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Emodin Inhibits TNF-α-Induced Human Aortic Smooth-Muscle Cell Proliferation Via Caspase- and Mitochondrial-Dependent Apoptosis

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ABSTRACT

Vascular smooth-muscle cell (VSMC) proliferation plays a vital role in hypertension, atherosclerosis and restenosis. It has been reported that emodin, an active component extracted from rhubarb, can stop the growth of cancer cells; however, it is not known if emodin exerts similar anti-atherogenic effects in TNF-α treated human aortic smooth-muscle cells (HASMC). In this study, emodin treatment showed potent inhibitory effects in TNF-α-induced HASMC proliferation that were associated with induced apoptosis, including the cleavage of poly ADP-ribose polymerase (PARP). Moreover, inhibitors of caspase-3, -8 and -9 (Ac-DEVD-CHO, Z-IETD-FMK and Z-LEHD-FMK) efficiently blocked emodin-induced apoptosis in TNF-α treated HASMC. Therefore, emodin-induced cell death occurred via caspase-dependent apoptosis. Emodin treatment resulted in the release of cytochrome *c* into cytosol and a loss of mitochondrial membrane potential ($\Delta\Psi_m$), as well as a decrease in the expression of an anti-apoptotic protein (Bcl-2) and an increase in the expression of an a pro-apoptotic protein (Bax). Emodin-mediated apoptosis was also blocked by a mitochondrial membrane depolarization inhibitor, which indicates that emodin-induced apoptosis occurred via a mitochondrial pathway. Taken together, the results of this study showed that emodin inhibits TNF-α-induced HASMC proliferation via caspase- and a mitochondrial-dependent apoptotic pathway. In addition, these results indicate that emodin has potential as an anti-atherosclerosis agent. J. Cell. Biochem. 105: 70–80, 2008.

KEY WORDS: EMODIN; APOPTOSIS; CASPASE ACTIVATION; MITOCHONDRIAL PATHWAY; ANTI-ATHEROSCLEROSIS

The proliferation of vascular smooth-muscle cell(s) (VSMC) plays a vital role in hypertension, atherosclerosis and restenosis [Ross, 1995]. In addition, abnormal VSMC growth contributes to vascular diseases such as atherosclerosis and restenosis following angioplasty [Ross, 1995]. In a normal artery, the VSMC exist in a non-proliferative quiescent state and show a well-differentiated contractile phenotype. However, following a vascular injury a loss of differentiated phenotype and a shift to a synthetic phenotype, such as proliferation, occurs [Owens et al., 2004; Zhang and Wang, 2006]. Vascular lesions form during several pathological processes including the accumulation of inflammatory cells and the production of inflammatory cytokines such as tumor necrosis factor-α (TNF-α) [Jovinge et al., 1997]. TNF-α is secreted by VSMC in the neointima after a balloon injury as well as by macrophages in atherosclerotic lesions [Tanaka et al., 1996; Jovinge et al., 1997], and previous studies have shown that TNF-α induces an increase in DNA synthesis and the proliferation of VSMC [Moon et al., 2003; Lee and Moon, 2005; Suh et al., 2006; Zhang and Wang, 2006; Kim et al., 2007].

Apoptosis, a type of programmed cell death, is one method of controlling immune responses such as cellular homeostasis as well as a variety of physiological processes. Apoptosis is often linked with carcinogenesis resulting from abrogation of the apoptotic process [Blagosklonny, 2003; Rodriguez-Nieto and Zhivotovsky, 2006] and is characterized by a number of well-defined features, including cellular morphological change, chromatin condensation, oligonucleosomal DNA cleavage, membrane blebbing and activation of a family of cysteine proteases called caspases [Thornberry,

Abbreviations used: TNF-α, tumor necrosis factor-α; VSMC, vascular smooth-muscle cells; HASMC, human aortic smooth-muscle cells; PARP, poly ADP-ribose polymerase; PBS, phosphate-buffered saline; DTT, dithiothreitol; MTS, [3-(4,5-dimethylthiazol-2-yl]-5-(3-carbo-xymethoxyphenyl)-2-(4-sulfenyl)-2H-tetrazolium]; BA, bongkrekic acid; PTCA, percutaneous transluminal coronary angioplasty.

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1998]. Activated caspase-8 can activate downstream caspases either by direct cleavage or by indirectly cleaving Bid and inducing the release of cytochrome *c* from the mitochondria. 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, which then cleaves and activates downstream caspases such as caspase-3, -6, -7 and -8 [Salvesen and Dixit, 1997; Thornberry and Lazebnik, 1998; Budihardjo et al., 1999]. Caspase-3 cleaves many substrates that respond to DNA-strand breaks, such as poly(ADP-ribose)polymerase (PARP), and eventually leads to apoptosis [Wyllie, 1980]. Additionally, protein Bcl-2 families affect the integrity of mitochondria and caspase activation [Cory and Adams, 2002]. The Bcl-2 family consists of the pro-apoptotic and apoptosic executioners, 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 the mitochondria in apoptotic cells and triggers disruption of the mitochondrial membrane potential [Jürgensmeier et al., 1998; Antonsson et al., 2001; Park et al., 2003]. In contrast, Bcl-2 and Bcl-xL bind to mitochondria and inhibit the release of cytochrome *c* [Finucane et al., 1999; Cory and Adams, 2002].

Emodin (1,3,8-trihydroxy-6-methylanthraquinone), a natural anthraquonoid compound, is an active component of the dried root of Rhei Rizoma (Rheum palmatum, Daehwang, Korea) that is also present in many herbs and vegetables such as cabbage, lettuce, beans and peas [Huang et al., 2007; Wang et al., 2007]. Emodin has anti-cancer, antiviral, antimicrobial, immunosuppressive, hepatoprotective, anti-inflammatory and wound healing properties; however, it does not possess anti-atherosclerosis properties [Huang et al., 1992, 2007; Chang et al., 1996; Lin et al., 1996; Hatano et al., 1999; Shuangsuo et al., 2006; Tang et al., 2007].

Emodin has been reported to stop the growth and induce the death of human VSMC through reactive oxygen species and p53 [Wang et al., 2007], and it has also been reported to inhibit TNF-induced NF-kB activation and IκB degradation in human vascular endothelial cells [Kumar et al., 1998]. Furthermore, it has been suggested that emodin induces cell death during TNF-α-induced human aortic smooth-muscle cell(s) (HASMC) proliferation. Therefore, we conducted this study to determine if emodin inhibits TNF-α-induced human aortic smooth-muscle cell proliferation via caspase- and a mitochondrial-dependant apoptotic pathway.

MATERIALS AND METHODS

MATERIALS

Emodin was purchased from Sigma Chemical Co. (St. Louis, MO). Recombinant human TNF-α and PDFG-BB were purchased from R&D Systems (Minneapolis, MN) and diluted in 0.1% bovine serum albumin (BSA)–phosphate-buffered saline (PBS) buffer. The CellTiter 96, Aqueous One Solution Cell Proliferation Assay (MTS) was purchased from Pr omega (Madison, WI). Aprotinin, leupeptin, DMSO, dithiothreitol (DTT), Ac-DEVD-CHO, bongkrekic acid and phenylmethylsulfonyl fluoride (PMSF) were purchased from Sigma Chemical Co. (St. Louis, MO) Z-IETD-FMK, Z-LEHD-FMK were purchased from R&D Systems (Minneapolis, MN). Anti-β-actin monoclonal antibody (mAb), cleaved Poly ADP-ribose polymerase (PARP), IκB-α and phosphorylated IκBα (p-IκBα) mAb were purchased from Cell Signaling Technology (Beverly, MA). Bax mAb was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-rabbit IgG horseradish peroxidase (HRP) and anti-mouse IgG HRP were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-cytochrome *c* and Bcl-2 mAb were purchased from BD PharMingen (San Jose, CA).

CELL CULTURES

HASMC, smooth-muscle cell medium (SMCM) and SMC growth supplement were purchased from ScienCell (Carlsbad, CA). HASMC were grown in SMCM with growth supplement at 37°C in a humidified 95% air/5% CO_2 atmosphere. For all experiments, HASMC were grown to 80–90% confluence and made quiescent by starvation for at least 24 h.

CELL PROLIFERATION ASSAYS

HASMC were incubated with various concentrations of emodin and 10 ng/ml TNF-α or PDFG-BB for 24 h at 37°C. Cell proliferation was measured by a Cell Titer 96 colorimetric assay using an MTS tetrazolium compound according to the manufacturer's instructions. Assays were performed by adding [3-(4,5-dimethylthiazol-2-yl]-5-(3-carbo-xymethoxyphenyl)-2-(4-sulfenyl)-2H-tetrazo-lium] (MTS) directly to the culture wells and then incubating them for 4 h at 37°C. In addition, cell proliferation was measured by a BrdU-(5′bromo-2-deoxyuridine) ELISA assay (Cell proliferation ELISA BrdU, Roche Diagnostics), according to the manufacturer's instructions. Cells cultivated under the same conditions were then exposed to BrdU (10 μM, 2 h). For the BrdU assay, cells were fixed and their DNA was then denatured and blocked, after which the samples were incubated with an anti-BrdU monoclonal antibody coupled to peroxidase and 3,3′,5,5′-tetramethylbenzidine (TMB). Next, the absorbance was evaluated at 490 nm in an enzyme-linked immunosorbent assay reader (Opsys MR, USA). The results were then expressed as percentage changes from the basal conditions using three to five culture wells for each experimental condition. In some experiments, HASMC were preincubated with cultured anti-human TNFR1 mAb and anti-human TNFR2 mAb (1 μg/ml, respectively) for 30 min at 37°C prior to treatment with TNF-α. After 24 h, the cells were measured by a BrdU-(5′bromo-2-deoxyuridine) ELISA assay.

ANALYSIS OF THE MITOCHONDRIAL MEMBRANE POTENTIAL

The mitochondrial membrane potential ($\Delta\Psi_m$) was assessed by the retention of $DiOC_6(3)$. Cationic lipophilic fluorochrome $DiOC_6(3)$ is a cell-permeable marker that specifically accumulates into mitochondrion depending on the $\Delta\Psi_m$ [Lin et al., 2003]. HASMC (3×10^5/ml) were treated with various concentrations of emodin for 24 h. The cells were then washed once in 0.2% BSA–PBS and incubated with 10 μM $DiOC_6(3)$ for 30 min at 37°C. Mitochondrial depolarization is indicated by a decrease in the $DiOC_6(3)$ fluorescence intensity. As many as 1×10^4 cells were then analyzed at FL1 with a Becton Dickinson FACS calibur flow cytometer and CELL-Quest Pro software (BD Biosciences, San Jose, CA).

DETERMINATION OF APOPTOSIS BY FLOW CYTOMETRY

To detect externalized phosphatidylserine (PS) as an early indication of apoptosis, HASMC (3×10^5/ml) were incubated with various concentrations of emodin and 10 ng/ml TNF-α for 24 h at 37°C. The cells were then harvested, washed two times with FACS buffer (0.2% BSA–PBS), stained with Annexin V–FITC according to the manufacturer's protocol (Clontech Laboratories, Palo Alto, CA), and then analyzed with a Becton Dickinson FACS calibur flow cytometer and CELL-Quest Pro software (BD Biosciences). Bongkrekic acid (50 μM) was added to HASMC for 2 h before the emodin and/or TNF-α treatment to inhibit disruption of the mitochondrial membrane potential (MMP). After 24 h, the cells were stained with Annexin V–FITC for FACS analysis [Hur et al., 2004]. In some experiments, 10 μM of Ac-DEVD-CHO, Z-IETD-FMK and Z-LEHD-FMK were added to the HASMC for 30 min before the emodin and/or TNF-α treatment. After 24 h, the cells were stained with Annexin V–FITC for FACS analysis.

PREPARATION OF CYTOSOLIC EXTRACTIONS FOR CYTOCHROME *C* ANALYSIS

The cells were washed with ice-cold PBS and then resuspended in a cold lysis buffer (250 mM sucrose, 20 mM HEPES, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 1 mM EDTA, 1 mM DTT, 0.1 mM PMSF, 10 μg/ml leupeptin and 10 μg/ml aprotinin). After incubation on ice for 30 min, the cells were homogenized with 15 strokes, and the homogenates were then centrifuged at 1,200*g* for 15 min at 4°C. Next, the supernatants were transferred to another tube and then centrifuged at 100,000*g* for 60 min at 4°C, after which they were subjected to 12.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). The cytochrome *c* release was then analyzed by immunoblotting using an anti-cytochrome *c* mAb. In some experiments, bongkrekic acid (50 μM) was added to HASMC for 2 h before the emodin and/or TNF-α treatment. After 24 h, the cells were washed with ice-cold PBS and resuspended in a cold lysis buffer for analysis by immunoblotting using an anti-cytochrome *c* mAb.

PREPARATION OF TOTAL CELL EXTRACTS AND WESTERN BLOTTING

HASMC were washed three times with ice-cold PBS and then harvested. The cells were then sonicated in 50 mM Tris–HCl buffer (pH 7.5, 5 mM $MgCl_2$, 5 mM NaCl, 5% (v/v) glycerol, 20 mM NaF, 2 mM Na_3VO_4 and various protease inhibitors). After centrifugation at 12,000*g* for 15 min at 4°C, the supernatants were collected. Hereafter, the supernatants are referred to as the cell extract. The protein concentrations were measured using the Bradford protein dye reagent (Bio-Rad). The total cell lysates from the HASMC were then separated by 10–12.5% SDS–PAGE, after which the proteins were transferred electrophoretically onto nitrocellulose membranes (0.2 μm, Schleicher and Schuell). The membranes were then blocked with 5% non-fat dried milk in Tris buffered saline (TBS) and subsequently probed with a primary antibody, anti-Bcl-2, anti-Bax, anti-IκB-α and anti-p-IκBα in TBS containing 3% non-fat dried milk. Antibody–antigen complexes were detected using goat anti-mouse IgG or goat anti-rabbit IgG-HRP conjugated antibodies and an enhanced chemiluminescence detection kit (Amersham Pharmacia Biotech, UK, USA).

STATISTICS

The data shown represent the mean ± SEM. The differences between groups were evaluated by a Student's *t*-test and the results are representative of at least three independent experiments.

RESULTS

EMODIN INHIBITS HASMC PROLIFERATION

Our group investigated the proliferation of HASMC in response to 0, 1, 10 and 100 ng/ml of TNF-α. TNF-α stimulated the proliferation of HASMC, as determined by a Cell Titer 96 Cell Proliferation Assay; with 10 ng/ml of TNF-α being maximally effective at promoting proliferation (Fig. 1A, left panel). We also performed a BrdU-(5′bromo-2-deoxyuridine) ELISA assay of the HASMC using neutralizing antibodies, anti-human TNFR1 mAb and anti-human TNFR2 mAb to show that TNF-α-induced proliferation occurred via TNFR1 and 2 (Fig. 1A, right panel). Emodin significantly inhibited the TNF-α-induced proliferation in a dose-dependent manner. In addition, inhibition of the TNF-α-induced proliferation in HASMC that had been treated with emodin was 59.9 ± 4.5%, 66.8 ± 4.1% and 79.5 ± 2.5% of that of HASMC that were treated with TNF-α alone (10 ng/ml; 100%) at final concentrations of 0.1, 1, 10 μM, respectively. We also confirmed the effect of emodin on TNF-α-induced proliferation by a BrdU-(5′bromo-2-deoxyuridine) ELISA assay. Emodin significantly and dose-dependently decreased the proliferation of HASMC (34%, 36% and 48% of TNF-α alone at final concentrations of 0.1, 1, 10 μM emodin, respectively; Fig. 1B, lower panel), whereas emodin alone (10 μM) had no effect on the proliferation and cytotoxicity of HASMC (Fig. 1B). Moreover, it is well known that PDGF is a strong inducer of VSMC proliferation. Therefore, we examined the effect of emodin on PDGF-induced proliferation by a BrdU-(5′bromo-2-deoxyuridine) ELISA assay. Emodin also significantly inhibited the PDGF-induced proliferation in a dose-dependent manner, with the inhibition of the PDGF-induced proliferation in HASMC by emodin being 22%, 30% and 57% of that of PDGF alone (10 ng/ml) at final concentrations of 0.1, 1 and 10 μM, respectively (Fig. 1C, right panel).

THE INHIBITORY EFFECT OF EMODIN ON THE TNF-α-INDUCED PHOSPHORYLATION OF IκBα

To further confirm the inhibitory effect of emodin on TNF-α-induced proliferation, we examined the phosphorylation state of IκBα. As shown in Figure 2, treatment with TNF-α; alone increased the phosphorylation of IκBα in HASMC that were stimulated with TNF-α, however, emodin dose-dependently inhibited the phosphorylation of IκBα, which may result in a reduction of the degradation of IκBα. This finding indicates that emodin may inhibit potential NF-κB activation via IκBα degradation in HASMC that are stimulated with TNF-α.

EMODIN-INDUCED APOPTOSIS IN HASMC IS 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 [Wyllie, 1980; Hur et al., 2004]. Apoptosis was measured by annexin V staining of PS

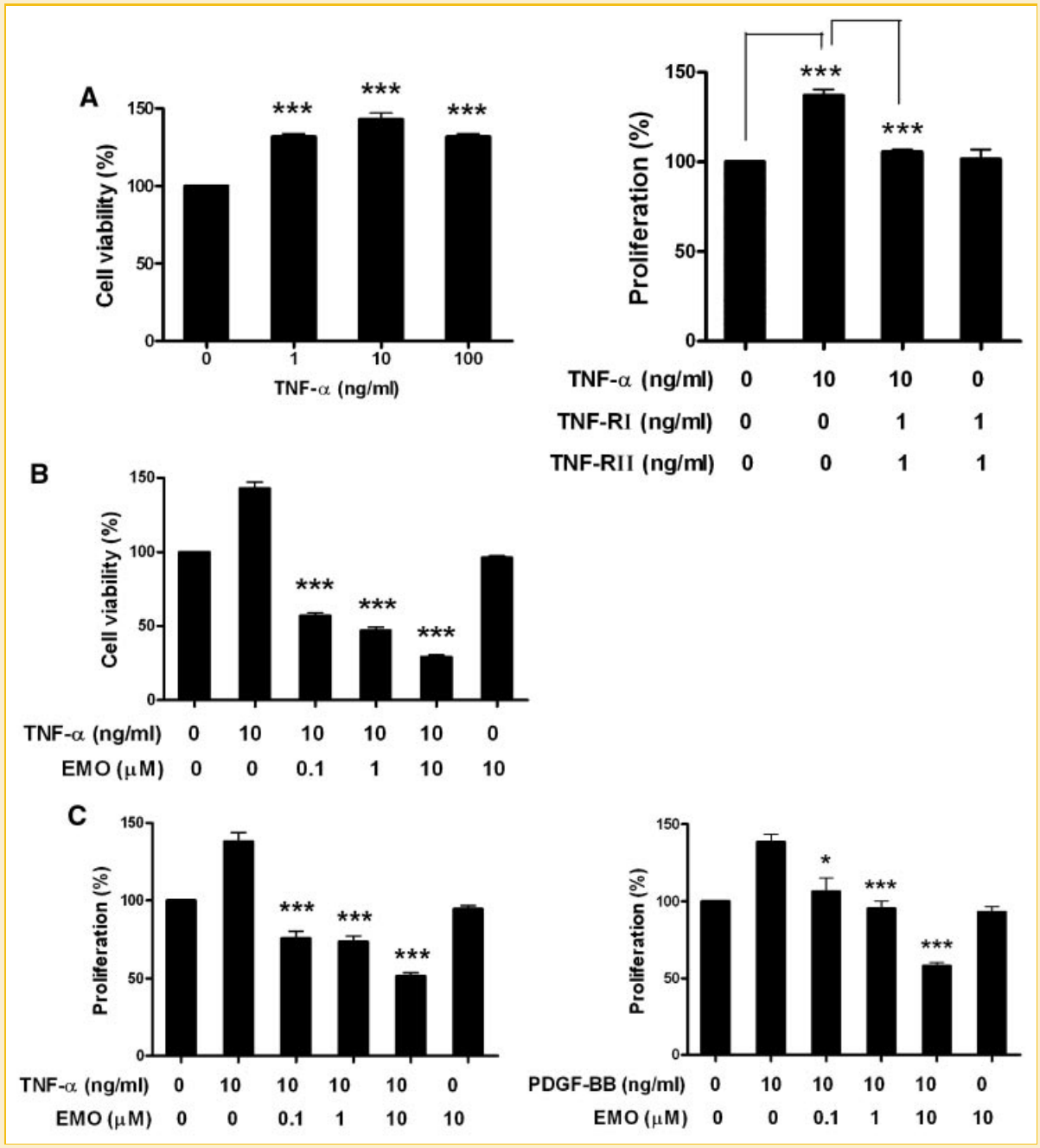

Fig. 1. Effect of HASMC proliferation induced by TNF-α and emodin. Arrested HASMC were stimulated with 0.1, 1 or 10 μM emodin (EMO) for 30 min prior to treatment without (A) or with (B and C) 10 ng/ml TNF-α for 24 h. The control group was not treated with TNF-α or EMO. HASMC proliferation was assayed by a CellTiter 96 Cell Proliferation Assay (B) and by a BrdU-(5′bromo-2-deoxyuridine) ELISA assay (C). Data shown are representative of at least three independent experiments. Data represent the means ± SEM. *Significantly different from control (0 ng/ml TNF-α, A) and TNF-α alone group (B and C), $^{*}P < 0.05$, $^{***}P < 0.001$. EMO, emodin.

following emodin treatment. HASMC were incubated with TNF-α (10 ng/ml) and various concentrations of emodin (0, 0.1, 1, 10 μM) for 24 h. Emodin was found to markedly induce apoptosis in TNF-α treated HASMC at a concentration of 10 μM (Fig. 3A; 0 μM, 2.8 ± 0.6%; 0.1 μM, 17 ± 3.1%; 1 μM; 28.1 ± 9.6%; 10 μM, 60.5 ± 8.7%). To further confirm that apoptosis was induced by emodin in TNF-α-treated HASMC the cleavage of poly (ADP-ribose) polymerase (PARP), which responds to DNA strand breaks and is used as another hallmark of apoptosis, was evaluated. As shown in Figure 3B, the cleavage of PARP was detected after 24 h of exposure to 1 μM emodin, however; the most prominent PARP activity of emodin in TNF-α-treated HASMC was attained in response to treatment with 10 μM emodin (relative band density; 10 μM emodin, 580-fold of control groups, Fig. 3B).

It has been reported that caspase-3, -8, and -9 play a pivotal role in the terminal phase of apoptosis [Lin et al., 2003]. To determine an

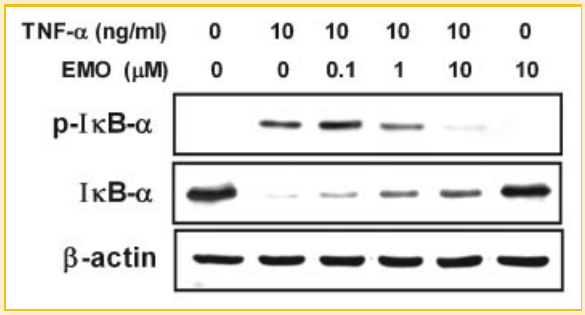

Fig. 2. The effects of emodin on TNF-α-induced NF-κB activation in HASMC. Cells were incubated with various concentrations of emodin and 10 ng/ml TNF-α for 24 h at 37°C. Cell lysate was then isolated and investigated to determine if emodin inhibited the phosphorylation of IκBα. The levels of phosphorylated IκBα (p-IκBα) and IκBα protein in the cell lysate were then evaluated by western blot. A western blot for p-IκBα and IκBα was conducted as described in Materials and Methods Section. EMO, emodin.

emodin-induced apoptosis pathway in TNF-α treated HASMC, our group used caspase-3, -8 and -9 inhibitors. The cells were pre-incubated 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 emodin and TNF-α to determine which cells were apoptotic. Inhibitors of caspase-3, -8 and -9 efficiently blocked emodin-induced apoptosis in TNF-α treated HASMC (percent of apoptotic cells: 10 μM emodin alone, 60.5 ± 8.7%; with 10 μM Ac-DEVD-CHO, 17.03 ± 5.6%; with 10 μM Z-IETD-FMK, 10.3 ± 2.2%; with 10 μM Z-LEHD-FMK, 8.9 ± 1%). In addition, the caspase-3 inhibitor, Ac-DEVD-CHO, inhibited emodin-induced apoptosis by up to 72 ± 7.7% in TNF-α treated HASMC, and the caspase-8 inhibitor, Z-IETD-FMK, and the caspase-9 inhibitor, Z-LEHD-FMK, also inhibited emodin-induced apoptosis by up to 82 ± 4.5% and 85 ± 2.9%, respectively, in TNF-α treated HASMC. These results indicate that emodin-induced cell death occurred via caspase-dependent apoptosis (Fig. 4).

EMODIN INDUCES APOPTOSIS VIA THE MITOCHONDRIAL PATHWAY

Mitochondria play a very important role in apoptosis [Petit et al., 1997]; therefore, the effect of emodin on the $\Delta\Psi_m$ was examined using the potential-sensitive dye, $DiOC_6(3)$. A remarkable loss of $\Delta\Psi_m$ was observed at 24 h in response to treatment with 10 μM of emodin in TNF-α treated HASMC (Fig. 5A). Additionally, it has been shown that translocation of cytochrome *c* from the mitochondrion is essential in apoptotic signaling because it leads to apoptosis by inducing caspase activation [Singh et al., 2005]. As shown in Figure 5B, a dose-dependent accumulation of cytochrome *c* in the cytosol was detected in emodin/TNFα-treated cells, and an efflux in mitochondrion was observed simultaneously. To inhibit MMP disruption, bongkrekic acid (BA, 50 μM) was added to HASMC for 2 h prior to emodin and/or TNF-α treatment. After 24 h, the cells were analyzed by immunoblotting using an anti-cytochrome *c* mAb. The BA efficiently blocked the emodin-induced cytochrome *c* release in TNF-α treated HASMC (relative band density: with 10 μM emodin, 100%; with 50 μM BA, 15.7%). In addition, the BA efficiently inhibited emodin-induced apoptotic cells by up to 80.4 ± 13.3% in TNF-α treated HASMC (Fig. 5C). These results suggest that emodin causes an efflux of cytochrome *c* from the mitochondrion as a result of the 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 overexpression of anti-apoptotic proteins such as Bcl-2 and Bcl-xL [Yang et al., 1997; Garland and Rudin, 1998; Finucane et al., 1999]. In contrast, translocation of the pro-apoptotic protein, Bax, to the mitochondrial membrane induces cytochrome *c* release [Jürgensmeier et al., 1998; Antonsson et al., 2001; Park et al., 2003; Gustafsson and Gottlieb, 2007]. This study indicated that emodin elicits MMP disruption by regulating the expression of Bcl-2 family members. As shown in Figure 6, the pro-apoptotic protein, Bax, increased as the concentration of emodin increased in TNF-α treated HASMC (relative band density: 10 μM emodin and TNF-α, 5-fold of control groups and TNF-α alone treated group). By comparison, the anti-apoptotic protein, Bcl-2, decreased as the concentration of emodin increased (relative band density: 10 μM emodin and TNF-α, 67% inhibition of TNF-α alone treated group). These data suggest that emodin-induced apoptosis might be regulated by mitochondrial disorganization. Therefore, emodin induces apoptosis via a mitochondrial pathway.

DISCUSSION

In addition to playing an important role in vascular diseases such as atherosclerosis and restenosis following angioplasty [Ross, 1995], the abnormal growth of VSMC also induces inflammation in blood vessels and/or arteries [Jovinge et al., 1997]. Inflammatory cytokines such as tumor necrosis factor-α (TNF-α) are secreted by VSMC in the neointima following a balloon injury as well as by macrophages in atherosclerotic lesions [Tipping et al., 1993]. They also play an important role in inducing inflammation [Tanaka et al., 1996; Jovinge et al., 1997]. Additionally, it is well known that TNF-α-induced HASMC proliferation contributes greatly to the development of atherosclerosis [Lee and Moon, 2005; Suh et al., 2006; Zhang and Wang, 2006]. Furthermore, many studies have shown that various materials, such as quercetin, resveratrol, gensenoside, ochnaflvone, and magnolol, inhibit TNF-α-induced HASMC proliferation by regulating cell cycle-related proteins and the suppression of extracellular signal-regulated kinases (ERK) 1/2 activity [Moon et al., 2003; Lee and Moon, 2005; Suh et al., 2006; Zhang and Wang, 2006; Kim et al., 2007]. However, it is not known if the inhibition of TNF-α-induced HASMC proliferation occurs via caspase- and a mitochondrial-dependent apoptotic pathway.

Emodin inhibits TNF-α-induced HASMC proliferation (Fig. 1), and it is well known that TNF-α functions as a survival signal, induces proliferation (via DNA synthesis), and acts as a death signal [Aggarwal, 2003]. However, in this study we attempted to confirm TNF-α-induced proliferation, not TNF-α-induced cell death. Cell proliferation was measured by a BrdU-(5′bromo-2-deoxyuridine) ELISA assay (Cell proliferation ELISA BrdU, Roche Diagnostics) and the results obtained were very similar to those of the cell viability

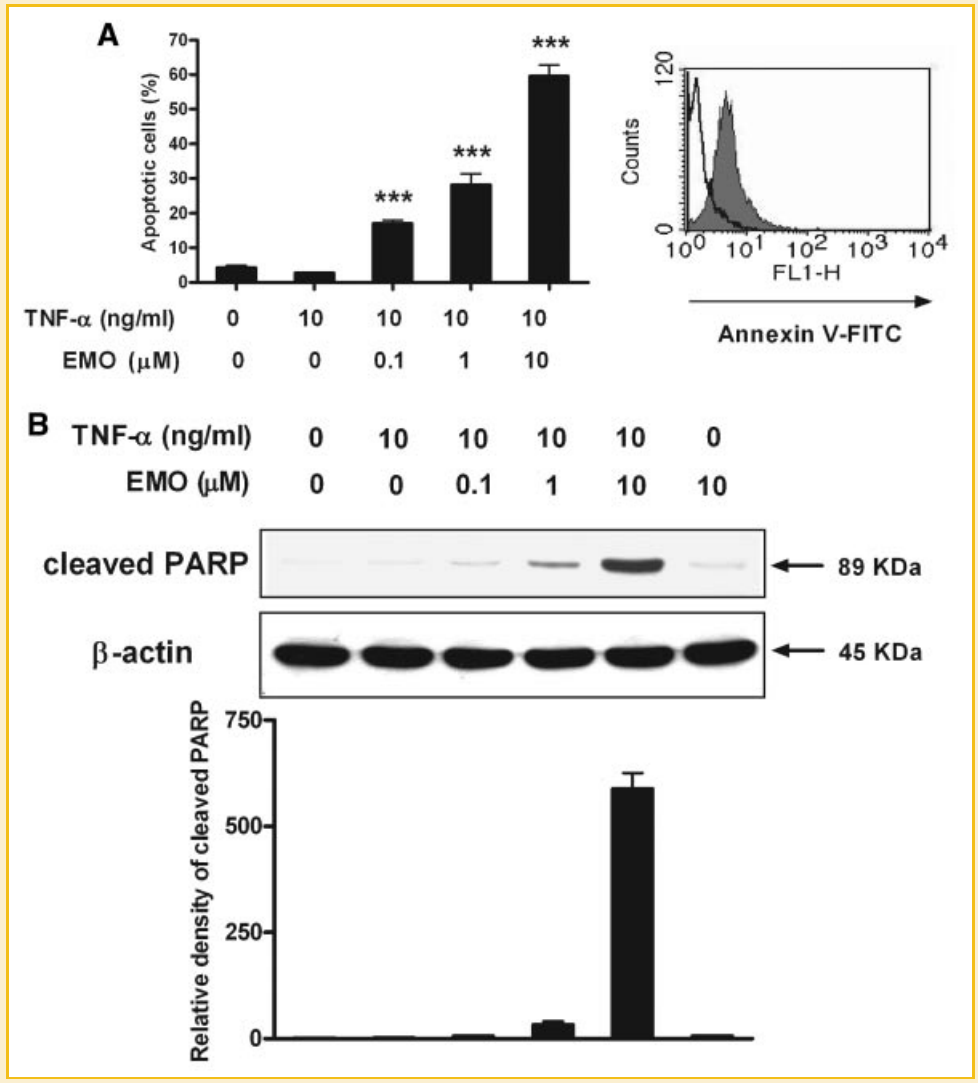

Fig. 3. Emodin induces apoptosis in TNF-α treated HASMC. HASMC were incubated with the indicated concentrations of emodin for 24 h. A: Emodin dose-dependently induces apoptotic cells. Apoptosis was measured by staining with FITC-labeled annexin V followed by flow cytometric analysis and the percentage of apoptotic cells was then determined (left panel). The open histogram represents cells treated with TNF-α alone (EMO, 0 μM) and the filled histogram represents cells treated with 10 μM EMO and TNF-α, respectively (right panel). B: Emodin induces PARP cleavage. HASMC incubated in the presence of the indicated concentrations of EMO for 24 h. Whole-cell lysate was obtained and used for Western blotting analysis with anti-cleaved PARP mAb, as described in Materials and Methods Section. The membrane was stripped and reprobed with anti-β-actin mAb to confirm equal loading. Data shown are representative of at least three independent experiments. Data represent the means ± SEM. *Significantly different from TNF-α alone, $^{***}P < 0.001$. EMO, emodin.

assay (Fig. 1B). Moreover, the results of previous studies that used thymidine incorporation assays to evaluate TNF-α-induced HASMC proliferation have shown that TNF-α induces an increase in DNA synthesis, and induces the proliferation of HASMC [Moon et al., 2003; Lee and Moon, 2005; Suh et al., 2006; Kim et al., 2007]. Although these studies have generally used 10 or 100 ng/ml of TNF-α, which is the same concentration used in this study, our system operates using a concept that is different from that of TNF-α-induced apoptosis. Therefore, to further confirm the inhibitory effect of emodin on TNF-α-induced proliferation, we examined the phosphorylation state of IκBα. As shown in Figure 2, treatment with TNF-α alone increased the phosphorylation of IκBα and completely degraded IκBα in TNF-α-treated HASMC, which indicates that TNF-α-induced proliferation via NF-kB activation. However, emodin dose-dependently inhibited the phosphorylation of IκBα, which may result in a reduction of the degradation of IκBα. This finding indicates that emodin inhibits a potential NF-κB activation by IκBα degradation in TNF-α-treated HASMC.

Emodin induces apoptotic cells (Fig. 3, upper panel), PARP cleavage (Fig. 3, lower panel) and caspase activation (data not shown, it was shown using caspase inhibitors in Fig. 4). Emodin also induces a cytochrome *c* release from mitochondria via a loss of $\Delta\Psi_m$ (Fig. 5A); a decreased Bcl-2 level; and an increased Bax protein level (Fig. 6). Therefore, activation of caspases and a mitochondrial

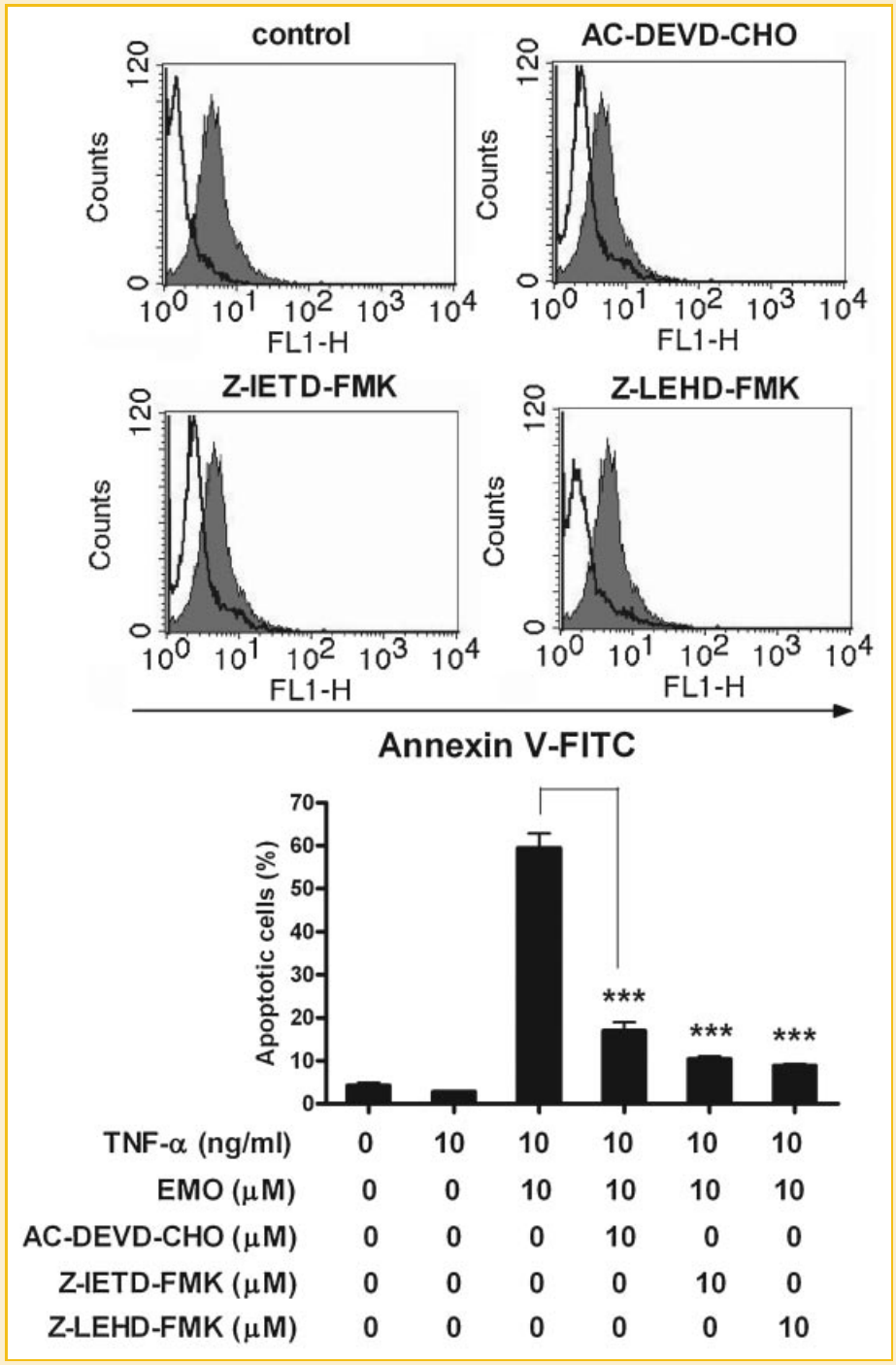

Fig. 4. Caspase-3, -8 and -9 are essential in emodin-induced apoptosis. HASMC were preincubated with a caspase-3 inhibitor, Ac-DEVD-CHO (10 μM); caspase-8 inhibitor, Z-IETD-FMK (10 μM); or caspase-9 inhibitor, Z-LEHD-FMK (10 μM) for 1 h, and then stimulated with EMO (10 μM) and TNF-α (10 ng/ml) for 24 h and harvested. Cells (3×10^5) were stained with FITC-labeled annexin V, followed by FACS analysis. Filled histogram represents apoptotic cells treated with 10 μM EMO and TNF-α; open histogram indicates cells incubated in the presence of the indicated caspase inhibitor, Ac-DEVD-CHO (10 μM), Z-IETD-FMK (10 μM), or Z-LEHD-FMK (10 μM), respectively (upper panel). Data show the percentage of apoptotic cells (lower panel). Data shown are representative of at least three independent experiments. Data are the means ± SEM. *Significantly different from TNF-α alone, $^{***}P < 0.001$. EMO, emodin.

pathway is required for emodin-induced apoptosis in TNF-α treated HASMC to occur. This study also presents evidence that caspase-3, -8 and -9 as well as mitochondria contribute directly to emodin-mediated apoptosis (Figs. 4 and 5). The caspase-3 inhibitor, Ac-DEVD-CHO, inhibited emodin-induced apoptosis by up to 72 ± 7.7% in TNF-α treated HASMC and the caspase-8 inhibitor, Z-IETD-FMK, and the caspase-9 inhibitor, Z-LEHD-FMK, also inhibited emodin-induced apoptosis by up to 82 ± 4.5% and 85 ± 2.9% in TNF-α

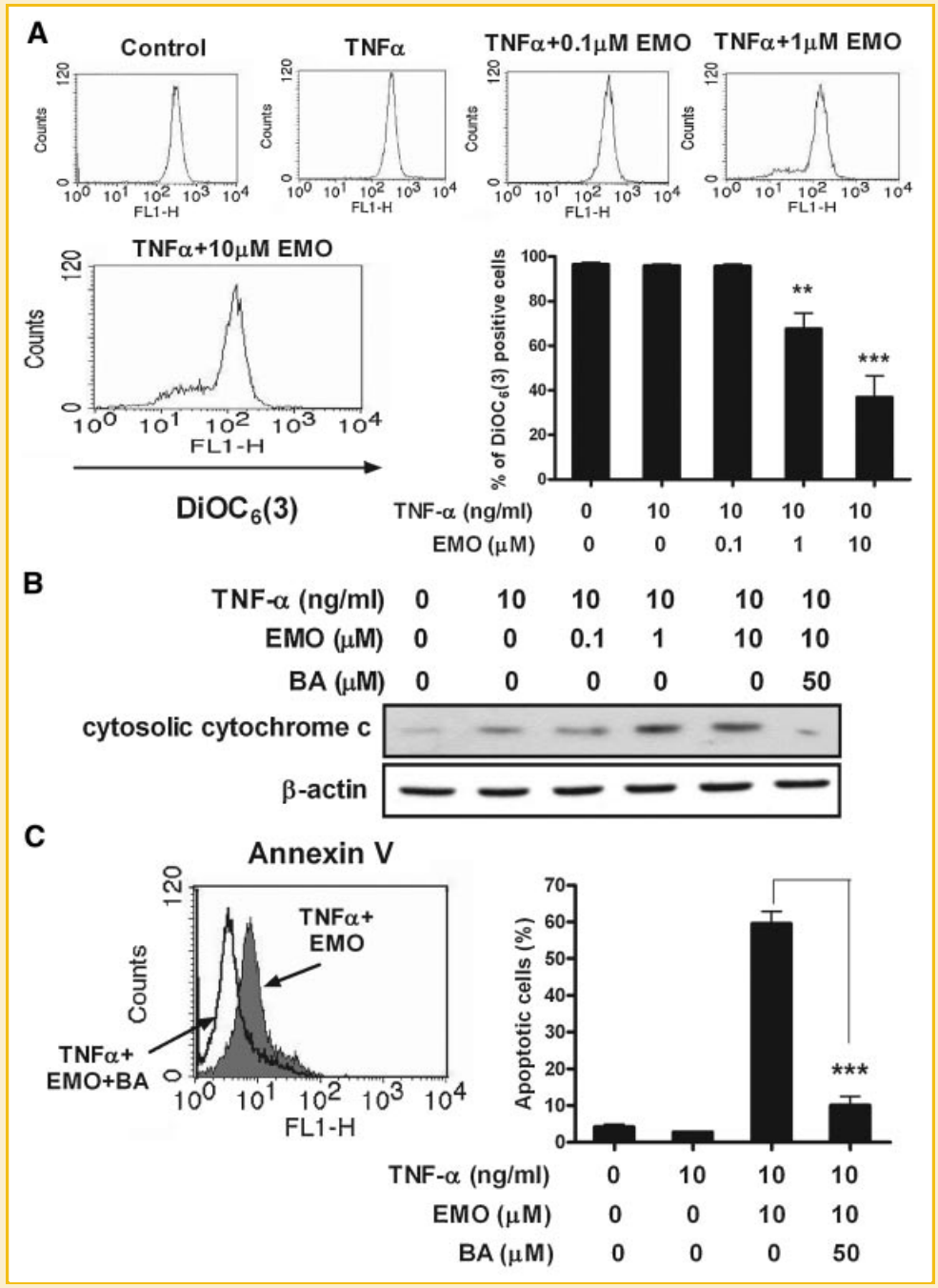

Fig. 5. Emodin-induced apoptosis involves the mitochondrial pathway. A: HASMC were treated with 0.1, 1 or 10 μM EMO in the presence or absence of TNF-α (10 ng/ml) for 24 h and then harvested. Cells (3×10^5) were stained with $DiOC_6(3)$ dye, followed by FACS analysizs. B: TNF-α-treated HASMC were incubated with 0.1, 1 or 10 μM EMO in the presence or absence of bongkrekic acid (BA, 50 μM) for 24 h and then harvested. Mitochondrial and cytosolic fractions were separated and 30 μg of each sample was then 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 HASMC were incubated with EMO (10 μM) in the presence (left panel, open histogram) or absence (left panel, filled histogram) of bongkrekic acid (50 μM) for 24 h and then harvested. Cells (3×10^5) were stained with FITC-labeled annexin V, followed by FACS analysis. Data show the percentage of apoptotic cells (right panel). Data shown are representative of at least three independent experiments. Data represent the means ± SEM. *Significantly different from TNF-α alone, $^{**}P < 0.01$ and $^{***}P < 0.001$. EMO, emodin. BA, bongkrekic acid.

treated HASMC, respectively (Fig. 4). Furthermore, BA (bongkrekic acid, inhibitor of MMP disruption) efficiently inhibited emodin-induced apoptotic cells by up to 80.4 ± 13.3% in TNF-α treated HASMC (Fig. 5B and C). Taken together, these results indicate that emodin-induced cell death occurred via caspase- and mitochondria-dependent apoptosis.

It has been suggested that emodin-treated HASMC were killed by apoptosis in atherosclerotic lesions and inflammation (TNF-α-

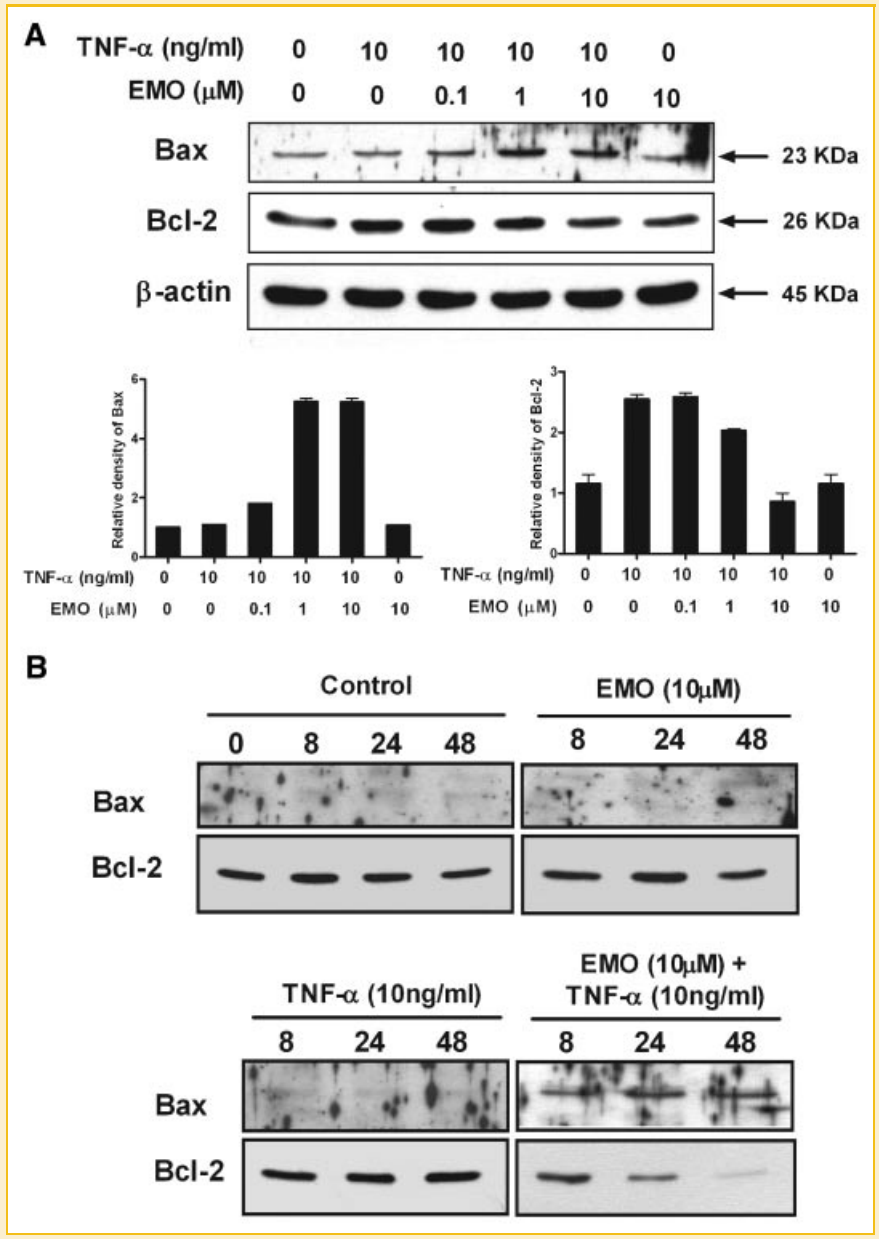

Fig. 6. Emodin regulates the expression of Bcl-2 family proteins. TNF-α-treated HASMC were incubated with EMO for the indicated dosages and times at 37°C. The supernatants of cell lysates were then analyzed by Western blot for the expression of Bcl-2 and Bax. The membrane was stripped and reprobed with anti-β-actin mAb to confirm equal loading. A: Dose-dependent responses in 24 h. B: Time-dependent responses in indicated dosages. EMO, emodin.

affected HASMC), which in turn promoted PARP cleavage and caspase activation, released cytochrome *c* from mitochondria via a loss of $\Delta\Psi_m$ (mitochondrial disorganization), decreased survival protein Bcl-2, and increased the apoptotic protein Bax (fivefold of control groups, Fig. 6). It is interesting to note that emodin alone (10 μM) had no effect on proliferation (Fig. 1B), NF-kB activation (Fig. 2), PARP cleavage (Fig. 3B), caspase activation (Fig. 4), cytochrome *c* release (Fig. 5B), or Bak expression (Fig. 6B) in HASMC. Taken together, these results indicate that emodin induces cell death in inflammatory sites only.

This study may have important pathological and therapeutic implications because overgrowth of VSMC is a pivotal etiologic factor in the development of atherosclerosis and restenosis following angioplasty. To date, inhibition of VSMC proliferation is among the most effective strategies for preventing their overgrowth and controlling neointimal thickening [Chien and Hoshijima, 2004; Guo et al., 2007]. Although VSMC apoptosis plays a critical role in the control of VSMC overgrowth and neointimal thickening, it can interrupt neointimal formation at defined time points and is inversely correlated with restenosis [Bochaton-Piallat et al., 1995;

Durand et al., 2002]. In addition, it is well known that the consequence of VSMC apoptosis depends on the stage and location in the plaque [Rakesh and Agrawal, 2005]. Furthermore, the consequences of VSMC apoptosis can also be detrimental to the overall stability of the plaque, thereby causing thrombosis and rupture [Bauriedel et al., 1999]. 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 emodin-mediated VSMC apoptosis has on thrombosis and plague rupture is not yet known and will be evaluated in a future study using animal models to better understand how they occur. However, it is obvious that emodin inhibits TNF-α-induced human aortic smooth-muscle cell proliferation via caspase- and mitochondrial-dependant apoptosis. Moreover, emodin prevents overgrowth of VSMC and the development of atherosclerosis. Therefore, emodin can be used to prevent atherosclerosis via induction of apoptosis in VSMC.

Emodin, a natural anthraquonoid compound, is an active component of the dried root of Rhei Rizoma (Rheum palmatum, Daehwang, Korea) and is also present in many herbs and vegetables (cabbage, lettuce, beans, peas) [Huang et al., 2007; Wang et al., 2007]. Emodin has anti-cancer, antiviral, antimicrobial, immunosuppressive, hepatoprotective, anti-inflammatory, and wound healing properties; however, little is known about the anti-atherosclerosis activity of emodin or its signal pathway [Huang et al., 1992, 2007; Chang et al., 1996; Lin et al., 1996; Hatano et al., 1999; Shuangsuo et al., 2006; Tang et al., 2007]. It was recently reported that emodin stopped the growth and induced the death of human VSMC through reactive oxygen species and p53 [Wang et al., 2007]. Emodin has also been shown to inhibit rabbit iliac artery smooth-muscle cell proliferation following percutaneous transluminal angioplasty (PTCA) [Guo et al., 1996]. However, little is known about emodin's anti-atherogenic effects or its signal pathway (and mechanism) in HASMC. In spite of this, other anthraquinone components of rhubarb, such as aloe-emodin, have been reported to have an anti-proliferative effect in VSMC after arterial injury [Yin and Xu, 1998]. Furthermore, rhein has also been isolated from rhubarb and identified as a compound that enhanced anti-cancer activity [Lin et al., 2003]. Therefore, further investigation regarding the effect of rhein should be conducted to determine if it has anti-atherosclerosis activity that leads to cytotoxicity or its signal in TNFα-treated HASMC.

In conclusion, emodin inhibits TNF-α-induced HASMC proliferation via caspase- and a mitochondrial-dependant apoptotic pathway. Emodin has the potential for use as an anti-atherosclerosis agent and may be particularly useful for preventing restenosis after PTCA. To the best of our knowledge, this is the first study to report that inhibition of TNF-α-induced HASMC proliferation is directly involved in caspase- and mitochondrial-dependant apoptosis.

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