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Induction of phospholipid hydroperoxide glutathione peroxidase in human polymorphonuclear neutrophils and HL60 cells stimulated with TNF-α

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Abstract

Phospholipid hydroperoxide glutathione peroxidase (PHGPx) is characterized as an important enzyme for protecting cells from oxidative stress-induced apoptosis and regulating the production of leukotrienes and prostanoids in cells overexpressing PHGPx. We studied whether the expression level of PHGPx fluctuates in polymorphonuclear leukocytes (PMNs) which were exposed to reactive oxygen species (ROS) and inflammatory cytokines at an inflammation site. Human peripheral PMNs up-regulated the expression level of PHGPx following culture with TNF-α, but not with IL-1β, IL-8, and GRO. The up-regulated PHGPx expression was also observed in neutrophil-like cells that differentiated from the human leukemia cell line HL60 only after stimulation with TNF-α. However, macrophage-like differentiated HL60 cells and other cell lines, A498, ECV304, HeLa, U937, and HEK293, showed no increase in the PHGPx expression. This up-regulation of PHGPx was inhibited by treatment with the anti-oxidants, pyrrolidine dithiocarbamate, and *N*-acetyl-L-cysteine, and by inhibitors of NFκB and Src kinases. The stimulation of neutrophil-like differentiated HL60 cells with TNF-α induced activation of NFκB and c-Src kinase, and the activation was attenuated by treatment with the anti-oxidants. Up-regulation in neutrophil-like HL60 cells was also observed following exposure to H_2O_2. These results indicate that activation of NFκB and/or Src kinases through ROS signaling may be involved in the up-regulation of the PHGPx in human PMNs stimulated by TNF-α.

Keywords: Phospholipid hydroperoxide glutathione peroxidase; Polymorphonuclear leukocyte; Growth-regulated oncogene; Gene regulation; TNF-α; Reactive oxygen species

Neutrophils, polymorphonuclear leukocytes (PMNs), are known to play important roles in inflammatory responses. They kill invading pathogens via the generation of reactive oxygen species (ROS) and release of lytic enzymes stored in granules [1,2]. PMNs also produce several inflammatory cytokines (e.g., TNF-α and IL-1β), chemokines (e.g., IL-8 and GRO), and lipid mediators to modulate inflammatory and immune responses [3–6]. In an inflammatory response, PMNs are thought to be exposed to these molecules produced by themselves and other inflammatory cells. Since ROS are potentially highly toxic, the ROS-mediated damage to intracellular molecules is considered to be limited by cellular anti-oxidant enzymes such as superoxide dismutases (SOD) and glutathione peroxidases (GPxs). SOD catalyze the dismutation of superoxide ions (O_2^-) to H_2O_2 and O_2, and GPxs convert hydrogen peroxide to H_2O. In GPxs, there are four types of isozymes known; all of them contain a selenocysteine at the active site that reduces hydrogen peroxide and lipid hydroperoxide. Intracellular GPx comprises three distinct proteins, cytosolic GPx (cGPx), gastrointestinal GPx

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(GPx-GI), and phospholipid hydroperoxide glutathione peroxidase (PHGPx). Among these, cGPx and PHGPx are widely distributed in various tissues and cells. cGPx is present predominantly in the cytosol and some are located in mitochondria. PHGPx exists in cytosolic, nuclear, and mitochondrial fractions, and is a known anti-oxidative enzyme that can directly reduce peroxidized phospholipids [7], fatty acids [8], and cholesterol [9] in membranes. We have investigated several functions of PHGPx with rat basophilic leukemia 2H3 (RBL2H3) cells overexpressing the enzyme. PHGPx-overexpressing RBL2H3 cells are reported to be resistant to necrotic and apoptotic cell death caused by various oxidative stresses [10–12], and to have reduced production of lipid mediators, such as leukotrienes, prostaglandin D_2 (PGD_2), and platelet-activating factor (PAF) [13–15]. Furthermore, human dermal fibroblast cells overexpressing PHGPx exhibited impaired NFκB activation, p65 phosphorylation, and nuclear translocation following UVA irradiation [16]. NFκB activation by linoleic acid hydroperoxide (LOOH) was also inhibited in rabbit abdominal aortic smooth muscle cells (SMC) transfected stably with the cDNA of PHGPx [17]. NFκB is a key molecule in the transcriptional regulation of inflammatory protein factors such as TNF-α and IL-8 [18,19]. These findings suggest that the expressed levels of PHGPx could modulate various cellular functions in PMNs. Our recent study demonstrated that PMNs collected from the peritoneal cavity of rats given an intraperitoneal injection of sodium casein showed an increase in the expression level of PHGPx during the further 24-h cultivation of these PMNs in vitro and in vivo [20]. The production of leukotriene B_4 and 5-HETE, lipid mediators, and intracellular hydroperoxide levels were reduced in the cultured casein-induced PMNs exhibiting PHGPx up-regulation. Furthermore, the up-regulation of PHGPx mRNA was attenuated by cycloheximide, a protein synthesis inhibitor, and this effect was canceled by culturing the cells in the conditioned medium of the cultured casein-induced PMNs, indicating that the up-regulation could be initiated by an autocrine mechanism involving a protein factor. The rat casein-induced PMNs, however, were thought to have already been activated during the process of migrating into the peritoneal cavity, and as well as being exposed to inflammation mediators in the site. Therefore, the cells were not appropriate for investigating in detail a signaling pathway involved in the up-regulation of PHGPx expression. The present study was designed to investigate whether the expression of PHGPx fluctuates in human peripheral PMNs or in cell lines stimulated with various cytokines. Here, we report that human peripheral PMNs showed an increase in expression level of PHGPx by cultivation with an inflammatory cytokine, TNF-α, and a similar response was observed in the human leukemia cell line HL60 differentiated into neutrophil-like cells. This up-regulation of PHGPx was inhibited by treatment with anti-oxidants and inhibitors for NFκB and Src kinases. These results indicate that activation of NFκB and/or c-Src kinase through ROS signaling may be involved in the up-regulation of PHGPx in human PMNs.

Materials and methods

Reagents. Recombinant human TNF-α, IL-1β, IL-6, and GM-CSF were obtained from R&D Systems, MN, USA, recombinant human IL-8 and GRO were from PeproTech EC (London, England), PolymorphoPrep was from Daiichi Pure Chemical Industries (Tokyo, Japan), SYBR Green PCR Master Mix was from Applied Biosystems (CA, USA), anti-CuZn-SOD and MnSOD antibodies were from Stressgen Biotechnology (BC, Canada), anti-Src [pY418] phosphospecific antibody was from BioSource International (CA, USA), and anti-c-Src kinase antibody was from Santa Cruz Biotechnology (CA, USA). Ammonium pyrrolidinecarbodithioate (PDTC), *N*-acetyl-L-cysteine (NAC), and phorbol 12-myristate 13-acetate (PMA) were from Sigma Chemical (MO, USA), Bay 11-7082, chelerythine, PD98059, SB20358, genistein, KT5720, Wortmannin, and PP2 were from Calbiochem (Darmstadt, Germany).

Cell preparations. PMNs were isolated from venous blood obtained from healthy volunteers using a heparinized syringe. PMNs were isolated by a density gradient centrifugation method with PolymorphoPrep. Contaminating erythrocytes were eliminated by hypotonic lysis with 0.2% NaCl for 1 min followed by addition of the same volume of 1.6% NaCl to restore osmolarity. Cells were then washed three times with phosphate-buffered saline (PBS). PMNs were more than 90% pure and viable as assessed by May–Giemsa staining and the Trypan blue exclusion test.

HL60 cells were obtained from the Japanese Collection of Research Bioresources Cell Bank (JCRB0085, Osaka, Japan) and were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum. Differentiation into neutrophil-like cells was induced by culturing HL60 cells in a CO_2 incubator for 7 days in the presence of 1.25% dimethyl sulfoxide and into macrophage-like cells by culturing for 3 days in the presence of 10 nM PMA.

RNA isolation and quantitative polymerase chain reaction. Total RNA was extracted by lysis of cells with ISOGEN (Wako Pure Chemical, Osaka, Japan) and purified according to the manufacturer's directions. Briefly, 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 RNAase-free distilled water. Total RNA (0.5 or 1 μg) was subjected to first-strand cDNA synthesis with 0.25 U/μl AMV reverse transcriptase XL, 2.5 μM random 9 mers, 1 U/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 polymerase chain reaction (PCR) was then performed with the ABS PRISM 7700 Sequence Detection System (Applied Biosystems). The following specific primers for PHGPx, cGPx, CuZnSOD, MnSOD, and 18S rRNA as an internal control were used: PHGPx: sense 5′-AAGGACATCGACGGGCACATGG-3′ and antisense 5′-TTGGATC TTCATCCACTTCCA-3′; cGPx: sense 5′-TTCCCGTGCAACCAGTT TG-3′ and antisense 5′-ATGTCAATGGTCTGGAAGCGG-3′; CuZn-SOD: sense 5′-GGGCATCATCAATTTCGAGC-3′ and antisense 5′-CC AACATGCCTCTCTTCATCC-3′; MnSOD: sense 5′-GGAGAAGTAC CAGGAGGCGTT-3′ and antisense 5′-CTCCCCTTTGGGTTCTCC AC-3′; 18S 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 undifferentiated HL60 cells. The quantity was normalized with that of 18S rRNA.

Immunoblot analysis of anti-oxidant enzyme and c-Src kinase proteins. Ten million PMNs were lysed with a lysis buffer (Passive Lysis Buffer, Promega, WI, USA) containing a protease inhibitor cocktail (Complete Mini, Roche Diagnostics GmbH, Mannheim, Germany). After that, the lysate was centrifuged at 10,000*g* for 10 min, and the supernatant was collected. Thirty micrograms of protein was solubilized by boiling for 5 min in the sampling buffer and then separated in 5–20% SDS–poly-