J. Biochem. 119, 817-822 (1996)

The Protective Role of Glutathione Peroxidase in Apoptosis Induced by Reactive Oxygen Species[1]

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Received for publication, December 6, 1995

Selenium-dependent glutathione peroxidase (GPx) plays a protective role in oxidative stress-induced apoptosis. In this study, we demonstrated that MDBK cells, a bovine renal epithelial cell line, exhibited internucleosomal DNA fragmentation characteristic of apoptotic cell death under selenium-deficient conditions with lower doses of hydrogen peroxide (H_2O_2) than under selenium-supplemented ones. This was due to a decreased amount of GPx in the cells under selenium-deficient conditions, because other antioxidative enzyme activities were not affected by the selenium supplementation. Cumene hydroperoxide also induced DNA fragmentation in selenium-deficient cells but no ladder formation was observed. Flow cytometric analysis showed that selenium-deficient cells were less capable of scavenging intracellular peroxides after exposure to exogenous H_2O_2 than selenium-supplemented ones. In contrast, there was no difference in viability between selenium-supplemented and non-supplemented cells in cell survival after exposure to menadione, which activates the electron transport system and increases intracellular superoxide radicals. Clofibrate, a peroxisomal proliferator and an inducer of catalase (CAT), partially protected both Se-deficient and Se-supplemented cells from exogenous H_2O_2. We concluded that selenium-deficient cells were more easily brought to apoptotic cell death by peroxides, but not by superoxide radicals, than selenium-supplemented ones and that CAT could compensate for the depletion of GPx to a certain degree by scavenging H_2O_2.

Key words: apoptosis, catalase, glutathione peroxidase, hydrogen peroxide, selenium deficiency.

Glutathione peroxidase (GPx) is a tetrameric seleno-enzyme (*1*) carrying one essential seleno-cysteine residue per subunit for its activity (*2*). The "classical" intracellular enzyme is mainly a cytosolic one, but it is also found in the mitochondrial matrix of mammalian cells (*3*). The enzyme catalyzes the reduction of hydrogen peroxide (H_2O_2) to water and that of a variety of organic hydroperoxides to the corresponding alcohols (*3-6*), with a specific requirement for the reductant substrate, glutathione (GSH) (*3*). Together with catalase (CAT), which also reduces H_2O_2, as well as superoxide dismutases (SODs), which scavenge superoxide radicals, GPx provides the main intracellular enzymatic defense system against reactive oxygen species (ROS) in the mammalian body.

It has been shown that selenium-deficient cells fail to synthesize GPx mRNA and protein. The addition of selenium (Se) to the culture medium of Se-deficient cells results in the induction of GPx mRNA and protein synthesis followed by the increase in GPx activity (*7*). The activities of CAT, Mn-SOD, and Cu,Zn-SOD remain essentially unchanged on Se-supplementation (*7*). Several Se-deficient cells are more sensitive to H_2O_2 and/or organic peroxides than Se-supplemented ones due to the loss of GPx activity (*8, 9*). *In vivo*, animals fed Se-deficient diets suffer from disorders of several organs, such as liver, heart, muscle, pancreas, and kidney (*10*). This organ failure might be due to the loss of GPx activity.

Recently, the involvement of ROS in apoptotic cell death was reported. Bcl-2, a protein that inhibits apoptosis, is reported to suppress lipid peroxidation through an antioxidant pathway (*11*). Cells transfected with GPx cDNA are more resistant to apoptosis following withdrawal of a growth factor (*11*). Lipid hydroperoxides induce apoptosis in T cells infected with the human immunodeficiency virus (HIV), suggesting an HIV-associated GPx deficiency (*12*). GPx also compensates for the hypersensitivity of Cu,Zn-SOD-overproducing cells to oxidant stress and suppresses the induction of apoptotic cell death (*13*).

In this study, we examined whether exogenous peroxides induce apoptosis of Se-deficient MDBK cells at lower doses

[1] This work was supported in part by a Grant-in-Aid for Scientific Research on Priority Areas and Specific Project Research on Cancer Bio-Science from the Ministry of Education, Science and Culture of Japan.

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Abbreviations: GPx, glutathione peroxidase; H_2O_2, hydrogen peroxide; GSH, glutathione; CAT, catalase; SOD, superoxide dismutase; ROS, reactive oxygen species; Se, selenium; HIV, human immunodeficiency virus; Cu-OOH, cumene hydroperoxide; DCFH-DA, 2′,7′-dichlorofluorescin diacetate; DCF, 2′,7′-dichlorofluorescein; FBS, fetal bovine serum; PBS(−), Mg^{2+},Ca^{2+}-free phosphate-buffered saline; NBT, nitroblue tetrazolium; TGF-β1, transforming growth factor-β1.

than in the case of Se-supplemented ones. We also investigated the effects of several reagents which affect antioxidative enzyme activities or change the level of ROS in Se-deficient and Se-supplemented cells.

MATERIALS AND METHODS

Materials—Selenious acid, clofibrate, and diethyldithiocarbamate were purchased from Wako Pure Chemical (Osaka). Menadione was from Sigma. H_2O_2 and cumene hydroperoxide (Cu-OOH) were from Nacalai Tesque (Kyoto). 2′,7′-Dichlorofluorescin diacetate (DCFH-DA) was from Molecular Probes.

Establishing Se-Deficient MDBK Cells—MDBK cells were obtained from the Japanese Cancer Research Bank, and were first cultured in Ham's F-12 (Gibco) supplemented with 5% fetal bovine serum (FBS), 100 U/ml penicillin G (Banyu), and 100 μg/ml streptomycin (Wako Pure Chemical, Osaka). The serum concentration was decreased stepwise in order to decrease the Se concentration in the medium. Finally, the cells maintained in 0.5% FBS-supplemented medium had substantially no GPx activity. Two days later, these cells were plated on 10-cm culture dishes, and the medium was changed to serum-free Ham's F-12 containing 5 μg/ml bovine transferrin, 10 μg/ml insulin, and the above antibiotics, with or without 10 ng/ml selenious acid. Viability assay of cells, agarose gel electrophoresis of DNA, flow cytometry, and measurement of enzyme activities were all performed 5 days after changing the medium.

Enzyme Assays and Protein Determination—For measuring the specific activities of antioxidative enzymes, 5×10^7 cells were pelleted and washed with PBS(−) twice. Then the pellets were suspended in PBS(−) and sonicated for 10 min. The samples were centrifugated to remove insoluble materials, then the supernatants were subjected to enzyme assay, as follows. GPx activity was determined by means of a coupled enzyme assay (*9*). One milliliter of assay mixture comprised 3 mM GSH, 0.2 mM NADPH, 2 U/ml glutathione reductase, 0.2 mM Cu-OOH (or 0.2 mM H_2O_2), and cellular proteins (0.1–0.2 mg) in 50 mM phosphate buffer (pH 7.4) at 37°C. The decrease in absorbance of NADPH at 340 nm was monitored. One unit of activity was defined as the amount of enzyme catalyzing the oxidation of 1 nmol NADPH/min.

CAT activity was determined by monitoring the rate of decomposition of H_2O_2, by measuring the decrease in absorbance at 240 nm (*14*). Calculations were based on an extinction coefficient of 43.6 $M^{-1} \cdot cm^{-1}$ for H_2O_2 at this wavelength. One unit of activity represents the consumption of 1 μmol of peroxide/min. The assay mixture (1 ml) comprised 19 mM H_2O_2 and cell sample (20–40 μg protein) in 50 mM phosphate buffer (pH 7.0) at 25°C.

Measurement of superoxide dismutase activity was based on inhibition of the superoxide mediated conversion of nitro-blue tetrazolium (NBT) to formazan, which was monitored at 560 nm (*15*). The assay mixtures, in a series of spectrophotometer cuvettes, consisted of 0.15 mM xanthine, 2 mU/ml xanthine oxidase, 1 mM diethylenetriamine-pentaacetate, and cell sample (100–500 μg/ml protein). One unit of activity is defined as the amount of cell protein required to inhibit the reduction of NBT by 50%. To determine Mn-SOD activity, KCN (1 mM) was added to the mixture to inhibit Cu,Zn-SOD activity.

Total protein in MDBK cells was determined with a BCA protein assay kit (Pierce, Rockford, IL).

Assessment of Cell Viability by the Methylene Blue Assay—Cell viability after exposure to several reagents was assessed by methylene blue assay (*16*). After incubation of cells in the presence of various reagents, 0.02 ml of a 25% glutaraldehyde solution was added to each well to fix the surviving cells for 25 min. After washing and removal of the dead cells, the fixed cells were stained with 0.1 ml of a 0.05% methylene blue solution for 15 min. Then 0.2 ml of 0.33 N HCl was added to each well, and the plate was agitated with a mixer. The absorbance at 665 nm was measured.

Analysis of DNA Integrity by Agarose Gel Electrophoresis—DNA fragmentation was assayed as described (*12*). Briefly, 10^7 cells were pelleted and lysed by the addition of ice-cold 20 mM Tris-HCl (pH 7.4) containing 10 mM EDTA and 0.2% Triton X-100. Following centrifugation to pellet high molecular weight DNA together with cell debris, the supernatant was treated sequentially with proteinase K and RNase A. The DNA remaining was extracted twice with phenol, and once with chloroform/isoamyl alcohol (24:1), then precipitated overnight in 75% ethanol. The recovered DNA fragments were separated by electrophoresis on a 1.5% agarose gel and visualized by ethidium bromide staining under UV irradiation.

Measurement of Intracellular Peroxides by Flow Cytometry—Intracellular peroxide levels were determined using an oxidation-sensitive fluorescent probe, DCFH-DA (*17*). In the presence of a variety of intracellular peroxides, DCFH is oxidized to the highly fluorescent compound DCF. Cells treated with H_2O_2 or Cu-OOH were incubated with 5 mM DCFH-DA. The cellular fluorescence intensity, which was directly proportional to the level of intracellular peroxides after 30 min DCFH-DA oxidation, was measured with a FACScan (Becton Dickinson, San Jose, CA). For each analysis, 10,000 events were recorded. For image analysis, cells were analyzed for fluorescence intensity using a lysis cell analysis system.

RESULTS

Effect of Selenious Acid on GPx-Free MDBK Cells without Affecting Other Antioxidative Enzyme Activities—When MDBK cells were fed with stepwise decreasing concentrations of FBS, the GPx activity in the cells, assayed by using H_2O_2 as a substrate, gradually decreased to a trace level (data not shown). This was due to the decrease in the Se content accompanying the decrease in the serum concentration, because serum is a major source of Se. Expression of GPx mRNA was also suppressed in Se-deficient conditions as described (*7*). We then measured the activities of GPx, CAT, and SOD in Se-deficient and Se-supplemented cells. Although there was only a trace amount of GPx activity, if any, with H_2O_2 as a substrate, it increased on the addition of 10 ng/ml selenious acid in Se-deficient cells (Table I). On the other hand, a slight GPx activity toward Cu-OOH was maintained after serum depletion, probably because certain GSH-S-transferases, which are not seleno-enzymes but exhibit GPx activity toward organic peroxides (*18*), exist in the cytosol of the cells. The activities of CAT, Mn-SOD, and Cu,Zn-SOD

were not affected by the addition of selenious acid, indicating that GPx is the only antioxidative enzyme induced by Se-supplementation.

DNA Fragmentation by Hydrogen Peroxide and Cu-OOH in Se-Deficient Cells—When the cytotoxic effects of H_2O_2 and Cu-OOH on both Se-deficient and Se-supplemented cells were assessed by the methylene blue method (*16*), Se-deficient cells were found to be 10-times more sensitive to both peroxides than Se-supplemented ones (Fig. 1). Then, we investigated the DNA damage to these cells by H_2O_2 and Cu-OOH by agarose gel electrophoresis. Figure 2A shows that 20 and 100 μM H_2O_2 caused internucleosomal DNA fragmentation, which is a characteristic pattern of DNA in apoptotic cell death, in Se-deficient cells, while no DNA damage occurred in Se-supplemented ones.

TABLE I **Antioxidative enzyme activities in Se(−) and Se(+) cells.** Cells were incubated with or without 10 ng/ml selenious acid for 5 days, then pelleted and washed with PBS(−) twice. They were then suspended in PBS(−) and sonicated for 10 min, and the activities of GPX, CAT, Mn-SOD, and Cu,Zn-SOD in cells were determined as described under "MATERIALS AND METHODS."

	GPx (H_2O_2)	GPx (Cu-OOH)	CAT	Mn-SOD	Cu,Zn-SOD
	(milliunits/mg protein)		(units/mg protein)		
Se(−)	0.6±1.0*	4.1±3.1**	0.99±0.08	4.2±2.1	7.4±2.8
Se(+)	32.2±3.4*	24.7±4.9**	0.98±0.29	3.9±1.6	6.7±3.4

Results are expressed as means±SD. *$p<0.001$, **$p<0.05$.

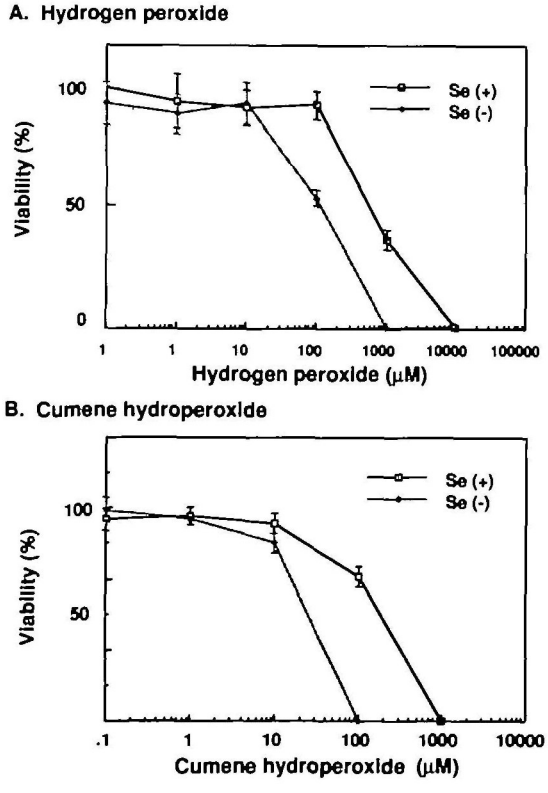

Fig 1. **Cytotoxicity of H_2O_2 and Cu-OOH under Se-deficient conditions.** The cytotoxicities of H_2O_2 (A) and Cu-OOH (B) toward both Se-deficient and Se-supplemented MDBK cells were assessed by the methylene blue method.

Judged from the ladder formation observed on electrophoresis of DNAs, apoptotic cell death occurred in Se-deficient cells but not in Se-supplemented cells. Also, 100 μM Cu-OOH caused DNA fragmentation in Se-deficient cells (Fig. 2B), but ladder formation did not occur.

Accumulation of Intracellular Peroxides after Exposure to H_2O_2—We examined how Se-deficient and Se-supplemented cells cope with intracellular peroxides after the addition of 100 μM H_2O_2. Flow cytometric analysis using DCFH-DA, a peroxidation-sensitive fluorescent dye, showed that Se-deficient cells failed to eliminate the increased intracellular peroxide after exposure to exogenous H_2O_2

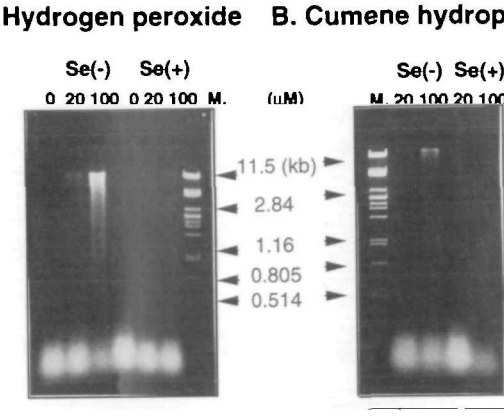

Fig 2 **DNA fragmentation by hydrogen peroxide and Cu-OOH in Se-deficient MDBK cells.** DNA damage to Se-deficient MDBK cells caused by H_2O_2 (A) and Cu-OOH (B) was investigated using agarose gel electrophoresis Se(+) indicated the presence of 10 ng/ml selenious acid and M indicated molecular marker.

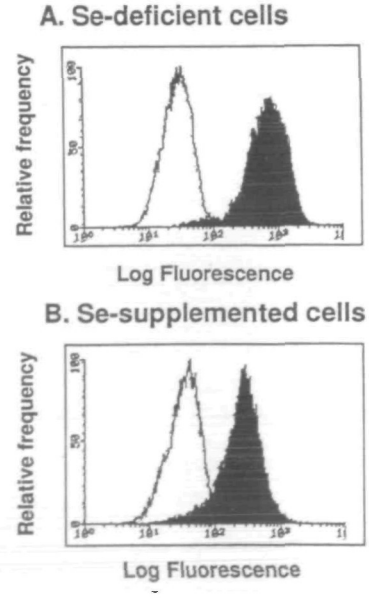

Fig. 3. **Accumulation of intracellular peroxides after exposure to H_2O_2.** After the addition of 100 μM H_2O_2, flow cytometric analysis of both Se-deficient and Se-supplemented cells using DCFH-DA was carried out. The fluorescence intensity of Se-deficient (A) or Se-supplemented (B) MDBK cells at 2 h after exposure to H_2O_2 (black area) was plotted relative to that of Se-deficient or Se-supplemented MDBK cells without incubation with H_2O_2 (white area), respectively.

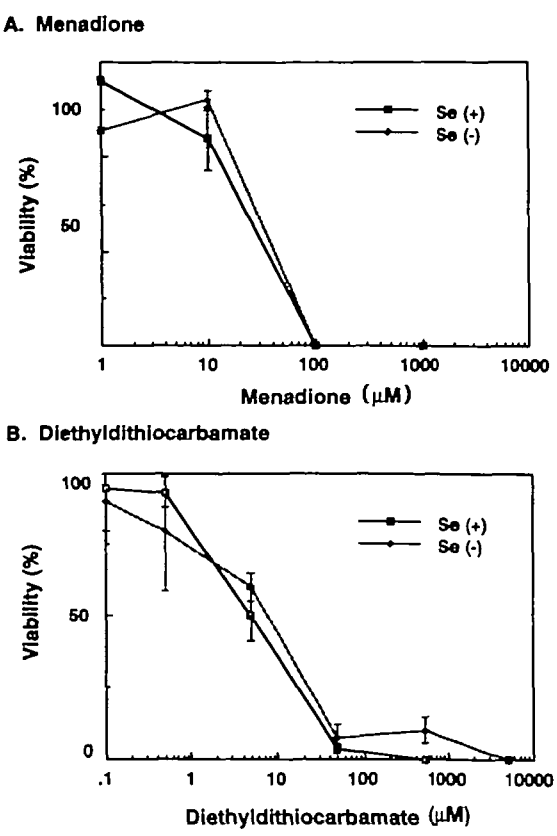

Fig. 4. **Cytotoxicity of superoxide radicals toward Se-deficient and Se-supplemented cells.** MDBK cells grown under Se-deficient and Se-supplemented conditions were incubated with given doses of menadione (A) or diethyldithiocarbamate (B) for 1 day, then cell viability was measured by the methylene blue method.

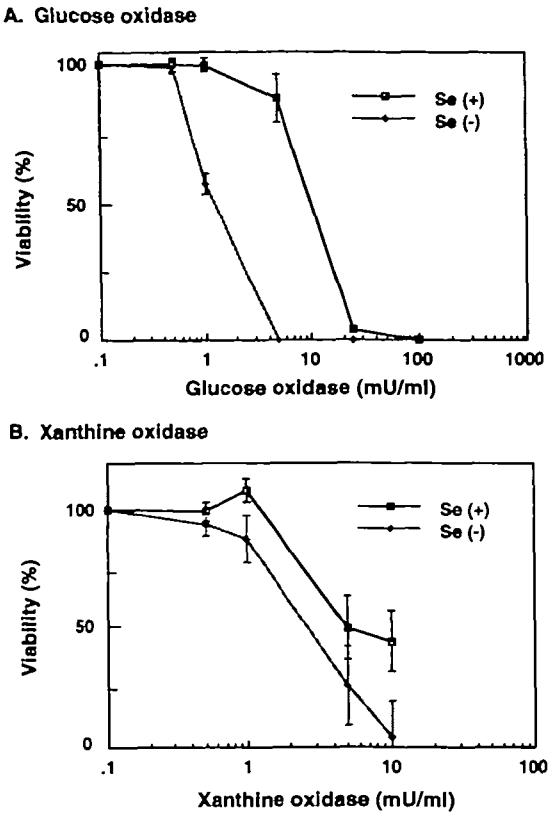

Fig. 5. **Influence of reactive oxygen species produced by glucose oxidase and xanthine oxidase on Se-deficient cells.** MDBK cells grown under Se-deficient and Se-supplemented conditions were incubated with given doses of glucose oxidase (A) and xanthine oxidase (B) for 1 day, then cell viability was assessed by the methylene blue method.

(Fig. 3) while Se-supplemented cells were able to partly reduce it at 2 h after exposure to H_2O_2.

Participation of GPx in Apoptotic Cell Death Caused by Superoxide Radicals—Since superoxide radicals are upstream ROS to H_2O_2, we investigated whether there was a difference in the effect of superoxide radicals on Se-deficient and Se-supplemented cells. Menadione, a quinone compound that alters intracellular redox cycles and converts oxygen to superoxide radicals (*11*), induced dose-related killing of both Se-deficient and Se-supplemented cells to similar extents (Fig. 4A). We also investigated the cytotoxicity of diethyldithiocarbamate, which is an inhibitor of Cu,Zn-SOD and thus results in an increased level of superoxide radicals (*19*), against Se-deficient and Se-supplemented cells. There was again no difference between Se-deficient and Se-supplemented cells in terms of cytotoxicity of diethyldithiocarbamate (Fig. 4B). Taken together, GPx seemed to have no protective effect against cellular damage caused by superoxide radicals produced in these systems.

H_2O_2 Is a Major ROS Exhibiting Cytotoxicity in Se-Deficient Cells—We further investigated the cytotoxicity of ROS produced exogenously by glucose oxidase, a H_2O_2 generator, and xanthine oxidase, a superoxide radical generator. Glucose oxidase decreased the viability of Se-deficient cells more severely than that of Se-supplemented ones (Fig. 5A). Treatment with xanthine oxidase resulted in damage to these cells to a similar extent (Fig. 5B), indicating that GPx could not effectively protect cells from death induced by exogenously produced superoxide radicals. Clofibrate is known to increase the production of catalase, a peroxisomal antioxidative enzyme, by proliferating peroxisomes (*20*). This reagent protected both Se-deficient and Se-supplemented cells from the cytotoxicity of H_2O_2 (Fig. 6). The increased amount of catalase might have compensated for the depletion of GPx, resulting in a decrease in intracellular H_2O_2.

DISCUSSION

Recently, many works on apoptosis induced by oxidative stress have been reported (*11-13, 21*). Among antioxidative enzymes, GPx is supposed to play a protective role in oxidative stress-induced apoptosis (*11-13*). Since both the content and activity of GPx are reduced under Se-deficient conditions (*7*), we prepared Se-deficient MDBK cells by decreasing FBS content stepwise in the medium. Only GPx activity was decreased in the Se-deficient cells, without affecting CAT, Mn-SOD, or Cu,Zn-SOD activity (Table I). It was, therefore, possible to evaluate the role of GPx in ROS-induced cell death by using Se-deficient conditions. Both H_2O_2 and Cu-OOH decreased the viability of Se-deficient cells more dramatically than that of Se-supplemented cells (Fig. 1). When the nature of death was

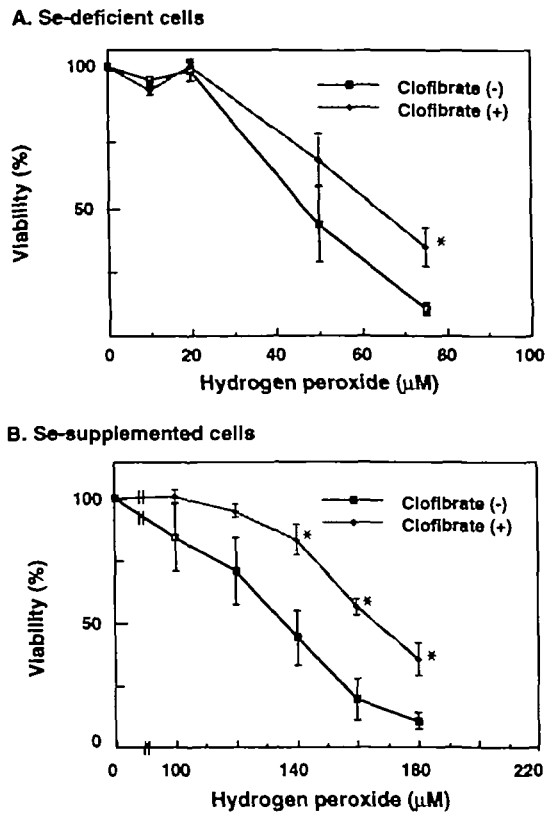

Fig. 6. **Protective role of clofibrate pretreatment against the cytotoxicity of H_2O_2 toward Se-deficient cells.** MDBK cells grown under Se-deficient (A) and Se-supplemented (B) conditions were incubated with or without pretreatment with 100 μM clofibrate for 1 week, then subjected to the given doses of H_2O_2 for 1 day. Cell viability was assessed by the methylene blue method. $^*p<0.01$.

evaluated by agarose gel electrophoresis of DNA extracted from cells, H_2O_2 induced ladder formation, while Cu-OOH induced only random DNA fragmentation (Fig. 2). This suggested that H_2O_2, but not Cu-OOH, induced apoptosis in Se-deficient MDBK cells. The higher susceptibility of Se-deficient cells than Se-supplemented ones to H_2O_2 was consistent with data obtained using H_2O_2 enzymatically produced by glucose oxidase (Fig. 5A). Flow cytometric analysis using a peroxide-sensitive dye, DCFH-DA, showed that Se-deficient cells were less capable of reducing intracellular peroxides (Fig. 3). Thus the accumulation of intracellular peroxides was more pronounced in Se-deficient cells than Se-supplemented ones, resulting in the killing of cells whose oxidant levels were beyond their scavenging capacity.

In contrast, there was no difference between Se-deficient and Se-supplemented cells in terms of the cytotoxicity of superoxide radicals produced either intracellularly by menadione (Fig. 4A) or extracellularly by xanthine oxidase (Fig. 5B). Superoxide is the primary ROS under physiological conditions, but its reactivity with cellular components is not high. The cytotoxic effect of superoxide radicals, therefore, would be mediated by H_2O_2, a product of the catalytic reaction of SOD or by the metal-driven Fenton reaction to form hydroxy radicals. A possible explanation is that H_2O_2 produced from superoxide *via* the SOD catalytic reaction was far beyond the scavenging capacity of GPx, because the rate-constant of GPx activity is much smaller than that of SOD. More precise analysis is necessary to distinguish such a subtle difference between Se-deficient and Se-supplemented cells.

Since Se-deficient cells are more likely to be killed by exogenous H_2O_2 than Se-supplemented ones, the organs of Se-deficient animals might be weaker in the case of infection, where a large amount of ROS is produced by neutrophils to defend against microorganisms. Moreover, accumulated peroxides, even at physiological levels might cause apoptosis and contribute toward organ failure in Se-deficient animals because of a GPx deficiency. Clofibrate is a peroxisome proliferator that induces catalase in hepatocytes and in renal proximal tubular epithelial cells (*20*). In this study, we demonstrated that clofibrate protected both Se-deficient and Se-supplemented cells from the toxicity of H_2O_2 to some extent (Fig. 6). Another peroxisome proliferator, nafenopin, suppressed both spontaneous and TGF-β1-induced apoptosis of primarily cultured hepatocytes (*22*). Recently, we showed that TGF-β1 suppressed antioxidative enzyme mRNA expression and increased the accumulation of intracellular peroxides (*23*). Taking our data and these findings together, the induction of catalase by peroxisome proliferators might render cells more resistant to apoptosis under peroxide-generating conditions such as inflammation and ischemia-reperfusion injury. In addition, since an HIV-associated GPx deficiency is suspected to contribute to the depletion of $CD4^+$ T cells, which occurs in acquired immune deficiency syndrome (AIDS) patients (*12*), some peroxisome proliferators in T cells might become useful therapeutic agents for AIDS.

REFERENCES

1. Rotruck, J.T., Pope, A.L., Ganther, H.E., Swanson, A.B., Hafeman, D.G., and Hoekstra, W.G. (1973) Selenium: Biochemical role as a component of glutathione peroxidase. *Science* **179**, 588-590
2. Forstrom, J.W., Zakowski, J.J., and Tapple, A.L. (1978) Identification of the catalytic site of rat liver glutathione peroxidase as selenocysteine. *Biochemistry* **17**, 2639-2644
3. Flohe, L., Günzler, W.A., and Ladenstein, R. (1976) Glutathione peroxidase in *Kroc Foundation Series Vol. 6 Glutathione: Method and Function* (Arias, I.M. and Jakoby, W.B., ed.) pp. 115-138, Raven Press, New York
4. Ursini, F., Maiorino, M., and Gregolin, C. (1985) The selenoenzyme phospholipid hydroperoxide glutathione peroxidase. *Biochim. Biophys. Acta* **839**, 62-70
5. Duan, Y.-J., Komura, S., Fiszer-Szafarz, B., Szafarz, D., and Yagi, K. (1988) Purification and characterization of a novel monomeric glutathione peroxidase from rat liver. *J. Biol. Chem.* **263**, 19003-19008
6. Maddipati, K.R., Gasparski, C., and Marnett, L.J. (1987) Characterization of the hydroperoxide-reducing activity of human plasma. *Arch. Biochem. Biophys.* **254**, 9-17
7. Sandstrom, B.E., Carlsson, J., and Marklund, S.L. (1989) Selenite-induced variation in glutathione peroxidase activity of three mammalian cell lines: No effect on radiation-induced cell killing or DNA strand breakage. *Radiat. Res.* **117**, 318-325
8. Geiger, P.G., Thomas, J.P., and Girotti, A.W. (1991) Lethal damage to murine L1210 cells by exogenous lipid hydroperoxides: Protective role of glutathione-dependent selenoperoxidases. *Arch. Biochem. Biophys.* **288**, 671-680
9. Geiger, P.G., Lin, F., and Girotti, A.W. (1993) Selenoperoxidases-mediated cytoprotection against the damaging effects of *tert*-butyl hydroperoxide on leukemia cells. *Free Radical Biol. Med.* **14**, 251-266

10. Combs, G.F., Jr. and Combs, S.B. (1986) *The Role of Selenium in Nutrition*, pp. 266-326, Academic Press, Orland, FL
11. Hockenbery, D.M., Oltvai, Z.N., Yin, X.-M., Milliman, C.L., and Korsmeyer, S.J. (1993) Bcl-2 functions in an antioxidant pathway to prevent apoptosis. *Cell* **75**, 241-251
12. Sandstrom, P.A., Tebby, P.W., Cleave, S.V., and Buttke, T.M. (1994) Lipid hydroperoxides induce apoptosis in T cells diplaying a HIV-associated glutathione peroxidase deficiency. *J. Biol. Chem.* **269**, 798-801
13. Amstad, P., Moret, R., and Cerutti, P. (1994) Glutathione peroxidase compensates for the hypersensitivity of Cu,Zn-superoxide dismutase overproducers to oxidant stress. *J. Biol. Chem.* **269**, 1606-1609
14. Claiborne, A. (1985) *Handbook of Methods for Oxygen Free Radical Research*, pp. 283-284, CRC Press, Boca Raton, FL
15. Beauchamp, C. and Fridovich, I. (1971) Superoxide dismutase: Improved assays and an assay applicable to acrylamide gels. *Anal. Biochem.* **44**, 276-287
16. Yamazaki, S., Onishi, E., Enami, K., Natori, K., Kohase, M., Sakamoto, H., Tanouchi, M., and Hayashi, H. (1986) Proposal of standardized methods and reference for assaying recombinant human tumor necrosis factor. *Jpn. J. Med. Sci. Biol.* **39**, 105-118
17. Bass, D.A., Parce, J.W., Dechatelet, L.R., Szejda, P., Seeds, M.C., and Thomas, M. (1983) Flow cytometric studies of oxidative product formation by neutrophils: A graded response to membrane stimulation. *J. Immunol.* **130**, 1910-1917
18. Meyer, D.J., Beales, D., Tan, H.K., Coles, B., and Ketterer, B. (1985) Glutathione transferase in primary rat hepatomas: The isolation of a form with GSH peroxidase activity. *FEBS Lett.* **184**, 139-143
19. Maitre, B., Jornot, L., and Junod, A.F. (1993) Effects of inhibition of catalase and superoxide dismutase activity on antioxidant enzyme mRNA levels. *Am. J. Physiol.* **265**, L636-L643
20. Kritchevsky, D. (1975) *Handbook of Experimental Pharmacology, 41, Hypolipidemic Agents*, Springer-Verlag, New York
21. Kaneto, H., Fujii, J., Seo, H.G., Suzuki, K., Matsuoka, T., Nakamura, M., Tatsumi, H., Yamasaki, Y., Kamada, T., and Taniguchi, N. (1995) Apoptotic cell death triggered by nitric oxide in pancreatic β-cells. *Diabetes* **44**, 733-738
22. Bayly, A.C., Roberts, R.A., and Dive, C. (1994) Suppression of liver cell apoptosis in vitro by the non-genotoxic hepatocarcinogen and peroxisome proliferator nafenopin. *J. Cell Biol.* **125**, 197-203
23. Kayanoki, Y., Fujii, J., Suzuki, K., Kawata, S., Matsuzawa, Y., and Taniguchi, N. (1994) Suppression of antioxidative enzyme expression by transforming growth factor-β1 in rat hepatocytes. *J. Biol. Chem.* **269**, 15488-15492