG27994	AJ277957.1	Aldose reductase-like protein	4.6	4.8
rCG28864	X98746.1	Alcohol dehydrogenase 7	4.5	4.4
rCG33135	AF058786.1	Chemokine (C-C motif) ligand 2	4.5	4.4
rCG23731	D45414.1	Protein tyrosine phosphatase, receptor type, N	2.3	4.1
rCG48289	AF450248.1	Actinin alpha 3	3.7	4.0
rCG26716	K01929.1	Catalase	3.3	4.0
rCG22225	AF394785.3	Solute carrier family39 (iron-regulated transporter)		
rCG42017	L01624.1	Serum/glucocorticoid-regulated kinase	4.3	3.6
rCG26062	BC061793.1	Aminolevulinic acid synthase 1	3.1	3.6
rCG52982	D88666.1	Phosphatidylserine-specific phospholipase A1	2.2	3.5
rCG23495	AF170284.1	Gap junction protein, beta 6 (connexin 30)	6.6	3.4
rCG27858	X05884.1	Aldehyde reductase 1	3.3	3.4
rCG25522	AF059030.2	Sodium channel, voltage-gated, type11	9.1	3.3
rCG42875	M27812.1	Synapsin 1	3.6	3.1
rCG27756	U66107.2	Bradykinin receptor B1	2.9	3.0
rCG23512	AY040223.1	Crystalline, lamda 1	2.7	3.0
rCG47144	BC059126.1	Transaldolase 1	2.3	2.9
rCG30772	AY230497.1	Aflatoxin B1 aldehyde reductase	2.0	2.9

transcripts (about 55% of total changed genes) were down-regulated. Among the altered genes, only 87 among up-regulated genes (Table 2) and 99 among down-regulated genes were annotated genes (Table 3), respectively, and the rest were unidentified genes. Although many genes were found to be regulated by low-level exposure to arsenic using the rat whole-genome microarray, most of them are unknown genes. The majority of the genes altered by arsenic treatment were associated with the functional categories of signal transduction, protein metabolism and modification, immunity and defense mechanism, nucleic acid metabolism, developmental processes, and cell structure and mobility. Transcriptions such as heme oxygenase-1, glutathione S-transferase-alpha 1, metallothionein, catalase, and thioredoxin reductase, which are modulated in response to increased levels of ROS, were increased in low-level arsenic-treated cells.[11–13] Adenosine monophosphate deaminase is an enzyme catalyzing the irreversible deamination of 5-adenosine 5′-monophosphate (AMP) to inosine 5′-monophosphate (IMP) and it is known to modulate the local production of adenosine, a cardioprotective mediator of vascular and cardiomyocyte function. ATP is essential for myocardial contraction and relaxation. Thus the activation of AMP deaminase may deplete ATP in cardiomyocytes and cause heart failure.[14,15] It is interesting that arsenic increased the mRNA transcript of AMP deaminase. At present, we do not know

Table 2. Continued

Gene ID	GB no.	Gene Name	1st	2nd
rCG30304	M55636.1	Guanylate cyclase 2C	6.4	2.9
rCG59667	AY059628.1	F-box only protein 32	3.8	2.9
rCG37885	M99252.1	Secreted phosphoprotein 1	2.1	2.9
rCG34954	AY394725.1	CIN85-associated multi-domain	2.4	2.9
rCG43004	AB001349.1	Steroidogenic acute regulatory protein	12.6	2.9
rCG58841	BC072478.1	Down syndrome critical region homologue 1 (human)	3.0	2.8
rCG30948	L08496.1	Gamma-aminobutyric acid A receptor, delta	2.8	2.8
rCG43441	L27651.1	Solute carrier family 22 (organic anion transporter)	2.5	2.8
rCG34740	AJ426052.2	ATP-binding cassette, subfamily A	2.6	2.7
rCG49701	X57281.1	Glycine receptor, alpha 2 subunit	4.5	2.6
rCG60749	BX883043.1	Proteosome (prosome, macropain) subunit, ...	4.8	2.6
rCG50170	L13600.1	Glycine transporter 1	2.2	2.5
rCG39375	AY149342.1	RSB-11-77 protein	2.4	2.5
rCG29552		Phosphodiesterase 6G, cGMP-specific, rod, ...	5.9	2.5
rCG36428	U37138.1	Steroid sulfatase	2.2	2.5
rCG30607	AF271156.1	CaM-kinase II inhibitor alpha	2.3	2.4
rCG45095		RE70703p-like	3.9	2.4
rCG55338	L32591.1	Growth arrest and DNAdamage-inducible 45 α	4.3	2.4
rCG57785	AF013144.1	MAP-kinase phosphatase (cpg21)	3.1	2.4
rCG43883	D10233.1	Renin-binding protein	2.2	2.4
rCG54208	M73553.1	Lectin, galactose binding, soluble 4	6.3	2.4
rCG30869		Inhibitor of DNA binding 3, dominant negative	2.5	2.4
rCG27876	BC063810.1	Islet cell autoantigen 1, 69 kDa	3.5	2.4
rCG51321	AF459021.1	Tubulin, beta 3	3.1	2.4
rCG41749	AF198442.1	Spleen protein 1 precursor	2.2	2.4
rCG61878	BC061829.1	Zinc finger protein 46	6.6	2.4
rCG42678	AF052042.1	Zinc finger protein Y1 (RLZF-Y)	7.7	2.3
rCG57506	AF003008.1	Max-interacting protein 1	2.9	2.3
rCG26381	U96367.1	Calpain3	3.5	2.3
rCG39721	BC061775.1	Adrenomedullin	3.3	2.3
rCG34318	AY379972.1	Chemokine (C-C motif) receptor 7	6.8	2.3
rCG27829	AF064869.1	Brain-enriched guanylate kinase-associated	2.3	2.3
rCG24733	U10279.1	Solute carrier family 28 (sodium-coupled .....)	2.5	2.3

whether the increase in AMP deaminase is related to oxidative stress, which is specifically induced only by arsenic, and further study is ongoing.

The functional categories of the down-regulated genes are very similar to those of the up-regulated genes. A variety of genes including the heat-shock protein 27-kD protein family, voltage-gated channel-like 1, and G protein-coupled receptor 30 showed a marked decrease in gene expression. Some reports on the gene profiles changed by arsenic compounds have been published in human fibroblasts[16] and in human keratinocytes.[17] In those reports, many genes categorized as signal transduction, transcriptional regulation, cell cycle control, and stress response genes were reported to be altered by treatment with arsenic. Many genes that were found to be increased in our study, such as metallothionein, thioredoxin reductase, glutathione S-transferase, and heme oxygenase, were also found to be increased in those studies even though the cell types are different. However, in the previous two studies, the induction or repression level was not provided, and only the names of the up-regulated or down-regulated genes were listed. Furthermore, the sizes of cDNA chips were relatively small, and the numbers of imprinted genes in the cDNA chips in the two studies were only 568 and 1906 genes, respectively. Other gene expression analyses have been done with mice using a 600 cDNA-imprinted microarray. In those studies, oxidative stress-related genes such as heme oxygenase-1, glutathione S-transferase, and metallothionein were also found to be induced in

Table 3. Partial List of Genes Significantly Down-Regulated in Rat Whole-Genome Microarray Analysis

Gene ID	GB no.	Gene Name	1st	2nd
rCG46475	BC061993.1	Norvegicus myosin-binding protein H	−38.2	−21.3
rCG36216	AF364071.1	Small muscle protein, X-linked	−3.8	−9.5
rCG44383	AF203374.1	Heat-shock 27 kD protein family, member3	−3.5	−8.2
rCG37070	AF078779.1	Voltage-gated channel-like 1	−5.9	−7.6
rCG42789	U92802.1	G protein-coupled receptor 30	−15.9	−6.1
rCG32182	AF259981.1	WNT1-inducible signaling pathway protein 2	−11.2	−5.6
rCG41150	U41453.1	A kinase (PRKA) anchor protein (gravin) 12	−13.5	−5.3
rCG24846	L03813.1	Neural receptor protein-tyrosine kinase	−2.2	−5.2
rCG34383	AB011531.1	Slit homologue 3 (*Drosophila*)	−19.8	−5.2
rCG32919	AB004638.1	Fibroblast growth factor 18	−8.8	−5.1
rCG34382	X04267.1	Myosin, heavy polypeptide 3	−20.0	−4.9
rCG45971	X82152.1	Fibromodulin	−3.1	−4.7
rCG22142	X71068.1	Phospholamban	−5.4	−4.6
rCG24195		Kinesin-related protein KRP5	−2.6	−4.1
rCG57056	BC062390.1	Toll-like receptor 6	−2.5	−4.1
rCG39776	X00975.1	Myosin, light polypeptide 2	−6.7	−4.0
rCG46533	J04993.1	Troponin 1, slow isoform	−4.6	−4.0
rCG52015	AF021806.1	Gap junction membrane channel protein alpha 5	−8.1	−3.8
rCG52352	U93092.1	Homeo box A1	−4.7	−3.8
rCG51318	AF109674.1	Late gestation lung protein 1	−5.1	−3.7
rCG54025	U25264.1	Selenoprotein W, muscle 1	−3.0	−3.6
rCG21136	D31873.1	LIM motif-containing protein kinase 1	−2.2	−3.5
rCG20836	U03491.1	Transforming growth factor, beta 3	−7.4	−3.5
rCG46454	AF054894.1	Myogenin	−9.6	−3.5
rCG52318	X67948.1	Aquaporin 1	−6.0	−3.4
rCG42789	U92802.1	G protein-coupled receptor 30	−2.8	−3.4
rCG42341	AB027562.1	SH3-domain-binding protein 5	−5.3	−3.3
rCG29718	Z54212.1	Epithelial membrane protein 1	−3.8	−3.3
rCG37899	D64085.1	Fibroblast growth factor 5	−3.0	−3.3
rCG44203	Y12502.1	Coagulation factor XIIIa	−8.7	−3.3
rCG48866	BC061878.1	Lumican	−5.0	−3.2
rCG44600	U48596.1	Mitogen-activated protein kinase 1	−3.0	−3.0
rCG47348	M73701.1	Troponin 1, type 2	−5.9	−2.9
rCG46652	M88111.1	Solute carrier family 6 (neurotransmitter .....)	−3.6	−2.9
rCG51188	BC063152.1	WAP four-disulfide core domain 1	−4.4	−2.9
rCG28268	AF439779.1	Caveolin	−5.1	−2.8
rCG31214	AF144090.2	Fatty acid-binding protein 3	−3.6	−2.8
rCG53876	AY277279.1	Histidin rich calcium-binding protein	−6.0	−2.8
rCG25122	AF292102.1	Chondroitin sulfate proteoglycan 5	−2.3	−2.7
rCG27687	D17512.1	Cysteine-rich protein 2	−2.0	−2.7
rCG54356	AF368269.1	Cytochrome P450 monooxygenase CYP2T1	−2.5	−2.6
rCG55205	AY232999.1	Collagen, type XXVII, alpha 1	−5.0	−2.6
rCG52359	BC061535.1	Protein disulfide isomerase-related protein	−2.7	−2.6
rCG22716	L20681.1	v-ets erythroblastosis virus E26 oncogene homologue	−2.8	−2.6
rCG29574		Matrix Gla protein	−2.3	−2.5
rCG26264	L38483.1	Jagged 1	−3.1	−2.5
rCG50808	U17254.1	Immediate early gene transcription factor NGFI-B	−2.9	−2.5
rCG34241	U73030.1	Pituitary tumor-transforming 1	−2.3	−2.5
rCG61735	AJ004858.1	SRY box-containing gene 11	−3.4	−2.5
rCG40512	U10188.1	Polo-like kinase homologue (*Drosophila*)	−5.6	−2.5
rCG36633	AB027143.1	Glycoprotein Ib (platelet), beta polypeptide	−5.2	−2.5
rCG42658	AF157595.1	FMS-like tyrosine kinase 1	−4.1	−2.5

Table 3. Continued

Gene ID	GB no.	Gene Name	1st	2nd
rCG50179	U05341.1	Cell cycle protein p55CDC	−5.4	−2.4
rCG56121	M96601.1	Solute carrier family 6, member 6	−2.8	−2.4
rCG23531	J02712.1	Mast cell protease 2	−2.5	−2.4
rCG28051	J03025.1	Muscarinic receptor m2	−5.2	−2.4
rCG32093	AF130457.1	Tyrosine protein kinase pp60-c-src	−6.1	−2.4
rCG50933	AF018957.1	Neuropilin	−2.9	−2.4
rCG50408	U44979.1	Kinesin-related protein 2	−5.4	−2.4
rCG61453	AF139518.1	A kinase (PRKA) anchor protein 6	−4.1	−2.4
rCG24610	AF003944.1	Nuclear receptor subfamily 2, group F, member 2	−2.4	−2.3
rCG61959	D10666.1	Visinin-like 1	−6.5	−2.3
rCG58763	AY231162.1	Solute carrier family 5 (inositol transporters), ...	−3.1	−2.3
rCG28138	X66845.1	Dynein, cytoplasmic, intermediate chain 1	−4.1	−2.3
rCG20388	J05107.1	Hydroxysteroid 11-beta dehydrogenase 1	−4.5	−2.3
rCG31411	AB050717.1	Lipocalin 7	−2.3	−2.3
rCG34167	AY192567.1	Protein kinase, lysine deficient 4	−3.3	−2.3
rCG35147	D45201.1	Neurofibromatosis 1	−2.2	−2.3
rCG42920	D78359.1	Sushi repeat-containing protein	−3.2	−2.3
rCG50093	AF205717.1	Transmembrane 4 superfamily member 4	−4.2	−2.2

liver cells by arsenic exposure.[18,19] In our study, we used Applied Biosystems Rat Genome Survey Arrays (35 K, oligo chip), and found many new genes that were regulated by arsenic exposure, including genes without annotation. Gene profile study using cardiomyocytes may provide valuable information to the mechanism studies of cardiac failure caused by environmental pollutants like arsenic.

Genes that showed alteration of more than 2-fold in two hybridizations are listed in Table 2 (up-regulated) and Table 3 (down-regulated). In the tables, the genes without annotation, which do not have a Gene Bank number with unidentified functions, are not listed.

Expression of mRNA for Oxidative-Related Genes in RT-PCR

To investigate the expression level of each mRNA related to the oxidative stress response in H_9C_2 cells, RT-PCR was performed. As shown in Fig. 2, various oxidative stress-related genes such as heme oxygenase-1, glutathione S-transferase, metallothionein, catalase, and thioredoxin reductase were shown to be induced by different doses of arsenic in H_9C_2 cells upon agarose gel electrophoresis. The gene expression was dose dependent. The heme oxygenase-1 system, which oxidatively cleaves heme to produce biliverdin, is a well-known biomarker of oxidative stress and it was reported to be induced in human cells by inorganic arsenic.[9,11] Actin mRNA was also amplified and shown as a loading control.

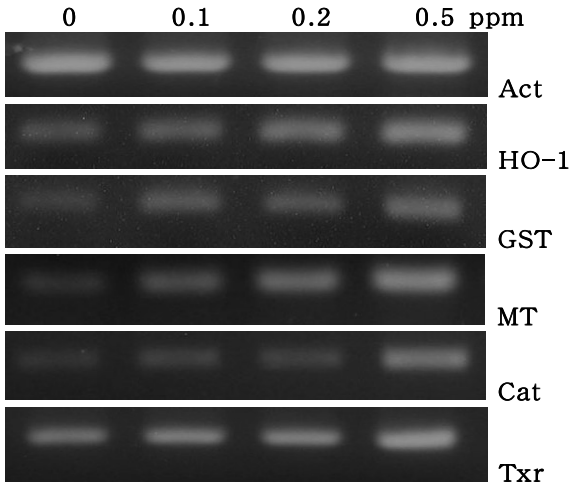

Fig. 2. Effects of Arsenic on the Induction of Oxidative Stress-Related Genes

Cells were treated with the indicated concentrations of arsenic for 24 hr. mRNA transcription was detected by RT-polymerase chain reaction (PCR) analysis using the respective primers described in Table 1. Act, actin; HO-1, heme oxygenase-1; GST, glutathione S transferase; Cat, catalase; MT, metallothionein; Txr, thioredoxin reductase.

Induction of ROS by Arsenic

Direct measurement of ROS was performed using a redox-sensitive fluorescence dye, DCF-DA.

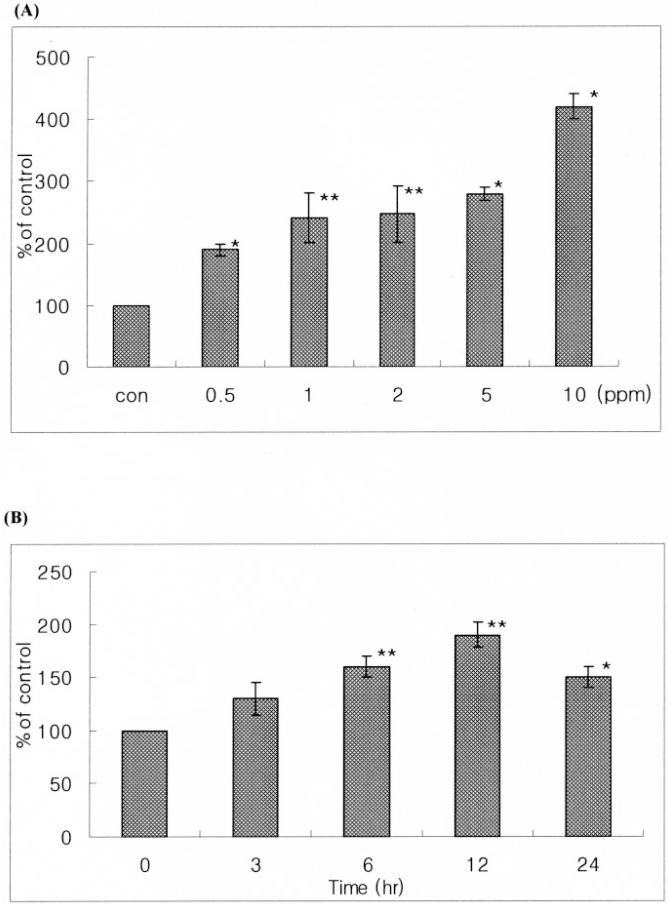

Fig. 3. Effects of Arsenic on ROS Production in Cardiomyocytes

Cells grown to confluence were pretreated with arsenic, washed with PBS, and then incubated with DCF-DA 40 μM. At the end of DCF-DA incubation, the cells were lysed with NaOH and the fluorescence of aliquots was measured. Results represent means of three independent experiments, and error bars represent the standard error of the mean. Asterisks indicate a statistically significant difference ($*p < 0.05$, $**p < 0.01$) from control group in Student's *t*-test. (A): Cells were treated with the indicated concentration of arsenic for 12 hr. (B): 0.5 ppm of arsenic was treated to the cells for 3, 6, 12 and 24 hr.

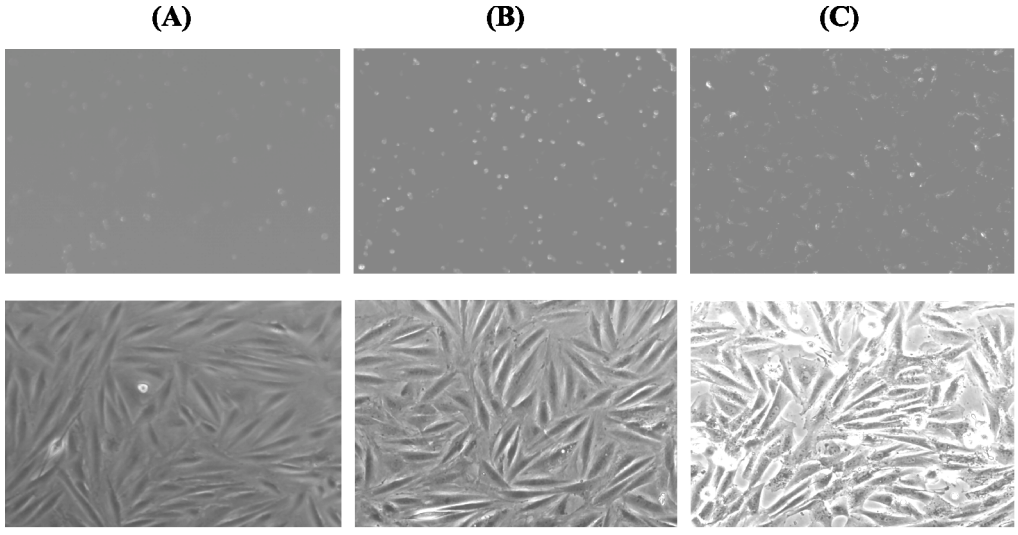

Fig. 4. Qualitative Characterization of ROS Generation using DCF-DA Staining and Fluorescence Microscopy

Cells were cultured in the presence or absence of arsenic, washed with PBS, and then loaded with DCF-DA 40 μM. After washing with PBS, cells were visualized by fluorescent microscopy (× 40). Images of phase-contrast microscopy (× 100) are shown in the lower panel. (A): control, (B): Cells treated with arsenic 0.5 ppm for 12 hr. (C): Cells treated with arsenic 0.5 ppm for 24 hr.

The fluorescent dye is known to detect hydrogen peroxide and other species such as organic hydroperoxides, nitric oxide, and peroxy nitrite. As shown in Fig. 3A and 3B, the fluorescence intensity of the oxidized product, DCF, was increased in H_9C_2 cells treated with arsenic 0.5 ppm. Previous reports showed that exposure of EC/CUHK1 cells to arsenic led to an increase in cellular ROS.[20] In one study, compared with arsenic alone, emodin(anthraquinone) plus arsenic induced more rapid and remarkable induction of ROS. Arsenic (2 μM) alone induced ROS less than 2-fold for 1.5-hr exposure. It seemed that the induction level of ROS measured by fluorescent dye with low-level arsenic treatment was as marked as the induction levels of molecular biomarkers identified by microarray (Table 2). This means that the transcription of oxidative stress-related genes is sensitively increased by ROS and the gene expression is a sensitive biomarker of low-level arsenic exposure. Visualization of ROS production under the fluorescent microscope showed that oxidized DCF fluorescence was increased in the cells cultured in the presence of arsenic, while a little fluorescence was observed in the control group (Fig. 4). ROS is an important factor in apoptosis and the excess generation of ROS induces mitochondrial membrane permeability and damages the respiratory chain.[21,22]

Direct measurement of ROS using fluorescent dye showed that arsenic produced oxidative stress at the subcytotoxic level. Although no direct comparison with other different cells was made, it appears that arsenic can cause physiologic adverse reactions in cardiomyocytes sensitively at a relatively low level. These results suggest that cardiomyocytes are also vulnerable targets of heart failure by arsenic compound like endothelial cells or smooth muscle cells of the artery. Furthermore, transcriptional profiling is useful in predicting the toxic mechanism of cardiomyocytes with low-dose exposure.

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