Up-regulation of phospholipid hydroperoxide glutathione peroxidase in rat casein-induced polymorphonuclear neutrophils

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Antioxidant enzymes play key roles in the protection of cells from oxidative damage. Little is known, however, about the expression of antioxidants and/or their roles in PMNs (polymorphonuclear leucocytes), which are thought to suffer from oxidative stress in an inflammation site. In the present paper, we report on the regulation of expression of PHGPx (phospholipid hydroperoxide glutathione peroxidase) and cGPx (cytosolic glutathione peroxidase) in rat PMNs in the inflammation site. PHGPx mRNA levels were much lower in casein-induced peritoneal and carrageenan-induced pleural PMNs just after their collection than in peripheral PMNs. cGPx mRNA was also reduced in the casein-induced PMNs, but not in carrageenan-induced PMNs. Both enzymes with decreased levels in the casein-induced PMNs were up-regulated during further 24 h cultivation *in vitro* and *in vivo*, with elevation of their protein levels and activities, and reduction of intracellular peroxides. Up-regulation of PHGPx mRNA was attenuated by cycloheximide, a protein synthesis inhibitor, and this effect was cancelled by culturing the cells in the conditioned medium of the cultured casein-induced PMNs. This latter effect was attenuated by pre-treatment with anti-GRO (growth-regulated oncogene) antibody. Recombinant rat GRO could also induce the up-regulation in the presence of cycloheximide, demonstrating that GRO may play an important role in the PHGPx up-regulation of casein-induced PMNs. Production of the lipid mediators leukotriene B_4 and 5-HETE (5-hydroxyeicosatetraenoic acid) was decreased in the cultured casein-induced PMNs exhibiting PHGPx up-regulation. The evidence obtained indicates that PHGPx activity in the activated PMNs would be related to the appearance of the intrinsic function of PMNs in the inflammatory site.

Key words: gene expression, growth-regulated oncogene, inflammation, peroxide tone, phospholipid hydroperoxide glutathione peroxidase, polymorphonuclear leucocyte.

INTRODUCTION

Neutrophils, PMNs (polymorphonuclear leucocytes), are known to play an important role in inflammatory responses. They are the first cells that migrate into tissues in response to invading pathogens from the bloodstream. Their principle role in inflammatory and immune responses has long been thought to be phagocytosis and killing of bacteria via the generation of ROS (reactive oxygen species) and release of lytic enzymes stored in granules [1,2]. Especially, since ROS are potentially highly toxic, they are essential molecules in the killing of bacteria. PMNs are thought to be exposed to ROS produced by themselves and other inflammatory cells, and to suffer from resultant damage, such as DNA cleavage, protein denaturation, and lipid peroxidation. The ROS-mediated damage to intracellular molecules is considered to be limited by cellular antioxidant enzymes, such as SODs (superoxide dismutases) and GPxs (glutathione peroxidases). Intracellular GPx comprise three distinct proteins, cGPx (cytosolic GPx), GPx-GI (gastrointestinal GPx) and PHGPx (phospholipid hydroperoxide glutathione peroxidase). Among these, cGPx and PHGPx are widely distributed in various tissues and cells. cGPx is present predominantly in the cytosol, and some is located in mitochondria. PHGPx exists in cytosolic, nuclear and mitochondrial fractions [3–5] and is known to be an antioxidative enzyme that can directly reduce peroxidized phospholipids [6], fatty acids [7] and cholesterol [8] in membranes. GST (glutathione S-transferase) and 1-Cys peroxiredoxin, a member of the peroxiredoxin superfamily, have also been shown to reduce phospholipid hydroperoxides [9,10]. The activity of GST for reducing phospholipid hydroperoxides, however, is relativity low as compared with that of the other two enzymes [9]. 1-Cys peroxiredoxin is distributed in the cytosol and mitochondria, but not in the nuclei and membranes [11,12]. We have reported that RBL-2H3 (rat basophilic leukaemia 2H3) cells overexpressing mitochondrial PHGPx are resistant to necrotic and apoptotic cell death caused by various oxidative stresses [3,13,14]. Overexpression of 1-Cys peroxiredoxin, cGPx or Mn-SOD (manganese-dependent SOD) also suppressed the oxidant-induced cell damage and the apoptosis induced by various stimuli [15–20]. Accordingly, the antioxidant enzymes may play a key role in the prevention of oxidative damage at an inflammation site.

On the other hand, the functional role of PMNs in the inflammatory responses is not limited to phagocytosis and bacteria killing via the ROS. They also produce several inflammatory cytokines, chemokines and lipid mediators to modulate inflammatory and immune responses. Eicosanoids, such as LTs (leukotrienes) and prostanoids, are a family of lipid mediators that are derived from the metabolism of arachidonic acid, and have a wide range of biological actions, including potent effects on inflammation and immunity. Activities of LOXs (lipoxygenases) or

Abbreviations used: AMV, avian myeloblastosis virus; CHX, cycloheximide; DCFDA, 5,6-carboxy-2,7-dichlorofluorescein diacetate; DHR 123, dihydrorhodamine 123; FBS, foetal bovine serum; GPx, glutathione peroxidase; cGPx, cytosolic GPx, GRO, growth-regulated oncogene; GST, glutathione S-transferase; H_2DCFDA, 5,6-carboxy-2,7-dichlorodihydrofluorescein diacetate; HBSS, Hanks balanced salt solution; 5-HETE, 5-hydroxyeicosatetraenoic acid; IL, interleukin; IL-1ra, IL-1 receptor antagonist; LOX, lipoxygenase; LT, leukotriene; MAPK, mitogen-activated protein kinase; PCOOH, phosphatidylcholine hydroperoxide; PG, prostaglandin; PHGPx, phospholipid hydroperoxide GPx; PMN, polymorphonuclear leucocyte; PT, pertussis toxin; RBL-2H3, rat basophilic leukaemia 2H3; ROS, reactive oxygen species; SOD, superoxide dismutase; TNFα, tumour necrosis factor α.

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COXs (cyclo-oxygenases), enzymes responsible for biosynthesis of LTs and PGs (prostaglandins), have been demonstrated to be regulated by the expression level of GPx through controlling the cellular peroxide tone [13]. Our previous results have shown that the intracellular PHGPx level is closely associated with the production of eicosanoids in RBL-2H3 cells. The levels of LTC_4 and LTB_4 produced in PHGPx-overexpressing RBL-2H3 cells were much lower than those in a control cell line [5]. Furthermore, production of PGD_2 and PAF (platelet-activating factor), which are also important mediators in the inflammatory response, was suppressed in the non-mitochondrial PHGPx-overexpressing cells [21,22]. Impairment of GPx activity by selenium deficiency or glutathione depletion led to enhanced activity of 5-lipoxygenase in leucocytes [23–25]. These findings suggest that the expressed levels of cellular antioxidant enzymes, especially PHGPx and cGPx, could modulate various cellular functions through the control of cellular redox states. Although many studies have focused on the regulation of ROS production in PMNs, little is known about the expression of GPx and/or their role in the cells. The present study was designed to clarify the regulation of PHGPx and cGPx mRNA expression in those PMNs that have migrated into inflammation sites. Intracellular levels of PHGPx and cGPx were determined in rat exudative PMNs and macrophages induced by the intraperitoneal injection of casein. We found dramatic down- and up-regulation of PHGPx and cGPx mRNA expression in rat exudative PMNs. Furthermore, a protein factor that is active in the up-regulation of PHGPx mRNA was identified.

EXPERIMENTAL

Reagents

ISOGEN, β-actin probe and recombinant rat GRO (growth-regulated oncogene) were obtained from Wako Pure Chemical Industries (Osaka, Japan); ATP, CTP and GTP were from Boehringer Mannheim (Mannheim, Germany); sodium metrizoate and NycoPrep were from Daiichi Pure Chemical Industries (Tokyo, Japan); heparin sodium salt injection was from Mitsubishi Pharma Co. (Osaka, Japan); dextran sulphate was from Amersham Biosciences (Little Chalfont, Bucks., U.K.); sodium casein was from Tokyo Kasei Kogyou Co. (Tokyo, Japan). Rabbit IgG as an isotype control and antibodies against rat GRO/CICN-1 (cytokine-induced neutrophil chemoattractant-1) C-terminal and N-terminal were purchased from IBL Co. (Gunma, Japan); recombinant rat TNF-α (tumour necrosis factor α), recombinant rat IL-1β (interleukin 1β), and recombinant rat IL-1ra (IL-1 receptor antagonist) were from PeproTech EC (London, U.K.); SYBR Green Master Mix (QuantiTect™) was from Qiagen GmbH (Hilden, Germany); AMV (avian myeloblastosis virus) reverse transcriptase XL and random nonomers were from TaKaRa Shuzo Co. (Shiga, Japan). CHX (cycloheximide) was obtained from Carbiochem (Darmstadt, Germany); PT (pertussis toxin) was from List Biological Laboratory (Campbell, CA, U.S.A.).

Collection and culture of PMNs and macrophages

Casein-induced PMNs and macrophages were collected by the method described in [26,27]. Briefly, male Sprague–Dawley rats (Charles River Japan, Kanagawa, Japan), aged from 9 to 17 weeks, were intraperitoneally injected with sterilized 5 % (w/v) casein dissolved in physiological saline at an injection volume of 100 ml/kg. Exudative cells in the peritoneal cavity were collected 16 h after the casein injection to obtain PMNs and macrophages, except in time-course experiments. After removing erythrocytes by hypotonic lysis with 0.2 % (w/v) NaCl for 1 min, followed by addition of the same volume of 1.6 % (w/v) NaCl to restore osmolarity, PMNs were separated by a density-gradient centrifugation method with sodium metrizoate or NycoPrep. PMNs were more than 90 % pure and were viable as assessed by May–Giemsa staining and the Trypan Blue dye-exclusion test. To obtain macrophages, peritoneal exudative cells were incubated in RPMI 1640 medium containing 5 % (v/v) foetal bovine serum (5 % FBS RPMI 1640 medium) in a CO_2 incubator for 2 h in a 6-cm polystyrene dish to allow adherence of macrophages. After removing non-adherent cells by washing three times with PBS, adherent cells were collected.

Peripheral blood was drawn from the abdominal vena cava of naive rats into a heparin-treated syringe and added to an equal volume of PBS and 3 % dextran. Blood was then allowed to stand at room temperature to separate out erythrocytes; the upper layer was collected and centrifuged at 200 ***g*** for 10 min at room temperature. After erythrocytes were removed by the shock-lysing method, the cells were washed with HBSS (Hanks balanced salt solution), and PMNs were separated by a density-gradient centrifugation method with Ficoll/sodium metrizoate.

Resident macrophages were collected from naive rats by peritoneal lavage with saline. Cells were resuspended in 5 % FBS RPMI 1640 medium after removing erythrocytes and were incubated for 2 h in a 6-cm polystyrene dish to allow adherence of macrophages. Non-adherent peritoneal cells were removed by washing the cells three times with PBS, and adherent peritoneal macrophages were then collected.

Carrageenan-induced PMNs were collected from the pleural cavity of rats 16 h after the intrathoracic injection of 2 % (w/v) carrageenan dissolved in saline at a constant volume of 0.1 ml/animal. PMNs were isolated by the same way as that used for isolating the casein-induced PMNs.

The numbers of purified PMNs and macrophages were counted by Trypan Blue staining. These cells were cultured for 24 h in 5 % FBS RPMI 1640 medium in the presence or absence of several inhibitors or antibodies, except in the time-course experiments. In the present study, all experimental procedures were performed in accordance with the in-house guidelines of the Institutional Animal Care and Use Committee of Daiichi Pharmaceutical Co.

RNA isolation and Northern blot analyses

Total RNA was extracted by lysis of cells with ISOGEN and was purified according to the manufacturer's directions. Briefly, $(2–5) \times 10^7$ cells were homogenized in ISOGEN. After the addition of chloroform and phase separation, the RNA was precipitated with isopropyl alcohol and washed with ethanol. The RNA was dissolved in RNase-free distilled water.

Total RNA (20 μg) from each sample was electrophoresed under denaturing conditions, blotted on to a nylon membrane and cross-linked by UV irradiation. Membranes were pre-hybridized at 42 °C for 2 h and hybridized overnight with 2×10^6 c.p.m./ml of a ^{32}P-labelled probe. Membranes were then washed twice at 42 °C for 15 min in 2×SSC [1×SSC = 0.15 M NaCl and 0.015 M sodium citrate (pH 7.0)] with 0.1 % SDS and twice at 65 °C for 30 min in 0.2×SSC with 0.1 % SDS, before being autoradiographed with a BioImaging Analyser (BAS2000; Fujifilm, Tokyo, Japan). The cDNAs for PHGPx and cGPx (GenBank® accession numbers AB030643 and NM_030826) were used as the probes.

Quantitative PCR

Total RNA (2 μg) was subjected to first-strand cDNA synthesis with 0.25 unit/μl AMV reverse transcriptase XL, 2.5 μM random nonomers, 1 unit/μl RNase inhibitor and 1 mM dNTP mixture. Thermal cycling conditions of 30 °C for 10 min, 42 °C for 30 min,

99 °C for 5 min and 5 °C for 5 min were used for the reverse transcription. cDNA and each primer were mixed with SYBR Green Master Mix, and quantitative PCR was then performed with the ABS PRISM 7700 Sequence Detection System (Applied Biosystems). The following specific primers for PHGPx, cGPx and 18 S rRNA as an internal control were used: PHGPx: sense, 5′-GGGCATCGTCCCCATTTAC-3′, and antisense, 5′-ATGGGCACATGGTTTGCCTGGATAAGT-3′; cGPx: sense, 5′-TTGAGAATGTCG CGTCCCTC-3′, and antisense, 5′-GCAGTGGGATCGTCACTGG-3′; and 18 S rRNA: sense, 5′-GCCCGAAGCGTTTACTTTGAA-3′, and antisense, 5′-GGCATCGTTTATGGTCGGAAC-3′. The relative quantity was calculated from a standard curve generated from diluted standard cDNA samples obtained from the rat testis. The quantity was normalized with that of 18 S rRNA.

Assays of PHGPx and cGPx activities

PMNs (10^8) were sonicated in 1 ml of 10 mM Tris/HCl (pH 7.4) containing 5 mg/ml leupeptin and 17 mg/ml PMSF. The homogenate was centrifuged at 10000 ***g*** for 10 min at 4 °C, and the supernatant was used for the assay. PHGPx activity was determined by using PCOOH (phosphatidylcholine hydroperoxide) as the substrate, according to our previous papers [5,28].

To measure the enzyme activity of cGPx in the supernatant, the reduction of H_2O_2 was monitored by the decrease in absorbance at 340 nm of NADPH in the presence of 3 mM GSH and 3 mM H_2O_2.

Immunoblot analysis of PHGPx and cGPx proteins

PMNs (2×10^8) were lysed with a lysis buffer (Passeve Lysis Buffer; Promega, Madison, WI, U.S.A.) containing a protease inhibitor cocktail (Complete Mini, Roche Diagnostics GmbH, Mannheim, Germany). After that, the lysate was centrifuged at 10000 ***g*** for 10 min, and the supernatant was collected. Protein (50 μg) was solubilized in the sampling buffer by sonication and boiling for 5 min in the sampling buffer, and then separated on SDS/12.5 % polyacrylamide gels and transferred on to a PVDF membrane (NEN, Boston, MA, U.S.A.). The membrane was blocked with 5 % (w/v) dried skimmed milk and then incubated with anti-PHGPx (6F10) monoclonal antibody or anti-cGPx polyclonal antibody as produced previously [29]. Anti-(p44/42 MAPK) (mitogen-activated protein kinase) antibody (Cell Signaling Technology, Beverly, MA, U.S.A.) was used as an internal control. Horseradish-peroxidase-conjugated anti-mouse IgG was used as a secondary antibody. Bands reactive with the antibodies were visualized by a horseradish-peroxidase enhanced chemiluminescence method and then analysed densitometically with an imaging analyser. The band intensity was normalized with that of p44/42 MAPK, and intensity of the enzyme relative to that of the casein-induced PMNs without cultivation (0 h) was calculated.

Nuclear run-on assay

Casein-induced PMNs and those cultured further for 24 h in 5 % FBS RPMI 1640 medium were resuspended in 10 mM Tris/HCl (pH 7.4), 10 mM NaCl, 3 mM $MgCl_2$ and 0.5 % Nonidet P40 and incubated on ice for 10 min. The nuclear pellets were spun down at 1700 ***g*** and resuspended in 50 mM Tris/HCl (pH 8.3), 40 % (v/v) glycerol, 5 mM $MgCl_2$ and 0.1 mM EDTA. The nuclei were mixed with 200 μl of 10 mM Tris/HCl (pH 8.0), 5 mM $MgCl_2$, 300 mM KCl, 1 mM each of ATP, GTP and UTP, and 3.7 MBq of [α-^{32}P]UTP and incubated for 30 min at 30 °C. RNase-free DNase I was added, and the incubation was continued for 10 min at 37 °C. After the incubation, 400 μl of 20 mM Tris/HCl (pH 7.4), 10 mM EDTA, 2 % (w/v) SDS and 10 μl of 20 mg/ml protease K was added to the reaction mixture, followed by incubation for 1 h at 50 °C. The labelled RNA transcripts were isolated by phenol extraction, phenol/chloroform extraction and ethanol precipitation, and were quantified by counting.

As hybridization templates, purified PHGPx, cGPx, β-actin cDNA fragments and the PTZ-18R plasmid, a cloning vector without the genes, as a negative control were applied to nylon membranes (Millipore) and fixed by UV cross-linking. The filters were pre-hybridized by 2 h of incubation at 42 °C in the pre-hybridization buffer, and then hybridized for 48 h at 42 °C with hybridization buffer containing the radioactively labelled mRNAs (25×10^7 c.p.m./filter) that had been denatured (5 min at 65 °C). Washing was performed three times for 15 min at 42 °C with 2 × SSC with 0.1 % SDS, and twice for 15 min at 65 °C with 0.2 × SSC with 0.1 % SDS. The filters were exposed to X-ray film for autoradiography. The membrane bands were then analysed densitometically with an imaging analyser, and the intensity of PHGPx and cGPx was normalized with that of β-actin, and the relative intensity of both enzymes to the casein-induced PMNs without cultivation (0 h) was calculated.

GRO production of casein-induced PMNs

Casein-induced PMNs were cultured in 5 % FBS RPMI 1640 medium for 4, 8, 16 and 24 h, and supernatants were collected at each time point. The GRO level in the culture supernatant was measured by using a commercial ELISA kit (IBL Co., Gunma, Japan).

Flow-cytometric analysis of intracellular peroxides

To assess the levels of intracellular peroxides, we performed flow-cytometric analysis with two oxidation-sensitive fluorescent probes, H_2DCFDA (5,6-carboxy-2,7-dichlorodihydrofluorescein diacetate) and DHR 123 (dihydrorhodamine 123), and an oxidation-insensitive probe, DCFDA (5,6-carboxy-2,7-dichlorofluorescein diacetate) (all from Molecular Probes, Eugene, OR, U.S.A.). Cells (2×10^6) were washed with PBS and incubated with 5 μM H_2DCFDA, 2 μM DHR 123 or 5 μM DCFDA in PBS in a final volume of 2 ml for 30 min at 37 °C in the CO_2 incubator. After washing with PBS, the cells were resuspended in HBSS containing 0.1 % (w/v) BSA and sodium azide, and the intensity of fluorescence in the cells was analysed with a flow cytometer (EPICS-XL; Beckman Coulter, Fullerton, CA, U.S.A.).

Quantification of LTB_4 and 5-HETE (5-hydroxyeicosatetraenoic acid)

Casein-induced PMNs isolated just after the collection from the peritoneal cavity and after 24 h cultivation were pre-incubated in PBS containing 1 mM $CaCl_2$ for 5 min at 37 °C and then stimulated with 5 μM A23187 for 5 min at 37 °C. After the incubation, supernatants were obtained by centrifugation at 200 ***g*** for 10 min at 4 °C. This was followed by extraction of LTB_4 and 5-HETE with ethyl acetate. PGB_2 (50 ng), as an internal standard, was added before the extraction. After the solvent in the extraction was removed by evaporation with nitrogen gas, 0.1 ml of ethanol was added. The supernatant was injected into an HPLC system equipped with a reverse-phase column (LiChrosorb RP-18; 240 mm × 0.4 mm inner diameter; Merck, Darmstadt, Germany) for the separation of LTB_4 and 5-HETE. Concentrations were calculated from the peak area of the internal control.

RESULTS

Expression of PHGPx and cGPx mRNA between carrageenan- and casein-induced PMNs

Levels of PHGPx and cGPx mRNA were determined in peripheral blood and in carrageenan-induced thoracic and casein-induced

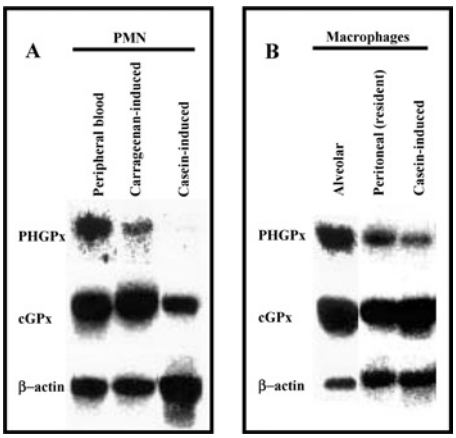

Figure 1 PHGPx and cGPx mRNA levels in rat PMNs and macrophages

Total cellular RNA was isolated from the cells indicated in the Figure, and mRNA levels were detected by Northern blot analysis. (**A**) mRNA levels of PHGPx and cGPx, and β-actin as an internal control, were compared for peripheral blood PMNs, carrageenan-induced PMNs, and casein-induced PMNs. (**B**) mRNA levels of both enzymes and β-actin were compared for alveolar, resident peritoneal and casein-induced peritoneal macrophages. Reproducibility of these results was confirmed in three experiments.

peritoneal PMNs by Northern blot analysis (Figure 1A). PHGPx expression in PMNs was much lower than that of cGPx. Striking differences in mRNA levels in both types of GPx were observed in peripheral blood PMNs, carrageenan-induced PMNs and casein-induced PMNs. The expressed levels of PHGPx and cGPx were markedly decreased in the casein-induced PMNs collected from the peritoneal cavity as compared with those levels in the peripheral blood PMNs. The PHGPx mRNA level was also reduced in the carrageenan-induced PMNs, but cGPx was expressed at the same level as that of the peripheral blood PMNs. These results indicate that PHGPx expression was extremely down-regulated in those PMNs that migrated to the inflammatory site. In contrast, no marked differences in the expression of cGPx and PHGPx were observed between the resident and casein-induced macrophages, although the casein-induced macrophages exhibited slightly decreased expression of PHGPx (0.8-fold) and increased expression of cGPx (1.1-fold). The alveolar macrophages expressed higher amounts of PHGPx mRNA than did the peritoneal macrophages (Figure 1B).

Elevation of PHGPx and cGPx expression of casein-induced PMNs *in vitro* and *in vivo*

Casein-induced PMNs collected from the peritoneal cavity were cultured in 5 % FBS RPMI 1640 medium for 1, 4, 8, 16 and 24 h, and mRNA levels of both enzymes were measured by Northern blot analysis and quantitative PCR. Northern blot analysis showed that the casein-induced PMNs maintained low levels of PHGPx and cGPx expression during the first 4 h of cultivation. Expressed levels of both PHGPx and cGPx mRNA were then elevated in the cells from 8 to 24 h as compared with those levels for the casein-induced PMNs without the cultivation (Figure 2A). Similar time-dependent up-regulation of PHGPx and cGPx mRNA could be detected by quantitative PCR. PHGPx and cGPx mRNA was significantly elevated in the cells collected at 8 and 24 h as compared with the cells without cultivation (0 h), and the levels of PHGPx and cGPx after 24 h of cultivation were approx. 10 and 2 times higher respectively than those levels for the casein-induced PMNs without cultivation (Figure 2B).

Next, we examined whether the up-regulation of PHGPx was also found in the casein-induced PMNs that existed in the peri-

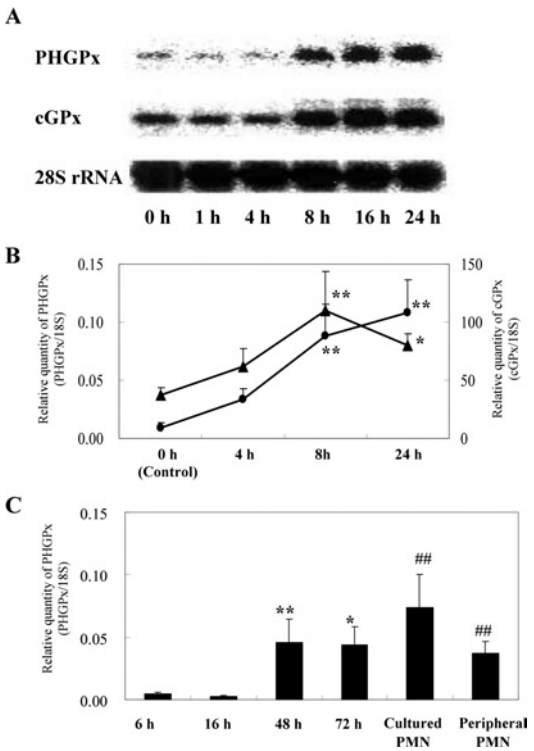

Figure 2 Time course for the up-regulation of PHGPx and cGPx mRNA in rat casein-induced PMNs cultured *in vitro* and *in vivo*

(**A**) Casein-induced PMNs were collected from the peritoneal cavity of rats 16 h after the intraperitoneal injection of 5 % casein. The cells were then cultured in 5 % FBS RPMI 1640 medium for 1, 4, 8, 16 and 24 h. Total cellular RNA was isolated from the cells, and mRNA levels of both enzymes and 28 S rRNA as an internal control were detected by Northern blot analysis. (**B**) Casein-induced PMNs were collected as described above, and the cells were then cultured in 5 % FBS RPMI 1640 medium for 4, 8 and 24 h. Total cellular RNA was isolated from the cells, and mRNA levels were detected by quantitative PCR. The relative quantity of PHGPx (●) and cGPx (▲) mRNA was normalized with that of 18 S rRNA as an internal control. Results are means $\pm$ S.D. Data were analysed statistically by Dunnett's test ($n = 4$, $^{*}P < 0.05$, $^{**}P < 0.01$ compared with 0 h). (**C**) Casein-induced PMNs were collected from the peritoneal cavity 6, 16, 48 and 72 h after the intraperitoneal injection of 5 % casein. As a control, PMNs were collected from peripheral blood and peritoneal cavity 16 h after the injection and thereafter cultured *in vitro* for 24 h (cultured PMNs). Total cellular RNA was isolated from the cells, and mRNA levels were detected by quantitative PCR. The relative quantity of PHGPx mRNA was normalized with that of 18 S rRNA. Results are means $\pm$ S.D. Data were analysed statistically by Dunnett's test ($n = 4$–6, $^{*}P < 0.05$, $^{**}P < 0.01$ compared with 16 h) or Student's *t* test ($n = 4$–6, $^{\#\#}P < 0.01$ compared with 16 h).

toneal cavity. Quantitative PCR was carried out with mRNA of the casein-induced PMNs collected from the peritoneal cavity at 6, 16, 48 and 72 h after the 5 % casein injection (Figure 2C). The casein-induced PMNs showed a low expression level of PHGPx mRNA by 16 h after the casein injection, but the level was subsequently up-regulated from 48 h after the injection. The casein-induced PMNs collected at 48 h exhibited 15 times higher expression of PHGPx than did those collected at 16 h, and this level was equivalent to that of peripheral PMNs, indicating that the casein-induced PMNs exhibited a transient down-regulation of PHGPx levels with a subsequent recovery phase. This up-regulation was sustained in the PMNs for up to 72 h.

Enzyme activities of PHGPx and cGPx were determined by using PCOOH and H_2O_2 as the respective substrates (Figure 3A). The activities of PHGPx and cGPx in the casein-induced PMNs cultured for 24 h were approx. 1.8- and 1.5-fold higher respectively than those in the casein-induced PMNs without cultivation.

PHGPx and cGPx in the casein-induced PMNs were detected by immunoblot analysis. The protein levels of PHGPx and cGPx

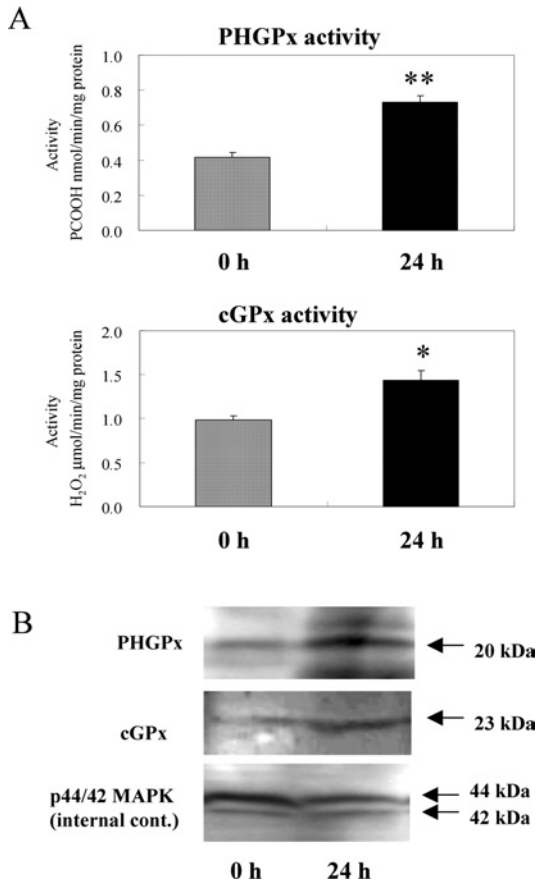

Figure 3 Enzyme activities and protein levels of PHGPx and cGPx in rat casein-induced PMNs and cultured PMNs

(**A**) Casein-induced PMNs were collected and cultured as described in Figure 2. PHGPx and cGPx activities were measured in casein-induced PMNs just after the collection (0 h) and at 24 h after the culture in the medium with phosphatidylcholine hydroperoxide as a substrate for PHGPx and H_2O_2 for cGPx. Results are means ± S.D. Data were analysed statistically by Student's t test ($n = 3$, $^*P < 0.05$, $^{**}P < 0.01$ compared with 0 h). Reproducibility of these results was confirmed in three experiments. (**B**) PHGPx and cGPx proteins were detected in the casein-induced PMNs just after the collection (0 h) and at 24 h after the culture in the medium by immunoblot analysis with anti-PHGPx monoclonal antibody and anti-cGPx polyclonal antibody. Anti-(p44/42 MAPK) polyclonal antibody was used as an internal control (internal cont.). Reproducibility of these results was confirmed in three experiments. Molecular-mass sizes are given in kDa.

in the cultured casein-induced PMNs were 2.1 and 2.0 times higher respectively than that of the casein-induced PMNs without cultivation, corresponding to the enzyme activity (Figure 3B).

Nuclear run-on assays were performed to determine whether the up-regulation of PHGPx and cGPx mRNA in the casein-induced PMNs was due to the activation of transcription (Figure 4). Transcription levels for both PHGPx and cGPx were enhanced in the cultured PMNs as compared with those levels in the uncultivated casein-induced PMNs. The intensities of the bands of PHGPx and cGPx after 24 h of incubation were approx. 1.6 and 1.8 times higher respectively than those of the cells without the cultivation.

Relevance of protein synthesis and cytokines to the up-regulation of PHGPx mRNA in the cultured casein-induced PMNs

To elucidate the mechanism for the PHGPx up-regulation in the casein-induced PMNs during the 24 h cultivation, the effects of a protein synthesis inhibitor (CHX) on the up-regulation of PHGPx mRNA were examined by quantitative PCR (Figure 5A). The up-

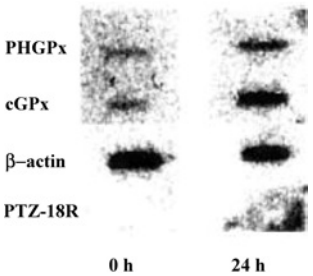

Figure 4 Elevation of PHGPx and cGPx transcription in casein-induced PMNs cultured for 24 h

Casein-induced PMNs were collected as described in Figure 2, and the cells were then cultured in the medium for 24 h. The cells were harvested, and nuclear run-on transcription assays were performed to analyse the transcription rates of PHGPx and cGPx mRNA. β-Actin and PTZ-18R were used as an internal control and a negative control respectively. Reproducibility of these results was confirmed in three experiments.

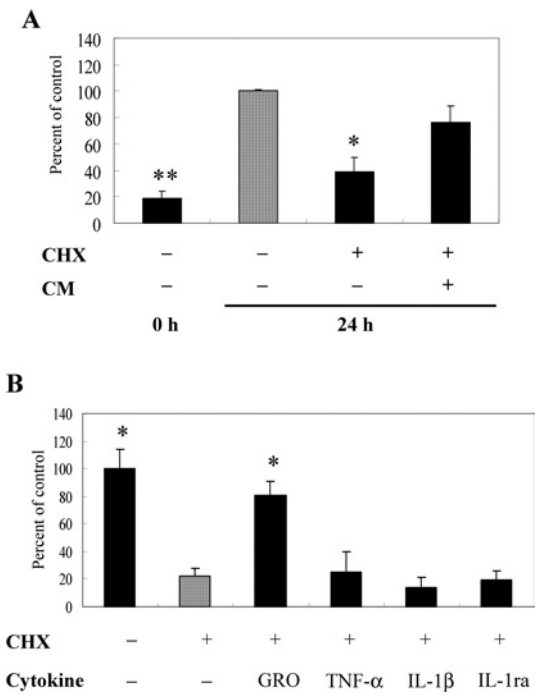

Figure 5 Relevance of protein synthesis and cytokines to the up-regulation of PHGPx mRNA in casein-induced PMNs

(**A**) Casein-induced PMNs were collected as described in Figure 2. The cells were cultured for 24 h in the medium or conditioned medium (CM), which was collected from the cells cultured for 24 h, with or without 10 μM CHX. Total cellular RNA was isolated from the cells, and mRNA levels were detected by quantitative PCR. Results are expressed as mean percentages ± S.D. of the medium control at 24 h (shaded bar). Data were analysed statistically by Student's t test ($n = 4$–6, $^*P < 0.05$ and $^{**}P < 0.01$ compared with the medium control). (**B**) Casein-induced PMNs were collected as described in Figure 2. The cells were cultured in the medium for 24 h in the presence of 10 μM CHX and a cytokine (100 ng/ml GRO, 20 ng/ml TNFα, 50 ng/ml IL-1β or 50 ng/ml IL-1ra). Total cellular RNA was isolated from the cells, and mRNA levels were detected by quantitative PCR. Results are expressed as mean percentages ± S.D. of the medium control without CHX and cytokine. Data were analysed statistically by Student's t test ($n = 4$–6, $^*P < 0.05$ compared with the CHX treatment group without cytokine, shaded bar).

regulation of PHGPx mRNA was significantly suppressed by the addition of 10 μM CHX. This inhibitory effect of CHX, however, was cancelled when the PMNs were cultured in conditioned medium, which was the culture supernatant of the casein-induced PMNs cultured for 24 h. These results suggest that a certain factor

secreted from the PMNs into the medium is involved in the up-regulation of PHGPx mRNA.

The effect of several cytokines on the expression of PHGPx mRNA in the cultured casein-induced PMNs was determined by quantitative PCR (Figure 5B). PHGPx mRNA expression was up-regulated by the addition of GRO in the presence of CHX as compared with that in the PMNs cultured without cytokine. No elevation of PHGPx mRNA was detected in the cells cultured with TNF-α, IL-1β or IL-1ra in the presence of CHX. The cytokines and chemokine did not show any cytotoxic effect in the presence of CHX during 24 h culture periods.

The effect of anti-GRO antibody on the up-regulation of PHGPx mRNA in the casein-induced PMNs cultured in the conditioned medium containing CHX was examined. Pre-treatment of the conditioned medium with anti-GRO antibody significantly suppressed the up-regulation of PHGPx mRNA in the presence of CHX, but pre-treatment with an isotype control antibody did not (Figure 6A). Furthermore, the up-regulation of PHGPx mRNA during the 24 h cultivation was significantly suppressed to approx. 66 % of the control value by the addition of 3 μg/ml PT, an inhibitor of G-protein-coupled membrane receptor signalling (Figure 6B).

Production of GRO in the casein-induced PMNs

Production of GRO was measured in the culture supernatant collected from the casein-induced PMNs at 4, 8, 16 and 24 h after the cultivation. GRO was secreted from the casein-induced PMNs during the first 4 h of culture; the concentration was then sustained for up to 24 h (Figure 6C).

Comparison of intracellular hydroperoxide level and LT production between casein-induced PMNs with or without cultivation

To elucidate whether the cultured casein-induced PMNs showed any different features as compared with the casein-induced PMNs without cultivation, the level of hydroperoxides was determined in the casein-induced PMNs by flow-cytometric analysis with H_2DCFDA and DHR 123 (Figure 7). The basal fluorescence intensities of H_2DCFDA and DHR 123 in the cultured casein-induced PMNs were lower than those of the casein-induced PMNs without the cultivation, indicating that the level of hydroperoxide was reduced in the PMNs concomitantly with the up-regulation of PHGPx and cGPx levels during the 24 h cultivation. No notable differences in the basal fluorescence intensity of the oxidation-insensitive probe DCFDA were observed between the cultured casein-induced PMNs and the casein-induced PMNs without the cultivation (results not shown).

Production of LTB_4 and 5-HETE was measured in the casein-induced PMNs with or without cultivation after exposure to A23187. Stimulated PMNs without cultivation produced LTB_4 and 5-HETE, which reached levels of 978 ± 350 and 1783 ± 606 ng/2×10^7 cells respectively, whereas synthesis of LTB_4 and 5-HETE was significantly decreased to 94 ± 21 and 292 ± 75 ng/2×10^7 cells respectively in the cultured casein-induced PMNs. In the casein-induced PMNs, the expressed levels of 5-LOX and cytosolic phospholipase A_2, which are key enzymes of LTB_4 synthesis, were not altered during cultivation (results not shown).

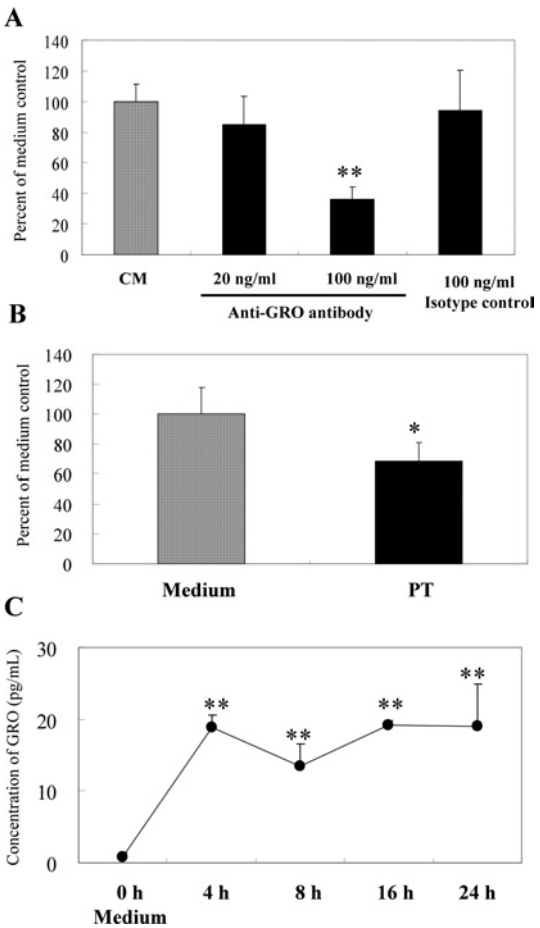

Figure 6 Effects of anti-(rat GRO) antibody and PT on the up-regulation of PHGPx mRNA, and GRO production of casein-induced PMNs

(**A**) Casein-induced PMNs were collected as described in Figure 2. The cells were cultured for 24 h in the presence of 10 μM CHX and conditioned medium (CM), which was incubated with an antibody against rat GRO or an isotype control antibody for 1 h at room temperature beforehand. Total cellular RNA was isolated from the cells, and mRNA levels were detected by quantitative PCR. Results are expressed as mean percentages $\pm$ S.D. of the CM control (shaded bar). Data were analysed statistically by Dunnett's test ($n = 4$–6, $^{**}P < 0.01$ compared with CM control). (**B**) Casein-induced PMNs were cultured in the medium for 24 h in the presence of 1 μg/ml PT and 10 μM CHX. Total cellular RNA was isolated from the cells, and mRNA levels were detected by quantitative PCR. Results are expressed as mean percentages $\pm$ S.D. of the medium control. Data were analysed statistically by Student's t test ($n = 4$–6, $^{*}P < 0.05$ compared with the medium control). (**C**) Casein-induced PMNs were cultured in 5 % FBS RPMI 1640 medium, and supernatants were collected at each culture period indicated in the Figure. GRO concentration in the supernatants was measured by ELISA. Results are expressed as the means $\pm$ S.D., and were analysed statistically by Dunnett's test ($n = 4$–5, $^{**}P < 0.01$ compared with the medium control).

DISCUSSION

The present study was focused on the expression of GPx, especially PHGPx, in PMNs that have migrated into the inflammatory site, which appears to be a potent oxidizing environment. The level of PHGPx mRNA was markedly reduced in the casein-induced PMNs collected from the peritoneal cavity as compared with peripheral PMNs (Figure 1A), but was elevated during further 24 h cultivation *in vitro* and *in vivo* (Figures 2B and 2C). Expressed levels of PHGPx and cGPx mRNA in the 24-h-cultured PMNs were 10 and 2 times higher respectively than those of the casein-induced PMNs without the cultivation (Figure 2B). A run-on assay revealed that the elevation of PHGPx mRNA was transcriptionally up-regulated (Figure 4). Three previous studies have only addressed changes in the intracellular PHGPx expression in spermatogenesis, embryogenesis and stimulation with cytokines. From these, PHGPx has been reported to be expressed at high levels in rat, mouse and human testis [29–32]. An extraordinarily high rate of transcription of the gene for PHGPx was detected in the layer of late spermatocytes in mice [31], rats [32]

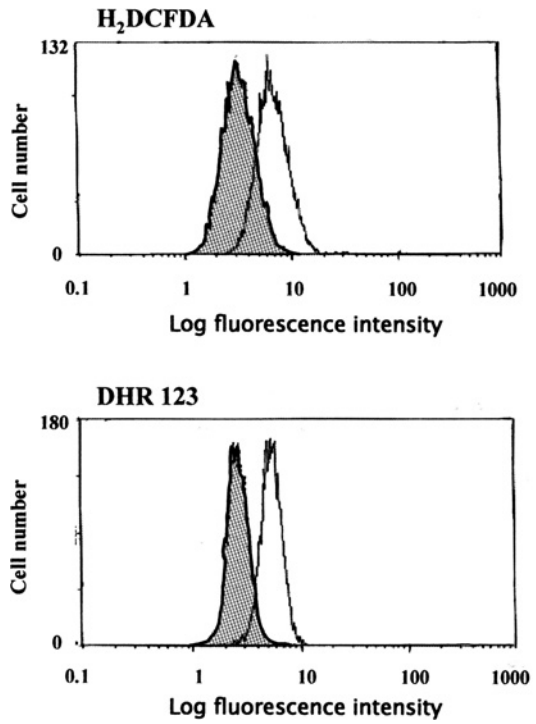

Figure 7 Intracellular hydroperoxide levels in rat casein-induced PMNs

Casein-induced PMNs were collected as described in Figure 2. Intracellular hydroperoxide levels were measured in the cells with (shaded areas) or without (open areas) cultivation by flow cytometric analysis with H_2DCFDA and DHR 123 as oxidation reporter molecules. The reproducibility of these results was confirmed in three experiments.

and humans [29]. Expression of PHGPx in rat testes disappeared after hypophysectomy and was partially restored by gonadotropin treatment [30]. Conversely, *in vitro* exposure of decapsulated whole testis to testosterone, human chorionic gonadotropin or forskolin did not induce any transcriptional activation or inhibition of the PHGPx gene [32]. Our recent study demonstrated that the expression of PHGPx was increased in the embryonic ectoderm and the yolk sac membrane from 7.5 days post-coitum during embryogenesis [33]. Targeted disruption of the PHGPx gene caused embryonic lethality between 7.5 and 8.5 days post-coitum [33]. Cytokines IL-4 and IL-13 have been reported to reduce the PHGPx expression in the human lung carcinoma cell line A549 during a 6-day cultivation, accompanied by an increase in 12/15-LOX level [34]. IL-4, however, seems to accelerate the decay of PHGPx mRNA, and IL-4-mediated down-regulation of PHGPx expression appears to be a post-transcriptional phenomenon [35]. The present study demonstrated that PHGPx expression was dramatically down- and up-regulated in the casein-induced PMNs. The mechanisms that affect these interesting changes of PHGPx are still not well known. However, CHX inhibited the up-regulation of PHGPx in the cultured casein-induced PMNs, and also the inhibitory effect of CHX was cancelled by culturing the cells in the conditioned medium obtained from the 24 h culture of the PMNs (Figure 5A). Furthermore, we have confirmed that the up-regulating effect of the conditioned medium was diminished by heating at 100 °C for 5 min with boiling water (results not shown), suggesting that the up-regulation of PHGPx mRNA could be initiated by an autocrine mechanism that involves a protein factor. PMNs expressed the receptors of TNFα, IL-8 and GRO [36,37]. To elucidate the implication of cytokines secreted from the casein-induced PMNs, the cells were cultured with several cytokines in the presence of CHX (Figure 5B). Among the cytokines tested, recombinant rat GRO caused significant elevation of PHGPx mRNA, and treatment of the conditioned medium with anti-GRO antibody effectively suppressed the PHGPx up-regulation (Figure 6A). The cultured casein-induced PMNs secreted GRO during the first 4 h of cultivation (Figure 6C). GRO is a member of the CXC family of chemokines in rodents that are selective for the recruitment and activation of PMNs. The CXC chemokine receptors CXCR1 and CXCR2 belong to a G-protein-coupled membrane receptor family, and its intracellular signalling via the G_α subunit has been reported to be inhibited by PT [38]. PT partially suppressed the up-regulation of PHGPx mRNA in the cultured casein-induced PMNs (Figure 6B). Taken together, GRO, at least in part, may be involved in the PHGPx mRNA up-regulation through the signalling pathway of the G-protein-coupled receptors. Other mechanisms, however, such as a lipid signalling activity are still not excluded from consideration as candidates involved in the up-regulation of PHGPx.

The protein levels of PHGPx and cGPx were increased approx. 2-fold in the cultured casein-induced PMNs as compared with those levels of the casein-induced PMNs without the cultivation (Figure 3B), corresponding to 1.8- and 1.5-fold increases in the enzyme activities of PHGPx and cGPx respectively (Figure 3A). Slightly lower efficiencies of the enzymatic activity of PHGPx and cGPx as compared with the protein levels might be due to the difficulties of measurement of the activity in the membrane-bound enzymes as described previously [4]. On the other hand, PHGPx and cGPx mRNA levels in the 24-h-cultured casein-induced PMNs were approx. 10- and 2-fold higher respectively than the casein-induced PMNs without the cultivation. This increased mRNA level of PHGPx was not consistent with increases in the protein level and the enzyme activity in the 24-h cultured casein-induced PMNs. This inefficient translation of PHGPx might be regulated by other mechanisms, such as selenium content in the medium.

When these experiments were undertaken, we hypothesized that the expression levels of PHGPx and cGPx might be elevated in the casein-induced peritoneal PMNs to protect them from cell injury by oxidative stress. Unexpectedly, however, PHGPx and cGPx mRNA levels in the casein-induced PMNs were excessively lower than those of peripheral PMNs (Figures 1A and 2C). In contrast, the casein-induced macrophages did not exhibit any notable change in the expression levels of PHGPx and cGPx mRNA (Figure 1B), suggesting that the decreased expression of mRNA for both enzymes was specific for PMNs. A mechanism for the down-regulation of PHGPx in the casein-induced PMNs is still unknown; however, peripheral blood PMNs were generally thought to be in an inactivated state, and they were reported to be stimulated during the migration process to an inflammation site from the bloodstream [39,40]. The level of PHGPx expression had already been decreased in the casein-induced PMNs collected 6 h after the casein injection (Figure 2C). The direct effect of casein on the expression level of PHGPx mRNA in the rat peripheral PMNs was examined in culture *in vitro* for 24 h. As a result, no down-regulation of PHGPx mRNA was observed (results not shown). Therefore the down-regulation of PHGPx in PMNs may be mediated through some signalling event during the migration process. However, the possibility that only peripheral PMNs expressing PHGPx at low levels invade the peritoneal cavity is not excluded from a mechanism for decreased expression of PHGPx in the casein-induced PMNs.

The CXC chemokine GRO has been shown not only to stimulate PMNs migration and production of ROS [36], but also to suppress PMNs apoptosis [41]. Our previous studies revealed that RBL-2H3 cells overexpressing mitochondrial PHGPx are resistant to apoptosis because of reduced intracellular hydroperoxides

[14,42]. In the present study, the intracellular hydroperoxide level was reduced in the cultured casein-induced PMNs (Figure 7). We have confirmed that the 24-h-cultured casein-induced PMNs with up-regulated PHGPx expression exhibited high viability (approx. 90 %), whereas the viability of PMNs collected from peripheral blood was significantly reduced to 30 % after 24 h of cultivation in the medium (results not shown). Accordingly, GRO, at least in part, appears to regulate the lifetime of PMNs by the up-regulation of PHGPx.

Intracellular expression levels of GPx have been demonstrated to modulate eicosanoid production by controlling the peroxide tone [23–25]. The rates of LTB_4 and 5-HETE production in the 24-h-cultured casein-induced PMNs exhibiting up-regulated expression of GPx and decreased hydroperoxide level are much lower than those in the PMNs without the cultivation (Figure 7). We have already revealed that the production levels of LTC_4 and LTB_4 were potentially suppressed in the non-mitochondrial PHGPx-overexpressing RBL-2H3 cells, with a reduction of intracellular hydroperoxides, as compared with those in a control cell line after exposure to A23187, even though the intracellular cGPx level of the overexpressing cells was equivalent to that of the control cells [5]. Production of 5-LOX metabolites was elevated by the inhibition of cGPx and PHGPx activities with the depletion of selenium, but the formation of metabolites was quickly recovered to the control level when the PHGPx activity was restored by the replenishment of selenium, even though cGPx activity was not restored [23]. Therefore lipophilic PHGPx, which has an affinity for biomembranes, appears to be involved in the regulation of the eicosanoid production, rather than cGPx. These results suggest that suppression of LTB_4 and 5-HETE in the casein-induced PMNs during cultivation partially results from the elevation of PHGPx level, and raises the possibility that PHGPx was involved in the modulation of the inflammatory response through control of eicosanoid production in PMNs.

In the present study, the casein-induced PMNs showed down- and up-regulation of PHGPx and cGPx expression, and we identified a protein factor, GRO, that is active in the up-regulation of PHGPx mRNA. Furthermore, the casein-induced PMNs showed the decreased intracellular hydroperoxide level and LT formation by up-regulation of the expression of PHGPx and cGPx. Since PHGPx has been reported to be an important molecule in the regulation of eicosanoid production in PHGPx-overexpressing cells, alternation of PHGPx expression in the activated PMNs might be related to the appearance of the intrinsic function of PMNs in the inflammatory site.

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REFERENCES

1 Root, R. K. and Cohen, M. S. (1981) The microbicidal mechanisms of human neutrophils and eosinophils. Rev. Infect. Dis. **3**, 565–598
2 Babior, B. M. (1978) Oxygen-dependent microbial killing by phagocytosis. N. Engl. J. Med. **298**, 659–668
3 Arai, M., Imai, H., Koumura, T., Toshida, M., Emoto, K., Umeda, M., Chiba, N. and Nakagawa, Y. (1999) Mitochondrial phospholipid hydroperoxide glutathione peroxidase plays a major role in preventing oxidative injury to cells. J. Biol. Chem. **274**, 4924–4933
4 Roveni, A., Maiorino, M. and Ursini, F. (1994) Enzymatic and immunological measurements of soluble and membrane-bound phospholipid-hydroperoxide glutathione peroxidase. Methods Enzymol. **233**, 202–212
5 Imai, H., Narashima, K., Arai, M., Sakamoto, H., Chiba, N. and Nakagawa, Y. (1998) Suppression of leukotriene formation in RBL–2H3 cells that overexpressed phospholipid hydroperoxide glutathione peroxidase. J. Biol. Chem. **273**, 1990–1997
6 Ursini, F., Maiorino, M. and Gregolin, C. (1985) The selenoenzyme phospholipid hydroperoxide glutathione peroxidase. Biochim. Biophys. Acta **839**, 62–70
7 Schnurr, K., Belkner, J., Ursini, F., Schewe, T. and Kuhn, H. (1996) The selenoenzyme phospholipid hydroperoxide glutathione peroxidase controls the activity of the 15-lipoxygenase with complex substrates and preserves the specificity of the oxygenation products. J. Biol. Chem. **271**, 4653–4658
8 Thomas, J. P., Maiorino, M., Ursini, F. and Girotti, A. W. (1990) Protective action of phospholipid hydroperoxide glutathione peroxidase against membrane-damaging lipid peroxidation: *in situ* reduction of phospholipid and cholesterol hydroperoxides. J. Biol. Chem. **265**, 454–461
9 Hurst, R., Bao, Y., Jemth, P., Mannervik, B. and Williamson, G. (1998) Phospholipid hydroperoxide glutathione peroxidase activity of human glutathione transferases. Biochem. J. **332**, 97–100
10 Fisher, A. B., Dodia, C., Manevich, Y., Chen, J. W. and Feinstein, S. I. (1999) Phospholipid hydroperoxides are substrates for non-selenium glutathione peroxidase. J. Biol. Chem. **274**, 21326–21334
11 Knoops, B., Clippe, A., Bogard, C., Arsalane, K., Wattiez, R., Hermans, C., Duconseille, E., Falmagne, P. and Bernard, A. (1999) Cloning and characterization of AOEB166, a novel mammalian antioxidant enzyme of the peroxiredoxin family. J. Biol. Chem. **274**, 30451–30458
12 Wang, X., Phelan, S. A., Forsman-Semb, K., Taylor, E. F., Petros, C., Brown, A., Lerner, C. P. and Paigen, B. (2003) Mice with targeted mutation of peroxiredoxin 6 develop normally but are susceptible to oxidative stress. J. Biol. Chem. **278**, 25179–25190
13 Imai, H. and Nakagawa, Y. (2003) Biological significance of phospholipid hydroperoxide glutathione peroxidase (PHGPx, GPx4) in mammalian cells. Free Radical Biol. Med. **34**, 145–169
14 Nomura, K., Imai, H., Koumura, T., Arai, M. and Nakagawa, Y. (1999) Mitochondrial phospholipid hydroperoxide glutathione peroxidase suppresses apoptosis mediated by a mitochondrial death pathway. J. Biol. Chem. **274**, 29294–29302
15 Manevich, Y., Sweitzer, T., Pak, J. H., Feinstein, S. I., Muzykantov, V. and Fisher, A. B. (2002) 1-Cys peroxiredoxin overexpression protects cells against phospholipid peroxidation-mediated membrane damage. Proc. Natl. Acad. Sci. U.S.A. **99**, 11599–11604
16 Manna, S. K., Zhang, H. J., Yan, T., Oberley, L. W. and Aggrarwal, B. B. (1998) Overexpression of manganese superoxide dismutase suppresses tumor necrosis factor-induced apoptosis and activation of nuclear transcription factor-κB and activated protein-1. J. Biol. Chem. **273**, 13245–13254
17 Bohler, T., Waiser, J., Hepburn, H., Gaedeke, J., Lehmann, C., Hambach, P., Budde, K. and Neumayer, H. H. (2000) TNF-α and IL-1α induce apoptosis in subconfluent rat mesangial cells: evidence for the involvement of hydrogen peroxide and lipid peroxidation as second messengers. Cytokine **12**, 986–991
18 Keller, J. N., Kindy, M. S., Holtsberg, F. W., St Clair, D. K., Yen, H. C., Germeyer, A., Steiner, S. M., Bruce-Keller, A. J., Hutchins, J. B. and Mattson, M. P. (1998) Mitochondrial manganese superoxide dismutase prevents neural apoptosis and reduces ischemic brain injury: suppression of peroxynitrite production, lipid peroxidation, and mitochondrial dysfunction. J. Neurosci. **18**, 687–697
19 Packham, G., Ashmun, R. A. and Cleveland, J. L. (1996) Cytokines suppress apoptosis independent of increases in reactive oxygen levels. J. Immunol. **156**, 2792–2800
20 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
21 Sakamoto, H., Imai, H. and Nakagawa, Y. (2000) Involvement of phospholipid hydroperoxide glutathione peroxidase in the modulation of prostaglandin D_2 synthesis. J. Biol. Chem. **275**, 40028–40035
22 Sakamoto, H., Tosaki, T. and Nakagawa, Y. (2002) Overexpression of phospholipid hydroperoxide glutathione peroxidase modulates acetyl-CoA, 1-*O*-alkyl-2-lyso-*sn*-glycero-3-phosphocholine acetyltransferase activity. J. Biol. Chem. **277**, 50431–50438
23 Weitzel, F. and Wendel, A. (1993) Selenoenzymes regulate the activity of leukocyte 5-lipoxygenase via the peroxide tone. J. Biol. Chem. **268**, 6288–6292
24 Werz, O. and Steinhilber, D. (1996) Selenium-dependent peroxidases suppress 5-lipoxygenase activity in B-lymphocytes and immature myeloid cells: the presence of peroxidase-insensitive 5-lipoxygenase activity in differentiated myeloid cells. Eur. J. Biochem. **242**, 90–97
25 Hatzelmann, A., Schatz, M. and Ullrich, V. (1989) Involvement of glutathione peroxidase activity in the stimulation of 5-lipoxygenase activity by glutathione-depleting agents in human polymorphonuclear leukocytes. Eur. J. Biochem. **180**, 527–533
26 Arai, M., Imai, H., Metori, A. and Nakagawa, Y. (1997) Preferential esterification of endogenously formed 5-hydroxyeicosatetraenoic acid to phospholipids in activated polymorphonuclear leukocytes. Eur. J. Biochem. **244**, 513–519
27 Quilley, J. and McGiff, J. C. (1985) Arachidonic acid metabolism and urinary excretion of prostaglandins and thromboxane in rats with experimental diabetes mellitus. J. Pharmacol. Exp. Ther. **234**, 211–216

28 Chiba, N., Imai, H., Narashima, K., Arai, M., Oshima, G., Kunimoto, M. and Nakagawa, Y. (1999) Cellular glutathione peroxidase as a predominant scavenger of hydroperoxyeicosatetraenoic acids in rabbit alveolar macrophages. Biol. Pharm. Bull. **22**, 1047–1051
29 Imai, H., Suzuki, K., Ishizaka, K., Ichinose, S., Oshima, H., Okayasu, I., Emoto, K., Umeda, M. and Nakagawa, Y. (2001) Failure of the expression of phospholipid hydroperoxide glutathione peroxidase in the spermatozoa of human infertile males. Biol. Reprod. **64**, 674–683
30 Roveri, A., Casasco, A., Maiorino, M., Dalan, P., Calligaro, A. and Ursini, F. (1992) Phospholipid hydroperoxide glutathione peroxidase of rat testis: gonadotropin dependence and immunocytochemical identification. J. Biol. Chem. **267**, 6142–6146
31 Nam, S. Y., Fujisawa, M., Kim, J. S., Kurohmaru, M. and Hayashi, Y. (1998) Expression pattern of phospholipid hydroperoxide glutathione peroxidase messenger ribonucleic acid in mouse testis. Biol. Reprod. **58**, 1272–1276
32 Maiorino, M., Wissing, J. B., Brigelius-Flohe, R., Calabrese, F., Roveri, A., Steinert, P., Ursini, F. and Flohe, L. (1998) Testosterone mediates expression of the selenoprotein PHGPx by induction of spermatogenesis and not by direct transcriptional gene activation. FASEB J. **12**, 1359–1370
33 Imai, H., Hirao, F., Sakamoto, T., Sekine, K., Mizukura, Y., Saito, M., Kitamoto, T., Hayasaka, M., Hanaoka, K. and Nakagawa, Y. (2003) Early embryonic lethality caused by targeted disruption of the mouse PHGPx gene. Biochem. Biophys. Res. Commun. **305**, 278–286
34 Schnurr, K., Borchert, A. and Kuhn, H. (1999) Inverse regulation of lipid-peroxidizing and hydroperoxyl lipid-reducing enzymes by interleukins 4 and 13. FASEB J. **13**, 143–154
35 Kuhn, H. and Borchert, A. (2002) Regulation of enzymatic lipid peroxidation: the interplay of peroxidizing and peroxide reducing enzymes. Free Radical Biol. Med. **33**, 154–172
36 Patel, L., Charlton, S. J., Chambers, J. K. and Macphee, C. H. (2001) Expression and functional analysis of chemokines receptors in human peripheral blood leukocyte populations. Cytokine **14**, 27–36
37 Menegazzi, R., Cramer, R., Patriarca, P., Scheurich, P. and Dri, P. (1994) Evidence that tumor necrosis factor α (TNF)-induced activation of neutrophil respiratory burst on biologic surfaces is mediated by the p55 TNF receptor. Blood **84**, 287–293
38 Molski, T. F., Naccache, P. H., Marsh, M. L., Kermode, J., Becker, E. L. and Sha'afi, R. I. (1984) Pertussis toxin inhibits the rise in the intracellular concentration of free calcium that is induced by chemotactic factors in rabbit neutrophils: possible role of the "G proteins" in calcium mobilization. Biochem. Biophys. Res. Commun. **124**, 644–650
39 Nathan, C. F., Srimal, S., Farber, C., Sanchez, E., Kabbash, L., Asch, A., Gailit, J. and Wright, S. D. (1989) Cytokine-induced respiratory burst of human neutrophils: dependence on extracellular matrix proteins and CD11/CD18 integrins. J. Cell Biol. **109**, 1341–1349
40 Berger, M., Budhu, S., Lu, E., Li, Y., Loike, D., Silverstein, S. C. and Loike, J. D. (2002) Different G_i-coupled chemoattractant receptors signal qualitatively different functions in human neutrophils. J. Leukocyte Biol. **71**, 798–806
41 Dunican, A., Grutkoski, P., Leuenroth, S., Ayala, A. and Simms, H. H. (2000) Neutrophils regulate their own apoptosis via preservation of CXC receptors. J. Surg. Res. **90**, 32–38
42 Imai, H., Koumura, T., Nakajima, R., Nomura, K. and Nakagawa, Y. (2003) Protection from inactivation of the adenine nucleotide translocator during hypoglycaemia-induced apoptosis by mitochondrial phospholipid hydroperoxide glutathione peroxidase. Biochem. J. **371**, 799–809

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