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PGK1 Induction by a Hydrogen Peroxide Treatment Is Suppressed by Antioxidants in Human Colon Carcinoma Cells

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Few protein biomarkers for oxidative stress have been reported. In this study, we attempted to identify the proteins selectively overexpressed in human colon tumor cells by treating with hydrogen peroxide as oxidative stress. A proteomic analysis followed by western blotting showed that phosphoglycerate kinase 1 (PGK1) was induced by hydrogen peroxide in a dose-dependent manner, while its expression was suppressed by a co-treatment with delphinidin, a known antioxidant. Furthermore, several antioxidants, including α-tocopherol, butylated hydroxytoluene (BHT), and Trolox, also inhibited the PGK1 induction caused by hydrogen peroxide. The data suggest that PGK1 might be a potential protein biomarker of intracellular oxidative status.

Key words: phosphoglycerate kinase 1 (PGK1); oxidative stress; antioxidant; biomarker

Oxidative stress is a cellular condition in which reactive oxygen species (ROS) far exceed the antioxidant defenses.[1] The imbalance may be a consequence of the reduced antioxidant capacity caused by disturbances in dietary intake, production and distribution of antioxidants, or an overabundance of ROS from an environmental or behavioral stressor, including tissue pathology.[2] Oxidative stress has been implicated in a growing list of human diseases, including colon pathologies such as cancer, inflammatory bowel disease (IBD) and diverticular disease.[3–5] A growing number of studies have recently reported the overall cellular and molecular responses to oxidative stress in biological systems.[1,6–10] However, the studies so far conducted have been limited in relation to the identification of biomarker protein(s) for oxidative stress which would facilitate the evaluation of oxidative activity and screening antioxidative agents which can be exploited for the prevention of ROS-induced diseases. In this study, we attempted to identify the protein(s) that are restored to untreated control levels by a treatment with delphinidin, an antioxidative anthocyanidin,[11–15] among the proteins up-regulated by H_2O_2 in HT29 cells, by coupling 2-D PAGE and MALDI TOF-MS. As delphinidin has been reported to be an anthocyanidin with good antioxidative potential,[11,13] this was used as a model compound for the study. The selectively regulated protein(s), by oxidative stress, could be developed into a useful tool for such purposes as screening anticancer or antioxidative agents and could provide a clue to the action mechanisms of ROS at the molecular level.

Materials and Methods

Reagents and materials. The reagents used for gel electrophoresis and IPG strips (17 cm, pH 3–10, Ready-Strip™ IPG Strip) were purchased from Bio-Rad (Hercules, CA, USA), while the other reagents were obtained from Sigma (St. Louis, MO, USA) and Merck (Darmstadt, Germany). The trypsin used for the in-gel digestion was purchased from Promega (Madison, WI, USA), and CHCA (α-cyano-4-hydroxy-trans-cinnamic acid) and other reagents used for the MALDI-TOF analysis were obtained from either Sigma or Merck. The water used in this study was purified with a Milli-Q water purification system to a resistance of 18.2 Ohm. The RPMI 1640 medium was obtained from Gibco-BRL (Rockville, MD, USA), the fetal bovine serum (FBS) was purchased from Hyclone (Logan, Utah, USA), and the urea, thiourea, CHAPS, DTT, iodoacetamide, Trizma-Base, SDS, and glycerol were all of electrophoresis grade. Hydrogen peroxide (H_2O_2) was purchased from Junsei Chemical Co., Ltd. (Japan). The

primary antibody and the horse radish peroxidase (HRP)-conjugated secondary antibody were obtained from Santa Cruz Biotecnology (Santa Cruz, CA, USA). Supersignal (Pierce, Rockford, IL, USA) was used as the chemiluminescent substrate.

Cell cultures. The human colon cancer HT29 cell line was obtained from Korean Cell Line Bank (Seoul, Korea). The cells were cultured in RPMI 1640 containing 10% FBS and grown at 37 °C under a 5% CO_2-95% air atmosphere. Cells were treated with 50 μM of H_2O_2, 25 μg/ml of delphinidin and H_2O_2 plus delphinidin for 24 h. The treated cells in φ 150-mm plates (Nunc, Rochester, NY, USA) were washed twice with 10 ml of a cold phosphate-buffered saline solution (PBS) and detached by scraping with a rubber policeman in a 6 ml of ice-cold PBS.

The collected cells were centrifuged at 20,000 × g for 25 min and 4 °C, and subjected to lysis with 10 ml of 1% Triton X-100. The cell pellet was suspended by pipetting and then incubated in ice for 1 h. After this incubation, the cell lysate was centrifuged at 20,000 × g and 4 °C for 20 min and the supernatant was collected in a 50-ml centrifuge tube. The protein was precipitated by adding trichloroacetic acid, amounting to one tenth of the total volume. The pellet was washed twice with 10 ml and then three times with 1 ml of ethanol. After washing, each pellet was dried in a Speed-Vac centrifugal evaporator (CVE-2000, Eyela, Japan) for 50 min. The protein pellet was redissolved in rehydration buffer II (6 M Urea, 2 M thiourea, 4% CHAPS, 130 mM DTT, 0.2% Ampholyte, and 0.001% bromophenol blue). The protein concentration was determined in the final supernatant by using a Bio-Rad protein assay dye reagent (Bio-Rad, Hercules, CA, USA), according to the manufacturer's instructions. The sample was then stored in Eppendorf tubes at −70 °C.

2-DE. The procedure for 2-DE was as previously described.[16] Briefly, proteins were dissolved in rehydration buffer II, and about 1.5 mg of protein was loaded on to a ReadyStrips™ IPG Strip. The IPG strips were then passively rehydrated for 10 min at 22 °C, and actively for 13 h at 50 V, before isoelectric focusing at 250 V for 15 min, ramping to 10,000 V for 4 h and focusing at 10,000 V up to 70,000 V. The IPG strips were equilibrated in 500 μl equilibration buffer I and II containing 0.375 M of Tris–HCl at pH 8.8 with 130 mM of DTT (buffer I) or 135 mM of iodoacetamide (buffer II), 2% (w/v) SDS, 20% (v/v) glycerol, and 6 M of urea.

The equilibrated IPG strips, after the first-dimensional electrophoresis, were placed on a second-dimensional disc gel that consisted of 12% acrylamide/bis at pH 8.8 for the separating gel, and 4% acrylamide at pH 6.8 for the stacking gel. Second-dimensional separation was run at 10 mA per gel and 15 °C overnight. After 2-D SDS PAGE, the gel was rinsed with distilled water for a minute and stained with 0.1% CBB R-250 in methanol/acetic acid/water (40:10:50, v:v:v) for 3 h. The stained gel was destained with methanol/acetic acid/water (40:10:50, v:v:v) until it reached a desirable background level. To perform subsequent analyses, each gel was washed with distilled water and stored in a refrigerator.

Image analysis. The CBB-stained gels were scanned with a GS-800 densitometer (Bio-Rad), and image files were exported to PDQuest 2-D gel analysis software (Bio-Rad, USA). Spots over certain levels of intensity were detected, and the detected spots were counted by using automatic spot-detection. The intensity of spots on the gel was compared in terms of molecular mass and isoelectric point values.

In-gel protein digestion. The protein spots of interest were excised from the CBB-stained gels, minced with a scalpel, and destained for 30 min by using the following destaining solution: 30% methanol (10 min), 50% ACN (10 min) and 100% ACN (10 min). The destained gels were dried in a Speed-Vac for one hour and subjected to in-gel digestion (37 °C, overnight) with a 20 μl of trypsin solution (10 ng/ml in 50 mM of NH_4HCO_3). Peptides were extracted for a total of 40 min with the following solutions; 50 mM of ABC (20 min), 50 mM of ACN (10 min) and 50% of ACN (10 min), consecutively, and dried in a Speed-Vac for 12 h. Dried peptides were re-dissolved in a resuspension solution (50% ACN in 0.5% TFA) and the solution was mixed with a matrix solution (5 mg of CHCA in 50% ACN in 0.5% TFA) at a ratio of 1:1, spotted on the MALDI plate, and dried entirely in the clean-bench.

MALDI-TOF MS. Measurements were performed with a Voyager DE-STR MALDI-TOF mass spectrometer and MALDI-TOF/TOF 4700 proteomics analyzer (Applied Biosystems, Framingham, MA, USA), equipped with a reflectron. The spectra were acquired in the delayed extraction, reflector mode under optimized conditions (20 kV acceleration voltage, 200 ns delay time). The mass scale was internally calibrated with trypsin autolytic products of a known amino acid sequence; m/z 842.51 (angiotensin I), 1045.56 (bradykinin), and 2211.10 (neurotensin).

Target identification by a database search. Mass values of the analyzed peptides were queried on protein search databases, using MS-fit from the Protein Prospector at the University of California at San Francisco (UCSF) (http://prospector.ucsf.edu/ucsfhtml 4.0/msfit. htm) and the Mascot search engine which uses raw MS/MS data to search the NCBI protein database. Protein identification was considered accurate when the MS/MS results from three or more peptides, in a given sample, identified the same protein. A maximum of one missed trypsin cleavage was allowed, although most matches did not contain any missed cleavages. A mass

A. Control
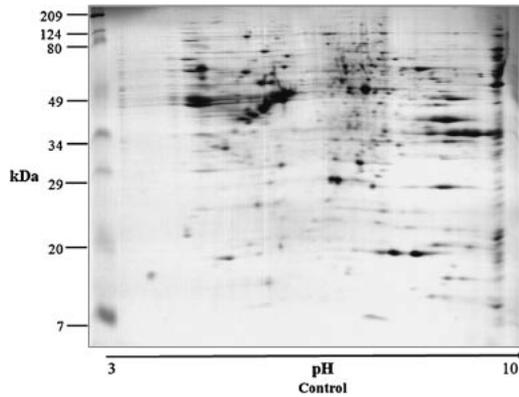
B. H₂O₂
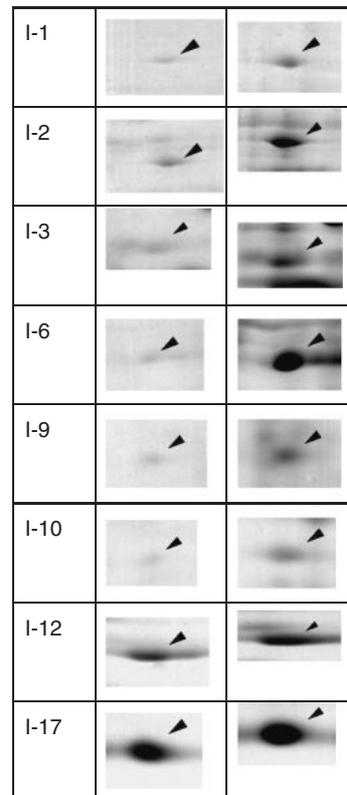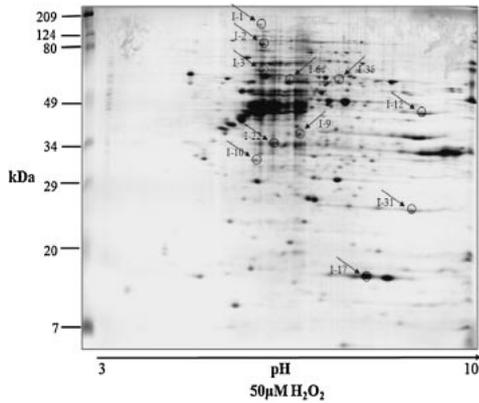
Fig. 1. *continued.*

tolerance of 50 ppm was allowed for matching the peptide mass values.

Western blot analysis. Cells were lysed with a SDS sample buffer (62.5 mM of Tris–HCl at pH 6.8, 2.3% SDS, 10% glycerol, 5% 2-mercaptoethanol, and 0.005% bromophenol blue). To confirm the expression of phosphoglycerate kinase 1 (PGK1) in the HT-29 cells, a western blot analysis was performed by using a primary antibody against PGK1. Proteins separated on 12% polyacrylamide gels were transferred on to a polyvinylidinedifluoride (PVDF) membrane with a transfer buffer (48 mM Tris, 39 mM glycine, and 20%

methanol at pH 9.2), using a wet blotting unit (Trans-Blot® electrophoretic transfer cell, Bio-rad). The blots were blocked by 5% skim milk in 0.1% Tween 20 in tris-bufffered saline (TBS/T) at room temperature for 1 h or at 4 °C overnight, and incubated in a dilution buffer (3% skim milk in TBS/T) with a primary antibody for 1 h. After washing 5 times with TBS/T, the blots were incubated in a dilution buffer (3% skim milk in TBS/T) with a secondary antibody for 1 h, followed by washing 8 times with TBS/T. The washed blots were incubated with a Supersignal West pico chemiluminescent substrate (Pierce, Rockford, IL, USA) and exposed on film. Protein concentrations were
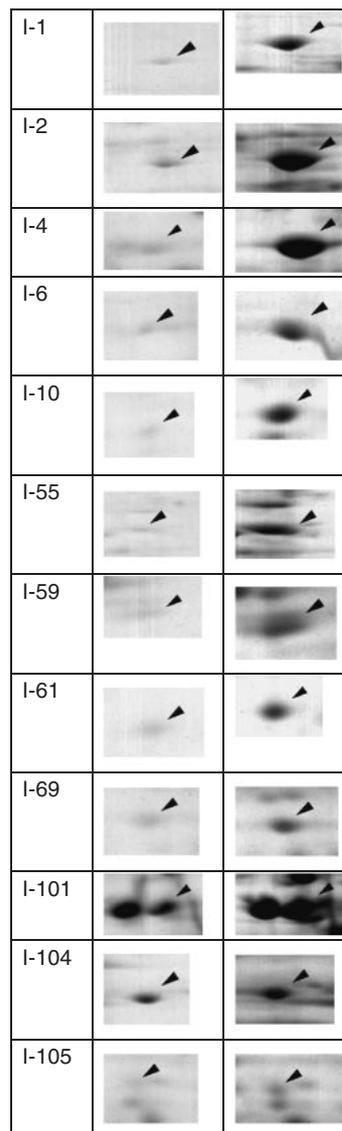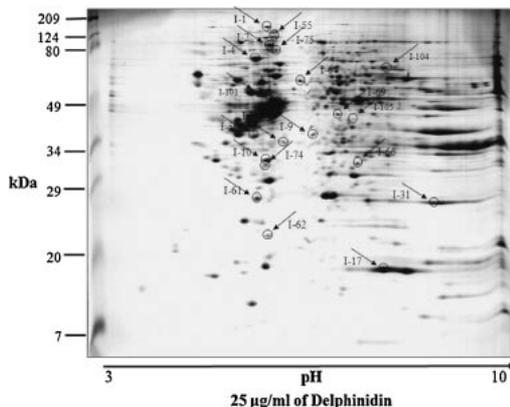
C. Delphinidin

Fig. 1. *continued.*

measured by Bradford's method, using BSA as a standard (Bio-Rad).

Results

In order to screen the protein(s) selectively regulated by oxidative stress and recovered to untreated control level by antioxidants, HT29 cells were subjected to H_2O_2 treatment, followed by proteome analyses using 2-D protein gel electrophoresis and peptide mass fingerprinting. By using Western blotting, protein(s) differentially expressed by oxidative stress was further investigated as to whether it returned to control levels by co-treatment with delphinidin, as an antioxidant.

Protein expression pattern in the H_2O_2-treated HT-29 cells

To profile the protein expression regulated by oxida-

tive stress, HT-29 human colon cancer cells were exposed to H_2O_2 at a concentration of $50\,\mu M$ for 24 h. The separated spots of the cellular proteins on two-dimensional electrophoresis were visualized by staining with CBB R-250 (Fig. 1). The scanned images were analyzed by the PDQuest image analysis program. Among the proteins differentially expressed by the H_2O_2 treatment for 24 h, 132 spots were up-regulated by more than 3-fold, while at least 227 spots were down-regulated in the HT29 cells. Tryptic digestion was performed, and protein identification was conducted on the differentially expressed spots by using MALDI-TOF-MS and a database search. Selected lists of the proteins significantly overexpressed in the HT29 cells treated with H_2O_2 and identified by the database search are summarized in Table 1. The proteins that were quantitatively up-regulated by the treatment included phosphoglycerate kinase 1 (PGK1), NADH-DH (ubi-

D. H₂O₂ + Delphinidin
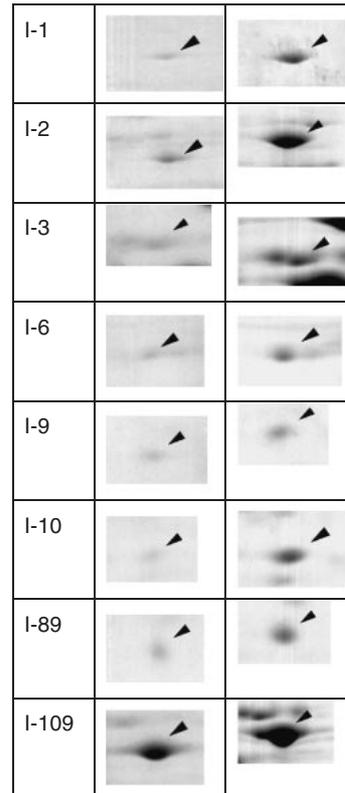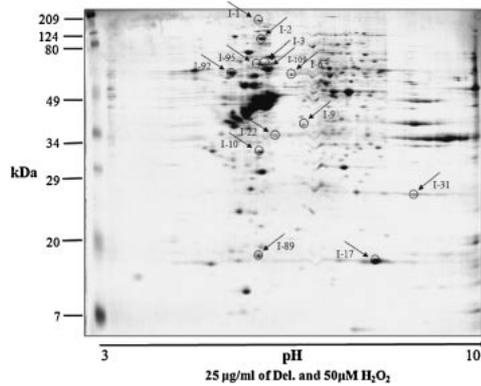
Fig. 1. Schematic Image of 2-D Gel and Location of Spots Excised for MALDI-TOF and Mass Spectrometry.

Proteins (100 µg) extracted from HT29 cells cultured in the absence and presence of H_2O_2 (A), delphinidin (B), or both (C) for 24 h were subjected to 2-DE (pH 3–10) and detected by CBB staining. Cropped images show the selected protein from 2-D gels. Arrowheads indicate the spots of interest, and the numbers serve to identify the proteins in Table 1.

quinone)-beta chaperonin, p120 catenin isoform 4 A, ribosomal protein 70, protein disulfide isomerase (ER60) precursor and caspase-like apoptosis regulatory protein 2. Among the proteins downregulated by the H_2O_2 treatment were NORI protein, retinoid X receptor, gamma, serine/threonine kinase, and thrombospondin 4 precursor. PGK1, however, was the only protein which was identified to have been selectively overexpressed by the H_2O_2 treatment in HT29 cells, while one protein spot (I-17) selectively overexpressed by the H_2O_2 treatment could not be identified. The other identified proteins were nonspecifically upregulated, demonstrating the increased expression by challenge with delphinidin as well as with H_2O_2. For instance, the expression of the PREX1 protein, protein disulfide-isomerase, ribosomal protein P0, caspase-like apoptosis regulatory protein 2, calsequestrin precursor, peroxiredoxin 1, and the CAP-binding protein complex interacting protein 1 isoform was induced by both H_2O_2 and delphinidin.

Therefore, these proteins could be classified as being nonspecifically regulated. To confirm the increased expression of PGK1 by H_2O_2, a western blot analysis was performed for PGK1 in the HT29 cells exposed to various levels of H_2O_2. As shown in Fig. 2A, PGK1 in p53-negative HT29 cells was up-regulated by H_2O_2 in a dose-dependent manner. Furthermore, the expression

pattern of PGK1 by H_2O_2 was also observed to be similar in another human colon carcinoma HCT116 cell line which is p53-positive (Fig. 2B), suggesting that PGK1 induction by H_2O_2 might be independent of p53 loss-of-function in the HT29 cells.

Suppression of H_2O_2-induced PGK-1 expression by antioxidants. The increased expression of PGK1 by H_2O_2 was attenuated by co-treating with delphinidin, an antioxidant (Fig. 3A). Furthermore, such other antioxidants such as α-tocopherol, Trolox, and butylated hydroxytoluene (BHT) suppressed the PGK1 induction mediated by H_2O_2. A similar trend in regulation of ROS-induced PGK1 expression by the antioxidants was seen with the HCT116 cell line, in which the increased expression of PGK1 by H_2O_2 was suppressed by the antioxidants, delphinidin, BHT, and Trolox (Fig. 3B).

This suggests that the expression of PGK1 was regulated by the redox state or ROS level inside the cells.

Discussion

Although there are numerous reports on biomarkers for oxidative damage, there have been only a few attempts to develop a protein biomarker for the

Table 1. 2-DE and MALDI-TOF MS Identification of Proteins That Were Overexpressed by the H_2O_2 or H_2O_2 Plus Delphinidin Treatment in HT29 Cells

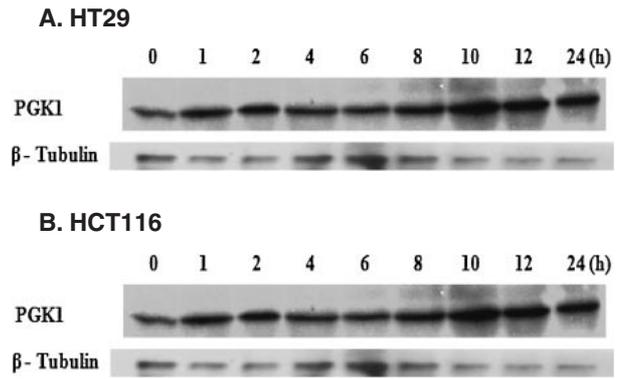| Spot	Proteins identified	Protein MW (Da)/pI	Accession #	# (%) Masses matched	Peptide coverage (%)	Mowse score
I-1	JMJD1 protein	132466/8.6	23468349	8 (16)	12	4.75E+04
I-2	PREX1 protein	166743/6.1	31657228	9 (18)	9	2.00E+04
I-3	catenin (cadherin-associated protein), delta 1	68004/8.3	30583505	6 (12)	10	482
	p120 catenin isoform 4A	68032/8.3	3152859M	6 (12)	10	482
I-4	BiP	72116/5.0	1143492M	17 (34)	33	7.11E+08
I-6	protein disulfide-isomerase (EC 5.3.4.1) ER60 precursor	56797/6.0	7437388M	9 (18)	21	6.02E+04
I-9	Ribosomal protein P0	34275/5.4	12654583	8 (16)	30	4288
I-10	caspase-like apoptosis regulatory protein 2	25418/7.7	2286147	7 (14)	24	6362
I-12	PGK1	44603/8.3	48145549	7 (14)	29	1.27E+05
I-22	CAPZA1 protein	32923/5.4	12652785M	4 (8)	22	1719
	calsequestrin precursor, fast skeletal muscle	44582/4.0	280770M	4 (8)	12	627
I-31	similar to proliferation associated gene (pag)	22111/8.3	55586231M	5 (10)	24	402
	peroxiredoxin 1	18976/6.4	55959887	5 (10)	28	166
I-35	KIAA1936 protein	61496/6.6	15620931	7 (14)	12	1.77E+04
I-55	heat shock 105 kDa protein 1	92117/5.4	55957725M	10 (20)	11	6.35E+05
I-61	cyclin-dependent kinase inhibitor 1B (p27, Kip1)	22031/6.5	54695978M	10 (20)	40	4.69E+04
I-66	DNAJC4 protein	27594/10.6	34785543M	7 (14)	13	4103
I-69	family with sequence similarity 46, member D	44500/6.2	22749287M	4 (8)	18	2070
I-74	PSMA3	28415/5.2	48145983	9 (18)	36	7998
I-75	CAP-binding protein complex interacting protein 1 isoform a	172931/8.7	38570107M	10 (20)	9	4.77E+04
	CAP-binding protein complex interacting protein 1, isoform b	155753/8.5	54673511	9 (18)	8	1.32E+04
I-87	tripartite motif-containing 45	62475/8.4	55959601	8 (16)	15	6740
I-89	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 8, 19 kDa	21766/6.3	4826854M	4 (8)	31	148
I-92	Prolyl 4-hydroxylase, beta subunit	57117/4.8	20070125M	10 (20)	23	8.16E+05
I-95	Ran-binding protein 10	67257/6.3	37703698M	5 (10)	7	817
	KIAA1464 protein	67385/6.4	7959189	5 (10)	7	815
	Protein kinase C, alpha	76765/6.6	4506067M	5 (10)	12	316
I-101	ATP synthase, H+ transporting, mitochondrial F1 complex, beta subunit precursor	56560/5.3	32189394M	12 (24)	32	2.90E+08
I-105	SEN54L protein	58877/7.7	31565520	7 (14)	14	1.98E+04
	Protein kinase C, alpha	76765/6.6	4506067M	10 (20)	16	1.74E+04
I-109	Chaperonin	61055/5.7	41399285M	10 (20)	28	5.98E+05

Numbered as in Fig. 1.

A. HT29
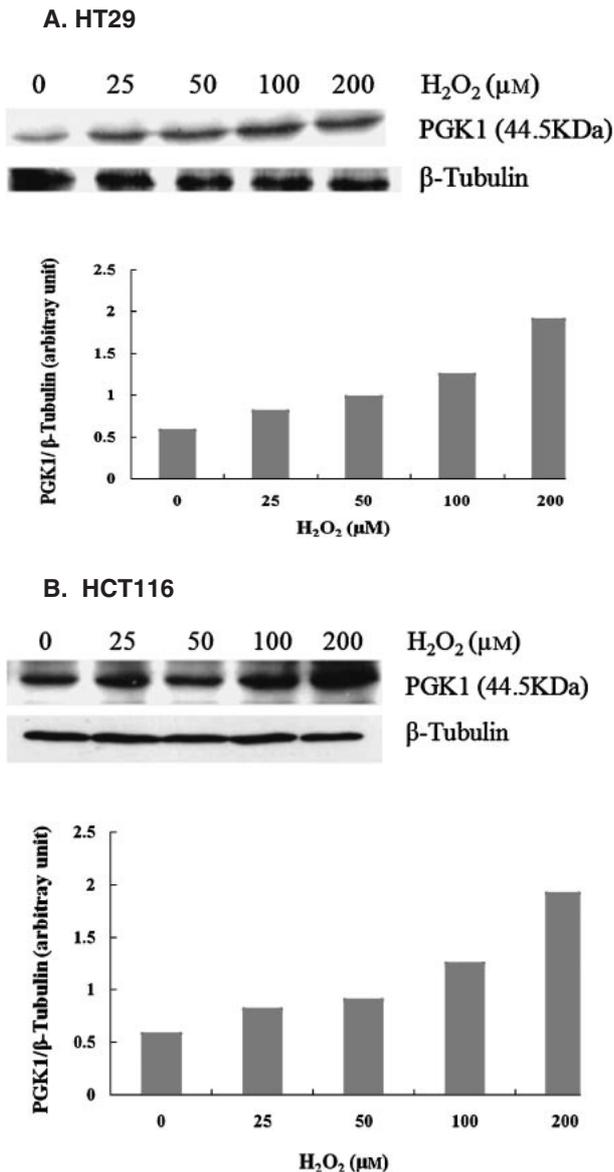
B. HCT116

Fig. 2. Modulation of PGK1 Expression by H_2O_2 in HT-29 (A) and HCT116 (B) Human Colon Cancer Cells.

The cells (3×10^5) were plated on a culture dish (100 mm diameter), incubated for 48 h, and cultured for another 24 h in the presence of various concentrations of H_2O_2, this being followed by Western blotting using the anti-PGK1 antibody.

A. HT29

B. HCT116

**Fig. 3.** Time-Dependent Regulation of PGK1 in HT29 (A) and HCT116 (B) Cells upon Exposure to Hydrogen Peroxide.

The cells (3×10^5) were plated on a culture dish (100 mm diameter), incubated for 48 h, and cultured in the presence of 100 μM H_2O_2 for various times, this being followed by Western blotting using the anti-PGK1 antibody.

identification of proteins selectively regulated in response to cellular oxidative stress.[1,2] The present study has demonstrated that PGK1 was the only protein selectively upregulated by H_2O_2, while its expression was attenuated by a co-treatment with several antioxidants. In fact, PGK1 expression was decreased consistently by the antioxidant treatment, implying its potential usefulness as a screening tool for antioxidants.

The increased expression of PGK1, one of the major glycolytic enzymes, by H_2O_2 exposure might be a response to compensate for an impaired glycolytic pathway caused by oxidative stress. A defective glycolytic pathway would divert glucose to the pentose phosphate pathway, thus generating additional NADPH which is required by antioxidant enzymes.[1,17] It has also been reported that ROS improved the stability of hypoxia-inducible factor-1 (HIF-1) which is involved in the transcriptional activation of several glycolytic enzymes including PGK1.[18]

The expression of PGK1 has been reported to be associated with the HER-2/*neu* status in breast cancer[19] and it has been upregulated in the serum of patients with pancreatic cancer,[20] suggesting its potential as a biomarker for cancer. PGK1 has also been shown to affect DNA replication and repair in mammalian-cell nuclei,[21,22] and several studies have linked its expression to tumor biology. The expression levels of PGK1 are controlled by oxygen tension, and such increased expression may reflect faster growth and more hypoxic tumors.[23,24] The increased expression of PGK1 at the protein and mRNA levels has been associated with a poor outcome for patients who have lung adenocarcinoma and who are multi-drug resistant.[25,26] PGK1 also functions as a protein secreted by tumor cells that participate in the angiogenesis process as a disulfide reductase.[27]

Interestingly enough, the enhancement of PGK1 expression by H_2O_2 was suppressed by the co-treatment with such antioxidants as Trolox, α-tocopherol, delphinidin, and BHT, suggesting that PGK1 induction might be sensitive to a cellular oxido-reductive state and a selective biomarker for oxidative stress. Furthermore, PGK1 expression was modulated in a similar fashion by other ROS-generating systems such as paraquat, a superoxide anion generator (unpublished data). As PGK1 appears to play a key role in tumor survival and angiogenesis, it may also be a good target for chemotheraphy.[27] Accordingly, antioxidants, including delphinidin, that have been shown to suppress the expression of PGK1 merit further evaluation as potential anticancer agents.

A. HT29 (PGK1)
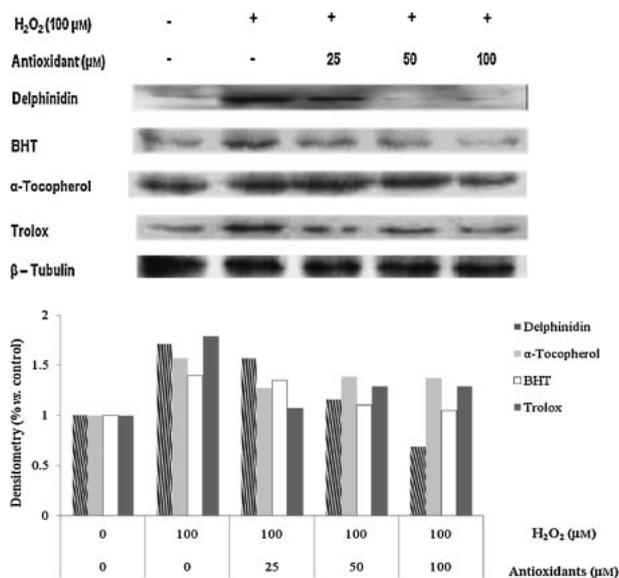
B. HCT116 (PGK1)
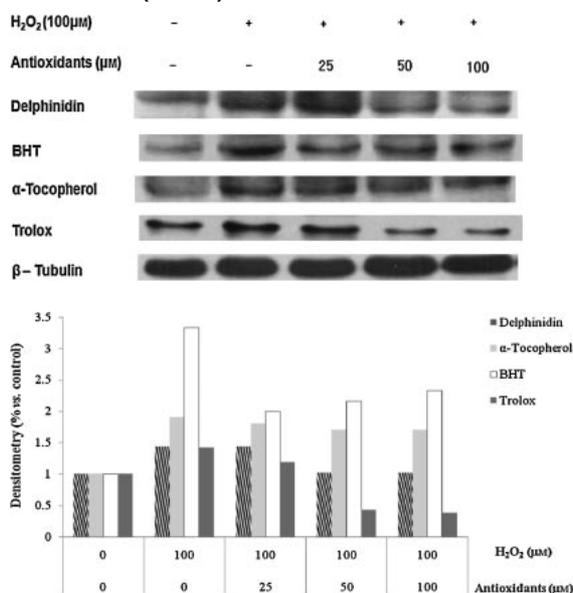
Fig. 4. Suppression of ROS-Induced PGK1 Induction by Antioxidants in HT-29 (A) and HCT116 (B) Human Colon Cancer Cells.
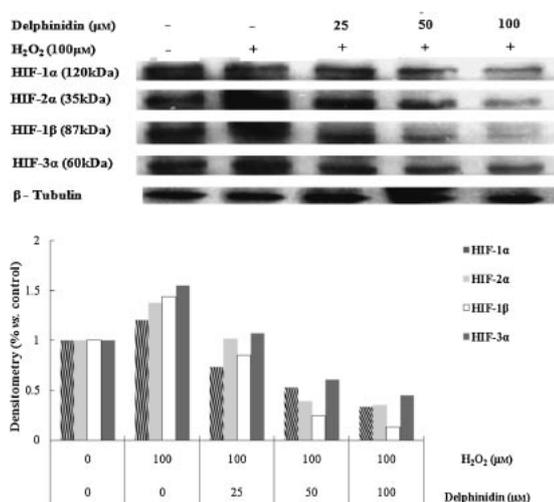
HT-29 or HCT116 cells (3×10^5) were plated on a culture dish (100 mm diameter), incubated for 48 h, and cultured for another 24 h in the presence and absence of 100 μM H_2O_2 with various concentrations of delphinidin, α-tocopherol, BHT, and Trolox. After exposure under various conditions, the cells were harvested, and subjected to protein extraction. The expression of PGK1 from each treatment was evaluated by western blotting as described in the Materials and Methods section. β-Tubulin was used as a loading control.

It is generally accepted that PGK1 expression is regulated by hypoxia-inducible factor 1α (HIF-1α).[28] HIF-1α is a transcriptional factor that functions as a major regulator of oxygen homeostasis. O_2-dependent hydroxylation of two proline residues in the HIF-1α subunit is necessary for the ubiquitination of the protein,

A. HT29
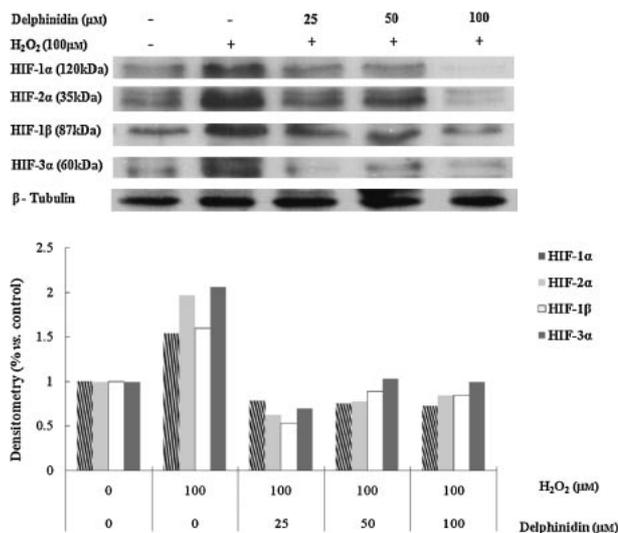
B. HCT116

Fig. 5. Effect of H_2O_2 and Delphinidin on the Expression of HIFs in HT29 (A) and HCT116 (B) Cells.

The cells (3×10^5) were plated on culture dish (100 mm diameter), incubated for 48 h, and cultured for another 24 h in the presence of H_2O_2 and various concentrations of delphinidin, this being followed by Western blotting using the anti-HIF1 antibody.

leading to its degradation by proteosome.[28] An intracellular accumulation of HIF-1α by the H_2O_2 treatment was observed, while delphindin, an antioxidant, decreased the level of most hypoxia inducible factors including HIF-1α, 2α, 3α and 1β (Fig. 4). Therefore, HIFs might be stabilized in the presence of such ROS as hydrogen peroxide, but destabilized by antioxidants. The increased stability of HIFs under hypoxic or dysoxic conditions may contribute to the accumulation of PGK1, while HIFs and PGK1 are downregulated or degraded in the presence of antioxidants or in a reductive environment.

Another possibility is the existence of a mechanism different from that on the HIF-1α-mediated pathway in regulating PGK1 expression. At least one upstream regulator of PGK1 expression and secretion has been

reported. C-X-C chemokine receptor type 4 (CXCR4) has been shown to regulate the expression of PGK1, in which the overexpression of PGK1 reduced the secretion of VEGF, interleukin-8, but increased the generation of angiostatin. As HIF-1α overexpression has been observed in many human cancers including colon cancer and has been associated with increased patient mortality in several cancer types, reducing the level of HIFs or glycolytic enzymes including PGK1, therefore, seems an appealing anticancer strategy.[18,29]

In conclusion, we attempted to identify a protein biomarker for oxidative and/or antioxidative activity by using proteomics technology. Our results suggest that PGK1 might be a potentially supplemental biomarker for oxidative stress, as it was counter-regulated by H_2O_2 and antioxidants, respectively.

Acknowledgment

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