Rhein Inhibits TNF- α -Induced Human Aortic Smooth Muscle Cell Proliferation via Mitochondrial-Dependent Apoptosis

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Key Words

Rhein • Apoptosis • Caspase activation • Mitochondrial pathway • Anti-atherosclerosis

Abstract

Background: Vascular smooth-muscle cell proliferation plays an important role in atherosclerosis and restenosis. Rhein is an active component extracted from rhubarb. In this study, rhein was found to exert potent inhibitory effects against tumor necrosis factor (TNF)- α -induced human aortic smooth-muscle cells (HASMCs) proliferation. **Method:** These effects were associated with induced apoptosis, including the induction of Annexin V-positive cells, the cleavage of poly(ADP-ribose)polymerase (PARP), and caspases 3, 8 and 9. **Results:** Inhibitors of caspases 3, 8 and 9 were efficiently blocked by rhein-induced apoptosis in TNF- α -treated HASMCs. In addition, treatment with rhein resulted in the release of cytochrome c into the cytosol, a loss of mitochondrial membrane potential ($\Delta\psi_m$), a decrease in Bcl-2 and Bcl-xL and an increase in Bax and Bak expression. However, rhein-mediated apoptosis was blocked by a mitochondrial membrane depolarization inhibitor. These findings indicate that rhein-induced apoptosis occurred via a mitochondrial pathway. Furthermore, the inhibition of mitochondrial membrane depolarization was efficiently blocked by rhein-in-

duced caspase-9 activity, which indicates that the rhein-induced caspase activation signal was downstream of the mitochondrial pathway. Taken together, the results of this study show that rhein inhibits TNF- α -induced HASMC proliferation via mitochondria-dependent apoptosis and that rhein has the potential to act as an anti-atherosclerosis agent.

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Introduction

The proliferation, differentiation or change in molecular regulation of vascular smooth muscle cells (VSMCs) induces diseases including atherosclerosis and restenosis [1, 2]. Following vascular injury, a loss of differentiated phenotype and a shift to a synthetic phenotype such as proliferation occurs [2, 3]. Vascular lesions form during several pathological processes, including the accumulation of inflammatory cells and the production of inflammatory cytokines such as tumor necrosis factor (TNF)- α , interleukin (IL)-8 and monocyte chemoattractant protein-1 (MCP-1) and so on [3]. Especially, TNF- α is secreted by VSMCs in the neointima after a balloon injury as well as by macrophages in atherosclerotic lesions [3, 4]. Previous studies have shown that TNF- α induces an in-

crease in DNA synthesis as well as the proliferation of VSMCs [5–9].

Apoptosis is one method of controlling immune responses. Apoptosis is characterized by a number of well-defined features, such as cellular morphological changes, chromatin condensation, oligonucleosomal DNA cleavage, membrane blebbing and activation of a family of cysteine proteases known as caspases [10]. Caspase activation is triggered in the mitochondrial-initiated pathway by the formation of a multimeric Apaf-1/cytochrome *c* complex that recruits and activates procaspase-9. Activated caspase-9 will then cleave and activate downstream caspases such as 3, 6, 7, 8 and 10 [11, 12]. Caspase-3, in turn, cleaves many substrates that respond to DNA-strand breaks, including poly(ADP-ribose)polymerase (PARP), and eventually leads to apoptosis [13]. Also, protein Bcl-2 families affect the integrity of mitochondria and caspase activation [14]. The Bcl-2 family consists of the pro-apoptotic proteins, Bax and Bak, as well as the anti-apoptotic proteins, Bcl-2 and Bcl-xL. Bax translocation directly induces the release of cytochrome *c* from mitochondria in apoptotic cells and triggers disruption of the mitochondrial membrane potential [15–17]. In contrast, Bcl-2 and Bcl-xL bind to mitochondria and inhibit the release of cytochrome *c* [14, 18].

Rhein (4, 5-Dihydroxyanthraquinone-2-carboxylic acid), a constituent enriched in the rhizome of rhubarb (*Rheum palmatum* L. or *Rheum tanguticum* Maxim, daehwang in Korean), is a traditional Chinese herb that is used as a laxative and stomach drug [19]. It is well known that rhein has anticancer activity in various cells, including human glioma cells [20], tumor cells in the rat liver [21], Ehrlich ascites tumor cells [22], human colonic adenocarcinoma [23], the mouse epidermal cell line JB6 [19], human promyelocytic leukemia HL-60 cells [24] and human cervical cancer Ca Ski cells [25]. However, little is known about the anti-atherogenic effects of rhein. Therefore, in this study, we evaluated rhein to determine if it inhibits TNF- α -induced human aortic smooth-muscle cell (HASMC) proliferation via a mitochondria-dependent apoptotic pathway.

Materials and Methods

Reagents

Rhein was purchased from Sigma Chemical Co. (St. Louis, Mo., USA). Recombinant human TNF- α was purchased from R&D Systems (Minneapolis, Minn., USA) and diluted in 0.1% bovine serum albumin (BSA)-phosphate-buffered saline (PBS) buffer. A CellTiter 96® Aqueous One Solution cell proliferation assay

(MTS) kit was purchased from Promega (Madison, Wisc., USA). Aprotinin, leupeptin, DMSO, dithiothreitol (DTT), Ac-DEVD-CHO, DiOC₆(3) dye, bongkrekic acid (BA) and phenylmethylsulfonyl fluoride (PMSF) were purchased from Sigma Chemical Co. Z-IETD-FMK and Z-LEHD-FMK were purchased from R&D Systems. Anti-Bcl-xL, Bax, cleaved PARP, β -actin monoclonal antibody (mAb) and cleaved caspase-3, 8, 9 were purchased from Cell Signaling Technology (Beverly, Mass., USA). Anti-Bak mAb, anti-rabbit IgG horseradish peroxidase (HRP) and anti-mouse IgG HRP were purchased from Santa Cruz Biotechnology (Santa Cruz, Calif., USA). Anti-cytochrome *c* and Bcl-2 mAb were purchased from BD Pharmingen (San Jose, Calif., USA). The caspase-9 assay system (colorimetric; Ac-LEHD-pNA) was purchased from Biomol (Plymouth Meeting, Pa., USA). A BrdU-(5'-Bromo-2-deoxyuridine) ELISA assay kit was purchased from Roche Diagnostics (Mannheim, Germany). A vibrant apoptosis assay kit was purchased from Invitrogen (Carlsbad, Calif., USA).

Cell Cultures

HASMCs, smooth muscle cell medium (SMCM), and SMC growth supplement were purchased from ScienCell (Carlsbad, Calif., USA). According to the manufacturer's instructions, SMCM is a complete medium designed for optimal growth of normal human SMCs in vitro. It contains essential and nonessential amino acids, vitamins, organic and inorganic compounds, hormones, growth factors, trace minerals and a low concentration of fetal bovine serum (2%). Thus, HASMCs were grown in SMCM including growth supplement and a low concentration of fetal bovine serum (2%) at 37°C in a humidified 95% air/5% CO₂ atmosphere. For all experiments, HASMCs were grown to 80–90% confluence and then made quiescent by starvation for at least 24 h.

Cell Proliferation Assays

Cell proliferation was measured with a CellTiter 96 colorimetric assay using an MTS tetrazolium compound according to the manufacturer's instructions. Briefly, cells were seeded in 96-well culture plates in SMCM, and then treated as described above for 24 h. Assays were then performed by adding [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-ulfenyl)-2H-tetrazolium] (MTS) directly to the culture wells, followed by incubation for 4 h. In addition, cell proliferation was measured by a BrdU-(5'-Bromo-2-deoxyuridine) ELISA assay (cell proliferation ELISA BrdU), which was performed according to the instructions of the manufacturer. Briefly, cells were seeded in 96-well culture plates in SMCM, and were then treated as described above for 24 h. Assays were then performed by adding BrdU (10 μ M, 2 h). For the BrdU technique, after cell fixation, DNA denaturation, and blocking, the cells were incubated with an anti-BrdU monoclonal antibody coupled to peroxidase and 3,3',5,5'-tetramethylbenzidine. The absorbance of both samples at 490 nm was then recorded using an enzyme-linked immunosorbent assay reader, Opsys MR (Dynex Technologies, Chantilly, Va., USA). The results were expressed as percentage changes from basal conditions using 3 to 5 culture wells for each experimental condition.

Analysis of Mitochondrial Membrane Potential

The mitochondrial membrane potential ($\Delta\psi_m$) was assessed based on the retention of DiOC₆(3). Cationic lipophilic fluorochrome DiOC₆(3) is a cell-permeable marker that specifically ac-

cumulates in the mitochondria depending on $\Delta\psi_m$ [24]. HASMCs (3×10^5 /ml) were treated with various concentrations of rhein for 24 h. The cells were then washed once in 0.2% BSA-PBS, after which they were incubated with $10 \mu\text{M}$ DiOC₆(3) for 30 min at 37°C. Mitochondrial depolarization is indicated by a decrease in the DiOC₆(3) fluorescence intensity. In addition, in some experiments, $10 \mu\text{M}$ of Ac-DEVD-CHO, Z-IETD-FMK and Z-LEHD-FMK were added to HASMCs for 30 min prior to rhein and/or TNF- α treatment. After 24 h, the cells were stained with $10 \mu\text{M}$ DiOC₆(3) and then subjected to FACS analysis. As many as 1×10^4 cells were analyzed at FL1 using a Becton Dickinson FACS-Calibur flow cytometer with the detection wavelength between 515 and 550 nm after excitation at 488 nm, and Cell-Quest Pro software (BD Biosciences, San Jose, Calif., USA).

Determination of Apoptosis by Flow Cytometry

To detect externalized phosphatidylserine (PS) as an early indication of apoptosis, HASMCs (3×10^5 /ml) were incubated with various concentrations of rhein for 24 h at 37°C. The cells were then harvested, washed twice with FACS buffer (0.2% BSA-PBS), stained with Annexin V-FITC and propidium iodide (PI) according to the protocol of the manufacturer, and then analyzed with a Becton Dickinson FACSCalibur flow cytometer and Cell-Quest Pro software. BA ($50 \mu\text{M}$) was added to the HASMCs for 2 h before rhein and/or TNF- α treatment in order to inhibit disruption of the mitochondrial membrane potential (MMP). After 24 h, the cells were stained with annexin V-FITC and PI for FACS analysis [26]. In some experiments, $10 \mu\text{M}$ Ac-DEVD-CHO, Z-IETD-FMK and Z-LEHD-FMK were added to HASMCs for 30 min prior to rhein and/or TNF- α treatment. After 24 h, the cells were stained with Annexin V-FITC and subjected to FACS analysis.

Preparation of Cytosolic Extractions for Cytochrome c Analysis

The cells were washed with ice-cold PBS, resuspended in cold lysis buffer (250 mM sucrose, 20 mM HEPES, 10 mM KCl, 1.5 mM MgCl₂, 1 mM EGTA, 1 mM EDTA, 1 mM DTT, 0.1 mM PMSF, $10 \mu\text{g/ml}$ leupeptin, and $10 \mu\text{g/ml}$ aprotinin) and then incubated on ice for 30 min. Next, the cells were homogenized with 15 strokes using a dounce homogenizer, after which they were centrifuged at $1,200 \text{ g}$ for 15 min at 4°C. The supernatants were then transferred to another tube and centrifuged at $100,000 \text{ g}$ for 60 min at 4°C, after which they were analyzed by 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The release of cytochrome c was analyzed by immunoblotting using an anti-cytochrome c mAb. In some experiments, BA ($50 \mu\text{M}$) was added to the HASMCs for 2 h prior rhein and/or TNF- α treatment. After 24 h, the cells were washed with ice-cold PBS and then resuspended in a cold lysis buffer for analysis by immunoblotting using an anti-cytochrome c mAb.

Preparation of Total Cell Extracts and Western Blotting

After HASMCs were cultured with a variety of concentrations of rhein and/or 10 ng/ml TNF- α for 24 h, they were washed 3 times with ice-cold PBS. The cells were then lysed in RIPA buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 10 mM NaF, 1 mM Na₃VO₄, 2 mM EDTA, 1% NP-40, and various protease inhibitors). Next, the cells were centrifuged at $12,000 \text{ g}$ for 15 min at 4°C, after which the supernatants were collected and referred to

as the cell extract. The protein concentrations were then measured using Bradford protein dye reagent (Bio-Rad). Next, the total cell lysates from the HASMCs were separated by approximately 10–12.5% SDS-PAGE, after which the proteins were electrophoretically transferred onto nitrocellulose membranes. The membranes were then blocked with 5% non-fat dried milk in Tris-buffered saline, and subsequently probed with primary antibodies, anti-cleaved PARP, anti-Bcl-xL, anti-Bax and anti-Bcl-2 in Tris-buffered saline containing 3% non-fat dried milk. Antibody-antigen complexes were then detected using goat anti-mouse IgG or goat anti-rabbit IgG-HRP-conjugated antibodies and an enhanced chemiluminescence detection kit.

Intracellular Staining for Bak Expression

HASMCs were incubated with various concentrations of rhein and/or 10 ng/ml TNF- α for 24 h at 37°C. The cells were then harvested and washed twice with FACS buffer (0.2% BSA-PBS). Next, cells were fixed with 4% paraformaldehyde in PBS, after which they were permeabilized with 0.1% Triton X-100 in PBS. The cells were then stained with anti-Bak mAb or isotype control mAb at 4°C for 30 min, after which they were analyzed with a Becton Dickinson FACSCalibur flow cytometer and Cell-Quest Pro software. The percentage of positive cells was recorded for each sample. In some experiments BA ($50 \mu\text{M}$) was added to HASMCs for 2 h prior to rhein and/or TNF- α treatment in order to inhibit disruption of the mitochondrial membrane potential.

Measurement of Caspase-9 Activity

Caspase activity was measured as described previously [27]. To accomplish this, the caspase-9 activity was determined by measuring the absorbance at 405 nm after cleavage of the Ac-LEHD-pNA substrate using the caspase-9 assay system and a colorimetric kit, respectively. Briefly, HASMCs were adjusted to 10^6 cells/ml, and various concentrations of rhein (0, 0.1, 1, 10 ng/ml), 10 ng/ml TNF- α , or anti-caspase-9 mAb ($5 \mu\text{M}$) were then added according to the experimental design (positive controls). The mixtures were then incubated for 24 h at 37°C, after which the cells were harvested by centrifugation at 2,000 rpm for 5 min at 4°C, washed twice with ice-cold PBS, and then resuspended in cell lysis buffer. The cells were then lysed by repeated freezing and thawing, followed by incubation on ice for 15 min. Next, the cell lysates were centrifuged at $16,000 \text{ g}$ for 15 min at 4°C, after which the supernatant fraction (cell extract) was collected. The caspase activities of the cell extracts were then measured in a total volume of $100 \mu\text{l}$ in 96-well plates that contained at least 1×10^6 cells/assay. Triplicate wells were prepared, with each set containing a blank, a negative control, induced extracts and inhibited extracts, according to the instructions of the manufacturer. Cell extracts were used as an enzyme source. Each substrate ($10 \mu\text{l}$) was added to all wells and the plate was then covered with Parafilm laboratory film and incubated at 37°C for 4 h. The absorbance was then read in the wells at 405 nm using an ELISA reader (Opsys). The enzyme activity was then calculated from a standard curve prepared using pNA standard. The relative levels of pNA were normalized against the protein concentration of each extract. The specific activity of caspase-9 was then calculated according to the number of micrograms of protein in each $100 \mu\text{l}$ sample volume, which was determined by the Bradford method using a BSA standard curve.

Fig. 1. Effect of HASMC proliferation induced by TNF- α and rhin. Arrested HASMCs were stimulated with 0.1, 1, or 10 μ M rhin for 30 min prior to treatment without (a) or with (b) 10 ng/ml TNF- α for 24 h. The control group was not treated with TNF- α or rhin. HASMC proliferation was evaluated by a CellTiter 96 cell proliferation assay (upper panel) and by a BrdU-(5'Bromo-2-deoxyuridine) ELISA assay (lower panel). Data shown are representative of at least 3 independent experiments. Data represent the means $\pm$ SEM. *** $p < 0.001$ vs. control (0 ng/ml TNF- α ; a) and TNF- α alone group (b).

Statistics

The data presented represent the means $\pm$ SEM of at least three independent experiments. All values were evaluated by one-way ANOVA followed by Duncan's multiple range tests using the GraphPad Prism 4.0 software. Differences were considered significant at $p < 0.05$.

Results

Rhein Inhibits the Proliferation of HASMCs

We investigated the proliferation of HASMCs in response to treatment with 0, 1, 10 and 100 ng/ml of TNF- α . The results of a CellTiter 96 cell proliferation assay revealed that TNF- α -stimulated the proliferation of HASMCs, with 10 ng/ml of TNF- α producing the maximum proliferation (fig. 1a). However, rhin significantly inhibited the TNF- α -induced proliferation in a dose-dependent manner, with only 33, 42 and 58% of the proliferation induced by treatment with TNF- α alone being observed in cells that were treated with TNF- α and rhin at final concentrations of 0.1, 1 and 10 μ M, respectively (fig. 1b, upper panel). In addition, the effect of rhin on TNF- α -induced proliferation was confirmed by a BrdU-

(5'Bromo-2-deoxyuridine) ELISA assay. Treatment with 0.1, 1 and 10 μ M rhin significantly and dose-dependently decreased the proliferation of HASMCs to 34, 36 and 48% of that of cells that were treated with TNF- α alone, respectively (fig. 1b, lower panel). In addition, treatment with rhin alone (10 μ M) had no effect on the proliferation or cytotoxicity of HASMCs (fig. 1b).

Rhein Induces Apoptosis in HASMCs Dependent on Caspase Activation

Cells undergoing apoptosis showed characteristic changes such as nuclear condensation, DNA fragmentation and translocation of PS to the outer leaflets of the plasma membrane [13, 26]. Apoptosis was measured by annexin V staining of PS following rhin treatment. HASMCs were incubated with TNF- α (10ng/ml) and various concentrations of rhin (0, 0.1, 1, 10 μ M) for 24 h. Rhin markedly induced apoptosis in TNF- α -treated HASMCs at a concentration of 10 μ M (0 μ M 1%, 0.1 μ M 21%, 1 μ M 32%, 10 μ M 71%; fig. 2a). To further confirm the apoptosis induced by rhin in TNF- α -treated HASMCs, we investigated the cleavage of PARP, which responds to DNA strand breaks and is used as another

Fig. 2. Rhein induces apoptosis in TNF- α -treated HASMCs. HASMCs were incubated with the indicated concentrations of rhein for 24 h. **a** Rhein dose-dependently induced apoptotic cells. Apoptosis was measured by staining with FITC-labeled annexin V and PI, followed by flow cytometric analysis and determination of the percentage of apoptotic cells. Normal = Annexin V negative and PI negative; early apoptosis = annexin V positive and PI negative; late apoptosis/necrosis = annexin V positive and PI positive; others = annexin V negative and PI positive. **b** Rhein induces PARP cleavage. HASMCs were incubated with the indicated concentrations of rhein for 24 h. Whole-cell lysate was obtained and used for Western blot analysis with anti-cleaved PARP mAb, as described in Materials and Methods. The membrane was stripped and reprobed with anti- β -actin mAb to confirm equal loading. Data shown are representative of at least 3 independent experiments. Data represent the means $\pm$ SEM. *** $p < 0.001$ vs. TNF- α alone.

hallmark of apoptosis. As shown in figure 2b, the cleavage of PARP was detected after 24 h of exposure to 0.1 μ M of rhein. The most prominent PARP activity of rhein in TNF- α -treated HASMCs was attained at a concentration of 10 μ M (relative band density with 10 μ M rhein was 190-fold that of the control or TNF- α alone group), while treatment with rhein alone (10 μ M) had no effect on the PARP cleavage of HASMCs (fig. 2b).

It has been reported that caspases 3, 8 and 9 play pivotal roles in the terminal phase of apoptosis [24]; therefore, the rhein-induced activation of these caspases was

investigated. Treatment with rhein for 24 h induced the cleavage and activation of caspases 3, 8 and 9 in a dose-dependent manner (fig. 3). The appearance of cleavage forms and the activities of the 3 caspases were observed after treatment with 0.1 μ M of rhein and the maximum caspase activation was observed in response to treatment with 10 μ M rhein (fig. 3). The relative band density is shown in figure 3b. Taken together, these data suggest that a caspase-dependent pathway is involved in rhein-induced apoptosis.

Fig. 3. Rhein induces activation of TNF- α -treated HASMCs by caspases 3, 8 and 9. HASMCs were treated with rhein (0, 0.1, 1, 10 μ M) for 24 h. Whole cell lysates were then obtained and used for Western blot analysis with various specific antibodies, as described in Materials and Methods (**a**). **b** Relative band density (fold of treatment with TNF- α alone). The membrane was stripped and reprobed with anti- β -actin mAb to confirm equal loading. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Furthermore, using inhibitors of caspases 3, 8 and 9, we confirmed that rhein-induced apoptosis is involved in the caspase pathway in TNF- α -treated HASMCs. HASMCs were preincubated with a caspase-3 inhibitor, Ac-DEVD-CHO, a caspase-8 inhibitor, Z-IETD-FMK, or a caspase-9 inhibitor, Z-LEHD-FMK, prior to stimulation with rhein and TNF- α , after which the presence of apoptotic cells was determined. Inhibitors of caspases 3, 8 and 9 efficiently blocked rhein-induced apoptosis in TNF- α -treated HASMCs (percent of apoptotic cells: 10 μ M rhein alone 71%, with 10 μ M Ac-DEVD-CHO 28%, with 10 μ M Z-IETD-FMK 17%, with 10 μ M Z-LEHD-FMK 23%). The caspase-3 inhibitor, Ac-DEVD-CHO, inhibited rhein-induced apoptosis by up to 61% in TNF- α -treated HASMCs. The caspase-8 inhibitor, Z-IETD-FMK, and the caspase-9 inhibitor, Z-LEHD-FMK, also inhibited rhein-induced apoptosis by up to 77% and 66%, respectively, in TNF- α -treated HASMCs. Taken together, these findings indicate that rhein-induced cell death occurred via caspase-dependent apoptosis (fig. 4).

Rhein Induces Apoptosis via a Mitochondrial Pathway

Mitochondria play a very important role in apoptosis [28]; therefore, we examined the effect of rhein on the $\Delta\psi_m$ of TNF- α -treated HASMCs by using a dye sensitive to potentials, DiOC₆(3). A remarkable loss of $\Delta\psi_m$ was observed at 24 h in response to treatment with 10 μ M rhein, while treatment rhein alone (10 μ M) had no effect on the $\Delta\psi_m$ of HASMCs (fig. 5a). Additionally, it has been shown that translocation of cytochrome *c* from the mitochondria is essential to apoptotic signaling, because it induces apoptosis via the activation of caspase [29]. As shown in figure 5b, a dose-dependent accumulation of cytochrome *c* in the cytosol was detected in rhein/TNF- α -treated cells, while an efflux was simultaneously observed in the mitochondria. To inhibit MMP disruption, BA (50 μ M) was added to HASMCs for 2 h prior to rhein and/or TNF- α treatment. After 24 h, the cells were analyzed by immunoblotting using an anti-cytochrome *c* mAb. BA efficiently blocked the rhein-induced release of cytochrome *c* in TNF- α -treated HASMCs (relative band density: with 10 μ M rhein 99.5%, with 50 μ M BA 0.5%; fig. 5b). In addition, BA efficiently inhibited rhein-in-

Fig. 4. Caspases 3, 8 and 9 are essential to rhein-induced apoptosis. HASMCs were preincubated with a caspase-3 inhibitor, Ac-DEVD-CHO (10 μ M), a caspase-8 inhibitor, Z-IETD-FMK (10 μ M), or caspase-9 inhibitor, Z-LEHD-FMK (10 μ M), for 1 h, after which they were stimulated with rhein (10 μ M) and TNF- α (10 ng/ml) for 24 h and then harvested. Cells (3×10^5) were stained with FITC-labeled annexin V, followed by FACS analysis. The filled histogram shows apoptotic cells in response to treatment with 10 μ M rhein in TNF- α -treated HASMCs; the open histogram indicates the presence of the indicated caspase inhibitor, Ac-DEVD-CHO (10 μ M), Z-IETD-FMK (10 μ M), or Z-LEHD-FMK (10 μ M) (upper panel). The lower panel shows data representing the percentage of apoptotic cells. Data shown are representative of at least 3 independent experiments and represent the means $\pm$ SEM. *** $p < 0.001$ vs. TNF- α alone.

duced apoptotic cells by up to 99.5% in TNF- α -treated HASMCs, while treatment with BA alone (50 μ M) had no effect on the apoptosis of HASMCs (fig. 5c). The results suggest that rhein causes an efflux of cytochrome *c* from mitochondria via a loss of $\Delta\psi_m$ and induces apoptosis via a mitochondrial pathway.

Mitochondrial membrane permeability is regulated by the equilibrium between pro-apoptotic and anti-apoptotic members of the Bcl-2 family. Mitochondrial apoptotic pathways can be negatively regulated by the over-expression of anti-apoptotic proteins such as Bcl-2 and Bcl-xL [18, 30, 31]. Conversely, translocation of the

pro-apoptotic protein, Bax, to the mitochondrial membrane induces the release of cytochrome *c* [15–17, 32]. We determined that rhein may elicit MMP disruption by regulating the expression of Bcl-2 family members. As shown in figure 6a, the pro-apoptotic protein, Bax, in TNF- α -treated HASMCs increased as the concentration of rhein increased (relative band density with 10 μ M rhein and TNF- α was 3.6-fold that of control groups or the group treated with TNF- α alone). By comparison, the anti-apoptotic proteins, Bcl-2 and Bcl-xL, decreased as the concentration of rhein increased (relative band density with 10 μ M rhein and TNF- α showed 80–90%

Fig. 5. Rhein-induced apoptosis involves the mitochondrial pathway. **a** HASMCs were treated with 0.1, 1, or 10 μM rhein in the presence or absence of TNF- α (10 ng/ml) for 24 h, and then harvested. Cells (3×10^5) were stained with DiOC₆(3) dye, followed by FACS analysis. **b** TNF- α -treated HASMCs were incubated with 0.1, 1, and 10 μM rhein in the presence or absence of BA (50 μM) for 24 h, after which they were harvested. The mitochondrial and cytosolic fractions were then separated, after which 30 μg of each sample was analyzed for the expression of cytochrome *c*. The membrane was stripped and reprobed with anti- β -actin mAb to confirm equal loading. **c** TNF- α -treated HASMCs were incubated with rhein (10 μM) in the presence or absence of BA (50 μM) for 24 h, and then harvested. Cells (3×10^5) were stained with FITC-labeled annexin V and PI, followed by FACS analysis. Data show the percentage of apoptotic cells. Normal = Annexin V negative and PI negative; early apoptosis = annexin V positive and PI negative; late apoptosis/necrosis = annexin V positive and PI positive; others = annexin V negative and PI positive. Data shown are representative of at least 3 independent experiments and represent the means $\pm$ SEM. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ vs. TNF- α alone.

Fig. 6. Rhein regulates the expression of Bcl-2 family proteins. TNF- α -treated HASMCs were incubated with the indicated dosages of rhein for 24 h at 37°C. The supernatants of the cell lysates were then analyzed by Western blotting for the expression of Bax, Bcl-2, and Bcl-xL. The membrane was stripped and reprobed with anti- β -actin mAb to confirm equal loading. **a** Dose-dependent responses of Bax, Bcl-2, and Bcl-xL. The relative band density is also shown in the lower panel. **b** Rhein induces Bak expression in a dose-dependent manner via MMP disruption. HASMCs were pretreated with the MMP disruption inhibitor (BA, 50 μ M) for 2 h, after which they were exposed to 10 μ M rhein and 10 ng/ml TNF- α for 24 h at 37°C. Cells underwent intracellular staining with anti-Bak mAb, as described in Materials and Methods. Data shown are representative of at least 3 independent experiments and represent the means $\pm$ SEM. * $p < 0.05$; *** $p < 0.001$ vs. TNF- α alone.

inhibition compared to the group treated with TNF- α alone). In addition, the pro-apoptotic protein, Bak, increased as the concentration of rhein increased in TNF- α -treated HASMCs (relative band density with 10 μ M rhein and TNF- α was 2.5-fold that of control groups or the group treated with TNF- α alone). Furthermore, the MMP disruption inhibitor, BA, efficiently blocked rhein-

induced Bak expression by up to 90% in TNF- α -activated HASMCs, as shown in figure 6b. Taken together, these findings indicate that rhein induces Bak expression via MMP disruption. Furthermore, these data suggest that rhein-induced apoptosis might be regulated by mitochondrial disorganization. Therefore, rhein induces apoptosis via a mitochondrial pathway.

Fig. 7. Effect of BA in rhein-induced caspase-9 activation. HASMCs were pretreated with MMP disruption inhibitor (BA) for 2 h, exposed to 10 μM rhein and 10 ng/ml TNF-α for 24 h at 37°C and then lysed. The caspase-9 activity was measured as described in Materials and Methods. Data shown are representative of at least 3 independent experiments and represent the means ± SEM. *** $p < 0.001$ vs. TNF-α alone. Cas-9 = Caspase-9 as a positive control.

Mitochondrial Membrane Depolarization Inhibitor Efficiently Blocked Rhein-Induced Caspase-9 Activity

The results of this study demonstrated that rhein-induced apoptosis occurs via caspases 3, 8 and 9 activation (fig. 3 and 4). Therefore, to determine the hierarchy of caspase and mitochondria, caspase-9 activity was measured in the presence or absence of the MMP disruption inhibitor, BA. BA efficiently blocked rhein-induced caspase-9 activation in TNF-α-activated HASMCs, as shown in figure 7, which indicates that rhein-induced caspase activation occurs downstream of the mitochondrial pathway. We also used caspase-9 as a positive control. The level of rhein-mediated caspase-9 activity in TNF-α-treated HASMCs was comparable with that of caspase-9-mediated induction.

Discussion

The abnormal growth of VSMCs plays an important role in the development of vascular diseases such as atherosclerosis and restenosis following angioplasty [1, 2]. In addition, abnormal growth of VSMCs also induces inflammation in blood vessels and/or arteries [3]. Inflammatory cytokines such as TNF-α are secreted by VSMCs

in the neointima after a balloon injury, as well as by macrophages in atherosclerotic lesions [33]. In addition, inflammatory cytokines also play an important role in the induction of inflammation, migration and proliferation in HASMCs [3–8].

Previous reports have shown that rhein induces apoptosis in promyelocytic leukemia HL-60 cells [24], and it is well known TNF-α-induced HASMC proliferation plays a critical role in development of vascular diseases including atherosclerosis [2, 5–9]. However, the effect of rhein on TNF-α-induced HASMC proliferation is not well understood. Moreover, most investigations have shown that some materials, such as quercetin [5], resveratrol [6], gensenoside [7], ochonaflavone [8], and magnolol [9], inhibit TNF-α-induced HASMC proliferation by regulating cell cycle-related proteins and by suppressing the activities of extracellular signal-regulated kinases 1 and 2. However, it is less well known that the inhibition of TNF-α-induced HASMC proliferation occurs via caspase and a mitochondrial-dependent apoptotic pathway.

Rhein inhibits TNF-α-induced HASMC proliferation (fig. 1). It is well known that TNF-α functions both as a survival signal including proliferation (via DNA synthesis) and as a death signal [34]. Therefore, we attempted to confirm the effects of rhein on TNF-α-induced proliferation of HASMCs, not TNF-α-induced cell death. Cell proliferation was measured by a BrdU-(5′Bromo-2-deoxyuridine) ELISA assay (cell proliferation ELISA BrdU), which produced results very similar to those obtained when the cell viability was assayed (fig. 1b). Moreover, other studies conducted to evaluate TNF-α-induced HASMC proliferation measured thymidine incorporation in conjunction with tritium and found that TNF-α induces an increase in DNA synthesis, as well as the proliferation of HASMCs [5, 6, 8, 9].

Rhein induces apoptotic cells (fig. 2a), PARP cleavage (fig. 2b), and caspase activation (fig. 3; also shown using caspase inhibitors in fig. 4) in TNF-α-treated HASMCs. Rhein also induces a release of cytochrome *c* from mitochondria through a loss of $\Delta\psi_m$ (fig. 5a, b), a decreased Bcl-2 and Bcl-xL level, and an increased Bax and Bak protein level (fig. 6). These findings indicate that the activation of caspases and a mitochondrial pathway is required for rhein-induced apoptosis in TNF-α-treated HASMCs. This study also demonstrated that caspases 3, 8 and 9 and mitochondria contribute directly to rhein-mediated apoptosis (fig. 4, 5c). The caspase-3 inhibitor, Ac-DEVD-CHO, inhibited rhein-induced apoptosis by up to 61% in TNF-α-treated HASMCs. Additionally, the caspase-8 inhibitor, Z-IETD-FMK, and the caspase-9 inhibitor, Z-

LEHD-FMK, also inhibited rhein-induced apoptosis by up to 77 and 66%, respectively, in TNF- α -treated HASMCs (fig. 4). Furthermore, BA (an inhibitor of MMP disruption) efficiently inhibited rhein-induced apoptotic cells by up to 74% in TNF- α -treated HASMCs (fig. 5c). It should be noted that inhibitors of caspases 3, 8 and 9 did not block rhein-induced $\Delta\psi_m$ (data not shown). In addition, the inhibitor of mitochondrial membrane depolarization efficiently blocked rhein-induced caspase-9 activity, as shown in figure 7. This finding indicates that caspase signals exist downstream of the mitochondrial pathway. Taken together, these results indicate that rhein-induced cell death occurred via mitochondria-dependent apoptosis.

It is well known that TNF- α acts as a double-edged sword by functioning as a survival signal that induces proliferation (via DNA synthesis), but also as a death signal [34]. Moreover, rhein has been shown to induce death during TNF- α -induced VSMC proliferation via the induction of apoptosis. It is possible that TNF- α -induced VSMC proliferation occurs via the recruitment of TNF-receptor-associated factors 1 and 2 in downstream of the TNF receptors; however, treatment of TNF- α -induced HASMC with rhein resulted in the proliferation signal changing to an apoptosis signal and causing the recruitment of Fas-associated death domain protein and caspase 8 [35]. We will evaluate these effects in a future study using several inhibitors to better understand how they occur. However, it is obvious that rhein inhibits TNF- α -induced human aortic smooth-muscle cell proliferation via caspase- and mitochondrial-dependant apoptosis. Moreover, rhein prevents overgrowth of VSMC and the development of atherosclerosis. Taken together, these findings indicate that rhein can be used to prevent atherosclerosis via the induction of apoptosis in VSMC.

The results of this study suggest that rhein-treated HASMCs are killed via apoptosis in atherosclerotic lesions and inflammation (TNF- α -affected HASMCs), which promotes PARP cleavage and caspase activation, subsequently releasing cytochrome *c* from the mitochondria as a result of a loss of $\Delta\psi_m$ (mitochondrial disorganization). This in turn decreases the level of the Bcl-2 and Bcl-xL survival proteins, and increases the level of Bax and Bak apoptotic proteins. It is interesting that treatment with rhein alone (10 μ M) had no effect on proliferation (fig. 1b), apoptosis (fig. 2a), PARP cleavage (fig. 2b), caspase activation (fig. 3), loss of $\Delta\psi_m$ (fig. 5a), cytochrome *c* release (fig. 5b), or Bax and Bak expression (fig. 6a, b) in HASMCs. This indicates that rhein only induces cell death in inflammatory sites.

It is well known that the consequences of VSMC apoptosis depend on the stage and location of the plaque SMC at the time of death [36]. Furthermore, the consequences of VSMC apoptosis can also be detrimental to the overall stability of the plaque, thereby causing thrombosis and rupture [37]. However, the exact role that apoptosis plays in the pathology and physiology of coronary diseases such as atherosclerosis is currently unknown. In addition, the effect that rhein-mediated VSMC apoptosis has on thrombosis and plaque rupture is not yet known and will be evaluated in a future study using animal models. However, it is obvious that rhein inhibits TNF- α -induced HASMC proliferation via caspase- and mitochondrial-dependant apoptosis. Moreover, rhein prevents the overgrowth of VSMC and the development of atherosclerosis. Therefore, rhein can be used to prevent atherosclerosis via the induction of apoptosis in VSMC.

Rhein, a constituent enriched in the rhizome of rhubarb (*R. palmatum* L. or *R. tanguticum* Maxim, daehwang in Korean), is a traditional Chinese herb that is used as a laxative and stomachic drug [19]. Although it is well known that rhein has anti-cancer activity in various cells, including human glioma [20], tumor cells in the rat liver [21], Ehrlich ascites tumor cells [22], human colonic adenocarcinoma [23], the mouse epidermal cell line JB6 [19], human promyelocytic leukemia HL-60 cells [24], and human cervical cancer Ca Ski cells [25], little is known about the anti-atherogenic effects of rhein or its signal pathway (and mechanism) in HASMCs. However, other anthraquinone components of rhubarb, such as aloe-emodin and emodin, also reportedly exert anti-proliferation effects in VSMCs following arterial injury [38, 39].

In conclusion, rhein inhibits TNF- α -induced HASMC proliferation via a mitochondria-dependent apoptotic pathway, which indicates that rhein has the potential for use as an anti-atherosclerosis agent. In addition, rhein may be particularly useful for the prevention of restenosis following percutaneous transluminal coronary angioplasty. To our knowledge, this is the first report to indicate that the inhibition of TNF- α -induced HASMC proliferation is directly involved in mitochondrial-dependent apoptosis.

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