Gene Expression, Vol. 11, pp. 77–83
Printed in the USA.

1052-2166/03 $20.00 + .00

Alteration of Gene Expressions by the Overexpression of Mitochondrial Phospholipid Hydroperoxide Glutathione Peroxidase (mtPHGPx)

JUN KITAHARA,* NOBUYOSHI CHIBA,‡ HIKARU SAKAMOTO,* AND YASUHITO NAKAGAWA*†[1]

**Radioisotope Research Laboratory, and †Department of Hygiene, Pharmaceutical Sciences Kitasato University, 5-9-1 Shirokane, Minato-ku, Tokyo 108-8641, Japan*
‡Japan Energy Corporation, 3-17-35 Niizo-Minami, Toda-shi, Saitama 335, Japan

To determine the effect on gene expression of trace levels of reactive oxygen species from mitochondria, we used the mRNA differential display technique to compare gene expression in two cell lines: M15, which overexpresses mitochondrial phospholipid hydroperoxide glutathione peroxidase (mtPHGPx), in rat basophilic leukemia RBL-2H3 cells, and a control cell line, S1. We isolated 27 differentially expressed genes, including 10 previously unreported sequences. These genes included cytoskeletal proteins (β-tubulin, nonmuscle myosin alkali light chain, and vimentin), growth or proliferation regulators [growth differentiation factor 1 (Gdf-1), Rap1a, and inhibitor of growth 3 (Ing3)], and others. Although the expression of most of the isolated genes did not respond to ROS (hydrogen peroxide) or antioxidant (pyrolidine dithiocarbamate) treatment, the expression of Gdf-1 was downregulated by hydrogen peroxide treatment. Thus, low levels of ROS produced in mitochondria during normal cellular metabolism can modulate gene expression.

Phospholipid hydroperoxide glutathione peroxidase (PHGPx) Mitochondria Reactive oxygen species (ROS) Differential display

IT is well known that reactive oxygen species (ROS), such as superoxide anions, hydrogen peroxide, and hydroxyl radicals, have damaging effects on biomolecules, such as DNA, membrane lipids, and proteins. Accumulated damage caused by ROS may cause cancer, aging, and degenerative disease, as well as other diseases. Several kinds of antioxidative enzymes exist in cells to protect them from ROS, including superoxide dismutases (SOD), catalase, peroxiredoxins, and glutathione peroxidases (GPx). In recent years, the roles of ROS in signal transduction have become more clear [reviewed in (21)]. In response to various kinds of cytokines and hormones, hydrogen peroxide is produced in cells and acts as a signaling molecule for cell growth, differentiation, and proliferation by activating transcription factors. In addition, the fact that exogenously added hydrogen peroxide mimics the action of cytokines suggests that ROS can control gene expression.

Mitochondria produce ATP by oxidative phosphorylation. During that process, small amounts of ROS are always formed (4). These ROS from mitochondria are the main source of intracellular ROS in the normal cellular metabolic state. It is possible that the trace amounts of hydrogen peroxide that are produced as the result of normal cellular metabolism act as signal transducers.

Phospholipid hydroperoxide glutathione peroxi-

Accepted March 10, 2003.
[1]Address correspondence to Yasuhito Nakagawa, Pharmaceutical Sciences, Kitasato University, 5-9-1 Shirokane, Minato-ku, Tokyo 108-8641, Japan. Tel: +81-3-3444-6161, ext. 3336; Fax: +81-3-3444-4944; E-mail: nakagaway@pharm.kitasato-u.ac.jp

dases (PHGPx) are members of the glutathione peroxidase family and are able to decompose phospholipid hydroperoxides (22,24). To date, at least two isotypes of PHGPx have been reported, one of which is located mainly in the cytosol and nucleus (non-mtPHGPx) and the other is located in mitochondria (mtPHGPx) (1,2, 10). We have developed several cell lines derived from rat basophilic leukemia RBL-2H3 that overexpress PHGPx (2,5,10). The mitochondrial-type PHGPx-overexpressing cell line M15 (2) and the nonmitochondrial PHGPx-overexpressing L9 (5) are tolerant of ROS producers such as hydrogen peroxide and *tert*-butyl hydroperoxide (2). M15 cells are also resistant to apoptosis mediated by the drugs that induce cytochrome c release from mitochondria, such as 2-deoxyglucose (5,10,11). It has also been shown that intracellular hydroperoxide levels in nonstimulated states are decreased by the overexpression of PHGPx (2). Thus, these cell lines are a good model for examining the physiological effects of trace amounts of constitutively formed ROS on gene expression.

In this report, we used the mRNA differential display technique (DD) (8) to search for differences in gene expression between the M15 and L9 cell lines, as well as a control line. We found 27 differentially expressed genes, including 10 previously unreported sequences. The isolated genes vary from cytoskeletal proteins to growth or proliferation regulators. We also found that the expression of Gdf-1, one of the isolated genes, responded to ROS treatment.

MATERIALS AND METHODS

Cell Culture

Rat basophilic leukemia RBL-2H3-derived mitochondrial PHGPx-overexpressing cells (M15), nonmitochondrial PHGPx-overexpressing cells (L9), and control cells (S1) were used. Cells were cultured in Dulbecco's modified Eagle's medium containing 5% fetal bovine serum with penicillin and streptomycin.

Differential Display (DD)

DD was carried out as described by Liang and Pardee (8). Briefly, RNA was extracted from exponentially growing M15 and S1 cells (4×10^6 cells) using an RNeasy Mini Kit (Qiagen GmbH, Germany) in combination with an RNase-free DNase Set (Qiagen GmbH, Germany) according to the manufacturer's instructions. RNA (200 ng) was reverse transcribed using MMLV reverse transcriptase (GenHunter, TN) with T_{12}VN primers (see Table 1). The cDNA yield was further amplified with the 48 different combinations of primer sets listed in Table 1 (4 arbitrary primers and 12 T_{12}VN primers) using ^{33}P-labeled dCTP. Amplified DNA was separated on a 6% denatured polyacrylamide gel and then visualized by autoradiography. Differentially expressed genes were excised from the dried gel and extracted in water at 100°C for 5 min. Extracted DNA was reamplified using primer sets with restriction enzyme recognition sites (5′ primers: an *Eco*RI recognition sequence was added to the primer sequence listed in Table 1; 3′ primer: T_{12} plus *Hin*dIII recognition sequence). Amplified DNA fragments were treated with *Eco*RI and *Hin*dIII overnight at 37°C and purified by a combination of agarose gel electrophoresis and phenol chloroform extraction. The extracted DNAs were ligated into pUC119 cloning vectors.

TABLE 1
LIST OF PRIMERS USED IN DIFFERENTIAL DISPLAY

#	Arbitrary primer	#	T_{12}VN Primer
E	GCC TCA TTG C	1	TTT TTT TTT TTT AA
F	ACG GCA GGT C	2	TTT TTT TTT TTT AG
G	GCA GGA GGA C	3	TTT TTT TTT TTT AC
H	ACC GCA CCA C	4	TTT TTT TTT TTT AT
		5	TTT TTT TTT TTT GA
		6	TTT TTT TTT TTT GG
		7	TTT TTT TTT TTT GC
		8	TTT TTT TTT TTT GT
		9	TTT TTT TTT TTT CA
		10	TTT TTT TTT TTT CG
		11	TTT TTT TTT TTT CC
		12	TTT TTT TTT TTT CT

Northern Blot Analysis

Total RNA was extracted from exponentially growing cells. Twenty micrograms of RNA was electrophoretically separated on a 1% agarose gel containing 2% formaldehyde and blotted onto a Biodyne nylon membrane (Pall BioSupport, NY). Prehybridization was carried out in 3× SSC, 10× Denhardt's solution, 40% formamide, 0.25 mg/ml denatured salmon sperm DNA, and 1% SDS at 42°C for more than 3 h. Hybridization was done overnight at 42°C in the same solution used for prehybridization except that it contained a ^{32}P-labeled probe. The membrane was washed twice: in 2× SSC and 0.1% SDS at 65°C for 30 min, and 0.2× SSC and 0.1% SDS at 65°C for 15 min. The membrane was autoradiographed using Hyperfilm MP (Amersham, UK) or analyzed using a BAS 2000 imaging system (Fuji Film, Tokyo).

DNA was sequenced using an ALFred automatic DNA sequencer (Amersham, UK) with a Cy5-labeled M13 primer. Other chemicals used in the experiments were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

RESULTS AND DISCUSSION

Characterization of Cloned Genes

A typical result of DD between S1 cells and M15 cells is shown in Figure 1. Because DD tends to give false-positive signals, the levels of gene expression were confirmed by Northern blot analysis. The gene expression of a total of 161 clones was examined by Northern blot analysis and scored according to the difference in expression levels. Among the 161 clones, 103 clones gave false-positive signals in DD or were not detected by Northern blot analysis. Some of these may have been true positives that gave very faint bands or had only very slight differences between S1 cells and M15 cells that we were not able to detect. Recently, the DNA microarray method was developed and it is becoming a popular method for investigating changes in gene expression. However, DNA chips can only give information about known sequences. One of advantages of DD compared with the DNA microarray method is the possibility of finding genes with unreported sequences.

We sequenced genes that were positive on both DD and Northern blot analysis and carried out a computer database search on these genes. The results are summarized in Table 2. Because some of the independent clones carried the same gene (e.g., the nonmuscle myosin alkali light chain), in the end 27 differentially expressed genes were obtained, including 10 with previously unknown sequences. Further experiments will be required to clarify the roles of these unknown genes.

Among the known genes that we obtained, vimentin, β-tubulin, and nonmuscle myosin alkali light chain are cytoskeletal proteins, and growth differentiation factor 1 (Gdf-1), inhibitor of growth 3 (Ing3), and Rap1a are growth or proliferation regulators.

Gdf-1 is a member of the TGF-β super family and is thought to have a role in determining the left and right sides of an embryo (16). According to a report by Lee (6), two different transcripts of Gdf-1 have been observed in mouse embryos, but the transcripts were observed at different gestational ages. In adult mouse tissues, Gdf-1 expression is limited to the central nervous system and the low molecular transcript is not found. It is interesting that we have detected both transcripts (Figs. 2 and 3) in RBL-2H3-derived cell lines that are not derived from the nervous system.

Rap1a is known as a member of the low molecular weight GTP-binding Ras protein family (14) and has an inhibitory effect on cell growth (13).

Ing3 was originally cloned as a new member of the

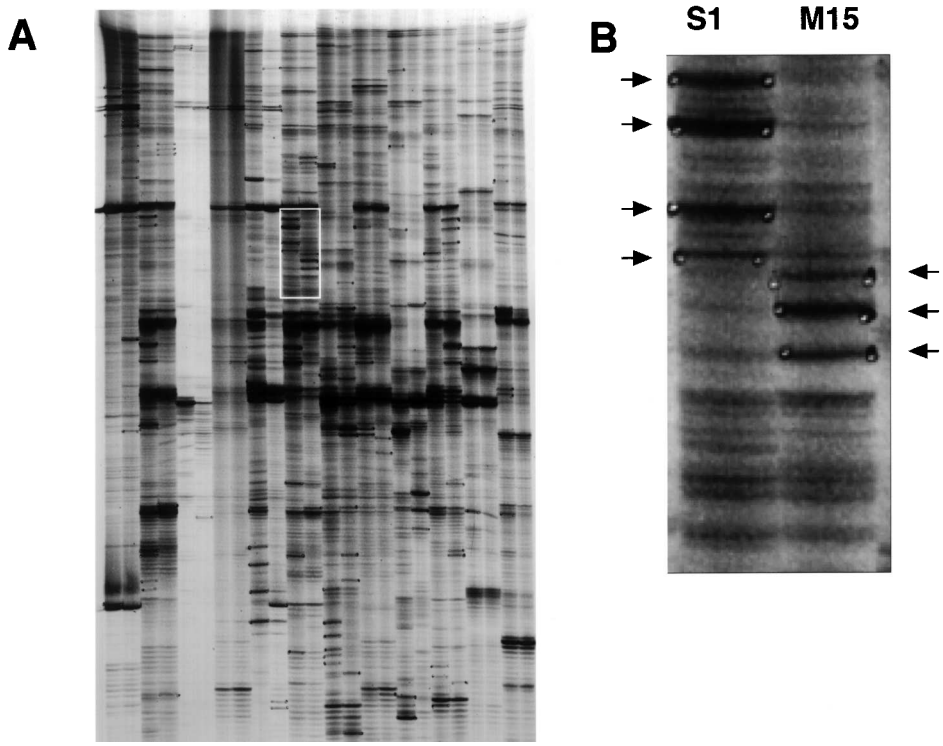

Figure 1. Typical result of differential display between S1 cells and M15 cells. The primers G and $T_{12}VN$ were used in combination. (B) An enlarged image of the portion of the gel surrounded by a white square in (A). Differentially expressed genes are indicated by arrows, and were extracted from the dried gel and cloned as described in Materials and Methods.

TABLE 2
DIFFERENTIALLY EXPRESSED GENES IN MITOCHONDRIAL PHGPx-OVEREXPRESSING CELL LINE (M15)

	Score	Name of Gene	Comment
1	↑	vimentin	cytoskeletal protein
2	↑	unknown	
3	↑	unknown	
4	↑	unknown	
5	↑	unknown	
6	↑	cellugyrin (synaptogyrin2)	synaptogyrin homolog
7	↑	gas-5	growth arrest homolog; nontranslated mRNA
8	↑	β-tubulin	cytoskeletal protein
9	↑	stearoyl CoA desaturase	lipid metabolism
10	↑	ribosomal protein S18	ribosomal protein
11	↑↑	mast cell function associated antigen (MAFA)	C-type lectin
12	↑↑	nonmuscle myosin alkali light chain	cytoskeletal protein
13	↑↑	unknown	
14	↑↑	unknown	
15	↓	unknown	
16	↓	unknown	
17	↓	unknown	
18	↓	growth differentiation factor 1 (Gdf-1)	member of TGF-β super family
19	↓	Rap1a	member of RAS oncogene family
20	↓	peroxiredoxin IV (PRxIV)	antioxidative enzyme
21	↓	steroid dehydrogenase (Ke 6)	steroid metabolism
22	↓	solute carrier family 25 member 17	mitochondrial carrier
23	↓	eukaryotic translation initiation factor 3 subunit 7 (Eifs7)	initiation of translation
24	↓↓	unknown	
25	↓↓	mast cell proteinase 3 (MCP3)	secretory granule proteinase
26	↓↓	tyrosine aminotransferase (TAT)	tyrosine metabolism
27	↓↓	inhibitor of growth 3 (Ing3) (high molecular weight)	growth inhibition
	↑	inhibitor of growth 3 (Ing3) (low molecular weight)	

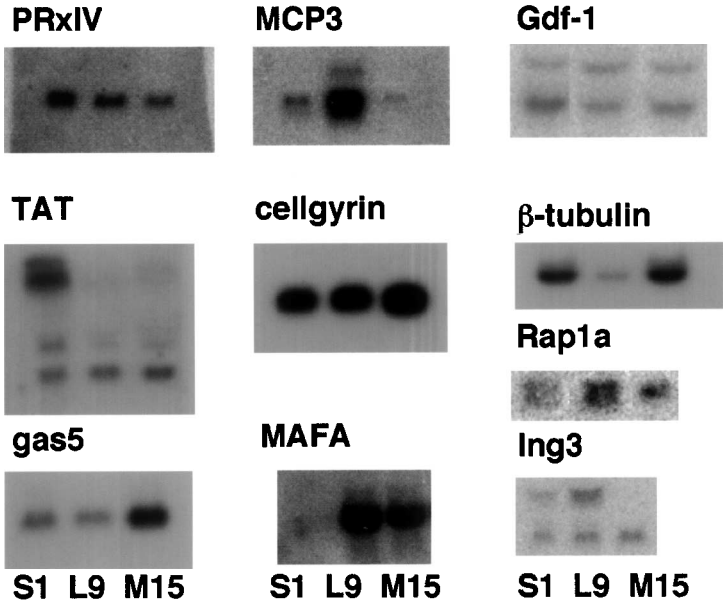

Figure 2. Comparison of gene expression in RBL-2H3-derived cell lines. RNA was extracted from S1, M15, and L9 cells. Hybridizations were carried out as described in Materials and Methods. The probes used are indicated in the figure.

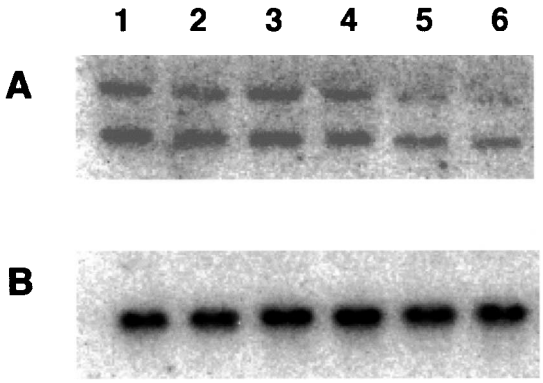

Figure 3. Changes in gene expression of Gdf-1 in response to hydrogen peroxide. S1 cells were treated with various concentrations of hydrogen peroxide for 6 h (lanes 1–6: 0, 62.5, 125, 250, 500, 1000 μM, respectively). (A) Gdf-1 probe, (B) G3PDH probe.

inhibitor of growth 1 (Ing1) family. The function of Ing3 itself has not yet been reported. However, Ing3 might act as a tumor suppressor because the related protein Ing1 acts as a p53 regulator and is a possible candidate for a tumor suppressor.

Gas-5 is also a growth-related gene. It is known as growth arrest homologue nontranslated mRNA, and was originally isolated as a transcript whose expression is increased when cell growth is arrested (18). Although Gas-5 mRNA is found in mammalian cells, no translated product has been detected (15). The only known function of this gene is as the host gene for a small nucleolar RNA that is involved in processing ribosomal RNA (20). It should be noted that, although we found differences in the expression of several growth-related genes as described above, no differences in growth rate were observed between S1, L9, and M15 cells (data not shown).

Peroxiredoxin IV (PRx IV) is a secreted type of peroxiredoxin that decomposes peroxide using thioredoxin as a cofactor (9,12). Downregulation of PRx IV was observed in both types of PHGPx-overexpressing cell lines (Fig. 2). Because PRx IV is also an antioxidative enzyme, this downregulation could reflect compensation for the antioxidant enzyme network, as has been observed for MnSOD-overexpressing NIH3T3 cells, which have lowered CuZnSOD activity (7). However, the levels of other antioxidative enzymes such as catalase and cGPx were not changed in the PHGPx-overexpressing lines of RBL2H3 cells (5).

Comparison of Gene Expression in mtPHGPx-Overexpressing Cell Lines With That in Non-mtPHGPx-Overexpressing Cells

Figure 2 shows the expression patterns of some of the genes obtained from S1, M15, and L9 cells. The expression patterns of differentially expressed genes were not always same in both PHGPx-overexpressing cell lines. Tyrosine aminotransferase (TAT) was strongly downregulated, and PRx IV was slightly downregulated, in both M15 cells and L9 cells. However, the expression of most of the genes was not the same in both types of PHGPx-overexpressing cell lines. The different expression patterns of genes in L9 cells and M15 cells might arise from the different localization of PHGPx in the two cell lines. Although both L9 cells and M15 cells have an increased ability to remove hydroperoxides compared with S1 cells, only M15 cells have an amplified capability to reduce hydroperoxides formed in the mitochondria. Thus, if signals other than ROS control the expression of these genes, but the signals were originally activated by hydroperoxides formed inside of mitochondria, only mtPHGPx will affect the gene expression. Other investigators have suggested a role for putative mitochondria-to-nucleus signaling in TNF-β-induced MnSOD gene expression (17), and a similar mechanism might be involved in controlling gene expression in our experiments.

Changes in Gene Expression in Response to Redox Modulation

Gdf-1 gene expression responded to the overexpression of mtPHGPx. In order to determine the effect of redox modulation on Gdf-1 gene expression, RNA was extracted from the control cell line S1 after a challenge by hydrogen peroxide or pyrolidine dithiocarbamate (PDTC), a well-known antioxidant, for 6 h. As shown in Figure 3, hydrogen peroxide treatment decreased the expression of Gdf-1 in a dose-dependent fashion from 62.5 to 1000 μM hydrogen peroxide. The high molecular weight band of Gdf-1 was only slightly sensitive to ROS treatment compared with the low molecular weight band. Under the same conditions, G3PDH and ribosomal protein S18 (data not shown) expressions were not affected by hydrogen

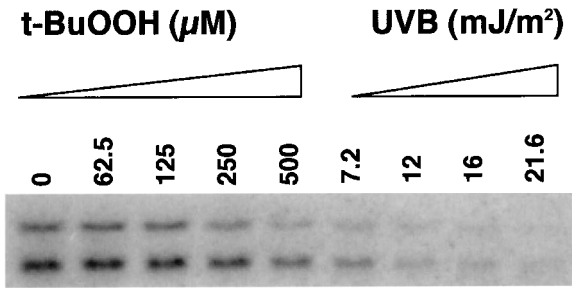

Figure 4. Changes in Gdf-1 gene expression in response to *t*-BuOOH and UVB. S1 cells were treated with *t*-BuOOH (lanes 1–5: 0, 62.5, 125, 250, 500 μM) or UVB (lanes 6–9: 7.2, 12, 16, 21.6 mJ/m^2) for 6 h.

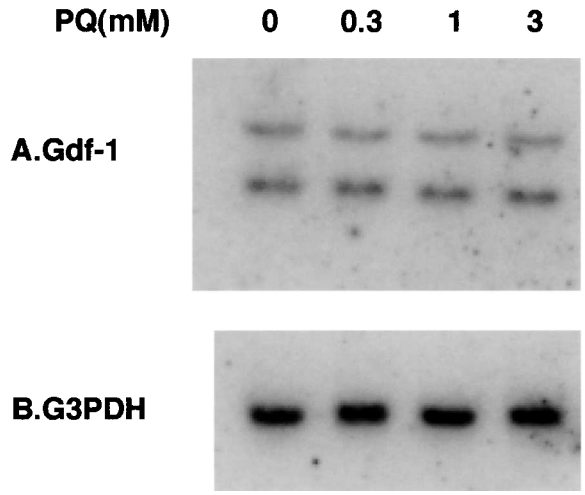

Figure 5. Effect of paraquat on Gdf-1 gene expression. S1 cells were treated with indicated concentrations of paraquat for 6 h.

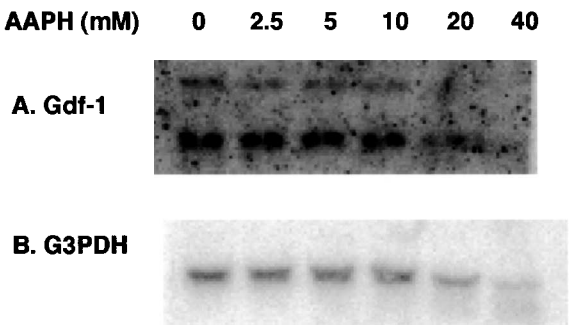

Figure 6. Effect of AAPH on gene expression in S1 cells. S1 cells were treated with AAPH in PBS for 3 h. AAPH was removed and replaced with DMEM containing 5% FCS and incubated for an additional 3 h. (A) Gdf-1 probe, (B) G3PDH probe.

peroxide. Treatment with the highest concentration of hydrogen peroxide for 6 h was slightly toxic to S1 cells, as we have observed small changes in cell morphology following treatment with hydrogen peroxide. However, we did not observe any morphological changes in S1 cells at concentrations under 250 μM that were sufficient to induce downregulation. In addition, no increase in the number of detached cells was observed at any concentration used in the experiment.

Downregulation of Gdf-1was also observed when cells were treated with *tert*-BuOOH or UVB (Fig. 4). However, neither paraquat, an intracellular superoxide generator, nor 2,2′-azobis(2-amdinopropane) dihydrochloride (AAPH), a water-soluble radical reaction inducer, changed Gdf-1 expression (Figs. 5 and 6). Because the highest concentration of paraquat used (3 mM) is enough to induce more than 70% of cell death when cells were treated for 16 h (data not shown), no change of Gdf-1 expression was caused by low concentration of paraquat. AAPH concentration higher than 20 mM induced degradation of mRNA, as can be seen in both Gdf-1 and G3PDH (Fig. 6, lanes 5 and 6). Thus, AAPH did not induce specific reduction of Gdf-1 mRNA as seen in hydrogen peroxide treatment. These results indicated that Gdf-1 expression is relatively susceptible to peroxide formed in the cell rather than superoxide or membrane peroxidation induced by AAPH. In addition, an antioxidant PDTC treatment did not alter any gene expression as far as we examined (data not shown). PRx IV expression was not changed by PDTC treatment, although low expression of PRx IV in PHGPx-overexpressing cells seems to be mediated by cellular reducing ambient. The fact that expression of Gdf-1 in S1 cells was downregulated by hydrogen peroxide treatment might be of interest because this gene product can control cell growth or proliferation. As previously noted, cells produce ROS in response to many types of cytokines, and ROS are involved in such effects of cytokines as cell proliferation, growth inhibition, and apoptosis (21). Additionally, it is known that some transcription factors, such as NF-κB and AP-1, are activated by ROS and increase transcription of growth- or proliferation-related genes [reviewed in (3)]. The production of ROS from mitochondria increases in metabolic disorders of mitochondria (19, 23), and genes whose expression is controlled by ROS might have some role in such disorders.

In conclusion, we have cloned 27 differentially expressed genes using DD between control cells and mtPHGPx-overexpressing cells. Further investigation will be required to clarify the mechanisms of the differential expression of such genes.

ACKNOWLEDGMENTS

The authors thank M. Sugiura, S. Watanabe, Y. Nakazawa, and Y. Kaminaga for their technical assistance.

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