Activated protein C inhibits bisphosphonate-induced endothelial cell death via the endothelial protein C receptor and nuclear factor-κB pathways

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Abstract. Bisphosphonates promote apoptosis of cancer cells as well as osteoclasts in bone metastatic sites, but several clinical trials and high concentration treatment have shown that bisphosphonates have side effects that include bone loss and damage to normal cells. The aim of this study was to elucidate the protective effect and the possible mechanism of activated protein C (APC) against bisphosphonate-induced cell damage using primary cultured human umbilical vein endothelial cells (HUVECs). HUVECs were treated with APC (10 μg/ml) for 1 h, and then treated with bisphosphonates including alendronate, zoledronate and pamidronate. Bisphosphonates induced cell death in HUVECs but the cell death was blocked by treatment with APC. Bisphosphonates markedly induced caspase-3 activation, which was diminished in cells exposed to APC. Matrix metalloproteinase-2 (MMP-2) reduction and nuclear factor-κB (NF-κB) activation induced by bisphosphonates in HUVECs were also blocked by APC treatment. Furthermore, APC highly induced the expression of the endothelial protein c receptor (EPCR) in HUVEC cells. In conclusion, the present study demonstrates that APC inhibits bisphosphonate-induced endothelial cell death via EPCR-induced inactivation of caspase-3 and NF-κB, and also suggests that APC has the potential to be a therapeutic drug in various vascular diseases induced by endothelial cell damage.

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Introduction

Activated protein C (APC) is a natural anticoagulant that is derived from its vitamin K-dependent plasma precursor protein C. Thrombin binds to thrombomodulin to activate protein C on the endothelial cell surface. The conversion to APC is augmented in the presence of its specific receptor, endothelial protein C receptor (EPCR) (1,2). APC is a physiological anticoagulant with anti-inflammatory and cytoprotective effects exerted through transcriptional regulation (3-5). APC manifests antiapoptotic activity both *in vitro* and *in vivo*, and this activity requires the enzymatic active site of APC and its receptors, EPCR and protease-activated receptor 1 (PAR-1). APC inhibits many characteristic apoptotic features, including DNA degradation, caspase activation and phosphatidylserine translocation to the outer cell membrane (3,6,7). APC also reduces the up-regulation of the proapoptotic Bax and maintains levels of the protective Bcl-2 protein during hypoxic stress of brain endothelial cells (6).

Bisphosphonates are used in the prevention and treatment of metastatic and myelomatous bone disease and osteoporosis (8-10). They primarily inhibit osteoclast-mediated bone resorption but also directly affect different types of tumor cells. Bisphosphonates, such as alendronate (Aln), inhibit cell adhesion to extracellular matrix proteins *in vitro* and secretion of matrix metalloproteinases (MMPs) by tumor cells (11,12). Other bisphosphonates including pamidronate (Pam), clodronate and zoledronate (Zol) induce apoptosis of breast cancer cells *in vitro* and inhibit angiogenesis and cell proliferation (13-15). However, high doses may cause oral avascular bone necrosis because of an antiangiogenic effect in patients receiving chemotherapy for malignancies (16,17). Moreover, several clinical trials have shown that bisphosphonates do not prevent local bone loss in patients with inflammatory arthritis (18,19). Ultimately, clinical use of biphosphonates requires more research *in vitro* and *in vivo* to reduce side effects.

The present study was undertaken to examine the direct effects of bisphosphonates on primary cultured human umbilical vein endothelial cells (HUVECs) and to elucidate APC's protective effect and possible mechanism against bisphosphonate-induced endothelial cell damage.

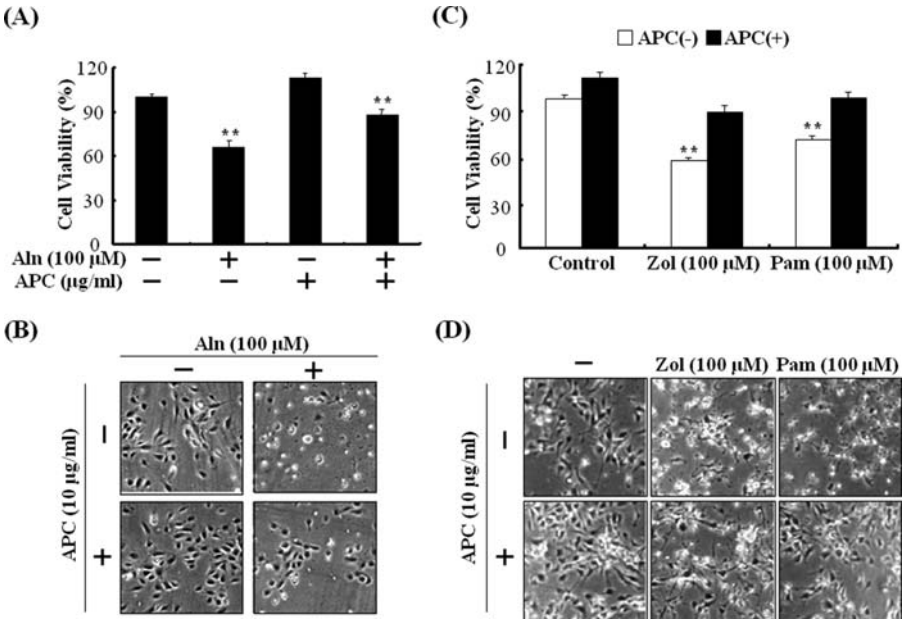

Figure 1. Protective effect of activated protein C (APC) on bisphosphonate-induced endothelial cell death. (A) HUVECs were untreated or pretreated with 10 μg/ml APC for 1 h and then treated with Aln (100 μM) for 48 h. Cell viability was determined by an established crystal violet staining method. Viability of control cells was set at 100%, and viability relative to the control was presented. The experiments were performed in triplicate, at least two independent times. The bars represent the mean ± standard deviation. **P<0.01 vs. control group. (B) Cell morphology under the conditions was photographed (x200). (C) HUVECs were untreated or pretreated with 10 μg/ml APC for 1 h and then treated with Zol (100 μM) and Pam (100 μM) for 48 h. Cell viability was determined as described above. (D) Cell morphology was photographed (x200). Aln, alendronate; Zol, zolendronate; Pam, Pamindronate.

Materials and methods

Cell isolation and culture. HUVECs were isolated from human umbilical veins as previously described (20) and were cultured in BioRich medium containing 20% fetal calf serum (FCS) plus 50 μg/ml endothelial cell growth supplement (Sigma-Aldrich, St. Louis, MO) and 50 μg/ml heparin (Sigma-Aldrich) at 37˚C in an atmosphere of 5% CO_2 in a humidified incubator. Twelve umbilical cords were used to obtain cells; there was little difference in the response of cells from different cords. Cells were starved for 24 h in their respective medium containing 2% FCS before treatment. Cells were treated with recombinant human APC (drotrecogin alfa, activated) Aln, Zol and Pam (Eli Lilly, Indianapolis, IN). Cells were used in experiments up to passage 4.

Cell viability. Cells were plated in wells of 12- or 24-well plates and were either untreated or pretreated with 10 μM APC for 1 h. The cells were then incubated with Aln (100 μM), Zol (100 μM) and Pam (100 μM) for 48 h. Cell morphology was observed and photographically recorded using light microscopy. Cell viability was determined by crystal violet (Sigma-Aldrich) dissolved in phosphate buffered saline (PBS). Briefly, cells were stained for 10 min at room temperature with a solution containing 0.5% crystal violet in 30% ethanol and 3% formaldehyde, washed four times with water and dried. The cells were then lysed with 1% sodium dodecyl sulfate (SDS) and the absorbance was measured at 550 nm. Cell viability was calculated based on the relative dye intensity compared to controls.

Protein extraction and Western blotting. HUVECs were washed three times with PBS and lysed in a cell lysis buffer (HEPES with 1% Triton X-100 and 10% glycerol) supplemented with protease and phosphatase inhibitors (Roche, Indianapolis, IN). Cell lysates were centrifuged at 10,000 x g for 15 min, and total protein concentration was determined using the BCA protein assay kit (Pierce Biotechnology, Rockford, IL). Equal amounts of protein were separated by 8-15% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting was performed as previously described (2). Primary monoclonal antibodies used were anti-cleaved caspase-3 antibody (R&D Systems, Minneapolis, MN) and the anti-human active form of NF-κB (Chemicon, Temucula, CA). Anti-human ß-actin antibody was included to normalize against unequal loading.

Zymography assay. MMP-2 secretion and activation were detected by gelatin zymography under non-reducing conditions as previously described (21). Cell culture supernatants were loaded onto a 10% SDS gel containing 0.5 mg/ml gelatin. Relative levels of MMP-2 were semi-quantified using the Gel-Pro Analyzer (Media Cybernetics, Bethesda, MD).

Immunofluorescent staining. HUVECs were cultured on gelatin (0.2% w/v)-coated four-chamber glass slides and processed for immunofluorescent staining as previously described (22). Briefly, HUVECs were fixed with cold acetone and blocked by 5% horse serum in PBS, and incubated with rabbit anti-human active caspase-3 antibody (R&D Systems), rabbit anti-human EPCR antibody (Santa Cruz Biotechnology, Santa Cruz, CA) or mouse anti-human active NF-κB antibody overnight at 4˚C. After three washes with PBS, cells were incubated with anti-rabbit IgG conjugated with Cy3 (red), anti-mouse IgG conjugated with AMCA (blue) and anti-goat IgG conjugated with fluorescein isothiocynate (FITC; green)

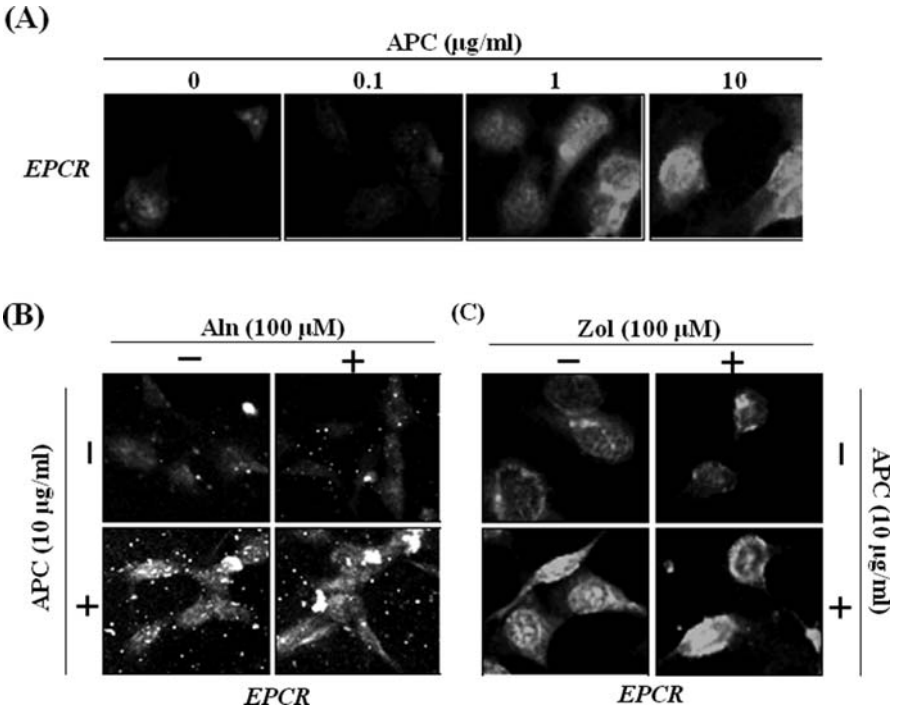

Figure 2. Endothelial protein C receptor (EPCR) expression in endothelial cells by bisphosphonates and/or activated protein C (APC). (A) HUVECs were untreated or treated with APC (0.1, 1 and 10 μg/ml) for 25 h, and cells were immunostained with an EPCR antibody and photographed by a fluorescence microscope. (B) HUVECs were treated with Aln (100 μM) or with (C) Zol (100 μM) for 24 h and either untreated or treated with 10 μg/ml APC. Cells were immunostained with EPCR antibody and photographed by a fluorescence microscope. Aln, alendronate; Zol, zolendronate.

(Invitrogen, Carlsbad, CA). Cells were washed with PBS, mounted with ProLong Gold antifade reagent with or without 4',6-diamidino-2-phenylindole (DAPI; Invitrogen) and observed with an Eclispse 80i fluorescence microscope (Nikon Corporation, Tokyo, Japan).

Statistical analysis. Statistical differences were analyzed using the Student's t-test. ANOVA was used to compare means among three or more independent groups, followed by the Newman-Keuls post-hoc test. Results are displayed as the mean ± standard deviation (SD).

Results and Discussion

APC inhibits bisphosphonate-induced endothelial cell death. Bisphosphonates directly induce apoptosis of osteoclasts through the mevalonate pathway and prevent prenylation of small GTP-binding proteins such as Ras and Rho. In addition, bisphosphonates inhibit growth of various cell types including cancer cells and chondrosarcoma (23). However, several clinical trials have shown that bisphosphonates fail to prevent local bone loss in patients with inflammatory arthritis (18,19). The reasons for this poor efficacy have yet to be elucidated. In contrast, APC acts directly on cells to exert multiple cyto-protective effects including alteration of gene expression profiles, anti-inflammatory activities, anti-apoptotic activity and protection of the endothelial barrier function. In cultured human skin keratinocytes and tenocytes, APC enhances cell proliferation and migration (24,25). Also, APC accelerates cutaneous wound healing due to increased cell proliferation and migration, and it attenuates keratinotyte apoptosis (26). Therefore, we examined the possibility of endothelial cell death by treatment with bisphosphonates and the protective effect of APC on bisphosphonate-induced endothelial cell death.

Primary cultured HUVECs were pretreated with or without 10 μg/ml APC for 1 h and then exposed to Aln (100 μM), Zol (100 μM) or Pam (100 μM) for 48 h prior to assessment of cell viability using an established crystal violet assay. APC alone accelerated cell proliferation, while Aln alone induced cell death by ~40%. Combined APC-Aln treatment markedly prevented Aln-induced HUVEC death (Fig. 1A). Examination of cell morphology also supported the protective effect of the APC-Aln combination in HUVECs (Fig. 1B). Zol and Pam treatments also induced cell death which was reversed by treatment with APC (Fig. 1C and D). Overall, the data show that bisphosphonates such as Aln, Zol and Pam could induce cell death in normal endothelial cells and APC could inhibit bisphosphonate-mediated endothelial cell damage.

EPCR is expressed in endothelial cells in response to APC, but not bisphosphonates. Many of the healing properties of APC are mediated by its specific receptor, EPCR, which is expressed on the surface of endothelial cells, keratinocytes and some leucocytes (25,26). Several studies have shown that by utilizing EPCR as a co-receptor, APC can cleave PAR-1 on the endothelial surface and exert cytoprotective effects (24,27). The present study probed how APC and/or bisphosphonates alter endothelial cell expression of EPCR. Primary cultured HUVECs were maintained in serum-free medium for 24 h to remove any soluble EPCR, followed by

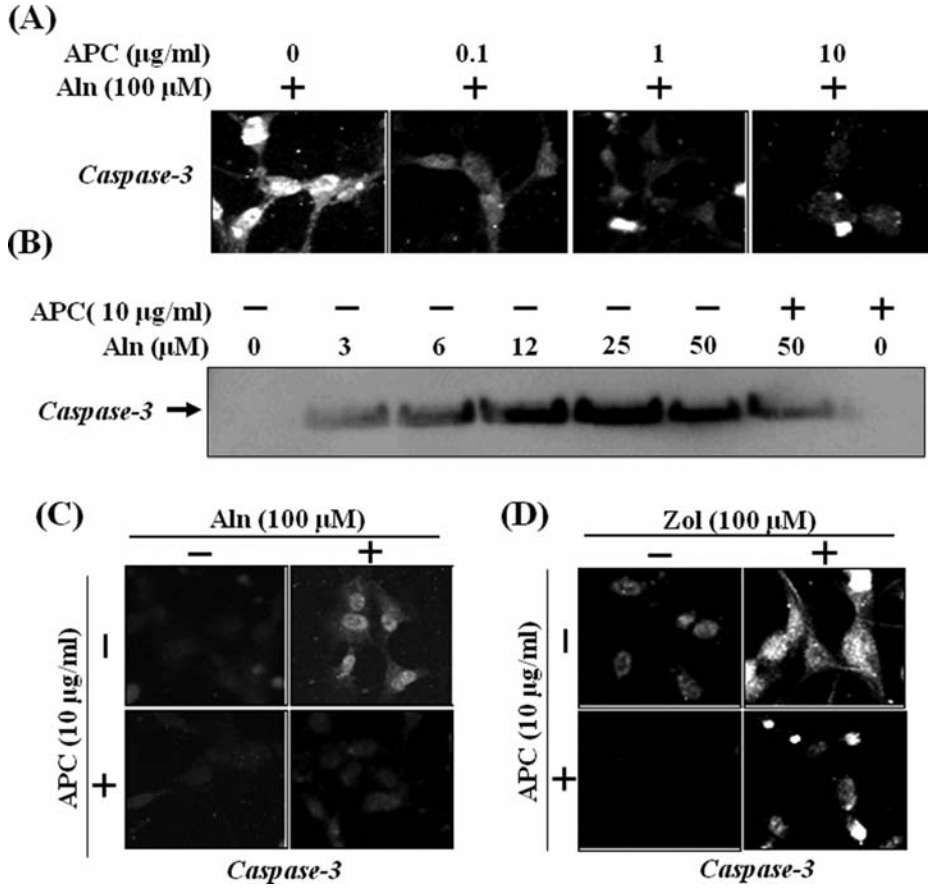

Figure 3. Inhibitory effect of activated protein C (APC) on bisphosphonate-induced caspase-3 activation in endothelial cells. (A) HUVECs were either untreated or pretreated with APC (0.1, 1 and 10 μg/ml) for 1 h, prior to either no treatment or treatment with 100 μM Aln for 24 h. Cells were immunostained with EPCR antibody and photographed by a fluorescence microscope. (B) HUVECs were pretreated with or without 10 μg/ml APC for 1 h, and were either untreated or treated with Aln (3, 6, 12, 25 and 50 μM) for 24 h. Active caspase-3 was detected in cell lysates using Western blotting. (C) HUVECs were treated with Aln (100 μM) or with (D) Zol (100 μM) for 24 h and then were either untreated or treated with 10 μg/ml APC. Active caspase-3 was detected by immunofluorescence staining. Aln, alendronate; Zol, zolendronate.

washing and assessment of EPCR by immunofluorescent staining. APC dose-dependently increased EPCR expression in HUVECs (Fig. 2A), whereas treatment of cells with Aln or Zol did not affect EPCR expression as compared to the untreated cells. Addition of APC in the presence of Aln or Zol, resulted in the highest EPCR staining intensity as compared to cells treated solely with Aln or Zol (Fig. 2B and C). Together, these data suggest that the APC-induced elevated level of EPCR in endothelial cells may play a potentially protective role in response to bisphosphonate-induced endothelial cell death.

APC inhibits bisphosphonate-induced cell death by inhibiting active caspase-3 in endothelial cells. Apoptotic signals coming from inside the cell activate the intrinsic pathway in response to cellular stress. Release of cyochrome c from the mitochondria and the subsequent procaspase-3 activation are key steps for the intrinsic pathway. Caspases play a key role in the execution phase of apoptosis. 'Initiator' caspases, such as caspase-8, activate 'effector' caspases, such as caspase-3 and caspase-7, which subsequently cleave cellular substrates, thereby precipitating the dramatic morphological changes of apoptosis. Several studies reported that bisphosphonates such as minodronate, decrease Bcl-2 expression and induce Bax expression, caspase-3 activity and degradation of poly(ADP-ribose) polymerase (PARP) in prostate cancer cells (28). Furthermore, as a clear indicator of apoptosis, strong cleavage of caspase-3, caspase-7 and PARP was detected in MDA-MB-231 cells exposed to Zol (29). Appropriately, the change of the key apoptotic regulator caspase-3 during activation by bisphosphonate treatment in endothelial cells and the change of this protein by APC pretreatment and subsequent treatment by bisphosphonates was assessed.

To examine whether active caspase-3 expression was changed by APC and/or bisphosphonates in endothelial cells, HUVECs were treated with 100 μM Aln or 100 μM Zol for 24 h after treatment with 0, 0.1, 1 or 10 μg/ml APC for 1 h. Cells treated with Aln alone for 24 h expressed high levels of active caspase-3, assessed using immunofluorescence, which was decreased by the addition of APC 1 h prior to Aln treatment in a dose-dependent manner (Fig. 3A). Western blotting showed that caspase-3 activation was increased by Aln in a dose-dependent fashion in HUVECs and was blocked in response to APC treatment (Fig. 3B). Furthermore, Zol treatment markedly induced caspase-3 activation in HUVECs which was diminished in response to APC treatment (Fig. 3C).

APC treatment notably reduces keratinocyte expression of active caspase-3, one of a group of intracellular proteases

that are responsible for the systematic disassembly of the cell into apoptotic bodies during apoptosis (30). APC has also been shown to attenuate calcium-induced cell death via prevention of cell apoptosis, as indicated by a decrease in both active caspase-3 and morphologically apoptotic cells (25). The results confirm that APC treatment in HUVECs decreases caspase-3 expression and reduces bisphoshonate-induced caspase-3 activation. These results support the suggestion that APC may attenuate endothelial cell damage by inhibition of active caspase-3 induced upon exposure to bisphosphonates.

APC protects endothelial cells from bisphosphonate-induced death via inhibition of NF-κB and enhancement of MMP-2 activity. NF-κB is a transcription factor that regulates the expression of genes involved in immune and inflammatory responses and plays a pivotal role in the regulation of inflammation (31). Aln inhibits the cell survival pathway stimulated by the phosphoinositide 3-kinase (PI3K)/Akt/NF-κB pathway by inhibiting the initial step, the activation of PI3K, thus causing apoptosis of osteosarcoma cells, and zoledronic acid induces the activation of NF-κB in dendritic cells (32,33). However, APC can directly suppress the production of tumor necrosis factor (TNF) and the activation of NF-κB and MAP kinase, p38 (34). In our study, activated NF-κB was strongly detected in cells treated solely with Zol compared to untreated cells (Fig. 4A). NF-κB activation by Zol was inhibited by the addition of APC. In addition, NF-κB activation by Aln in cells was blocked in response to APC treatment (Fig. 4B).

MMPs are a family of zinc-dependent endopeptidases capable of degrading all major components of the extracellular matrix. A number of studies have demonstrated that MMPs are important mediators in inflammatory and connective tissue diseases (35,36). MMP-2 is constitutively expressed by most cell types and is usually not induced by cytokines or growth factors (36). In contrast, basal levels of MMP-9 are usually low and its expression can be induced by different cytokines including TNF-α. In addition to extracellular matrix degradation, MMP-9 has stimulatory and MMP-2 has inhibitory effects on inflammation (37,38). Treatment with Zol dose-dependently down-regulated pro- and active-MMP-2 (Fig. 4C and D). APC had minimal effect on pro-MMP-2 but enhanced MMP-2 activation after bisphosphonate treatment in HUVECs. APC actually increased MMP-2 production and activation in HUVECs, but Aln and Zol decreased MMP-2 production and activation as compared to untreated HUVECs, and the addition of APC blocked these effect (Fig. 4D). Aln has been shown to reduce the activity and mRNA levels of MMP-2 and inhibit cell invasion in the chondrosarcoma cell line (39). APC can inhibit MMP-9 via NF-κB, while at the same time promoting MMP-2 activity (34). The present results also demonstrate that MMP-2 production and activation were reduced, and NF-κB expression was increased in HUVECs by Aln and Zol treatment. However, MMP-2 reduction and NF-κB activation by Aln and Zol were blocked in response to APC treatment. The present findings suggest that APC could protect endothelial cells from bisphosphonate-induced death via NF-κB inhibition and regulation of MMP-2 activity.

In conclusion, this study revealed that bisphosphonates such as Aln, Zol and Pam induce cell death through the activation of caspase-3 in endothelial cells. These findings suggest that APC has the potential to be part of a combined therapeutic drug regimen to reduce side effects such as bone loss and normal cell damage. This potential must be assessed in clinical trials of bisphosphonates.

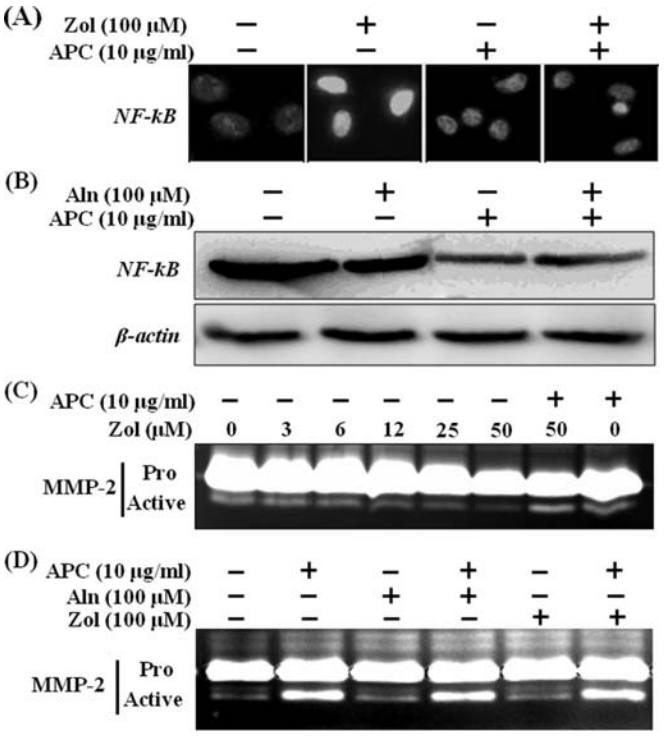

Figure 4. Protective effect of activated protein C (APC) by NF-κB inhibition and MMP-2 activity in endothelial cells. (A) HUVECs were treated with 100 μM Zol for 24 h with or without 10 μg/ml APC, and the active form of NF-κB was detected by immunofluorescence staining. (B) HUVECs were untreated or pretreated with 10 μg/ml APC for 1 h, prior to a 24 h treatment with 100 μM Aln. The active form of NF-κB in whole cell lysates of HUVECs was detected by Western blotting using an antibody against active NF-κB. ß-actin was used as the loading control. (C) MMP-2 was detected in supernatants of untreated cells or of cells treated with Zol (3, 6, 12, 25 and 50 μM) with or without treatment of 10 μg/ml of APC using gelatine zymography. (D) MMP-2 was detected in supernatants of cells untreated or treated with Aln (100 μM) or Zol (100 μM) for 24 h and following no treatment or treatment 10 μg/ml APC using gelatine zymography. Aln, alendronate; Zol, zolendronate.

Acknowledgements

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