Journal of Cellular Biochemistry 99:1628–1641 (2006)

A Proteomic Approach to the Identification of Early Molecular Targets Changed by L-Ascorbic Acid in NB4 Human Leukemia Cells

Seyeon Park,[1]* Joomin Lee,[2] and Chang-Hwan Yeom[3]**

[1]Department of Applied Chemistry, Dongduk Women's University, Seoul 136-714, Korea
[2]Department of Food and Nutrition, Yonsei University, Seoul 134, Korea
[3]Department of Family Medicine, Myongji-Hospital,
Kwandong University College of Medicine, Kyunggi-do 412-270, Korea

Abstract The pro-oxidant effect of L-ascorbic acid (LAA) is toxic to leukemia cells. LAA induces the oxidation of glutathione to its oxidized form (GSSG) and this is followed by a concentration-dependent H_2O_2 accumulation, which occurs in parallel to the induction of apoptosis. To identify early protein targets of LAA in leukemia cells, we used a differential proteomics approach in NB4 human leukemia cells treated with 0.5 mM of LAA for 30 min. This exposure was determined to efficiently block cellular proliferation and to activate oxidative stress-inducible apoptosis. We identified nine proteins that sensitively reacted to LAA treatment by using two-dimensional (2-D) gel electrophoresis and matrix-assisted laser desorption ionization time-of-flight-MS. A subunit of protein-disulfide isomerase (a thiol/disulfide exchange catalyst) and immunoglobulin-heavy-chain binding protein (BiP, identical to Hsp70 chaperone) showed quantitative expression profile differences. A myeloid leukemia associated antigen protein (a tropomyosin isoform) showed changes in pI as a result of phosphorylation. Our studies demonstrate for the first time that the addition of LAA to cells results in an immediate change in the intracellular thiol/disulfide condition and that this includes an increase in the GSH oxidation with changes in the superfamily of thiol/disulfide exchange catalysts. These results suggest that LAA oxidizes intracellular reduced glutathione and modulates disulfide bond formation in proteins. J. Cell. Biochem. 99: 1628–1641, 2006.

Key words: proteomic approach; L-ascorbic acid; NB4; tropomyosin, thiol/disulfide condition

A number of studies have demonstrated that L-ascorbic acid (LAA), one of the major water-soluble anti-oxidants present in cells and plasma, can under certain conditions function as a pro-oxidant and hence increase DNA damage [Stich et al., 1976; Speit et al., 1980]. Increasing evidence also indicates that LAA is selectively toxic to some types of tumor cells, and that when doing so it functions as a pro-oxidant, rather than as an anti-oxidant [Bram et al., 1980; Bruchelt et al., 1993]. LAA at concentrations of 10 nM–1 mM induced apoptosis in

Abbreviations used: AML, acute myeloid leukemia; APL, acute promyelocytic leukemia; BiP, immunoglobulin-heavy-chain binding protein; BSO, Buthionine sulfoximine; CHAPS, 3-((3-cholamidopropyl)dimethylammonium)-1-propanesulfonate; DCF, dichlorodihydrofluorescein; DHA, dehydroascorbic acid; DTT, dithiothreitol; ERK, extracellular signal-regulated kinases; FBS, fetal bovine serum; FITC, fluorescein-isothiocyanate; GSH, reduced glutathione; H_2DCFDA, 2′, 7′-dichlorodihydrofluorescein diacetate; HPLC, high performance liquid chromatography; IEF, isoelectric focusing; LAA, L-ascorbic acid (vitamin C); MALDI-TOF, matrix-assisted laser-desorption ionization-time of flight; MAPK, mitogen-activated protein kinases; MDS, myelodysplastic syndromes; MPA, meta-phosphoric acid; PDI, protein disulfide isomerase; P4-H, prolyl 4-hydroxylase; propidium iodide; SD, standard deviation; TBP, tributylphosphine.

Grant sponsor: Dongduk Women's University.

*Correspondence to: Seyeon Park, PhD, Department of Applied Chemistry, Dongduk Women's University, 23-1 Wolgok-dong, Sungbuk-ku, Seoul 136-714, Korea.
E-mail: sypark21@dongduk.ac.kr; seypark21@hotmail.com

**Correspondence to: Chang-Hwan Yeom, MD, PhD, Department of Family Medicine, Myongji-Hospital, Kwandong University College of Medicine, Kyunggi-do 412-270, Korea.

Received 17 January 2006; Accepted 29 March 2006
DOI 10.1002/jcb.20971

neuroblastoma and melanoma cells [Fujinaga et al., 1994; De Laurenzi et al., 1995], and it was found that LAA modulates mouse myeloma cell growth in an in vitro colony assay, and that LAA modulates leukemic progenitor cell growth, in cells from patients with acute myeloid leukemia (AML) and myelodysplastic syndrome (MDS) [Park, 1985; Park et al., 1992; Park et al., 2004].

LAA is a well-known reducing agent, and is easily oxidized to dehydroascorbate (DHA) by molecular oxidants in solution. Moreover, LAA is generally transported into cells in this oxidized form via facilitative glucose transporters or as ascorbic acid by sodium dependent ascorbic acid transporters [Nishikimi and Yagi, 1991; Vera et al., 1993; Tsukaguchi et al., 1999; Rumsey et al., 2000; Liang et al., 2001]. When DHA is transported via glucose transporters, it is rapidly reduced using intracellular glutathione (GSH), and then accumulates as ascorbic acid [Vera et al., 1993]. Thus, LAA acts as a pro-oxidant [Halliwell and Foyer, 1976; Heikkila and Cabbat, 1983]. The direct role of oxidative stress in the induction of apoptosis in AML cells was clearly demonstrated by the finding that catalase could completely abrogate LAA-induced apoptosis [Park et al., 2004]. However, the oxidative mechanism induced by ascorbate has not been elucidated.

To understand the molecular basis of LAA-induced oxidative stress, it is important to identify the expression or function of proteins which are modified by LAA. Little is known about the protein products produced by leukemia cells exposed to LAA since most studies have been performed at the cellular effect of LAA. In the present study, we analyzed molecular modifications induced by LAA in leukemia cells using a proteomic approach. This methodology provides important qualitative information on post-translational modifications to each protein and quantitative data on protein expressions in response to a particular stimulus. This information is particularly important because it provides data on early cellular events, such as, on the stimulus and signaling cascades triggered independently of protein neosynthesis.

MATERIALS AND METHODS

Cell Culture

Human leukemia cell line NB4 cells were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin) at 37°C in a humidified 5% CO_2 incubator. NB4 cell line was a gift from Dr. M. Lanotte (Hospital St. Louis, Paris, France). All cell culture reagents were obtained from Gibco (Carlsbad, CA).

Measurement of Intracellular Ascorbate

After incubation with LAA, cells were washed three times with PBS and harvested. Cell pellets were resuspended in 5% meta-phosphoric acid (MPA) and centrifuged at 14,000 rpm for 10 min. The supernatant was applied to high performance liquid chromatography (HPLC) system (Shimadzu Corp., Tokyo, Japan) equipped with Shim-pack CLC-ODS column (6 mm × 15 cm) connected to a Shim-pack G-ODS guard column (4 mm × 1 cm) (Shimadzu) and L-ECD electrochemical detector. The mobile phase was 50 mM potassium phosphate with 100 μM ethylenediaminetetraacetic acid and was adjusted at pH 2.5 with phosphoric acid (Iwase and Ono, 1994). The column was equilibrated with the mobile phase and analyzed at a flow rate of 1.0 ml/min. Ascorbate concentration was calculated from a standard curve for each experiment.

[^{3}H]-Thymidine Uptake Cell Proliferation Assay

Leukemic cells (3×10^4 cells/well) were cultured in 96-well flat bottom microtiter plates (Costar, Cambridge, MA) in 0.2 ml of RPMI 1640 containing antibiotics and 10% FBS. Cultures were incubated at 37°C with 5% CO_2 for 24 h and were pulsed with 1 μCi of [^{3}H] thymidine (2 Ci/mmol; New England Nuclear, Boston, MA) during the last 4 h of the culture period. Cultures were harvested and [^{3}H]-thymidine incorporation was determined by liquid scintillation counter. The statistical significance of the differences between treated and untreated samples were evaluated using Student's test. The difference was judged to be statistically significant if $P < 0.05$.

Measurement of Cell Apoptosis

For early apoptosis detection, cells were harvested and stained with fluorescein-isothiocyanate (FITC)-labeled annexin V and propidium iodide (PI) (Roche, Mannheim, Germany) according to the manufacturer's manual, and analyzed with a four-color fluorescence flow cytometer (Becton Dickinson, Franklin Lakes, NJ).

Measurement of Intracellular Total Glutathione (GSH_t) and Oxidized Form (GSSG)

Intracellular GSH contents were measured using a GSH:GSSG ratio assay kit (Calbiochem, San Diego, CA). In brief, for the accurate determination of the low amount of GSSG in cells, 5×10^6 cells were harvested and treated with a thiol-scavenging reagent to prevent oxidation of GSH to GSSG during sample preparation. Cells were freeze-thawed and the specimen centrifuged. Supernatant was used for GSSG measurement according to the manufacturer's instruction, while the pellet was dissolved in 1 M NaOH and analyzed for protein by Bio-Rad protein assay (Bio-Rad, Hercules, CA). To measure the total GSH level, cells were assayed using the same methods without treatment with thiol-scavenging reagent.

Determination of Intracellular H_2O_2 Accumulation

Intracellular H_2O_2 production was measured using H_2DCFDA (Sigma, St. Louis, MO), an H_2O_2 sensitive fluorescent dye. Briefly, cells (5×10^5) were incubated in 0.5 μM H_2DCFDA for 30 min and then, incubated in the presence or absence of LAA for 30 min. Cells were washed and resuspended in PBS, and then analyzed by flow cytometry. H_2DCFDA diffuses into cells and after conversion by non-specific esterases, reacts with H_2O_2 to form a fluorescent molecule [Grad et al., 2001]. The channel number of the peak of the fluorescence intensity distribution is used as a measure of the intracellular H_2O_2 content.

Preparation of Total Protein Extracts

To obtain total protein extracts, 5×10^7 cells were resuspended in a total volume of 500 μl buffer containing 8.0 M urea, 2 mM tributylphosphine (TBP), 4% (w/v) CHAPS, 0.2% carrier ampholyte (pH 4-7, Bio-Rad) and a protease inhibitor cocktail (Sigma). Cells were disrupted by ten strokes with Sonicator (XL-2020, MISONIX, Farmingdale, NY). After 1 h incubation at room temperature, cell lysates were centrifuged at 13,000 rpm for 1 h at 13°C. The supernatant was collected, and protein concentration was determined by using Detergent Compatible Protein Assay (Bio-Rad).

Two-Dimensional PAGE and Gel Analysis

Proteins (1.5 mg) were hydrated overnight in 8 M urea, 4% CHAPS, 0.2% carrier ampholyte and 0.0002% bromophenol blue at 4°C and applied on to isoelectric focusing (IEF) gel strips (pH 4–7 linear, 17 cm longer, Bio-Rad). The gel strips were rehydrated overnight in 8 M urea, 4% CHAPS, 0.2% carrier ampholyte and 0.0002% bromophenol blue at 20°C. IEF was initiated at 100 V for 2 h, 250 V for 2 h, and then gradually increased to 10,000 V for 10 h. The focusing process was carried out for 10,000 Vh and held at 500 V for 24 h. After IEF, IPG strips were equilibrated with 0.05 M Tris-HCl (pH 8.8), 0.6 mM TBP, 5% iodoacetamide, 6 M urea, 20% glycerol, and 2% SDS for 20 min. Strips were then transferred onto vertical slab 12% SDS–PAGE gels and sealed with 1% low melting point agarose. SDS–PAGE was run for 30 min at a constant current of 16 mA per gel, and then at 24 mA/gel at 15°C. After electrophoresis, the proteins were transferred to an Immobilon-P PVDF membrane or visualized by Coomassie brilliant blue G-250 staining of the gels. Images were digitalized using GS-800 Calibrated Densitometer (Bio-Rad) and analyzed by PDQuest 2-D Analysis software (Bio-Rad).

Evaluation of Differentially Represented Spots

Samples were analyzed both for quantitative and qualitative differences. Quantitative differences were determined only when a matched spot was found to show the same degree of down- or upregulation in duplicate experiments, whereas we used the term qualitative difference to indicate the presence of expressed spots in only one of two matched samples. Matching spots in gels from the same sample were identified and their intensities were measured using an Image Master 2-D system. Analysis was performed on approximate 240 different protein spots per sample. For each spot, its intensity value in LAA-treated gel was divided by its intensity value obtained in the control gel. The logs of these ratios were then calculated (LR). LR means and median values were clustered around the 0 value, which was as expected if errors were associated with our analysis were random and normally distributed. For spots showing an expression 2.0-fold less or greater than the control was considered a

statistically significant differentially expressed protein species.

In-Gel Enzymatic Digestion and Mass Spectrometry

In gel digestion was performed mainly as described by Rosenfeld et al. (1992). Spots were excised from the stained gel. Destained with 0.1 M ammonium bicarbonate/50% acetonitrile (Sigma) and dried with a Speed Vac plus SC1 10 (Savant Holbook, HY). The gel was rehydrated in a solution containing 1 M DTT and 0.1 M ammonium bicarbonate (pH 7.8) for 30 min at 56°C. After the subsequent incubation in a solution containing 1% iodoacetamide and 0.1 M ammonium bicarbonate (pH 7.8) for 30 min under dark condition, the gel was washed with 0.1 M ammonium bicarbonate/50% acetonitrile and dried with Speed Vac. The gel was rehydrated in trypsin solution (Promega, Madison, WI). After incubation overnight at 37°C, peptides were sonicated for 30 min.

MS Analysis

Digested samples were removed and subjected to a desalting/concentration step on a μZipTipC18 column (Millipore, Bedford, MA) using acetonitrile as an eluent before matrix-assisted laser-desorption ionization (MALDI)-MS analysis. Peptide mixtures were loaded on the MALDI system, using the dried-droplet technique and α-cyano-4-hydroxycinnamic acid (Sigma) as matrix, and were analyzed using a Voyager-DE-PRO mass spectrometer (Applied Biosystems, Framingham, MA). Internal mass calibration was performed using peptides derived from enzyme autoproteolysis. The Dataexplorer software package was used to identify spots from MS-Fit (Protein Prospector, CA) and the Mascot server (Matrix science, London) by mass searching human sequences. Candidates identified by peptide mapping analysis were evaluated further by comparing their calculated mass and pI using the experimental values obtained by two-dimensional (2-D) gel electrophoresis.

Western-Blot Analysis

Amounts of protein extracts were obtained from NB4 cells treated or untreated with LAA and electrophoresed on an SDS/10% (w/v) polyacrylamide gel. Then, proteins were transferred to nitrocellulose membranes (Schleicher and Schuell, Keene, NH). After transfer, membranes were saturated by incubation, at 4°C overnight, with 10% (w/v) non-fat dry milk in PBS/0.1% Tween-20 and then incubated with the indicated antibody (Becton and Dickinson Biosciences, San Jose, CA) for 3 h. After three washes with PBS/0.1% Tween-20, membranes were incubated with an anti-rabbit immunoglobulin coupled with peroxidase (Santa Cruz Biotechnology, Santa Cruz, CA). After 60 min of incubation at room temperature, the membranes were washed three times with PBS/0.1% Tween-20 and the blots were developed by using the ECL® (Enhanced Chemiluminescence) procedure (Amersham Biosciences, Milan, Italy). Normalizations were performed with the polyclonal anti-GAPDH antibody (Santa Cruz Biotechnology). Blots were quantified by using the Gel Doc 2000 densitometer (Bio-Rad).

Statistical Analysis

Statistical analysis was performed using MINITAB. Asterisk (*) indicated $P < 0.05$ compared to the control.

RESULTS

NB4 Cells Rapidly Respond to High Doses of LAA Treatment

Treatment of NB4 cell line with LAA (0.25–1 mM) for 24 h led to a marked dose-dependent reduction in cell proliferation as determined by [^{3}H]-thymidine uptake assays (Fig. 1A). As deduced by FACS analysis followed by FITC/PI double staining, treatment with 0.5 mM LAA for 30 min induced apoptosis in 35% cells (Fig. 1B). To determine whether the cytotoxic and apoptosis-inducing effect is directly related to LAA accumulation, the early uptake of LAA by NB4 cells was measured. A 30 min incubation of NB4 cells with LAA resulted in the uptake of LAA in a concentration-dependent manner (in the 0–2 mM range) (Fig. 2).

Redox Status Modulation Induced by LAA

It is known that GSH participates in the intracellular elimination of ROS and the reduction of DHA to LAA (Meister, 1994). Therefore, to determine whether LAA rapidly affects the redox status of NB4 cells, we investigated intracellular GSH/GSSG ratio changes and the H_2O_2 accumulation induced by LAA. Treatment

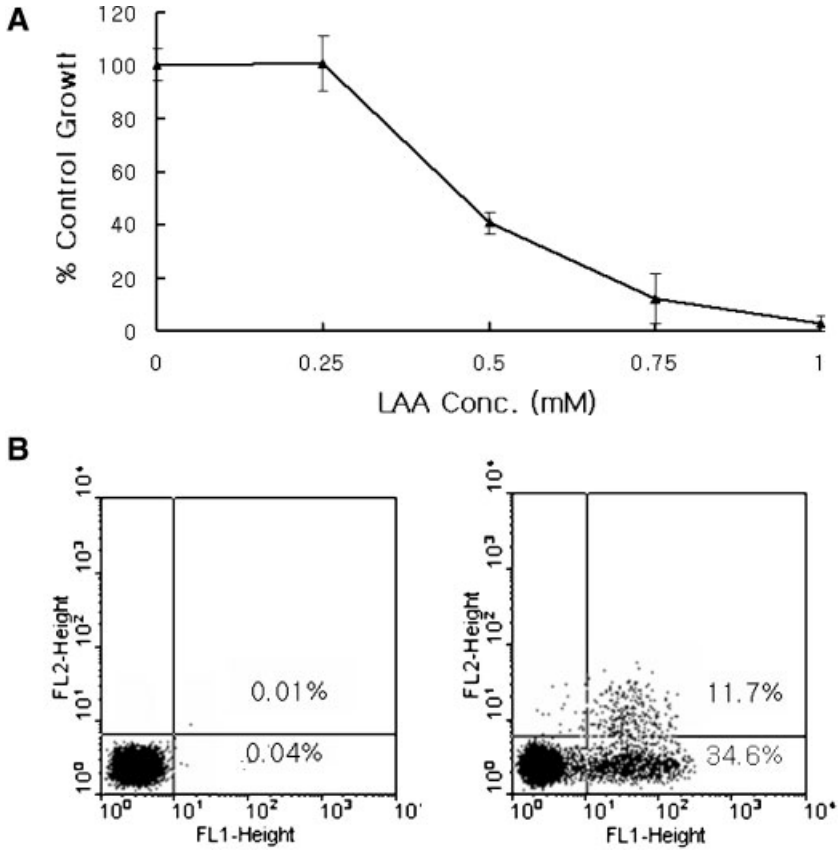

Fig. 1. **A**: Effect of LAA on the proliferation of NB4 cells. Cells were incubated in culture media with the indicated concentrations of LAA for 24 h. Cell proliferation was determined by measuring [H^3]-thymidine uptake. Data represent the means ± SD of triplicate determinations and are representative of three experiments. Results are presented relative to cell growth under control conditions (i.e., in the absence of LAA). **B**: Effect of LAA on apoptotic cell induction. After cells were incubated with LAA for 24 h, cells were harvested, stained with Annexin V-FITC (FL-1) and propidium iodide (FL-2), and analyzed by flow cytometry. Viable cells (lower left), apoptotic cells (lower right), and necrotic cells (upper right) are shown. An increase in apoptotic cells was evident after incubating NB4 cells in the presence of LAA. The results shown are representative of at least five experiments.

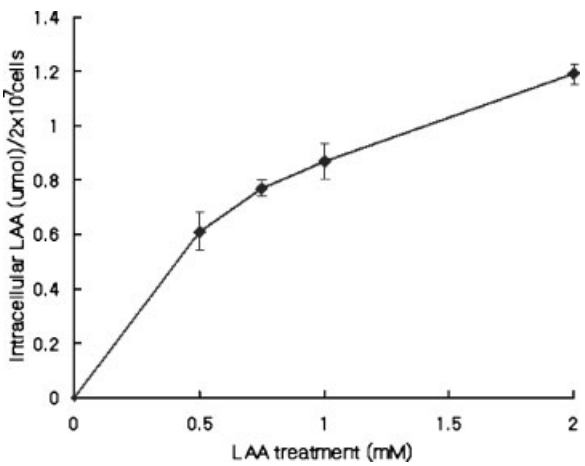

Fig. 2. Intracellular uptake of LAA. Cells were supplemented with the indicated concentrations of LAA for 30 min; intracellular LAA concentrations were measured by HPLC. The means ± SD of three determinations are shown.

with LAA of 0.5 mM for 30 min or 1 h decreased the intracellular GSH/GSSG ratio (Fig. 3A) and increased intracellular H_2O_2 levels (Fig. 3B). The oxidation of H_2DCFDA in NB4 cells treated with 0.5 mM LAA for 30 min represented as right-shifted cell population as shown in Fig. 3B. After treatment with 0.5 mM LAA, the marker area percentage (Fig. 3B) representing oxidized DCF increased versus untreated controls from 0.45 to 8.9% in NB4 cells within 30 min. Co-treatment of LAA with DTT, which prevented the GSH oxidation by LAA, caused the cells representing oxidized DCF to decrease compared with LAA-treated cells (Figs. 3A,B). In accordance with its proposed pro-oxidant activity, LAA-mediated reduction in the GSH/GSSG ratio correlated functionally with an intracellular H_2O_2 increase in the NB4 cell line.

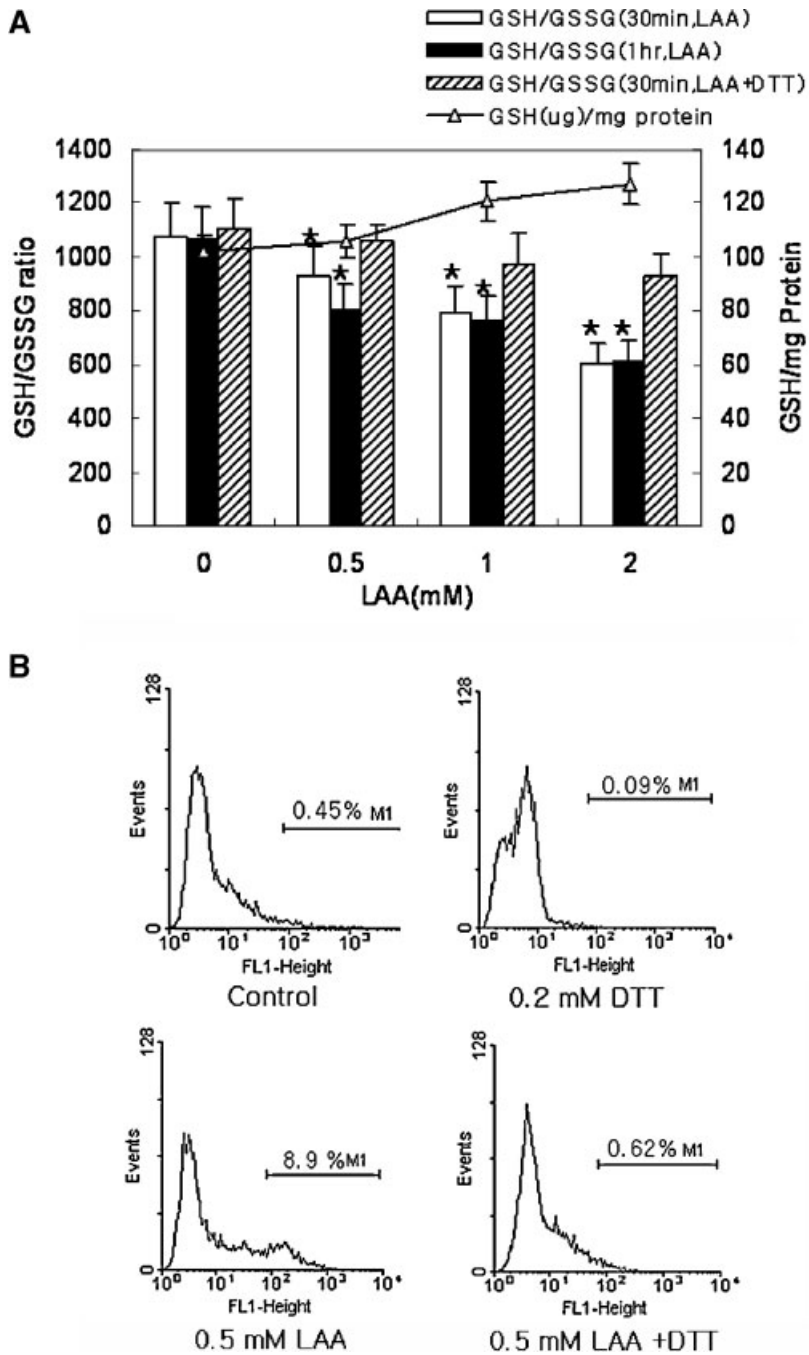

Fig. 3. **A**: Effect of LAA on GSH/GSSG ratio. NB4 cells were incubated with the indicated concentrations of LAA for 30 min or 1 h, and intracellular GSH and GSSG levels were estimated as described under Methods. The values shown are means ± SD of four experiments. LAA induced GSH/GSSG ratio changes were also measured in the presence of 0.2 mM DTT within 30 min. The MINITAB program was used to perform analysis by ANOVA followed by Dunnett's test. * indicates significantly different for LAA-treated and -untreated samples ($P < 0.05$). **B**: H_2O_2 accumulation induced by LAA. NB4 cells were pre-treated with H_2DCFDA probe for 30 min and then treated with 0.5 mM LAA with/without 0.2 mM DTT for 30 min. Oxidized DCF was analyzed by flowcytometry. The M1 gate represents cells undergoing H_2O_2 accumulation and values are presented as percentages of total collected events. Results are from one of three representative experiments. Standard deviations (not shown) were less than ±10%.

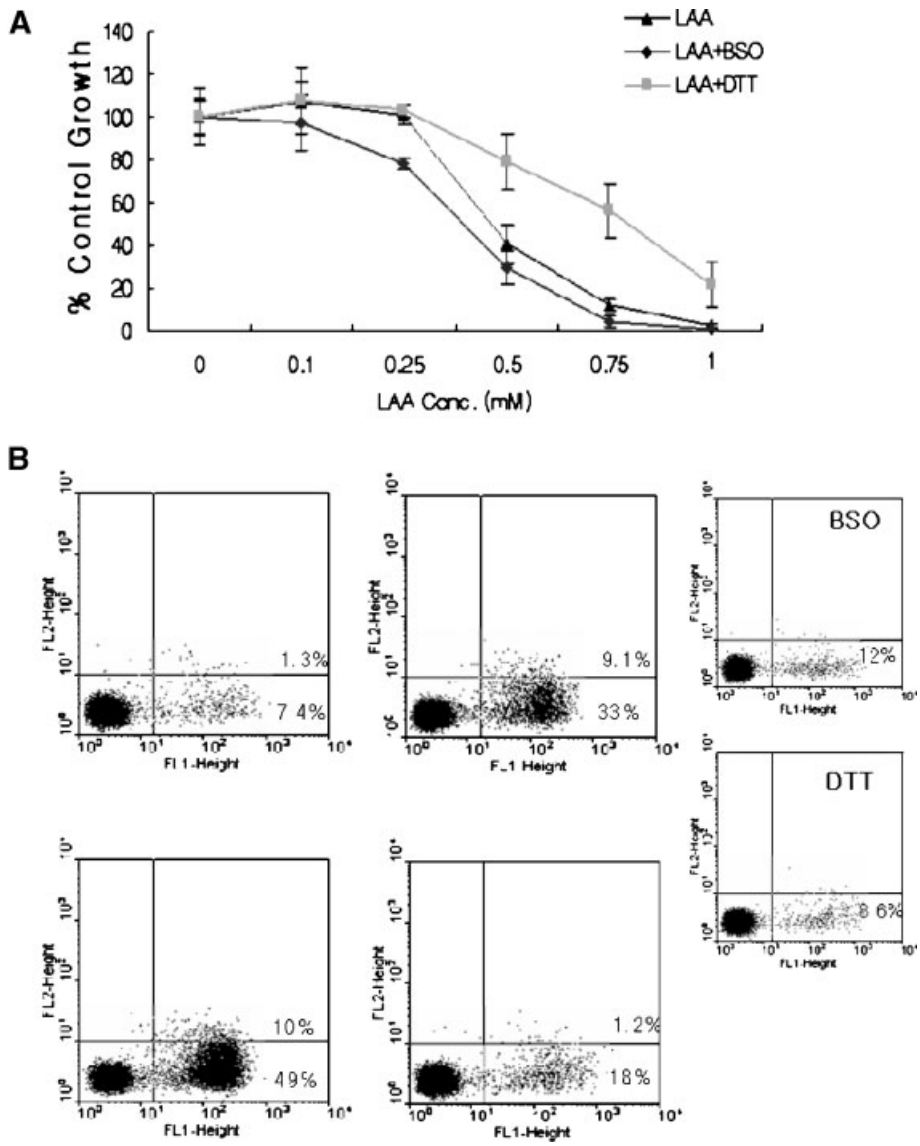

Fig. 4. **A**: Effects of BSO or DTT on the LAA-induced inhibition of cell proliferation. Data represent means ± SD of three experiments performed in triplicate. Results present growth relative to cell growth under control conditions (i.e., in the absence of LAA or other chemicals). NB4 cells (3×10^4/well) were cultured in the presence of various concentrations of LAA with/without 1 mM BSO or 0.2 mM DTT for 24 h. Cell proliferation was determined by measuring [H^3]-thymidine uptake. **B**: Effects of BSO or DTT on LAA-induced apoptosis. NB4 cells were cultured with the indicated concentrations of LAA with/without 1 mM BSO or 0.2 mM DTT. Proportions of apoptotic cells were determined by flowcytometry. The values shown are representative of at least four experiments. Standard deviations (not shown) were less than 10%.

We investigated the correlation between GSH levels and the inhibitory effect of LAA on the proliferation of NB4 cells in the presence of various concentrations of LAA and with/without 1 mM BSO or 0.2 mM DTT for 24 h. As shown in Fig. 4A, the addition of 1 mM BSO, which inhibits GSH synthesis and thus reduces GSH levels, increased the growth inhibition of NB4 cells induced by LAA. However, treatment with 0.2 mM DTT, which reduces GSH oxidation, decreased LAA-induced NB4 cell growth inhibition. Consistent with cell proliferation assay results, LAA-induced apoptosis was also affected by modulating the cellular redox status (Fig. 4B). This observation indicates that these early changes in intracellular redox status are correlated with the apoptotic activity of LAA. Based on these results, we decided to

identify early protein modifications associated with LAA treatment in the NB4 human leukemia cell line.

Changes in NB4 Proteome Profile After LAA Treatment

The immediate effects of cell stimuli are associated with protein post-translational modifications, such as, phosphorylation, glutathionylation and cysteine oxidation. To study these early modifications, NB4 human leukemia cells were treated with 0.5 mM LAA and then collected for 2-D electrophoresis analysis. Figure 5 shows the typical results of 2-D gel electrophoresis performed on total cellular extracts before and after LAA treatment. Approximate 240 different spots that were focused in the 4–7 pH range were analyzed per sample. After exposing cells to LAA, we observed; one new spot, three intensified spots, and five attenuated spots (Figs. 5 and 6). Each of these spots were excised, digested with trypsin and analyzed by MALDI–TOF. Peptide mass fingerprint analysis and non-redundant sequence database matching allowed the unambiguous identification of all of the analyzed species. Figure 5 and Table I detail the natures of each identified spot, their 2-D gel electrophoresis co-ordinates and relative sequence coverage.

Modification of Tropomyosin on Exposure of NB4 Cells to LAA

In the present study, tropomyosin was identified as a marker of the oxidative effect of LAA in NB4 cells. The spot corresponding to tropomyosin was positioned at a pI value of approximately 5.0 (Spot No 4903) (Fig. 5). This spot was attenuated in LAA-treated cells, in addition, a new spot was detected at pI 4.9 (Spot No 4902) of almost the same molecular weight. This new spot was also identified as tropomyosin, which suggested that some chemical modification had affected its pI value.

It has been reported that the extracellular signal-regulated kinase (ERK) mediated phosphorylation of tropomyosin-1 promotes cytoskeleton remodeling in response to oxidative stress [Houle et al., 2003]. To identify whether the phosphorylation of tropomyosin is responsible for the observed acidic shift (from Spot 4903 to 4902), the MEK inhibitor PD98059 was co-administered with LAA. As shown in Figure 7, the spot with a pI of ~4.9 and a molecular weight of ~27 kDa (pT) was weakened in cells co-administered with LAA and PD98059. The acidic shift of the spot with a pI 5.0 (T) to the pT spot by the treatment with LAA was found to be abrogated by the co-treatment with PD98059. Database searching followed by MALDI-TOF

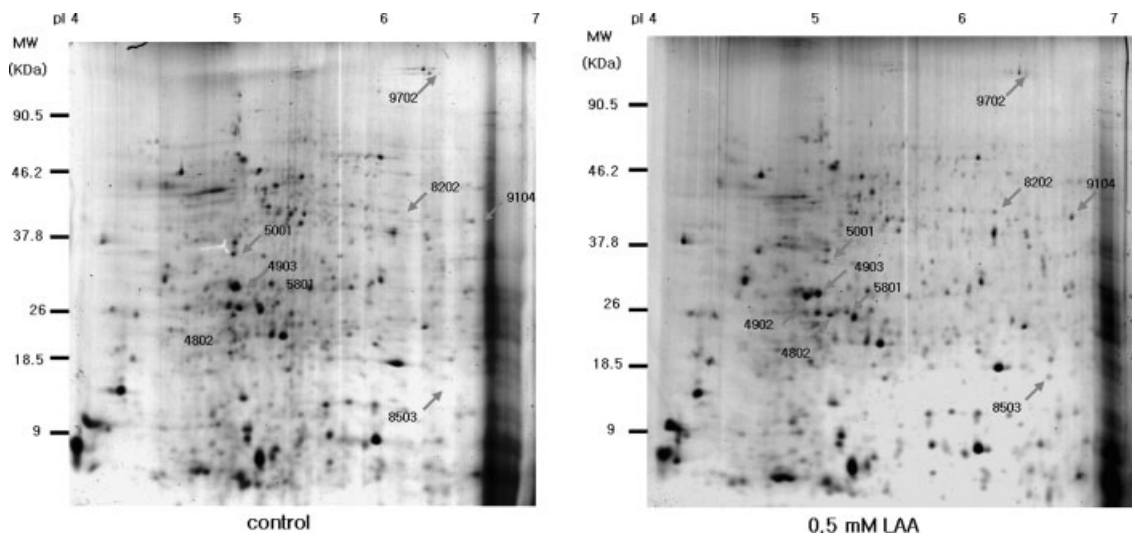

Fig. 5. NB4 cell protein patterns after treatment with LAA by 2-D gel electrophoresis. NB4 cells were incubated with/without 0.5 mM LAA for 30 min at 37°C, harvested, and lysed. Intracellular proteins were analyzed by 2-D gel electrophoresis and gels were stained with Coomassie brilliant blue G-250. **Right panel**: control cells; **left panel**: LAA-treated cells. Vertical axes represent apparent molecular mass (kDa) and horizontal axes pH values. Acquired images were repetitive. The data shown are representative of four separate experiments. Spot numbers are the same as those in Table I.

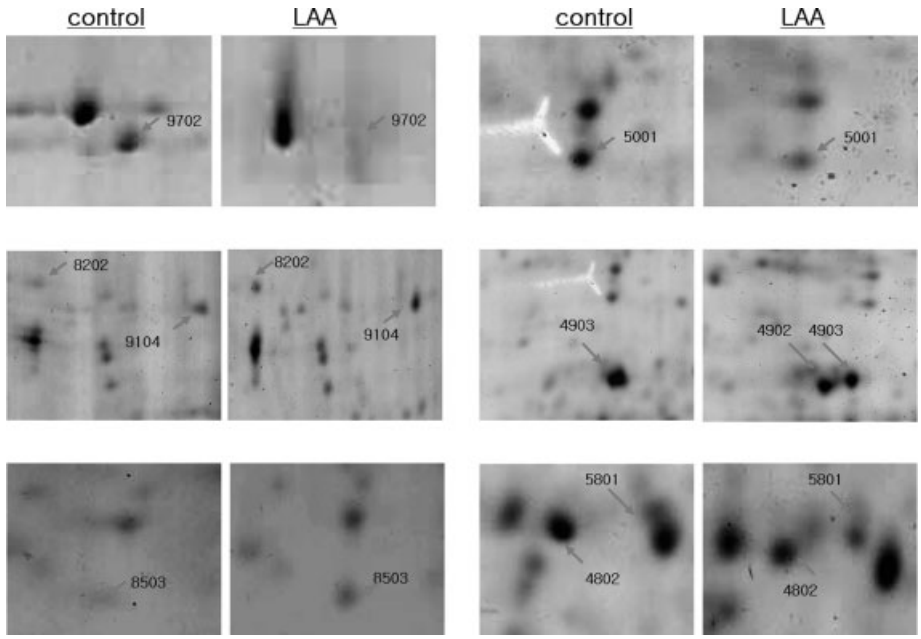

Fig. 6. Composition of 2-D gel images of NB4 cells exposed to LAA. Regions comprising statistically significant protein spots were cropped. The protein, Spot #4902 was the only one detected in this region of the gel.

analysis indicated that these spots sensitive to PD98059 were also tropomyosin.

Modifications of Protein Disulfide Isomerase (PDI) b Subunit and BiP Protein on Exposure of NB4 Cells to LAA

Proteomic analysis identified a thiol/disulfide exchange catalyst as a marker of the effect of LAA in NB4 cells. PDI belongs to the superfamily of thiol/disulfide exchange catalyst which acts as protein-thiol oxidoreductase enzymes and shares sequence similarities with thioredoxin [Noiva, 1999]. PDI is composed of four domains which have similarities with thioredoxin folds (a-b-b′-a′) [Kemmink et al., 1997]. In the present study, the spot corresponding to the PDI b subunit was positioned at a pI value of approximately 5.4 (calculated pI value, 5.6) representing a mass of 26 kDa (Fig. 5). Its intensity was weakened in LAA-treated cells versus control cells. To confirm the identities of proteins identified by MALDI-TOF, PDI protein

TABLE I. List of Spots/Proteins Sensitive to LAA Treatment in NB4 Cells, Detected by 2-D Gel Electrophoresis and Identified by Peptide Mass Fingerprint Analysis

Spot number	Protein description	Theoretical Mr(kDa)/pI	Measured Mr(kDa)/pI	Intensity variation	Accession number	MALDI-TOF coverage (%)
9702	SUPT6H protein	122.8/5.7	155/6.3	Downregulated (36 ± 13)%	13277574	16
5001	Homo sapiens heat shock 70 kDa protein 8	64.7/5.4	37/5	Downregulated (20 ± 9)%	48257068	28
8503	Matrilin-4	64/5.5	15.2/6.4	Upregulated (221 ± 41)%	3927992	43
8202	Heat shock 70 kDa protein 8 isoform 2	53.5/5.6	42/6.1	Upregulated (261 ± 64)%	24234686	34
9104	54 kDa Nuclear RNA- and DNA-binding protein	54.1/9.0	40.5/6.6	Upregulated (201 ± 81)%	13124797	40
4902	Tropomyosin isoform	30.2/4.71	30/4.9		Q5HYB6	65
4903	Tropomyosin isoform	30.2/4.71	30/5.1	Downregulated (36 ± 21)%	Q5HYB6	67
4802	BiP protein	70.9/5.2	26/5.3	Downregulated (34 ± 18)%	6470150	56
5801	Protein disulfide isomerase b subunit	13.2/5.94	26/5.4	Downregulated (48 ± 11)%	1MEK	63

Proteins identified were the only proteins detected in a given spot. The spot numbers are the same as those used in Figure 5.

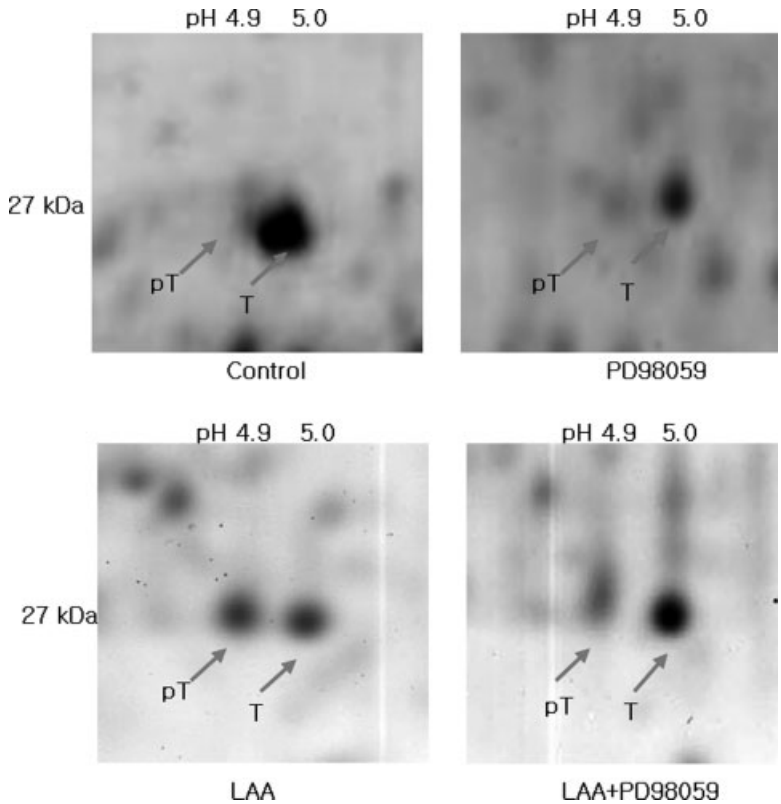

Fig. 7. 2-D gel identification of tropomyosin as a downstream target of ERK. NB4 cells were pretreated with vehicle (DMSO) or 5 μM PD98059 for 15 min and then treated with 0.5 mM LAA for 30 min. Proteins were extracted and analyzed by 2-D gel electrophoresis and stained with Coomassie brilliant blue G-250. **Right panel**: control cells (treated with vehicle and LAA); (**left panel**): cells treated with PD98059 and LAA. Vertical axes represent apparent molecular mass (kDa) and horizontal axes pH. Shifts of tropomyosin spots in the acidic direction are indicated by arrows. Acquired images were repetitive. The data shown are representative of four separate experiments.

levels were analyzed by Western blotting. As shown in Figure 8A, 55, 26, and 13 kDa PDI subunits, which were putatively ascribed to PDI represented a tetramer, a homodimer, and a monomer, respectively, were detected. The levels of the PDI 26 and 55 kDa forms were decreased in Western blotting, in accord with the 2-D gel data.

Interestingly, of the proteins with expressions changed by LAA treatment, immunoglobulin-heavy-chain binding protein (BiP, identical to Hsp70 chaperone: Spot No 4802) like PDI was also a multi-domain chaperone. BiP was associated with the α-subunit of prolyl 4-hydroxylase (P4-H) by a disulfide bond. P4-H consists of two distinct polypeptides, the catalytically more important α-subunit and the β-subunit, which is identical to the multifunctional enzyme, PDI [John and Bulleid, 1996]. Thus, BiP was associated with α-subunit of P4-H, which is another partner of PDI.

To verify the proteomic identification of BiP, Western blot analysis was performed. Immunodetection using BiP antibody revealed the presence of a 70 kDa protein and a 44 kDa domain. All Hsp 70 chaperones contain a conserved N-terminal 44 kDa nucleotide binding domain and the other substrate-binding domain [Bukau and Horwich, 1998]. The other substrate-binding domain of 26 kDa C-terminal domain seemed to be detected as Spot number 4802 in the 2-D gel data. Both species decreased in level after the LAA treatment (Fig. 8B).

It remains to be determined whether the interaction of PDI with its substrates is due to a change in disulfide bonds. Hence, we investigated the liberation of PDI in the presence of GSH. Accordingly, the purified protein was treated with GSH and separated by SDS–PAGE under non-reducing conditions, and subsequent Western blots were probed with a PDI antibody. Under these conditions, a

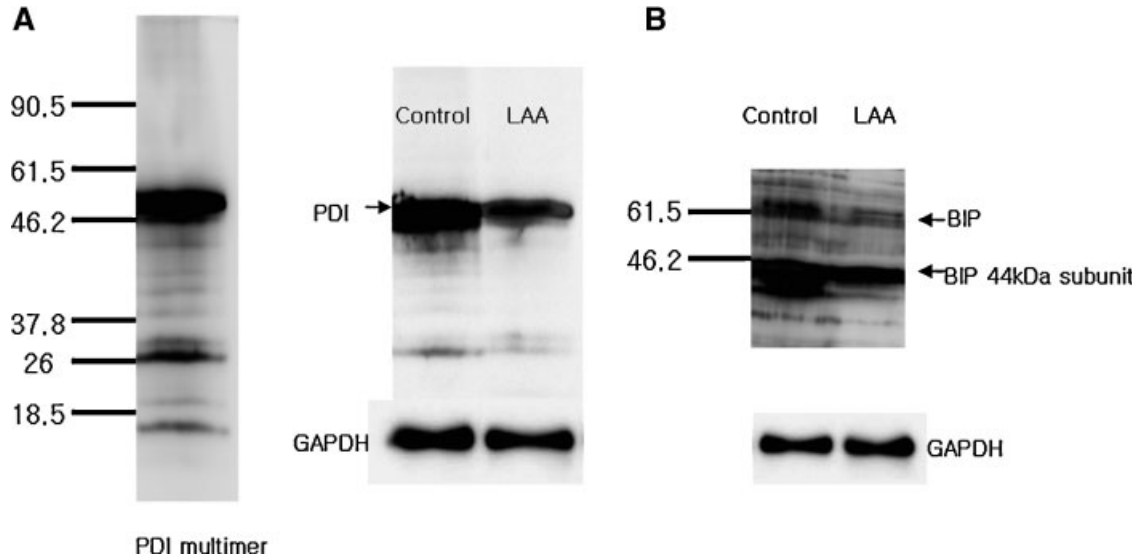

Fig. 8. Western blot analysis of PDI and BiP proteins. **A**: NB4 cells treated with 0.5 mM LAA for 30 min. Proteins were extracted, run into 10% SDS–PAGE, and transferred on nitrocellulose membranes. Data immunodetected with PDI antibody are shown. **B**: BiP protein in NB4 cells treated with 0.5 mM LAA for 30 min.

dissociation of PDI from substrates, as assessed by the increase of the PDI forms in the gel was apparent, indicating that intra-chain disulfide bonds between domains and substrates had been reduced (Fig. 9).

DISCUSSION

Our previous studies demonstrated the inductive effect of LAA on the apoptosis of AML cell lines, that is, HL-60, NB4, and NB4-R1 [Park et al., 2004]. However, LAA was found to have no significant effect on three ovarian cancer cell lines (SK-OV-3, OVCAR-3, 2774), thus demonstrating that the effect of LAA is cell-type specific. This differential sensitivity could be partly ascribed to differences in LAA uptake versus AML cell lines, which show higher uptake efficiency than ovarian cell lines. Our previous studies in three human AML cell lines and in the myeloid cells of AML patients showed that LAA induced GSH oxidation and subsequent H_2O_2 accumulation within 1 or 3 h and indicated that LAA was a putative apoptosis initiator.

The aim of the present study was to identify early mechanisms whereby changes in the intracellular GSH/GSSG ratio and H_2O_2 level

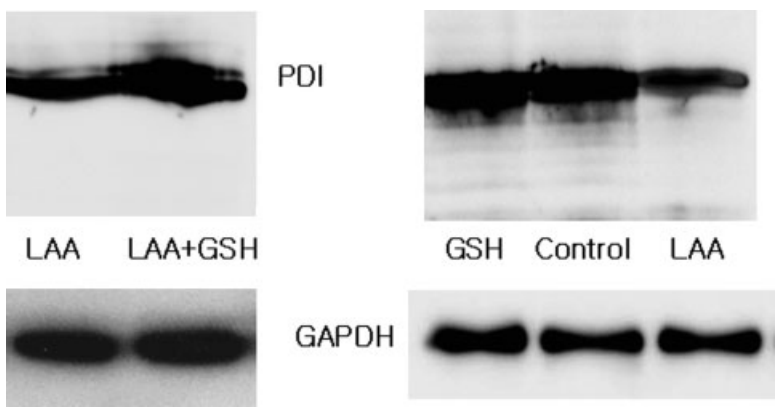

Fig. 9. Western blot analysis of PDI under the condition of GSH. NB4 cells were treated with 0.5 mM LAA for 30 min, and proteins were extracted and treated with 50 mM GSH. Proteins were separated by SDS–PAGE under non-reducing conditions and Western blots were probed with b-domain antibody.

induce a molecular response in the NB4 cell line, which is susceptible to LAA. First, we determined whether LAA treatment immediately changes the GSH oxidation state in the NB4 cell line. As shown in Figure 3, incubation with LAA for just 30 min decreased the GSH/GSSG ratio in a concentration-dependent manner. However, the amount of GSH per total amount of protein was not reduced indicating a change in oxidation state only. Just within 30 min, LAA-induced GSH/GSSG ratio reductions were found to be correlated with an increase in the intracellular H_2O_2 level in the NB4 cell line. In addition, a correlation between the GSH oxidation state and the inhibitory effect of LAA on the proliferation of NB4 cells was apparent (Fig. 4).

The proteomic approach was employed to screen the number of proteins differentially regulated by LAA treatment. The proteins identified are shown in Table I. One of the changes observed concerned post-translational modifications of tropomyosin. From our result, the acidic shift of the spot with a pI 5.0 (T) to the pT spot by the treatment with LAA was observed to be weakened by the co-treatment with the MEK inhibitor PD98059. Thus, tropomyosin was identified as a PD98059-sensitive phosphoprotein. Our previous study provided evidence that the ERK MAP kinase pathway is activated in response to LAA (at <1 mM) in AML cell lines [Park et al., 2005]. Moreover, LAA treatment of cells induced a dose-dependent phosphorylation of ERK and resulted in activation of its catalytic domain. Our data also demonstrated that the small G protein Raf1 and MAPK activated protein kinase 2 upstream and downstream regulators of ERK, respectively, are activated by LAA. Thus, it was ascertained that tropomyosin is phosphorylated by LAA-mediated oxidative stress and that this phosphorylation is the result of ERK activation. As judged by the present study, ERK activation induced by LAA treatment occurred within 30 min.

Tropomyosin-1 is known to associate with actin in vitro [Sen et al., 2001; Cooper, 2002]. However, the present study suggests that another tropomyosin isoform, not tropomyosin-1, is phosphorylated by LAA in non-muscle myeloid cells. Tropomyosin exists in a large number isoforms; over 20 have been identified [Perry, 2001]. Many of the isoforms are tissue and filament specific in their distribution. The tropomyosin identified in the present study was an unspecified isoform. Its sequence was registered as Q5HYB6, and identical with that of tropomyosin-3 except for the 87 amino acid residue. Our MALDI-TOF data showed a peak corresponding to a fragment containing Glu-87 instead of the Lys-87 found in the tropomyosin-3 isoform.

Here, we propose the phosphorylation of a tropomyosin isoform downstream of ERK in response to LAA in non-muscle myeloid cells. The significance of this is unclear but may be related to differences in the regulation of the actomyosin contractile system in non-muscle cells as compared with that present in muscle cells. One additional point worth noting is that the proteins, which specifically reacted with sera from chronic myeloid leukemia patients, included structural proteins, such as, beta-tubulin and tropomyosin isoforms [Zou et al., 2005]. Although the function of these proteins in myeloid leukemia needs further investigation, tropomyosin may have value as a leukemia-associated antigen and as a molecular target in antigen-based immunotherapy. From this point of view, it is important that LAA causes a tropomyosin isoform to be modified during the immediate early response.

The present results demonstrate that thiol/disulfide exchange proteins are regulated in NB4 cells after LAA exposure. This is not surprising because our study also shows that intracellular GSH/GSSG exchange occurs shortly after LAA exposure. Therefore, we speculate that the biochemical pathway leading to thiol/disulfide redox regulation could be triggered by LAA. PDI has two inter-related activities, that is, the ability to catalyze the formation, reduction, and isomerization of disulfide bonds, and the ability to bind to polypeptide chains [Freedman et al., 1994]. This latter activity enables PDI to function as a molecular chaperone and to assist in the folding of polypeptides. It has been suggested that PDI forms more permanent associations with specific proteins, for example, to become a subunit of prolyl 4-hydroxylase (P4H) and microsomal triglyceride transfer protein (MTP) [Pihlajaniemi et al., 1987; Wetterau et al., 1990]. The role of PDI as a molecular chaperone or as a polypeptide-binding protein is mediated primarily through interaction between its b domain and substrates. It has been suggested that this binding is regulated by the redox state of PDI, and that its association requires

the presence of GSH, and its dissociation the presence of glutathione disulfide [Freedman et al., 1994]. It has been reported that wild-type PDI is a homodimer in the active state, and that this 26-kDa PDI is a disulfide-linked dimer [Noiva, 1999]. Actually, when proteins from untreated or LAA-exposed cells were submitted to Western blot analysis using PDI antibody, which was generated against the b domain, 55, 26, and 13 kDa PDI subunits were detected. These species were ascribed to tetramer, homodimer and monomer in PDI, respectively. Moreover, the levels of the 26 and 55 kDa forms were lower in LAA-exposed cells than in control according to the 2-D gel data.

As shown in Figure 9, our data demonstrates that the binding between PDI domains and substrates is dependent on the thiol buffer condition, and that the interactions between PDI domains and substrates are increased and levels of liberated PDI domains are decreased in an intracellular GSH redox states favoring oxidation. When PDI was bound to its substrate as a chaperone, as previously suggested, it should be oxidized upon association. The decrease in the GSH/GSSG ratio following treatment with LAA observed in the present study could lead to oxidizing condition and an association of PDI with substrates. These relations are consistent with our results, which show a decrease in the GSH/GSSG ratio after LAA treatment led to a less liberated PDI domain.

PDI is a constituent of prolyl 4-hydroxylase (P4-H), an enzyme localized in the endoplasmic reticulum (ER), which catalyses the co- and post-translational hydroxylation of collagen and protein proline residues [Kivirikko et al., 1992]. Vertebrate P4-H consists of two distinct polypeptides, a catalytically more important, glycosylated α-subunit (64 kDa) and a β-subunit (60 kDa) which is identical to the multifunctional enzyme PDI. Moreover, unassembled α-subunits are maintained in an assembly competent form by interaction with the molecular chaperone BiP [John and Bulleid, 1996]. However, in the present study, we did not examine the assembly of P4-H from its constituent subunits. Nevertheless, considering the possible role of the LAA-mediated oxidation of sulfide on protein assembly/disassembly, the association of BiP with P4-H α-subunits appears to be dependent upon the formation of intramolecular disulfide bonds between β-subunits and other substrates. Moreover, if correct this speculation provides a reason why BiP protein is regulated by LAA.

REFERENCES

Bram S, Froussard P, Guichard M, Jasmin C, Augery Y, Sinoussi-Barre F, Wray W. 1980. Ascorbic acid preferential toxicity for malignant melanoma cells. Nature 284: 629–631.

Bruchelt G, Baader L, Reith AG, Holger NL, Gebhardt S, Niethammer D. 1993. Rationale for the use of ascorbic acid in neuroblastoma therapy. In: Schwab M, editor. Human Neuroblastoma. Newark: Harwood Academic Publishers; pp 34–40.

Bukau B, Horwich AL. 1998. The Hsp70 and Hsp60 chaperone machines. Cell 92:351–366.

Cooper JA. 2002. Actin dynamics: Tropomyosin provides stability. Curr Biol 12:R523–R525.

De Laurenzi V, Melino G, Savini I, Annicchiarico-Petruzzelli M, Finazzi-Agro A, Avigliano L. 1995. Cell death by oxidative stress and ascorbic acid regeneration in human neuroectodermal cell lines. Eur J Cancer 31A:463–466.

Freedman RB, Hirst TR, Tuite MF. 1994. Protein disulphide isomerase: Building bridges in protein folding. Trends Biochem Sci 19:331–336.

Fujinaga S, Sakagami H, Kuribayashi N, Takahashi H, Amano Y, Sakagami T, Takeda M. 1994. Possible role of hydrogen peroxide in apoptosis induction by ascorbic acid in human myelogenous leukemic cell lines. Showa Univ Med Sci 6:135–144.

Grad JM, Bahlis NJ, Reis I, Oshiro MM, Dalton WS, Boise LH. 2001. Ascorbic acid enhances arsenic trioxide-induced cytotoxicity in multiple myeloma cells. Blood 98:805–813.

Halliwell B, Foyer CH. 1976. Ascorbic acid, metal ions and the superoxide radical. Biochem J 155:697–700.

Heikkila RE, Cabbat FS. 1983. Ascorbate-induced lipid peroxidation and inhibition of [^{3}H]spiroperidol binding in neostriatal membrane preparations. J Neurochem 41: 1384–1392.

Houle F, Rousseau S, Morrice N, Luc M, Mongrain S, Turner CE, Tanaka S, Moreau P, Huot J. 2003. Extracellular signal-regulated kinase mediates phosphorylation of tropomyosin-1 to promote cytoskeleton remodeling in response to oxidative Stress: Impact on membrane blebbing. Mol Biol Cell 14:1418–1432.

Iwase H, Ono I. 1994. Determination of ascorbic acid in human plasma by high performance liquid chromatography with electrochemical detection using a hydroxyapatite cartridge for precolumn deproteinization. J Chromatogr B Biomed Appl 655:195–200.

John DC, Bulleid NJ. 1996. Intracellular dissociation and reassembly of prolyl 4-hydroxylase: The alpha-subunits associated with the immunoglobulin-heavy-chain binding protein (BiP) allowing reassembly with the beta-subunit. Biochem J 317:659–665.

Kemmink J, Darby NJ, Dijkstra K, Nilges M, Creighton TE. 1997. The folding catalyst protein disulfide isomerase is constructed of active and inactive thioredoxin modules. Curr Biol 7:239–245.

Kivirikko KI, Myllyla R, Pihlajaniemi T. 1992. Hydroxylation of proline and lysine residues in collagens and other

animal and plant proteins. In: Harding JJ, Crabbe MJC, editors. Post-translational modification of proteins. London: CRC Press, pp 1–51.

Liang WJ, Johnson D, Jarvis SM. 2001. Vitamin C transport systems of mammalian cells. Mol Membr Biol 18:87–95.

Meister A. 1994. Glutathione-ascorbic acid antioxidant system in animals. J Biol Chem 269:9397–9400.

Nishikimi M, Yagi K. 1991. Molecular basis for the deficiency in humans of gulonolactone oxidase, a key enzyme for ascorbic acid biosynthesis. Am J Clin Nutr 54: 1203S–1208S.

Noiva R. 1999. Protein disulfide isomerase: The multifunctional redox chaperone of the endoplasmic reticulum. Semin Cell Dev Biol 10:481–493.

Park CH. 1985. Biological nature of the effect of ascorbic acids on the growth of human leukemic cells. Cancer Res 45:3969–3973.

Park CH, Kimler BF, Bodensteiner D, Lynch SR, Hassanein RS. 1992. In vitro growth modulation by L-ascorbic acid of colony-forming cells from bone marrow of patients with myelodysplastic syndromes. Cancer Res 52:4458–4466.

Park S, Han SS, Park CH, Hahm ER, Lee SJ, Park HK, Lee SH, Kim WS, Jung CW, Park K, Riordan HD, Kimler BF, Kim K, Lee JH. 2004. L-Ascorbic acid induces apoptosis in acute myeloid leukemia cells via hydrogen peroxide-mediated mechanisms. Int J Biochem Cell Biol 36:2180–2195.

Park S, Park CH, Hahm ER, Kim K, Kimler BF, Lee SJ, Park HK, Lee SH, Kim WS, Jung CW, Park K, Riordan HD, Lee JH. 2005. Activation of Raf1 and the ERK pathway in response to l-ascorbic acid in acute myeloid leukemia cells. Cell Signal 17:111–119.

Perry SV. 2001. Vertebrate tropomyosin: Distribution, properties and function. J Muscle Res Cell Motil 22:5–49.

Pihlajaniemi T, Helaakoski T, Tasanen K, Myllyla R, Huhtala ML, Koivu J, Kivirikko KI. 1987. Molecular cloning of the beta-subunit of human prolyl 4-hydroxylase. This subunit and protein disulphide isomerase are products of the same gene. EMBO J 6:643–649.

Rosenfeld J, Capdevielle J, Guillemot JC, Ferrara P. 1992. In-gel digestion of proteins for internal sequence analysis after one- or two-dimensional gel electrophoresis. Anal Biochem 203:173–179.

Rumsey SC, Daruwala R, Al-Hasani H, Zarnowski MJ, Simpson IA, Levine M. 2000. Dehydroascorbic acid transport by GLUT4 in Xenopus oocytes and isolated rat adipocytes. J Biol Chem 275:28246–28253.

Sen A, Chen YD, Yan B, Chalovich JM. 2001. Caldesmon reduces the apparent rate of binding of myosin S1 to actin-tropomyosin. Biochemistry 40:5757–5764.

Speit G, Wolf M, Vogel W. 1980. The SCE inducing capacity of ascorbic acid: Investigations in vitro and in vivo. Mutat Res 78:273–278.

Stich HF, Karim J, Koropatnick J, Lo L. 1976. Mutagenic action of ascorbic acid. Nature 260:722–724.

Tsukaguchi H, Tokui T, Mackenzie B, Berger UV, Chen XZ, Wang Y, Brubaker RF, Hediger MA. 1999. A family of mammalian Na+-dependent L-ascorbic acid transporters. Nature 399:70–75.

Vera JC, Rivas CI, Fischbarg J, Golde DW. 1993. Mammalian facilitative hexose transporters mediate the transport of dehydroascorbic acid. Nature 364:79–82.

Wetterau JR, Combs KA, Spinner SN, Joiner BJ. 1990. Protein disulfide isomerase is a component of the microsomal triglyceride transfer protein complex. J Biol Chem 265:9801–9807.

Zou L, Wu Y, Pei L, Zhong D, Gen M, Zhao T, Wu J, Ni B, Mou Z, Han J, Chen Y, Zhi Y. 2005. Identification of leukemia-associated antigens in chronic myeloid leukemia by proteomic analysis. Leuk Res 29:1387–1391.