The overexpression of phospholipid hydroperoxide glutathione peroxidase (PHGPx) by RBL-2H3 cells was used as the basis for an investigation of the effects of PHGPx on the formation of leukotrienes. The rates of production of leukotriene C₄ (LTC₄) and leukotriene B₄ (LTB₄) in cells that overexpressed PHGPx were 8 times lower than those in a control line of cells. The reduction in rates of production of leukotrienes apparently resulted from the increase in the PHGPx activity since control rates of formation of leukotrienes could be achieved in PHGPx-overexpressing cells upon inhibition of PHGPx activity by diethyl malate. The conversion of radioactively labeled arachidonic acid to intermediates in the lipoxygenase pathway, such as 5-hydroxyeicosatetraenoic acid (5-HETE), LTC₄, and LTB₄, was strongly inhibited in PHGPx-overexpressing cells that had been prelabeled with [¹⁴C]arachidonic acid. PHGPx apparently inactivated the 5-lipoxygenase that catalyzed the conversion of arachidonic acid to 5-hydroperoxyeicosatetraenoic acid (5-HPETE) since 5-HPETE is a common precursor of 5-HETE, LTC₄, and LTB₄. The rates of formation of LTC₄ and LTB₄ in PHGPx-overexpressing cells returned to control rates upon the addition of a small amount of 12-HPETE. Flow cytometric analysis revealed that the rapid burst of formation of lipid hydroperoxides induced by A23187 was suppressed in PHGPx-overexpressing cells as compared with the control lines of cells. Subcellular fractionation analysis showed that the amount of PHGPx associated with nuclear fractions from PHGPx-overexpressing cells was 3.5 times higher than that from the control line of cells. These results indicate that PHGPx might be involved in inactivation of 5-lipoxygenase via reductions in levels of the fatty acid hydroperoxides that are required for the full activation of 5-lipoxygenase. Thus, in addition to its role as an antioxidant enzyme, PHGPx appears to have a novel function as a modulator of the production of leukotrienes.

Leukotrienes are important mediators both in host defense and inflammatory disease states since they have potent effects on cell migration, muscle contraction, vascular permeability, and the release of lysosomal enzymes (1–3). Leukotrienes can be formed in mast cells, granulocytes, and monocytes/macrophages in response to extracellular stimulation. The biosynthesis of leukotrienes is initiated by the release of arachidonic acid from membrane phospholipids (4). The liberated arachidonic acid is oxidized to hydroperoxyeicosatetraenoic acid (5-HPETE) and subsequent dehydration yields an unstable epoxye intermediate, leukotriene A₄ (LTA₄). These sequential reactions are catalyzed by 5-lipoxygenase that is the primary enzymatic regulator of the biosynthesis of leukotrienes (5). LTA₄ is further converted to a variety of leukotrienes, such as LTB₄ and LTC₄. The nuclear membrane is the site of leukotriene synthesis, and cytosolic phospholipase A₂ and 5-lipoxygenase are translocated to this site from the cytosol after the activation of cells (6, 7). The activity of 5-lipoxygenase is regulated by several factors, such as Ca²⁺, ATP, and 5-lipoxygenase-activating protein (FLAP) (8–12). There is evidence that fatty acid hydroperoxides might also activate 5-lipoxygenase (13, 14). A requirement for fatty acid hydroperoxides in the activation of lipoxygenase suggests that the activity of 5-lipoxygenase might be regulated by its own product, namely 5-HPETE (15). Intracellular 5-HPETE can be reduced to 5-hydroxyeicosatetraenoic acid (5-HETE) by lipid peroxidases such as glutathione peroxidase (GPx). However, the lipid peroxidase responsible for the reduction of 5-HPETE remains to be defined. This reduction seems to be a key reaction in the control of the production of leukotrienes since it can determine the level of 5-HPETE, which is utilized as an activator of 5-lipoxygenase and as a precursor of LTA₄.

GPx reduces cellular lipid hydroperoxides. Two types of selenium-dependent GPx are widely distributed in various tissues. Cytosolic glutathione peroxidase (cGPx, GPx1) is predominantly present in the cytosol of tissues, and it reduces fatty acid hydroperoxides and H₂O₂ at the expense of glutathione (16). Phospholipid hydroperoxide glutathione peroxidase (PHGPx, GPx4) is localized in both the cytosol and the membrane fraction (17). PHGPx can directly reduce the phospholipid hydroperoxides, fatty acid hydroperoxides (18, 19), and unsaturated epoxide intermediate, leukotriene A₄ (LTA₄). This reduction seems to be a key reaction in the control of the production of leukotrienes since it can determine the level of 5-HPETE, which is utilized as an activator of 5-lipoxygenase and as a precursor of LTA₄.
cholesterol hydroperoxides (20) that are produced in peroxi-
dized membranes and oxidized lipoproteins. Although direct evidence for the involvement of GPx in the reduction of 5-hpETE, required for the activation of 5-lipoxygenase, is still lacking, it has been shown that impairment of GPx activity by selenium deficiency or glutathione depletion leads to enhanced activity of 5-lipoxygenase in rat polymorphonuclear leukocytes, rat basophile leukemia cells (RBL-1) (21), B lymphocytes (22, 23) and human granulocytes (24, 25). These results suggest that glutathione peroxidase might regulate the activity of 5-li-
pyoxenase in the cellular level. The question now arises as to whether cGPx or PHGPx might be responsible for the regulation of the formation of leukotrienes. We are interested in the possible role of PHGPx in the regulation of leukotriene synthe-
sis since PHGPx is unique as an isozyme of GPx, being able to interact with the nuclear membrane in which the synthesis of leukotrienes occurs.

Stable transfectants that overexpress PHGPx provide useful model systems with which to attempt to clarify the ability of PHGPx to modulate leukotriene synthesis. We reported previ-
ously the cloning of a cDNA for rat PHGPx (26, 27) and the establishment of PHGPx-overexpressing stable transfectants of rat basophile leukemi4 2H3 (RBL-2H3) cells (28). PHGPx-
overexpressing cells exhibit resistance to cell death that is due to oxidative damage. RBL-2H3 cells are a neoplastic cell line from rats. They are derived from basophils, which are among the cells that have been used most extensively in studies of 5-lipoxygenase. RBL-2H3 cells produce LTC4 and LTB4 predominantly in response to stimulation by calcium ionophores (29, 30).

In the present study, the PHGPx-overexpressing RBL-2H3 cells were used to examine the effects of PHGPx on the forma-
tion of leukotrienes. We showed that PHGPx significantly sup-
pressed the activity of leukotrienes via a reduction in the level of intracellular lipid hydroperoxides, which was due to suppression of the activity of 5-lipoxygenase.

**EXPERIMENTAL PROCEDURES**

**Reagents**—Antibodies against PHGPx were prepared previously (28). Antibodies against 5-lipoxygenase and FLAP were a kind gift from Dr. M. Murakami (Showa University, Tokyo) (31). Anti-histone H1 monocl-
onal antibodies and 125I-protein A (2.60–3.70 TBq/g) were purchased from Cosmobio Co. Ltd. (Tokyo, Japan) and ICN Biochemicals Inc. (Irvine, CA), respectively. Antibodies against cPLA2, diethyl malate (DEM), LTB4, LTC4, LTA4, methyl ester, A23187, and 5,6-carboxy-2,7-
dichlorofluorescein-diacetate (DCFH-DA) were obtained from Funakoshi Co. Ltd. (Tokyo, Japan).

**Cell Cultures**—RBL-2H3 cells that overexpressed PHGPx were gen-
erated by transfection with p5Ro-PHGPx and pSV2neo as described previously (28). Antibodies against 5-lipoxygenase and FLAP were a kind gift from Dr. M. Murakami (Showa University, Tokyo) (31). Anti-histone H1 monocl-
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dichlorofluorescein-diacetate (DCFH-DA) were obtained from Funakoshi Co. Ltd. (Tokyo, Japan).

**Cell Cultures**—RBL-2H3 cells that overexpressed PHGPx were gen-
erated by transfection with p5Ro-PHGPx and pSV2neo as described previously (28). In brief, fragments of a rat cDNA for PHGPx, namely the insert in pRHGPx2 (761 base pairs) (26), were subcloned into pSRo as the expression vector (32). RBL-2H3 cells were harvested by trypsinization and were resuspended in the Dulbecco’s modified essen-
tial medium that contained 20 mM HEPES, 2 mM glutamine, penicillin (100 units/ml), and streptomycin (100 units/ml) at a concentration of 4 x 10^6 cells/ml. The suspension of cells (0.25 ml) was transferred to an electrocution cuvette (0.4-cm gap; Bio-Rad) with a total of 20 m

used to label the selenoproteins of RBL-2H3 cells. PHGPx-overexpress-

RBL-2H3 cells (5 x 10^6 cells) were labeled with 140 nCi/ml (75Se)
sodium selenite for 96 h. Labeled cells were collected after washing with ice-cold phosphate-buffered saline and sonicated in 1 ml of 10 mM Tris-HCl buffer (pH 7.4) containing 5 mg/ml leupeptin and 17 mg/ml phenylmethylsulfonyl fluoride (PMSF). The extract was centrifuged at 10,000 x g for 10 min at 4 °C, and the supernatant was collected for the analysis of selenoproteins. Labeled selenoproteins in transformants were separated by SDS-PAGE (12.5% acrylamide gel) under non-reducing conditions, as described by Laemmli (34). Gels were stained for 10 min with 0.1% (w/v) Coomassie Brilliant Blue R that had been dissolved in glacial acetic acid, methanol, and water (10:50, 30:1). Bands of dried gels were analyzed densitometrically with a Bio-

**Immunoblot Analysis**—Cell homogenates and nuclear fractions were frac-
tioned by SDS-PAGE on 12.5% acrylamide gels and transferred to PVDF membrane filter (Millipore Co., Bedford, MA) at 50 V for 150 min in 25 mM Tris, 192 mM glycine, 10% (w/v) methanol at 4 °C in a protein transfection system (Bio-Rad), as described previously (35). Each PVDF membrane with blotted protein was blocked by incubation with 3% (w/v) skim milk in 10 mM Tris-HCl (pH 7.4) that contained 150 mM NaCl and 0.1% Tween 20 (TBS-T) for 1 h. The PVDF membrane was then incubated with antiserum against cPLA2, 5-lipoxygenase, and FLAP that had been diluted with TBS-T at an appropriate concentration for 2 h. Then, the PVDF membrane was incubated for 1 h with horseradish peroxidase-conjugated goat antibodies against rabbit IgG (Zymed, South San Francisco, CA). The binding of antibodi-
es to the antigen on the PVDF membrane was detected with an enhanced chemiluminescence Western blotting analysis system (Amersham Corp.).

**Assays of Enzymatic Activities**—RBL-2H3 cells (2 x 10^6 cells) were sonicated in 1 ml of 10 mM Tris-HCl buffer (pH 7.4) that included 5 mg/ml leupeptin and 17 mg/ml PMSF. The homogenate was centrifuged at 10,000 x g for 10 min at 4 °C, and the supernatant was used for assays of enzymatic activity. PHGPx activity was measured as de-
scribed previously (28). The reaction mixture contained, in a final volume of 0.8 ml, 0.1 mM Tris-HCl (pH 7.4), 5 mM EDTA, 1.5 mM sodium azide, 3 mM glutathione, 0.25 mM NAPDH, 1 unit of glutathione reductase, 0.1% Triton X-100, and 10 μM phosphatidylcholine hydroperoxide (PCOOH), which had been prepared from egg yolk phosphatidylcholine as described previously (36). The reaction was started by the addition of PCOOH at 37 °C after preincubation for 10 min. The rate of the reaction was measured by following the decrease in the absorbance at 340 nm over a 10-min period. The reaction was stopped by the addition of methanol, and the total lipids were extracted by the method of Bligh and Dyer (37). The lipid extract was dried under a stream of nitrogen gas, and the residue was dissolved in 0.05 ml of methanol. PCOOH without reduction by PHGPx was quantitated by the methylene blue method (38). The decrease in the amounts of PCOOH during the incubation was calculated by subtracting the amounts of non-reduced PCOOH from the original amount of PCOOH. cGPx activity was meas-
ured after spectrophotometrically monitoring the formation of glutathione at 340 nm with 0.25 mM hydrogen peroxide as substrate (39). Glutathione S-transferase activity was measured spectrophotometrically by monitoring the formation of the conjugate of reduced glutathione and 1-chloro-2,4-dinitrobenzene at 340 nm (40). Catalase activity was de-
termined as described by Clairborne (41) with 10 mM hydrogen peroxide as the substrate.

**Quantitation of LTB4 and LTC4 by Enzyme Immunosorbent Assays (ELAs)—** RBL-2H3 cells (5 x 10^5 cells) were preincubated in phosphate-buffed saline (PBS) that contained 1 mM CaCl2 for 10 min at 37 °C and then stimulated with 5 μM A23187 for 5 min. Supernatants were prepared for the quantitation of LTB4 and LTC4 by enzyme immunoas-

**Liberation of [14C]-arachidonic Acid from A23187-stimulated Cells—** RBL-2H3 cells (4 x 10^5 cells) were grown in 24-well plates for 3 days. Cells then were incubated with 0.1 μCi of [14C]-arachidonic acid (60–100 C/mmol; NEN Life Science Products) for 24 h at 37 °C. Labeled cells were washed three times with PBS and then preincubated with PBS that contained 1 mM CaCl2 for 10 min at 37 °C and then stimulated with 5 μM A23187 for 5 min. Supernatants were prepared for the quantitation of LTB4 and LTC4 by enzyme immunoas-

**Labeling with [75Se] of RBL-2H3 Cells—** [75Se]Sodium selenite (10 mM/cm; Amersham Corp., Little Chalfont, Buckinghamshire, UK) was
The Formation of LTC₄ and LTB₄ from Exogenously Added LTA₄—LTA₄ was prepared by the hydrolysis of the methyl ester of LTA₄ with 0.02 M lithium hydroxide at room temperature in darkness described previously (42, 43). Cells were incubated in the presence of 1 μM LTA₄ for 10 min. The amounts of LTC₄ and LTB₄ in the supernatant were quantified by ELISA.

The Formation of LTC₄, LTB₄, and 5-HETE from Exogenous [¹⁴C]Arachidonic Acid—RBL-2H3 cells (5×10⁶ cells) were incubated with 0.1 μCi of [¹⁴C]arachidonic acid for 24 h at 37 °C. Labeled cells were stimulated with A23187 for 10 min in the presence of 1 mM CaCl₂ as described above. After the incubation, supernatants were obtained by centrifugation, and 3 ml of ethyl acetate (pH 3.0), supplemented with 0.1 ml of 0.09 M HCl, were added to each supernatant for the extraction of metabolites. Non-labeled LTC₄ (100 ng), LTB₄ (100 ng), and 5-HETE (200 ng), as carriers, and prostaglandin B₂ (PCG₂; 50 ng) as an internal standard, were added before the extraction. The supernatant was collected and injected into an HPLC system equipped with a reverse-phase column (LiChrosorb RP-18; 240×0.4 mm inner diameter; Merck, Darmstadt, Germany) for the separation of LTC₄, LTB₄, and 5-HETE.

Flow Cytometric Analysis of Intracellular Peroxides—To assess levels of intracellular peroxides, we performed flow cytometric analysis using an oxidation-sensitive fluorescent probe, 5,6-carboxy-2′,7′-dichlorofluorescein diacetate (CFDA-DA). After preincubation with 12-HETE and/or A23187, cells were washed with PBS and incubated with 2.5 μM CFDA-DA in PBS for 30 min. CFDA-DA was deacylated to the non-fluorescent compound 2′,7′-dichlorofluorescein (DCFH) within the cells, and DCFH was oxidized to the fluorescent compound 2′,7′-dichlorofluorescein by a variety of peroxides (44). The fluorescent intensity of dichlorofluorescein in the cells was analyzed with a flow cytometer (EPICS® Elite Flow cytometer; Coulter, Hialeah, FL).

Subcellular Fractionation of Cells—[⁷⁵Se]Sodium selenite-labeled cells were metabolically labeled with [⁷⁵Se]sodium selenite for 4 days. Then the ⁷⁵Se-labeled cells were sonicated, and 50 μg of protein were loaded in each lane for analysis by SDS-PAGE (12.5% polyacrylamide) with subsequent autoradiography for detection of the ⁷⁵Se-labeled selenoproteins.

RESULTS

Expression of Selenoproteins and the Activities of Antioxidant Enzymes in PHGPx-overexpressing RBL-2H3 Cells—The distribution of selenoproteins was determined in RBL-2H3 cells that had been labeled with [⁷⁵Se]sodium selenite for 4 days. Fig. 1 shows the electrophoretic profiles of [⁷⁵Se]-labeled proteins from control cells and PHGPx-overexpressing RBL-2H3 cells. Three major selenoproteins were detected, with estimated molecular masses of 57, 25, and 14 kDa respectively. The band of a 20-kDa protein that corresponded to PHGPx was quite faint in the control cells. The selenoproteins of 20 and 25 kDa were identified as cGPx and PHGPx, respectively, by immunoprecipitation with corresponding antibodies (data not shown). These results indicate that the level of PHGPx was quite low in the control cells of cells as compared with that of cGPx. PHGPx was extensively expressed in all lines of cells transfected with the cDNA for PHGPx, whereas the levels of expressions of the other three major selenoproteins in PHGPx-overexpressing cells were similar to those in the control lines of cells.

The specific activities of antioxidant enzymes were determined in the PHGPx-overexpressing cells and control lines of cells as shown in Table I. The activity of PHGPx in the PHGPx-overexpressing cells was three times that in the control lines of cells. No significant differences in the respective activities of cGPx, glutathione S-transferase, and catalase between the PHGPx-overexpressing cells and the control lines of cells were observed.

Production of Leukotrienes in the PHGPx-overexpressing RBL-2H3 Cells—Levels of leukotrienes were determined in the control and PHGPx-overexpressing cells after exposure to A23187 for 10 min by enzyme immunoassay kit (24). Immunoprecipitated protease were separated by SDS-PAGE (12.5% polyacrylamide) under non-reducing conditions. Gels were stained and dried for autoradiography. The distribution of PHGPx was calculated from scanning densitometry with a Bio-Imaging Analyzer (BAS2000; Fuji Film, Tokyo).

Quantitation of Proteins—Concentrations of protein were determined with the BCA protein assay reagent (Pierce) with bovine serum albumin as the standard.

Statistical Analysis—All data for which n ≥ 3 are expressed as mean values ± S.D.
The accumulated LTA4 was preferentially utilized for the

synthesis of LTB4 by LTA4 hydrolase. The amount of LTB4 in DEM-treated control cells was approximately 7 times that in non-treated control cells, in which the production of LTB4 was markedly inhibited by the treatment with DEM (Table II).

Inhibition of 5-Lipoxygenase Activity in PHGPx-overexpressing Cells—The rates of production of individual metabolites in the 5-lipoxygenase pathway, such as free arachidonic acid, 5-HETE, LTC4, and LTB4, were determined in control line of cells and in PHGPx-overexpressing cells that had been prelabeled with [14C]arachidonic acid to determine the step at which inhibition occurs in the 5-lipoxygenase pathway in PHGPx-overexpressing cells (Fig. 3 and 4). A23187 induced the activation of phospholipase A2 and provoked the liberation of free arachidonic acid from membrane phospholipids (Fig. 3A). No significant difference in the release of arachidonic acid was observed between the control line of cells and the PHGPx-overexpressing cells. Levels of cytosolic phospholipase A2 (cPLA2) were determined by immunoblot analysis with anti-cPLA2 (Fig. 3B). The levels of cPLA2 in PHGPx-overexpressing cells were almost the same as that in the control line of cells. The rates of formation of 5-HETE, LTC4, and LTB4 originated from [14C]arachidonic acid were determined by reverse-phase HPLC (Fig. 4). Peaks I and II contained LTC4 and LTB4, respectively. Peak III contained 5-HETE that had been produced by the reduction of 5-HPETE. The formation of three 5-lipoxygenase metabolites was facilitated in the control line of cells after the challenge with A23187. By contrast, the rate of formation of LTC4 and LTB4 from radioactive arachidonic acid was significantly reduced in the PHGPx-overexpressing cells. The rate of production of 5-HETE, formed by the reduction of 5-HPETE, was also reduced in the PHGPx-overexpressing cells, although the capacity for the reduction of 5-HPETE to 5-HETE was clearly elevated in the PHGPx-overexpressing cells. Immunoblot analysis with anti-5-lipoxygenase and 5-lipoxygenase activating protein (FLAP) revealed that levels of 5-lipoxygenase and FLAP were the same in PHGPx-overexpressing cells and in the control line of cells (Fig. 5B). These results indicated that activity of 5-lipoxygenase was inhibited in the PHGPx-overexpressing cells and, as a result, the production of 5-HPETE, the common precursor of LTC4, LTB4, and 5-HETE, was significantly suppressed.

To determine the effects of PHGPx on the activities of LTC4 synthase and LTA4 hydrolase, the PHGPx-overexpressing cells and control lines of cells were incubated with 1 μM exogenous LTA4 for 10 min. The productions of LTC4 in the PHGPx-overexpressing cells (107 cells) and control lines of cells (107 cells) were 46 ± 5 and 50 ± 3 ng, respectively. The productions of LTB4 in the PHGPx-overexpressing cells were almost same as that in the control lines of cells accounting for approximately 8 ng/107 cells. These results indicate that no significant differences of activities of LTC4 synthase and LTA4 hydrolase were observed in the PHGPx-overexpressing cells and the control lines of cells.

Involvement of HPETE in the Suppression of 5-Lipoxygenase
LTC4 in the PHGPx-overexpressing cells was significantly increased by the addition of 12-HPETE, and it returned to the basal level in the control line of cells at a concentration of 12-HPETE (Fig. 5A). 12-HETE, a reduced form of 12-HPETE, failed to restore the production of LTC4 in PHGPx-overexpressing cells after the addition to the control line of cells (Fig. 5B). These results indicate that the levels of hydroperoxides in PHGPx-overexpressing cells were much lower than those in the control line of cells without stimulation and also after stimulation with A23187. The levels of hydroperoxide in PHGPx-overexpressing cells after the addition to the medium of 10 ng of 12-HPETE were the same as the basal level.

Activity in PHGPx-overexpressing Cells—We determined the effects of hydroperoxides on the production of leukotrienes in an attempt to estimate whether the inhibition of the activity of 5-lipoxygenase was due to insufficient levels of hydroperoxides in PHGPx-overexpressing cells (Fig. 6). Levels of LTC4 in A23187-stimulated cells were determined in the presence of various concentrations of 12-HPETE, which is not utilized as a substrate for the synthesis of LTC4. The rate of formation of LTC4 in the PHGPx-overexpressing cells was significantly increased by the addition of 12-HPETE, and it returned to the rate in the control line of cells at a concentration of 12-HPETE of 5 ng (Fig. 5B). By contrast, 12-HPETE had no effect in the control line of cells (Fig. 5A). 12-HETE, a reduced form of 12-HPETE, failed to restore the production of LTC4 in PHGPx-overexpressing cells.

The levels of hydroperoxides were determined in the control line of cells and in the PHGPx-overexpressing cells by flow cytometric analysis after incorporation by cells of 2',7'-dichlorofluorescin diacetate (DCFH-DA), a hydroperoxide-sensitive fluorescent dye (Fig. 6). The basal fluorescence intensity of DCFH in PHGPx-overexpressing cells was lower than that observed in the control line of cells (Fig. 6A). A23187 provoked the production of intracellular hydroperoxides, and the intensities of peaks of fluorescence increased. The increase in fluorescence intensity in A23187-stimulated control cells was larger than that in similarly stimulated PHGPx-overexpressing cells (Fig. 6B). These results indicate that the levels of hydroperoxides in PHGPx-overexpressing cells were much lower than those in the control line of cells without stimulation and also after stimulation with A23187. The levels of hydroperoxide in PHGPx-overexpressing cells after the addition to the medium of 10 ng of 12-HPETE were the same as the basal level.

**TABLE II**

| DEM | Control line of cells | PHGPx-overexpressing cells |
|-----|-----------------------|----------------------------|
|     | LTC4                  | PHGPx-overexpressing cells |
|     | 246 ± 12              | 172 ± 28                   |
|     | LTB4                  | 58 ± 20                    |
|     | Total                 | 230 ± 48                   |

*Effects of diethyl malate on the production of leukotrienes in a control line of cells and PHGPx-overexpressing cells.* Control cells (S2) and PHGPx-overexpressing cells (L28) were preincubated for 3 h at 37°C with (+) or without (−) 0.5 mM diethyl malate (DEM) for 3 h at 37°C and then stimulated with 5 μM A23187 for 5 min at 37°C. LTC4 and LTB4 (pg/10⁶ cells), released into the supernatant, were quantitated by EIAbs. Values are given as means ± S.D. of results from three independent experiments.

**DISCUSSION**

In the control line of cells (Fig. 6C). The intensity of fluorescence after stimulation with A23187 of PHGPx-overexpressing cells that had been pretreated with 12-HPETE was the same as that in the control line of cells after stimulation with A23187 (Fig. 6D). These results indicated that the increase in levels of intracellular hydroperoxides, caused by the addition of 12-HPETE, in PHGPx-overexpressing cells had dramatically increased the rate of formation of intracellular hydroperoxides.

The subcellular localization of PHGPx in PHGPx-overexpressing cells—To investigate the amounts of PHGPx in nuclear fractions of the control line of cells and PHGPx-overexpressing cells, cells prelabeled with [75Se]sodium selenite for 4 days were fractionated into their organelle by the centrifugation, solubilized, and immunoprecipitated with anti-PHPx. PHGPx was mainly enriched both in the mitochondrial and the nuclear fraction in the control line of cells (Fig. 7, A and C). In PHGPx-overexpressing cells, the amounts of PHGPx were significantly enhanced in nuclear, microsomal, and cytoplasmic fractions (Fig. 7, B and D). The amount of PHGPx associated with nuclear fractions from PHGPx-overexpressing cells was 3.5 times higher than that from the control line of cells (C and D).
trienes (29, 30). The present study demonstrated that the over-expression of PHGPx in RBL2H3 cells caused a dramatic suppression of the production of leukotrienes in response to stimulation by A23187 (Fig. 2). The production of individual metabolites of 5-lipoxygenase was examined in PHGPx-overexpressing cells that had been preincubated with [14C]arachidonic acid by reverse-phase HPLC to define the step in the 5-lipoxygenase pathway where inhibition occurs (Fig. 4). The rates of production of radioactive 5-HETE, LTC₄, and LTB₄ in PHGPx-overexpressing cells were much lower than in the control line of cells, whereas the rates of release of [14C]arachidonic acid were unchanged (Fig. 3). No esterification of 5-HETE in phospholipid was also detected in PHGPx-overexpressing cells (data not shown). Suppression of the production of 5-HETE in PHGPx-overexpressing cells indicated that inhibition of the synthesis of leukotrienes was not due to acceleration of the reduction of 5-HPETE to 5-HETE but was, rather, due to the inhibition of the production of 5-HPETE from arachidonic acid by 5-lipoxygenase. Levels of 5-lipoxygenase did not change in PHGPx-overexpressing cells (Fig. 4), an indication that the activity of 5-lipoxygenase was suppressed in such cells.

The question remains as to how PHGPx down-regulates the activity of 5-lipoxygenase. The results obtained with PHGPx-overexpressing cells clearly indicate that PHGPx is intimately involved in the suppression of the activity of 5-lipoxygenase. Previous reports suggest that lipid hydroperoxides have dual effects on the activity of 5-lipoxygenase. In studies of enzyme kinetics, a small amount of HPETE activated 5-lipoxygenase (48), and a relatively high concentration (1.75 \( \mu M \)) of HPETE caused inactivation (49). Although the precise mechanisms of
the activation and inactivation of 5-lipoxygenase have not been elucidated, it seems likely that the state of the iron in 5-lipoxygenase might modulate the activity of the enzyme. When lipoxygenase is inactive, the enzyme contains a single non-heme iron that is in the ferrous oxidation state (Fe^{2+}). The active form of lipoxygenase contains iron in the ferric oxidation state (Fe^{3+}) (50, 51). Thus, the conversion of iron to the ferric from the ferrous oxidation state is necessary for the activation of lipoxygenase (52–55). Therefore, the activity of lipoxygenase might be regulated by a small amount of hydroperoxy lipids, acting as essential activators of the enzyme. In the present study, preincubation of PHGPx-overexpressing cells with 12-HPETE led to increased production of leukotrienes, whereas 12-HPETE did not affect the production of leukotrienes in the control line of cells (Fig. 5). Our results indicate that PHGPx is primarily responsible for the reduction of lipid hydroperoxides and thereby cause the down-regulation of 5-lipoxygenase in RBL-2H3 cells.

Flow cytometric analysis revealed that the basal level of intracellular hydroperoxides was significantly reduced in PHGPx-overexpressing cells (Fig. 6). A rapid and transient increase in levels of intracellular hydroperoxides induced by stimulation with A23187 was observed. The increase in fluorescence intensity was probably due to the production of intracellular lipid hydroperoxides, including 5-HPETE produced by 5-lipoxygenase. The increase in the levels of intracellular lipid hydroperoxides induced by A23187 was suppressed by overexpression of PHGPx. This effect by PHGPx was abolished by the addition of a small amount of 12-HPETE, which probably restored the production of LTC4. Thus, small variations in the levels of intracellular hydroperoxides can dramatically modulate the production of leukotrienes, and PHGPx seems to be able to regulate the activity of 5-lipoxygenase by modulating the levels of intracellular lipid hydroperoxides.

It is assumed that levels of lipid hydroperoxides are controlled by two kinds of intracellular GPx isozyme. Previous studies suggested that GPx might modulate the activity of 5-lipoxygenase because depleters of glutathione such as 1-chloro-2,4-dinitrobenzene or diamide induce the enhanced production of leukotrienes in human polymorphonuclear leukocytes (24) and B lymphocytes (22, 23). To evaluate whether it is cGPx or PHGPx that has such an effect, we examined the effects of an inhibitor of GPx on the production of leukotrienes in the control line of cells and in PHGPx-overexpressing cells (Table II). The production of LTC4 and LTB4 in the control line of cells was not influenced by the treatment with DEM, even though the activity of cGPx, as the predominant GPx in the control line of cells, was inhibited by the depletion of glutathione. By contrast, the production of leukotrienes was significantly increased in the PHGPx-overexpressing cells when PHGPx activity was inhibited by DEM. These results indicate that PHGPx was involved in the inhibition of the activity of 5-lipoxygenase, whereas cGPx did not affect this activity.

PHGPx is located in the cytosol and membrane fraction, and cGPx is known to be present predominantly in the cytosol. Godeas et al. (56) demonstrated the specific localization of PHGPx in nuclear and mitochondrial fractions of rat testis. PHGPx has the highest ability to reduce lipid hydroperoxides in membranes of the four known isozymes of GPx (57). We showed subcellular localization of PHGPx in the control line of cells and PHGPx-overexpressing cells (Fig. 7). Control line of RBL2H3 cells contained the small amounts of PHGPx in the cytosol, whereas the large amounts of PHGPx are localized in the mitochondria and nucleus. In PHGPx-overexpressing cells, the amounts of PHGPx were significantly enhanced in nuclear, microsomal, and cytosolic fractions. The amount of PHGPx in nuclear fractions from PHGPx-overexpressing cells was 3.5 times higher than that from the control line of cells (Fig. 7). The synthesis of leukotrienes occurs at the nuclear membrane where cytosolic phospholipase A2 and 5-lipoxygenase have been translocated from the cytosol (6, 7). The expression of PHGPx in the nucleus might be critical for modulation of the trace amounts of lipid hydroperoxide required for the activation of 5-lipoxygenase. Overexpression of PHGPx did not affect the translocation of 5-lipoxygenase and cytosolic phospholipase A2 from the cytosol to the nucleus in RBL-2H3 cells (data not
shown). These results indicate that inactivation of 5-lipoxygenase was due to high level expression of PHGPx in the nucleus.

Metabolites of arachidonic acid seem to play an important physiological role in the signal transduction system that includes activation of NF-kB (58) and AP-1 (59), as well as in the regulation of cell survival and apoptosis (60, 61). Furthermore, LTB4 is not only a powerful chemotactic factor through LTB4 receptor in inflammation (62) but is also an activator of the transcription factor PPARα (63). Thus, metabolites of lipoxygenase have a variety of biological activities in addition to their functions as chemical mediators. The present study suggests that PHGPx might be involved in the regulation of cellular functions and signal transduction through modulation of the production of leukotrienes.

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