

Protein Kinase C Delta-mediated Cytoskeleton Remodeling Is Involved in Aloe-emodin-induced Photokilling of Human Lung Cancer Cells

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Abstract. Photodynamic therapy is becoming a widely accepted form of cancer treatment using a photosensitizing agent and light. Our previous study has demonstrated that photoactivated aloe-emodin induced anoikis and changes in cell morphology, which were in part mediated through its effect on cytoskeleton in lung carcinoma H460 cells. However, the molecular mechanisms of these photoactivated aloe-emodin-induced changes remain unknown. The present study demonstrated that the expression of protein kinase C δ (PKC δ) was triggered by aloe-emodin and irradiation in H460 cells. Furthermore, the photoactivated aloe-emodin-induced cell death and translocation of PKC δ from the cytosol to the nucleus was found to be significantly inhibited by rottlerin, a PKC δ -selective inhibitor. Western blot analysis demonstrated that rottlerin also reversed the decrease in protein expression of cytoskeleton-related proteins, such as rat sarcoma (RAS), ras homolog gene family member A (RHO), p38, heat shock protein 27 (HSP27), focal adhesion kinase (FAK), α -actinin and tubulin, induced by photoactivated aloe-emodin. Our findings suggest that the regulation of cytoskeleton-related proteins mediated by PKC δ may be the mechanisms for the protective effects of rottlerin against the photoactivated aloe-emodin induced H460 cell death.

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Photodynamic therapy is an effective therapy for local malignant tumors and involves a tumor-localizing photosensitizing agent, light and molecular oxygen (1, 2). Using fiberoptic bronchoscope to deliver light to tumor tissue, photodynamic therapy is a treatment option for lung cancer (3, 4).

Adequate fruit and vegetable consumption is considered important in preventing the development of carcinoma. Many dietary compounds have also been demonstrated to have anticancer activities, including genistein, resveratrol, diallyl sulfide, S-allyl cysteine, capsaicin and curcumin (5-10). Furthermore, since such dietary chemopreventive agents are derived from natural sources, they are considered pharmacologically safe. However, further exploration of the exact mechanism for cancer chemoprevention mediated by dietary compounds is needed.

Rheum palmatum rhubarb has been used in Chinese medicine primarily for its diuretic and purgative effects for a long time. Recently, it was also utilized as a herbal preparation for weight loss and slimming. In addition, since the stem of *Rheum palmatum* is very juicy with a sour taste, it is also used as a flavoring agent mainly for desserts, sauces, jams and jellies. Therefore, *Rheum palmatum* is extensively cultivated for both its culinary and its great medicinal advantages and uses.

Aloe-emodin, an active component of the root and rhizome of *Rheum palmatum*, has been demonstrated to possess antitumor effects (11, 12). It is important to determine the mechanisms of the anticancer activity of aloe-emodin for defining aloe-emodin derived from the natural plant as a promising chemopreventive agent against human cancer. Our previous study demonstrated that aloe-emodin is a photosensitizer and exerts antitumor activity against human lung carcinoma H460 cells (13). Furthermore, cytoskeletal alteration is potentially involved in anoikis, induced by photosensitized aloe-emodin (13).

The cytoskeleton plays important roles in the cellular division, intracellular transport and cell shape regulation. It has been shown that activation of focal adhesion kinase (FAK), and proto-oncogene serine/threonine-protein kinase (RAF) and ras homolog gene family member A (RHO) family proteins is associated with actin cytoskeleton assembly and these molecules have been implicated in the formation of actin stress and focal adhesion complexes (14-16). Protein kinase C delta (PKC δ) has been demonstrated to be involved in cytoskeleton regulation and apoptosis (17, 18). Apoptosis is a major form of cell death, which involves many factors and signal transduction molecules. Expression of PKC δ has been reported as a proapoptotic response to various apoptotic stimuli, such as radiation and chemotherapeutic agents (19, 20). The mechanisms by which PKC δ regulates cellular apoptosis and cytoskeleton have been investigated in many cell types, and may include proteolytic activation by caspase-3 and translocation of the activated PKC δ to the mitochondria and cytoskeleton (17, 20, 21).

Rottlerin, also called mallotoxin, is a natural compound isolated from the tree *Mallotus philippinensis* (the monkey-faced tree). Many studies have employed rottlerin as a PKC δ -selective inhibitor and mitochondrial uncoupler (21, 22). The inhibition of PKC δ is an important cellular mechanism leading to rottlerin-mediated antiapoptotic signaling pathway. Rottlerin was also reported to act as mitochondrial uncoupler, which can promote apoptosis (21, 22). In this study, we found that rottlerin blocked lung cancer H460 cell death induced by photoactivated aloe-emodin. Therefore, we used rottlerin to investigate the mechanisms of photoactivated aloe-emodin-induced H460 cell death.

The overall objective of the present study was to further explore the integrated mechanism of photoactivated aloe-emodin-induced apoptosis of human lung carcinoma H460 cells.

Materials and Methods

Materials. Aloe-emodin, antipain, aprotinin, dithiothreitol (DTT), ethyleneglycol-bis(β -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA), leupeptin, pepstatin, phenylmethylsulfonyl fluoride (PMSF), rottlerin (1-[6-[(3-acetyl-2,4,6-trihydroxy-5-methylphenyl)methyl]-5,7-dihydroxy-2,2-dimethyl-2H-1-benzopyran-8-yl]-3-phenyl-2-propen-1-one), 2-amino-2-hydroxymethyl-propane-1,3-diol (Tris) and tetramethylrhodamine isothiocyanate (TRITC)-conjugated phalloidin were purchased from Sigma Chemicals Company (St. Louis, MO, USA). FAK, β -actin and heat shock protein 27 (HSP27) antibodies were from Sigma Chemicals Company. RAS, RAF, RHO, p38, α -actinin and PKC δ antibodies were purchased from BD Biosciences (San Diego, CA, USA). Alpha-tubulin antibody was purchased from Calbiochem (San Diego, CA, USA). Horseradish peroxidase (HRP)-conjugated goat anti-mouse and -rabbit IgG were from Abcam (Cambridge, MA, USA).

Cell culture. H460 cells, obtained from American Type Culture Collection (ATCC, Manassas, VA, USA), were grown in monolayer

culture in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA) containing 5% fetal bovine serum (HyClone, Logan, UT, USA), 100 U/ml penicillin and 100 μ g/ml streptomycin (Invitrogen) and 2 mM glutamine (Merck, Darmstadt, Germany) at 37°C, in a humidified atmosphere comprised of 95% air and 5% CO₂. When H460 cells were treated with aloe-emodin, culture medium containing 1% fetal bovine serum was used.

Light source. The irradiation source was a set of fluorescent lamp (2×20 W; China Electric MFG Corporation, Taiwan, ROC) located in a made-to-measure box. The wavelength of the fluorescence lamp was in the range of 400-700 nm. The intensity of light was measured as Lux and Lux was converted to light dose (J/cm²). The cells were irradiated at 40 W for 60 min, corresponding to 1.6 J/cm² light dose.

Morphological investigation. Cells were seeded at a density of 7×10⁴ cells per well onto a 12-well plate 48 h before being treated. H460 cells were incubated with 0.1% dimethylsulfoxide or 20 μ M aloe-emodin for 2 h and then irradiated with a 1.6 J/cm² light dose. For rottlerin treatment, cells were pre-treated at a final concentration of 10 μ M rottlerin for 1 h. After irradiation, the cells were immediately photographed with an Olympus IX 70 (Olympus Optical Co., Germany) phase-contrast microscope. A field was chosen in the center of each well at approximately the same location for photography.

Cell viability assay. Cells were seeded at a density of 7×10⁴ cells per well onto a 12-well plate 48 h before being treated. After treatment, the cells were washed with phosphate-buffered saline (PBS). Cellular mitochondrial reductase activity of live H460 cells was determined by measuring the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). At each end point, the treatment medium was replaced with fresh serum-free medium containing 2.4×10⁻⁴ M MTT at pH 7.4. Cells were incubated with MTT medium for 1 h at 37°C. After solubilization in dimethylsulfoxide, the absorbance was measured at 550 nm.

Localization of F-actin. Cells grown on coverslips were incubated with 0.1% dimethylsulfoxide or 20 μ M aloe-emodin for 2 h and then irradiated with a 1.6 J/cm² light dose. For rottlerin treatment, cells were pre-treated with a final concentration of 10 μ M rottlerin for 1 h. After irradiation, the medium was aspirated and cells were fixed in 3.7% formaldehyde in PBS for 15 min, followed by 3 min in acetone at -20°C. Cells were permeabilized with 1% Triton X-100 in PBS for 10 min. The cells were then gently washed with PBS. To visualize F-actin, the cells were incubated with 1.9×10⁻⁷ M TRITC-phalloidin in PBS for 40 min at room temperature. After three washings in PBS, the cells were observed under fluorescence microscopy (Nikon H600L; Chiyoda-Ku, Tokyo, Japan).

Immunostaining. Cells grown on coverslips were incubated with 0.1% dimethylsulfoxide or 20 μ M aloe-emodin for 2 h and then irradiated with 1.6 J/cm² light dose. For rottlerin treatment, cells were pre-treated with a final concentration of 10 μ M rottlerin for 1 h. After irradiation, cells were washed with PBS, fixed with formaldehyde for 10 min and then permeabilized with 1% Triton X-100 in PBS for 10 min. Fixed cells were subsequently incubated with a blocking solution (2.5% bovine serum albumin) for 1 h at room temperature. Cells were then incubated for 1 h at 37°C with α -tubulin, α -actinin or PKC δ antibodies diluted at 1:50 in Tris-buffered saline (10 mM Tris-HCl

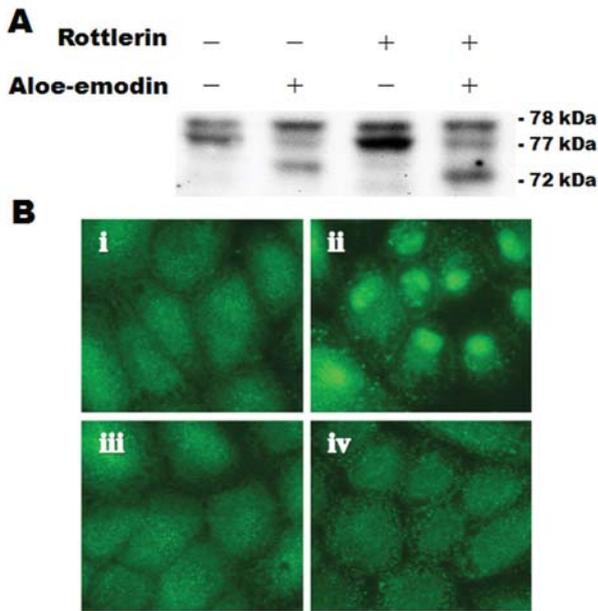


Figure 1. H460 lung cancer cells were incubated with 0.1% dimethylsulfoxide or 20 μ M aloe-emodin for 2 h and then irradiated with a 1.6 J/cm² light dose. For rottlerin treatment, cells were pre-treated with a final concentration of 10 μ M rottlerin for 1 h. A: The effect of photoactivated aloe-emodin on the protein expression of protein kinase C δ (PKC δ) in H460 cells. After irradiation, cell lysates were analyzed by 7% SDS-PAGE and then probed with an primary anti-PKC δ antibody. -: Control cells; +: aloe-emodin- or rottlerin-treated cells. B: Immunofluorescence localization of PKC δ in H460 cells. After irradiation, fixation and permeabilization, immunostaining of cells was performed with anti-PKC δ antibody, as described in the Materials and Methods. The specimens were observed under fluorescence microscopy. i: Control cells; ii: aloe-emodin-treated cells; iii: rottlerin-treated cells; iv: cells pre-treated with rottlerin and then with aloe-emodin. Results are representative of three independent experiments.

and 150 mM NaCl) with 0.05% Tween 20 (TBST). The cells were washed three times with TBST and incubated for 30 min at 37°C with fluorescein-conjugated anti-mouse or -rabbit IgG antibodies diluted at 1:50 in TBST. After washing with TBST, the specimens were mounted in glycerin and observed under fluorescence microscopy (Nikon H600L; Chiyoda-Ku, Tokyo, Japan).

Localization of microtubules. In this study, the detection of α - and β -tubulin was used to examine the microtubule formation in H460 cells. Cells grown on coverslips were incubated with 0.1% dimethylsulfoxide or 20 μ M aloe-emodin for 2 h and then irradiated with 1.6 J/cm² light dose. For rottlerin treatment, cells were pre-treated with a final concentration of 10 μ M rottlerin for 1 h. To visualize α -tubulin, the cells were incubated with a mouse anti- α -tubulin antibody, washed, and subsequently stained with FITC-conjugated goat anti-mouse IgG. To detect β -tubulin, the cells were incubated for 30 min at 37°C with 250 nM of the Tubulin Tracker Green reagent (Molecular Probes, Eugene, OR, USA). After three washings in PBS, the cells were observed under fluorescence microscopy (Nikon H600L; Chiyoda-Ku).

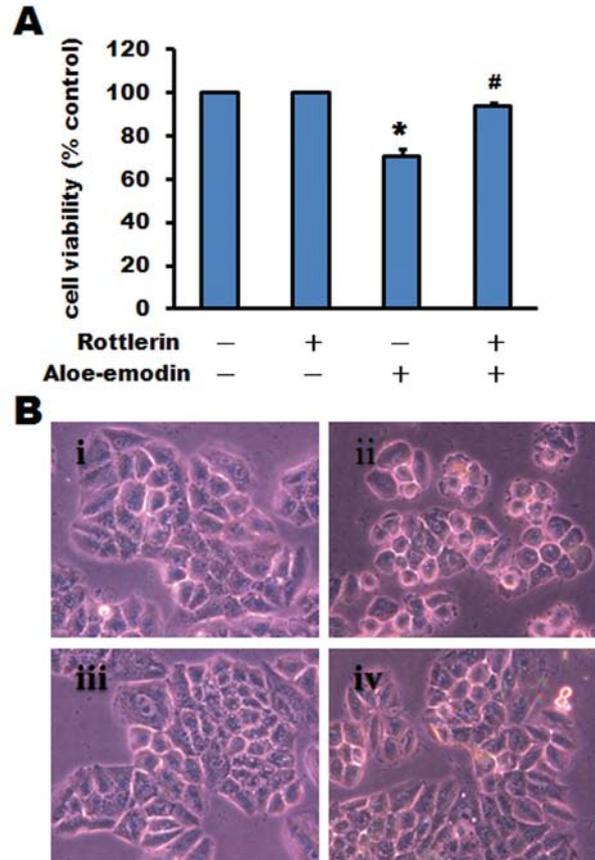


Figure 2. Effects of rottlerin on photoactivated aloe-emodin-induced cell death and changes in cell morphology. Cells were pre-treated with 10 μ M rottlerin for 1 h and then treated with 0.1% dimethylsulfoxide or 20 μ M aloe-emodin for 2 h and a 1.6 J/cm² light dose. A: After irradiation, the viable cells were measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Results are expressed as the mean percentage of the corresponding control \pm S.D. * p <0.01 compared to the control values; # p <0.01 compared to the photoactivated aloe-emodin-treated cells. B: Morphological analysis by phase-contrast microscopy of H460 cells. After irradiation, the cells were immediately photographed. i: Control cells; ii: aloe-emodin-treated cells; iii: rottlerin-treated cells; iv: cells pre-treated with rottlerin and then with aloe-emodin. All results are representative of three independent experiments.

Protein preparation. Cells were seeded at a density of 1.3×10^6 cells onto a 10-cm dish 48 h before being treated. H460 cells were incubated with 0.1% dimethylsulfoxide or 20 μ M aloe-emodin for 2 h and then irradiated with a 1.6 J/cm² light dose. For rottlerin treatment, cells were pre-treated with a final concentration of 10 μ M rottlerin for 1 h. After irradiation, adherent and floating cells were collected and washed twice in ice-cold PBS. Cell pellets were resuspended in cell lysis buffer consisting of 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, 0.25% sodium deoxycholate, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, 1 mM sodium orthovanadate, 1 mM sodium fluoride, 5 μ g/ml aprotinin, 5 μ g/ml leupeptin and 5 μ g/ml antipain, for 30 min at 4°C. Lysates were

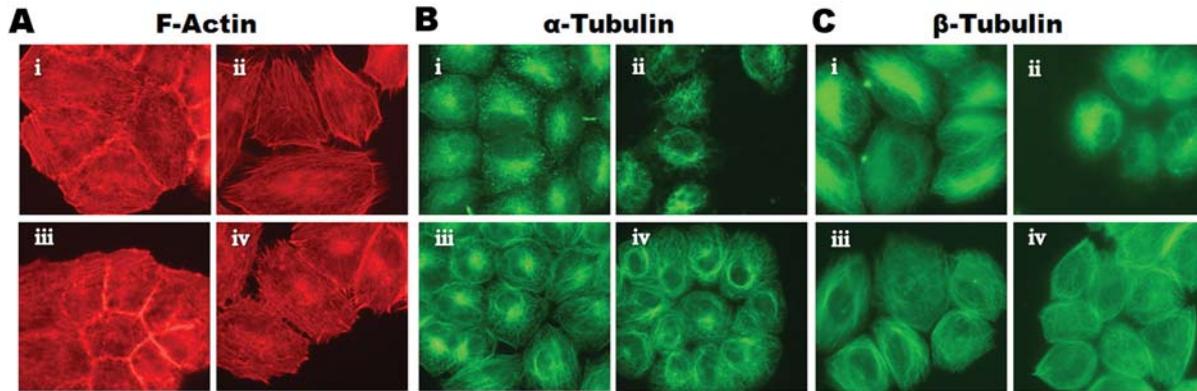


Figure 3. Effects of rotterlin on changes in cytoskeleton in H460 cells induced by photoactivated aloe-emodin. Cells were pre-treated with 10 μ M rotterlin for 1 h and then treated with 0.1% dimethylsulfoxide or 20 μ M aloe-emodin for 2 h and a 1.6 J/cm² light dose. A: F-Actin microfilaments in H460 cells were visualized by tetramethylrhodamine isothiocyanate (TRITC)-labeled phalloidin binding. B: Immunofluorescence localization of α -tubulin was performed with an anti- α -tubulin antibody, as described in the Materials and Methods. C: To detect β -tubulin distribution, cells were incubated for 30 min with 250 nM of the Tubulin TrackerTM Green reagent. The specimens were observed under fluorescence microscopy (\times 1000). i: Control cells; ii: aloe-emodin-treated cells; iii: rotterlin-treated cells; iv: cells pre-treated with rotterlin and then with aloe-emodin. Results are representative of three independent experiments.

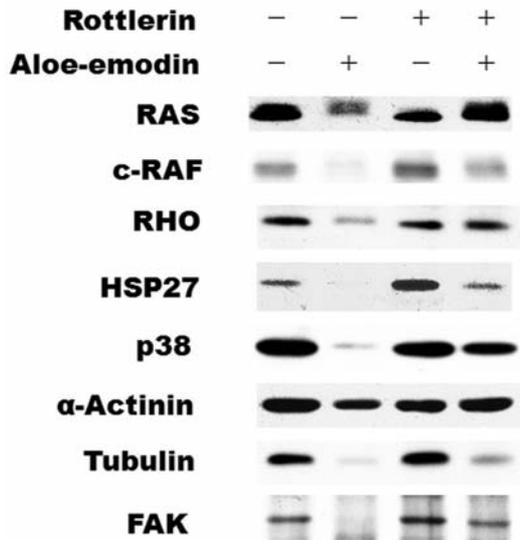


Figure 4. Effects of photoactivated aloe-emodin on the expression of cytoskeleton-related proteins in H460 cells. Cells were pre-treated with 10 μ M rotterlin for 1 h and then treated with 0.1% dimethylsulfoxide or 20 μ M aloe-emodin for 2 h and 1.6 J/cm² light dose. After irradiation, cell lysates were analyzed by 5% (focal adhesion kinase, FAK), 6% (α -actinin), 8% (proto-oncogene serine/threonine-protein kinase, Raf), 10% (α -tubulin), 12% (p38), 13% (heat shock protein 27, HSP27) and 14% (rat sarcoma, Ras and ras homolog gene family member A, Rho) sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then probed with primary antibodies. -: Control cells; +: aloe-emodin- or rotterlin-treated cells. Results are representative of three independent experiments.

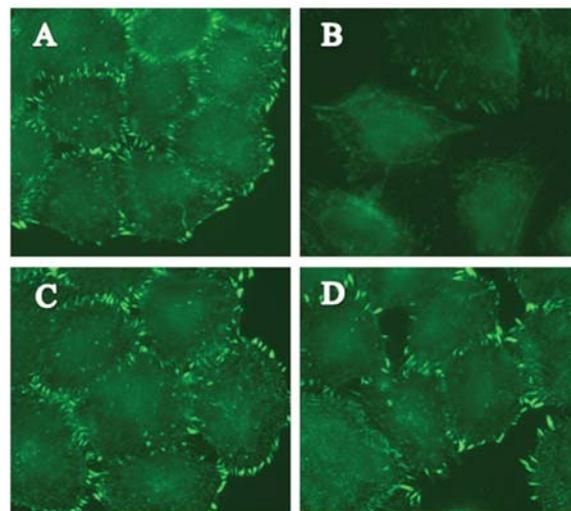


Figure 5. The effect of photoactivated aloe-emodin on the protein localization of α -actinin. The localization of α -actinin was performed by immunostaining in H460 cells. Cells were incubated with 0.1% dimethylsulfoxide or 20 μ M aloe-emodin for 2 h and then irradiated with a 1.6 J/cm² light dose. In rotterlin treatment, cells were pre-treated with a final concentration of 10 μ M rotterlin for 1 h. After irradiation, fixation and permeabilization, immunostaining of cells was performed with an anti- α -actinin antibody. The specimens were observed under fluorescence microscopy. A: Control cells; B: aloe-emodin-treated cells; C: rotterlin-treated cells; D: cells pre-treated with rotterlin and then with aloe-emodin. Results are representative of three independent experiments.

clarified by centrifugation at 1500 \times g for 30 min at 4°C and the resulting supernatant was collected, aliquoted and stored at -80°C until assay. The protein concentrations were measured with the Bradford method.

Western blot analysis. Samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; Bio-Rad, Hercules, CA, USA). The SDS-separated proteins were equilibrated in transfer buffer (50 mM Tris-HCl, pH 9.0-9.4, 40 mM glycine,

0.375% SDS and 20% methanol) and electrotransferred to Immobilon-P Transfer Membranes (Millipore Corporation, Bedford, MA, USA). The blot was blocked with a solution containing 5% non-fat dry milk in Tris-buffered saline (10 mM Tris-HCl and 150 mM NaCl) with 0.05% Tween 20 (TBST) for 1 h, washed and incubated with antibodies. Secondary antibodies consisted of a 1:20,000 dilution of HRP-conjugated goat anti-mouse IgG (for α -actinin, RAS, RAF, RHO, p38 and α -tubulin) or anti-rabbit IgG (for PKC δ , FAK and HSP27). The enhanced chemiluminescent (PerkinElmer Life and Analytical Sciences, Waltham, MA, USA) detection system was used for immunoblot protein detection.

Statistical analysis. All experiments were carried out at least three times. Each sample was tested in triplicate. The results are expressed as the mean percentage \pm S.D. of control. Statistically significant differences from the control group were identified by the Student's *t*-test for paired data. A *p*-value less than 0.05 was considered significant for the all tests.

Results

Protein kinase C delta expression is involved in aloe-emodin-induced photokilling in H460 cells. To investigate the role of PKC δ in modulating H460 cell apoptosis induced by photoactivated aloe-emodin, this study detected the protein expression of PKC δ by western blot analysis. After H460 cells were treated with 20 μ M aloe-emodin for 2 h and then irradiated with a 1.6 J/cm² light dose, the expression of PKC δ proteins significantly changed in cells treated with photoactivated aloe-emodin (Figure 1A). In control cells, PKC δ can be clearly identified as two distinct bands with molecular masses of about 77 kDa and 78 kDa, respectively. The amount of 78 kDa of PKC δ significantly increased after treatment of H460 cells with aloe-emodin and light, whereas the protein expression of 77 kDa of PKC δ was decreased (Figure 1A). Furthermore, photoactivated aloe-emodin induced a new protein band with a molecular weight of 72 kDa in this study (Figure 1A).

We also examined the effect of photoactivated aloe-emodin on the localization of the PKC δ in H460 cells after treatment with aloe-emodin and light, using immunofluorescence studies with anti-PKC δ antibody. Photoactivated aloe-emodin induced PKC δ translocation from the cytosol into the nucleus and nuclear PKC δ levels in H460 cells increased significantly (Figure 1B-ii). As shown in Figure 1B-i, PKC δ is localized diffusely throughout the cells, with no obvious organization in control cells.

Effects of rottlerin on H460 cell death induced by photoactivated aloe-emodin. The above data indicated that PKC δ protein is involved in photoactivated aloe-emodin-induced cell death. To further investigate whether H460 cell death, induced by treatment with aloe-emodin and light, could be linked to the activation of PKC δ expression, we examined the effect of PKC δ -selective inhibitor rottlerin on

photoactivated aloe-emodin-induced H460 cell death. As shown in Figure 2A, H460 cell death induced by photoactivated aloe-emodin was blocked by pre-treatment with 10 μ M rottlerin for 1 h. Furthermore, pre-treatment of H460 cells with 10 μ M rottlerin inhibited photoactivated aloe-emodin-induced changes in cell morphology (Figure 2B-iv). In western blot analysis, rottlerin had no significant effect on the decrease in protein expression of PKC δ mediated by photoactivated aloe-emodin (Figure 1A). It is noteworthy that the change in localization of PKC δ from the cytosol into the nucleus, commonly seen with photoactivated aloe-emodin, was largely prevented by rottlerin treatment (Figure 1B-iv).

Effects of rottlerin on the photoactivated aloe-emodin-induced changes in the localization of actin microfilaments in H460 cells. Our previous study demonstrated that the alteration of cytoskeleton is potentially involved in H460 cell anoikis induced by photosensitized aloe-emodin. In this study, the effect of rottlerin on the photoactivated aloe-emodin-induced changes in H460 cell actin microfilaments was examined. As shown in Figure 3A-iv, rottlerin (10 μ M, 1 h) had no significant effect on the photoactivated aloe-emodin-induced disruption of actin microfilaments. Rottlerin-treated cells had a similar appearance to those of the control (Figure 3A-iii). The effect of rottlerin on tubulin in H460 cell was also examined. Rottlerin (10 μ M) significantly inhibited photoactivated aloe-emodin-induced changes in the organization and distribution of tubulin (Figure 3B and C).

Effects of rottlerin on photoactivated aloe-emodin-induced changes in expression of cytoskeleton-related proteins in H460 cells. Photoactivated aloe-emodin-induced apoptosis is mediated by a change in the distribution of cytoskeleton-related proteins. To further investigate whether the regulation of cytoskeleton-related proteins was involved in prevention of H460 cells from photoactivated aloe-emodin-induced cell death of rottlerin, we focused our attention on the effect of rottlerin on the expression of cytoskeleton-related proteins, such as RAS, RHO, RAF, tubulin, FAK and α -actinin. As shown in Figure 4A, the expression of RAS, RAF, RHO, HSP27, p38, α -actinin, tubulin and FAK significantly decreased after treatment with 20 μ M aloe-emodin and irradiation with a 1.6 J/cm² light dose. These photoactivated aloe-emodin-induced decreases in protein levels were partly reversed by pre-treatment with 10 μ M rottlerin (Figure 4A). Since α -actinin binds cytoplasmic signaling proteins and may be critical for mediating cytoskeleton remodeling, the localization of α -actinin was examined by immunostaining during photosensitized aloe-emodin-induced H460 cell death. Figure 5A illustrates the distribution of α -actinin in H460 control cells, where α -actinin, organized into continuous aggregates, dispersed randomly throughout the cells and

aligned in parallel to each other, especially at the cell periphery. After treatment of H460 cells with 20 μM aloe-emodin and a 1.6 J/cm^2 light dose, α -actinin was diffusely localized throughout the cells, with no obvious organization (Figure 5B). However, changes induced by photoactivated aloe-emodin in the distribution of α -actinin were significantly reversed by rottlerin in this study (Figure 5D).

Discussion

Our previous study demonstrated that the cytoskeletal alteration is potentially involved in anoikis induced by photosensitized aloe-emodin in human lung carcinoma H460 cells. However, the regulatory mechanisms involved in this phenomenon are incompletely understood. PKC δ , a member of the PKC family, has been demonstrated to be involved in cytoskeleton regulation and apoptosis (17, 18). The present study demonstrated that the expression of PKC δ was involved in aloe-emodin-induced cell death. Furthermore, photoactivated aloe-emodin induced the formation of a new protein band with a molecular weight of 72 kDa. It is generally believed that the proteolytic activation of PKC δ is responsible for apoptotic execution (19, 20). In western blotting data, rottlerin had no effect on photoactivated aloe-emodin-induced changes in the expression of PKC δ in this study. However, it is noteworthy that the PKC δ translocation from the cytosol into the nucleus in H460 cells, induced by photoactivated aloe-emodin, was blocked by pre-treatment with 10 μM rottlerin. In addition, rottlerin also reversed the photoactivated aloe-emodin-induced cell death and changes in cell morphology in this study. Therefore, we suggest that the rottlerin-induced effects are associated with PKC δ translocation and assembly of cytoskeleton during cell death, induced by photoactivated aloe-emodin.

Several reports have indicated that the cytoskeleton may play a primary role in the initiation phase of apoptosis in certain circumstances (13, 23). Microtubules and actin microfilaments are the major proteins of cytoskeleton and both regulate cell shape. The involvement of the cytoskeleton in photoactivated aloe-emodin-induced apoptosis has been suggested by our previous study (13). In this study, rottlerin prevented photoactivated aloe-emodin-induced rearrangements of the microtubules, but not of actin microfilaments. This indicates that rottlerin, directly or indirectly, prevents the aggregation of cytoskeleton and stabilizes the H460 cell cytoskeleton. This finding suggests that the effect of rottlerin on photoactivated aloe-emodin-induced cell death and changes in cell morphology are mediated in part through its effect on cytoskeleton in H460 cells.

Alpha-actinin is an actin-cross-linking protein that may be one of the proteins involved in the attachment of the actin cytoskeletal framework to the plasma membrane. Our

previous study demonstrated that expression of the actin binding protein α -actinin was strongly correlated with the cell death of H460 cells, induced by photoactivated aloe-emodin (13). The present study has demonstrated that the changes in the distribution of α -actinin were significantly reversed by rottlerin, using western blotting analysis and immunostaining. It is well-known that α -actinin binds the cytoplasmic signaling proteins phosphatidylinositol 3-kinase, RHO effector kinase and integrins, the cytoplasmic domains of transmembrane receptors (14-16). The RHO family members are small GTPases and key regulators of actin dynamics, together with the related proteins, cell division control protein 42 homolog (CDC42), coordinating formation of stress fibers, focal adhesions, lamellipodia and filopodia, and thus regulating overall cellular movement and cell morphology (24, 25). In this study, there was a significant decrease in protein expression of RAS, RAF and RHO after treatment with aloe-emodin and light. This response was significantly antagonized by 1-h pre-treatment of the H460 cells with 10 μM rottlerin. The results of analysis of RAS, RAF and RHO protein expression are consistent with those of other studies reporting that the RAS superfamily is implicated in the regulation of cytoskeleton remodeling (14-16).

It has been suggested that p38-mediated F-actin reorganization is associated with translocation of HSP27 from the cytosol to the cytoskeleton (26). Our previous study has suggested that protein expression of p38 and HSP27 was involved in the photoactivated aloe-emodin-induced cell death (13). In this study, the photoactivated aloe-emodin-induced decrease in p38 and HSP27 protein expression was significantly reversed by rottlerin. We have also demonstrated that the photosensitized aloe-emodin-induced changes in the protein expression of cytoskeleton-related proteins, such as tubulin and FAK, were significantly reversed after pre-treatment of H460 cells with 10 μM rottlerin. These results suggested that regulation of cytoskeleton-related proteins may be mechanisms for the protective effects of rottlerin against the H460 cell death, induced by photoactivated aloe-emodin.

Overall, our findings suggested that promotion of the cytoskeleton-related signaling cascade following rottlerin treatment occurs by up-regulation of cytoskeleton-related mediators (RAS, RAF, RHO, p38, HSP27, FAK, α -actinin and tubulin), reducing H460 cell death, thus indicating that the cytoskeleton is the potential target of rottlerin on H460 cell apoptosis, induced by photoactivated aloe-emodin.

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References

- 1 Castano AP, Mroz P and Hamblin MR: Photodynamic therapy and anti-tumour immunity. *Nat Rev Cancer* 6: 535-545, 2006.
- 2 Morton CA, Brown SB, Collins S, Ibbotson S, Jenkinson H, Kurwa H, Langmack K, McKenna K, Moseley H, Pearse AD, Stringer M, Taylor DK, Wong G and Rhodes LE: Guidelines for topical photodynamic therapy: Report of a workshop of the British Photodermatology Group. *Br J Dermatol* 146: 552-567, 2002.
- 3 McCaughan JS Jr.: Survival after photodynamic therapy to non-pulmonary metastatic endobronchial tumors. *Lasers Surg Med* 24: 194-201, 1999.
- 4 Jones BU, Helmy M, Brenner M, Serna DL, Williams J, Chen JC and Milliken JC: Photodynamic therapy for patients with advanced non-small cell carcinoma of the lung. *Clin Lung Cancer* 3: 37-41, 2001.
- 5 Welch C, Wuarin L and Sidell N: Antiproliferative effect of the garlic compound S-allyl cysteine on human neuroblastoma cells *in vitro*. *Cancer Lett* 63: 211-219, 1992.
- 6 Han H, Zhong C, Zhang X, Liu R, Pan M, Tan L, Li Y, Wu J, Zhu Y and Huang W: Genistein induces growth inhibition and G₂/M arrest in nasopharyngeal carcinoma cells. *Nutr Cancer* 62: 641-647, 2010.
- 7 Bhattacharya S, Darjatmoko SR and Polans AS: Resveratrol modulates the malignant properties of cutaneous melanoma through changes in the activation and attenuation of the antiapoptotic protooncogenic protein AKT/PKB. *Melanoma Res* 21: 180-187, 2011.
- 8 Gogada R, Amadori M, Zhang H, Jones A, Verone A, Pitarresi J, Jandhyam S, Prabhu V, Black JD and Chandra D: Curcumin induces Apaf-1 dependent, p21-mediated caspase activation and apoptosis. *Cell Cycle* 10: 4128-4137, 2011.
- 9 Huh HC, Lee SY, Lee SK, Park NH and Han IS: Capsaicin induces apoptosis in cisplatin-resistant stomach cancer cells by causing degradation of cisplatin-inducible aurora-A protein. *Nutr Cancer* 63: 1095-1103, 2011.
- 10 Lai KC, Kuo CL, Ho HC, Yang JS, Ma CY, Lu HF, Huang HY, Chueh FS, Yu CC and Chung JG: Diallyl sulfide, diallyl disulfide and diallyl trisulfide affect drug resistant gene expression in colo 205 human colon cancer cells *in vitro* and *in vivo*. *Phytomedicine* 19: 625-630, 2012.
- 11 Pecere T, Gazzola MV, Mucignat C, Parolin C, Vecchia FD, Cavaggioni A, Basso G, Diaspro A, Salvato B, Carli M and Palù G: Aloe-emodin is a new type of anticancer agent with selective activity against neuroectodermal tumors. *Cancer Res* 60: 2800-2804, 2000.
- 12 Lai MY, Hour MJ, Leung WC, Yang WH and Lee HZ: Chaperones are the target in aloe-emodin-induced human lung non-small carcinoma H460 cell apoptosis. *Eur J Pharmacol* 573: 1-10, 2007.
- 13 Lee HZ, Yang WH, Hour MJ, Wu CY, Peng WH, Bao BY, Han PH and Bau DT: Photodynamic activity of aloe-emodin induces resensitization of lung cancer cells to anoikis. *Eur J Pharmacol* 648: 50-58, 2010.
- 14 Rodriguez-Viciano P, Warne PH, Khwaja A, Marte BM, Pappin D, Das P, Waterfield MD, Ridley A and Downward J: Role of phosphoinositide 3-OH kinase in cell transformation and control of the actin cytoskeleton by Ras. *Cell* 89: 457-467, 1997.
- 15 Hall A: Rho GTPases and the actin cytoskeleton. *Science* 279: 509-514, 1998.
- 16 Mackay DJ and Hall A: Rho GTPases. *J Biol Chem* 273: 20685-20688, 1998.
- 17 Liedtke CM, Hubbard M and Wang X: Stability of actin cytoskeleton and PKC δ binding to actin regulate NKCC1 function in airway epithelial cells. *Am J Physiol Cell Physiol* 284: C487-496, 2003.
- 18 You BJ, Wu YC, Bao BY, Wu CY, Yang YW, Chang YH and Lee HZ: Rottlerin inhibits *Lonicera japonica*-induced photokilling in human lung cancer cells through cytoskeleton-related signaling cascade. *Evid Based Complement Alternat Med* 2011;2011:193842. Epub 2011 Feb 8.
- 19 Reyland ME, Anderson SM, Matassa AA, Barzen KA and Quissell DO: Protein kinase C δ is essential for etoposide-induced apoptosis in salivary gland acinar cells. *J Biol Chem* 274: 19115-19123, 1999.
- 20 Mitsutake N, Namba H, Shklyayev SS, Tsukazaki T, Ohtsuru A, Ohba M, Kuroki T, Ayabe H and Yamashita S: PKC δ mediates ionizing radiation-induced activation of c-Jun NH(2)-terminal kinase through MKK7 in human thyroid cells. *Oncogene* 20: 989-996, 2001.
- 21 Xie MJ, Chang H, Wang YY, Zhang L, Song Z, Guo WG, Wang T, Che HL and Yu ZB: Evidence that apoptotic signalling in hypertrophic cardiomyocytes is determined by mitochondrial pathways involving protein kinase C δ . *Clin Exp Pharmacol Physiol* 37: 1120-1128, 2010.
- 22 Soltoff SP: Rottlerin is a mitochondrial uncoupler that decreases cellular ATP levels and indirectly blocks protein kinase C delta tyrosine phosphorylation. *J Biol Chem* 276: 37986-37992, 2001.
- 23 Godman G, Woda B, Kolberg R and Berl S: Redistribution of contractile and cytoskeletal components induced by cytochalasin. II. In HeLa and HEp2 cells. *Eur J Cell Biol* 22: 745-754, 1980.
- 24 Nobes CD and Hall A: Rho, Rac, and Cdc42 GTPases regulate the assembly of multimolecular focal complexes associated with actin stress fibers, lamellipodia, and filopodia. *Cell* 81: 53-62, 1995.
- 25 Hall A: Rho GTPases and the control of cell behaviour. *Biochem Soc Trans* 33: 891-895, 2005.
- 26 Okada T, Otani H, Wu Y, Kyo S, Enoki C, Fujiwara H, Sumida T, Hattori R and Imamura H: Role of F-actin organization in p38 MAP kinase-mediated apoptosis and necrosis in neonatal rat cardiomyocytes subjected to simulated ischemia and reoxygenation. *Am J Physiol Heart Circ Physiol* 289: H2310-2318, 2005.

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