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Biphasic effect of aspirin on apoptosis of bovine vascular endothelial cells and its molecular mechanism

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Key words

aspirin; hydroperoxide; apoptosis; mitogen-activated protein kinase; endothelial cells

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Abstract

Aim: To investigate the effect of aspirin on the apoptosis of cultured bovine aortic endothelial cells (BAEC) and the signal pathways involved in this process. **Methods:** BAEC were cultured and passaged in Dulbecco's modified Eagle's medium culture medium. Morphologic changes and quantification of apoptotic cells were determined using fluorescence microscope after staining the cells with Hoechst 33258. Cell viability was measured by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) method. DNA fragmentation was visualized by agarose gel electrophoresis. Phospho-p38 mitogen-activated protein kinase (MAPK) expression was detected by Western blotting. **Results:** Aspirin at low concentrations from 1×10^{-10} mol/L to 1×10^{-8} mol/L decreased the apoptosis and p38 MAPK phosphorylation induced by H_2O_2 in BAEC, while high doses of aspirin (1×10^{-7}–1×10^{-4} mol/L) induced typical apoptotic changes in BAEC and stimulated the expression of phospho-p38 MAPK in a concentration-dependent manner. SB203580, a specific p38 MAPK inhibitor, blocked such effects. **Conclusion**: Aspirin exhibits a biphasic effect on the apoptosis in BAEC, reducing apoptosis at low concentration and inducing apoptosis at high concentration. p38 MAPK may be an important signal molecule mediating the effects of aspirin.

Introduction

Aspirin (acetylsalicylic acid, ASA), a traditional analgesic-antipyretic drug, is now widely used in the prevention of cardiovascular diseases[1] and may even reduce the risk of Alzheimer's disease[2] and cancers[3]. The classic mechanism of aspirin in the prevention of cardiovascular diseases has been known to inhibit the activity of cyclooxygenase (COX) in platelets, which leads to the reduction of prostaglandin and thromboxane A_2 production[4]. However, recent studies show that the COX-inhibiting mechanism can not provide a full explanation of the cardiovascular protective effects of aspirin. Accumulating experimental evidence have indicated that many other effects of aspirin contribute to its cardiovascular protection, such as modulating the activity of the intracellular metabolism of ATP, inhibiting inducible nitric oxide synthase, modulating the activity of nuclear factor (NF)-kappa B and mitogen-activated protein kinases (MAPK)[5–7].

Apoptosis, or programmed cell death, is an active progress of cell elimination in physiological or pathological conditions[8]. Pathological apoptosis of endothelial cells not only damages the integrity of endothelium, but also affects the cytokine secretion of endothelial cells. Impaired endothelium function will then facilitate the formation of thrombus and promote atherogenesis[9]. However, inducing apoptosis of endothelial cells is potentially an effective strategy for blocking the progression of tumor development by inhibiting angiogenesis[10].

MAPK [extracellular signal-regulated kinase, Jun N-terminal kinase (JNK) and p38 MAPK] are well-known signal molecules involved in the regulation of cell proliferation and apoptosis. Among the 3 MAPK family members, p38 MAPK is activated by cell stress promoting cell apoptosis[11]. Aspirin has been shown to modulate MAPK expression in various cell types, including vascular endothelial cells[12–14]. In the present study, we investigated the direct effect of aspi-

rin on the apoptosis of bovine aorta endothelial cells and the role of p38 MAPK in this process.

Materials and methods

Cell culture Bovine aortic endothelial cells (BAEC) were harvested as previously described[15] and cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% heat-inactivated fetal bovine serum (FBS), 100 kU/L benzylpenicillin, and 100 mg/L streptomycin. Confluent cells were subcultured by trypsin digestion. Experiments were performed with cells from passages 4 to 10.

Cell viability assay Cell viability was measured through a 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. BAEC were seeded into 24-well plates and grown to 80% confluence in DMEM with 10% FBS. After starved in phenol-free M1640 medium for 24 h, the cells were incubated with the respective test substance. MTT dissolved in phenol red free M1640 at a concentration of 5 g/L was added to the cell cultures at the end of the experi-ments. After incubation for 1 h at 37 °C, solubilization solution containing 10% (*v*/*v*) Triton X-100 and 0.1 mol/L HCl in isopropanol were added into the cell cultures to stop the reaction and dissolve formazan crystals. Metabolic activity was quantified by measuring light absorbance at 570 nm.

Morphologic determination and quantification of apoptosis After the respective treatment, BAEC were stained with Hoechst 33258 and then observed under a fluorescence microscope. Under the fluorescence microscope, apoptotic cells with condensed or fragmented nuclei were easily distinguished from normal cells with intact nuclei. Quantification of apoptosis was routinely determined by counting the number of apoptotic cells: for each plate, we randomly chose 6 fields of view, and counted the total cells and apoptotic cells with a minimum number of 500 cells scored.

DNA electrophoresis At the end of experiments, the cells were harvested and DNA was extracted with standard phenol-chloroform extraction. Electrophoresis of DNA was performed in ethidium bromide-stained 1.5% agarose gel and visualized by exposure under UV light.

Western blotting BAEC cultured in 12-well culture plates were grown to 80%–90% confluence and then starved for 24 h in serum-free M1640 medium. After different treatment, the cells were scraped into lysis buffer containing (in mmol/L) NaCl 50, Na_3VO_4 2, phenylmethylsulfonyl fluoride 0.5, and HEPES 10 at pH 7.4, along with 0.01% Triton X-100 and 10 mg leupeptin. The cells were then disrupted by sonication, after which they were centrifuged for 20 min at 12 000×*g* to separate particular proteins from soluble fractions. The amount of proteins in each fraction was determined by the bicinchoninic acid (BCA) method.

Proteins (10 mg) were subjected to SDS-PAGE and electrophoretically transferred to a nitrocellulose membrane. The membranes were blocked with Tris-buffered saline Tween-20 (TBST) containing 5% bovine serum albumin (BSA) and immunoblotting was performed using either an antitotal-p38 MAPK antibody or antiphospho-p38 MAPK antibody. The membranes were then incubated with a second antibody conjugated to horseradish peroxidase and the blot was visualized by the Phototope Western Detection System (New England Biolabs, Ipswich, MA, USA) To control equal protein concentration in the experiments, 2 gels for each group were loaded parallely with the same protein samples and blotted for activated, phosphorylated p38 MAPK or total p38 MAPK. Bands of protein were quantitatively determined by thin-layer chromatography with Shimadzu Dual-Wavelength Chromato-Scanner (Model CS-930, Tokyo, Japan).

Reagents BSA, DMEM medium, SB203580, and Hoechst 33258 were purchased from Sigma Chemical Co (St Louis, MO, USA). phospho-p38 MAPK monoclonal antibody, horseradish peroxidase (HRP)-conjugated anti-rabbit secondary antibody, and a Phototope-HRP Western Detection kit were purchased from New England Biolabs Inc (New England Biolabs, Ipswich, MA, USA).

Statistical analysis Values were expressed as mean±SD and assessed by one-way ANOVA and Student's *t*-test. Values of $P<0.05$ were considered to be statistically significant.

Results

Cell viability Low doses of aspirin protected BAEC from apoptosis induced by H_2O_2. Co-incubation of aspirin 1×10^{-10}–1×10^{-8} mol/L reversed the decrease of BAEC viability induced by H_2O_2 at 200 μmol/L. Interestingly, aspirin at 1×10^{-9} mol/L exhibited a stronger protective effect than 1×10^{-10} and 1×10^{-8} mol/L (Figure 1).

Unexpectedly, at relatively high concentrations, aspirin from 1×10^{-7} mol/L to 1×10^{-4} mol/L, showed no protection effect against the apoptosis of BAEC (data not shown), and it by itself decreased the viability of BAEC. After incubation with 1×10^{-4} mol/L aspirin for 24 h, cell viability decreased to 78.0% of the control (absorbance: 0.401±0.011 *vs* 0.514±0.019 of the control, $P<0.01$). SB203580 (10 μmol/L), a specific p38 MAPK inhibitor, markedly reduced the toxic effect of aspirin and increased cell viability by about 12.7% (absorbance: 0.452±0.012 *vs* 0.401±0.011 of 1×10^{-4} mol/L aspirin, $P<0.01$; Figure 2).

Morphologic changes After 24 h incubation of the indicated treatments, BAEC showed typical morphologic changes of apoptosis, such as reduction of cell volume and conden-

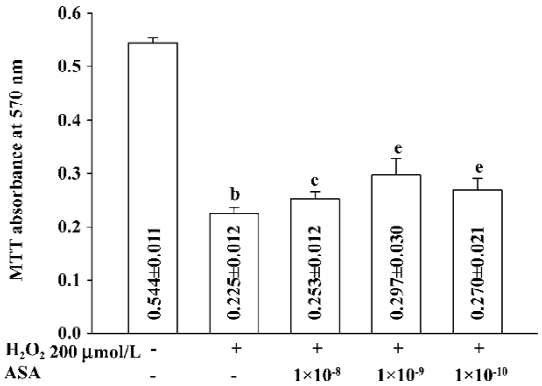

Figure 1. Effect of aspirin on bovine aortic endothelial cells viability induced by H_2O_2 (200 μmol/L). Cells were treated with the indicated treatments for 24 h and then analyzed by MTT assay. n=3 experiments. Average of triplicate constitutes 1 determination. Mean±SD. $^bP<0.05$ *vs* control. $^eP<0.05$ *vs* H_2O_2 200 μmol/L.

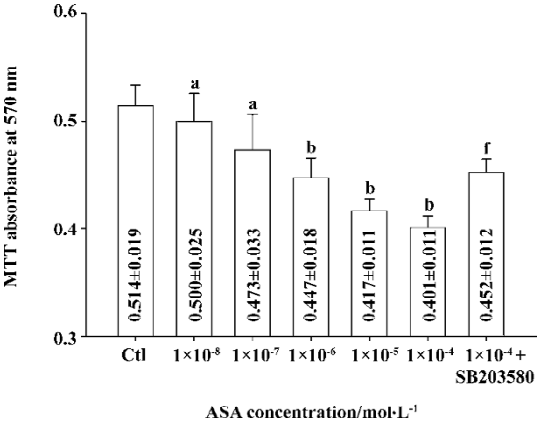

Figure 2. Effect of aspirin on bovine aortic endothelial cells viability. Cells were treated with the indicated concentrations of aspirin with or without 10 μmol/L SB203580 for 24 h and then analyzed by MTT assay. n=3 experiments. Average of triplicate constitutes 1 determination. Mean±SD. $^aP>0.05$, $^bP<0.05$ *vs* control. $^fP<0.01$ *vs* aspirin 1×10^{-4} mol/L.

sation and fragmentation of chromosomes; normal cells showed uniformed nuclei (Figure 3).

Quantification of apoptosis To further determine that the viability change of BAEC was mainly caused by apoptosis rather than necrosis, the apoptotic rate was directly measured under fluorescence microscope as described in Materials and methods.

At low concentration, aspirin from 1×10^{-10} mol/L to 1×10^{-8} mol/L reduced the apoptotic rate induced by 200 μmol/L H_2O_2 (Table 1). Incubation with aspirin (1×10^{-4} mol/L) for 24 h increased the apoptotic rate of BAEC to

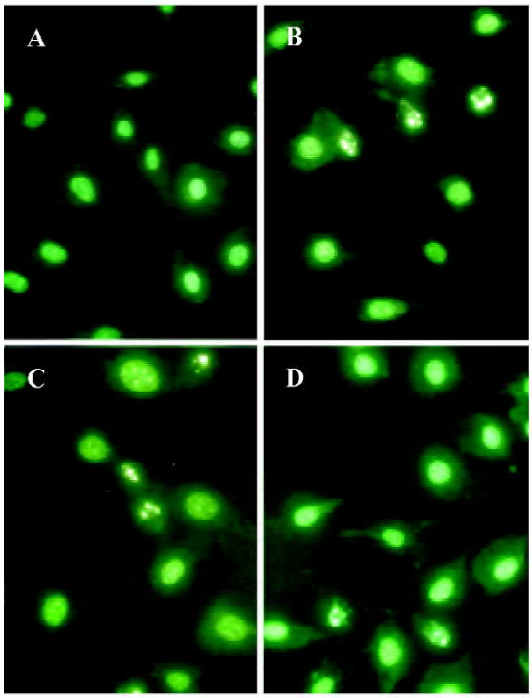

Figure 3. Fluorescence photomicrograph of BAEC stained with Hoechst 33258. magnification ×400. (A) control, nuclei of control (untreated) BAEC were stained uniformly. (B) BAEC treated with aspirin 1×10^{-4} mol/L showed typical apoptotic nuclei. (C) BAEC treated with 200 μmol/L H_2O_2 showed condensed or fragmented nuclei, typical of apoptosis. (D) compared with 200 μmol/L H_2O_2 alone, co-incubation of aspirin 1×10^{-9} mol/L and 200 μmol/L H_2O_2 showed a much less density of apoptotic cells.

Table 1. Effect of low concentration aspirin on H_2O_2 (200 μmol/L induced BAEC apoptosis. Cells were treated with or without the indicated treatments for 24 h and then the apoptotic cells were counted under a fluorescence microscope. n=3 experiments. Mean± SD. $^cP<0.01$ *vs* control. $^eP<0.05$ *vs* H_2O_2 (200 μmol/L).

Treatment (mol/L)	Apoptotic rate (%)
Control	3.3±2.1
H_2O_2	61.0±3.6^c
H_2O_2+ASA 1×10^{-8}	54.3±1.5^e
H_2O_2+ASA 1×10^{-9}	44.9±2.0^e
H_2O_2+ASA 1×10^{-10}	51.7±5.4^e

26.0%±1.8%, while pretreatment with SB203580 reduced the apoptotic rate to 13.9%±2.0% (Table 2). These results were consistent with the results in the cell viability assay.

DNA electrophoresis Following analyses with 1.5% agarose gel, DNA isolated from BAEC treated with 200 μmol/L H_2O_2 for 24 h showed a typical DNA ladder which represents

Table 2. Concentration-dependent effect of aspirin on apoptosis of BAEC and the effect of SB203580 (10 μmol/L)on aspirin-induced apoptosis in BAEC. n=3 experiments. Mean±SD. [a]P>0.05, [b]P<0.05 *vs* control. [c]P<0.05 *vs* aspirin 1×10^{-4} mol/L.

Aspirin concentration (mol/L)	Apoptotic rate (%)
0 (Control)	4.0±1.0
ASA 1×10^{-8}	4.4±0.7[a]
ASA 1×10^{-7}	10.0±2.6[b]
ASA 1×10^{-6}	14.9±2.9[b]
ASA 1×10^{-5}	21.3±3.8[b]
ASA 1×10^{-4}	26.0±1.8[b]
ASA 1×10^{-4}+SB203580	13.9±2.0[c]

integer multiples of the internucleosomal DNA length (about 180–200 base pair). Aspirin at 1×10^{-9} mol/L greatly reduced DNA fragmentation (Figure 4). Incubation with aspirin (1×10^{-4} mol/L) for 24 h also induced a typical DNA ladder which was reversed by 10 μmol/L SB203580, a specific inhibitor of p38 MAPK (Figure 5).

Phospho-p38 MAPK expression We previously showed that H_2O_2 activated p38 MAPK in BAEC[16]. In this study, aspirin from 1×10^{-10} to 1×10^{-8} mol/L significantly decreased p38 MAPK phosphorylation induced by 200 μmol/L H_2O_2 (Figure 6). Interestingly, aspirin at 1×10^{-9} mol/L exhibited the strongest effect, which paralleled well with its anti-apoptotic effect.

Aspirin from 1×10^{-7} mol/L to 1×10^{-4} mol/L dose-dependently increased the phosphorylation of p38 MAPK in BAEC, while SB203580 at 10 μmol/L blocked such change (Figure 7). Aspirin (1×10^{-4} mol/L) stimulated the phosphorylation of phospho-p38 MAPK in a time-dependent manner with a peak effect at 10 min, returning back to baseline at 1 h (Figure 8).

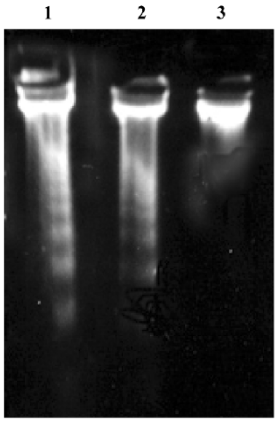

Figure 4. Effect of aspirin on H_2O_2-induced DNA fragmentation. Lane 1: 200 μmol/L H_2O_2 induced a typical DNA ladder; Lane 2: co-incubation of Aspirin 1×10^{-9} mol/L with 200 μmol/L H_2O_2 attenuated DNA fragmentation induced by H_2O_2; Lane 3: control (untreated BAEC).

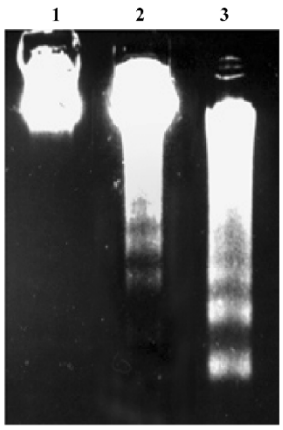

Figure 5. Aspirin induced DNA fragmentation in BAEC. Lane 1: control (untreated BAEC); Lane 2: co-incubation of aspirin 1×10^{-4} mol/L with 10 μmol/L SB203580. SB 203580 attenuated DNA fragmentation induced by aspirin; Lane 3: aspirin 1×10^{-4} mol/L induced a typical DNA ladder.

Discussion

Aspirin was introduced as an anti-inflammatory and analgesic drug in 1892, and the inhibition of COX has been thought to be the major mechanism mediating this effect. During the past decades, novel actions of aspirin have been discovered, which therefore largely expands the clinical use of aspirin. Experimental evidence has shown that aspirin can protect endothelial cells from oxidant damage through the NO–cGMP pathway, which provides a base for its use as a cardiovascular protective drug to reduce the occurrence of heart attack and stroke[7]. Aspirin has also been found to promote the apoptosis of tumor cells, and can be used to protect against the development of colon cancer and other digestive system cancers[5,17,18]. In the present study, we demonstrate that aspirin exhibits a biphasic effect on the apoptosis of bovine endothelial cells; at low concentrations, it protects BAEC from apoptosis induced by H_2O_2, while at relatively high concentrations, aspirin by itself induces apoptosis in BAEC. The endothelium protective effect of aspirin observed at low concentrations is consistent with its clinical practice that only a low dose of aspirin is required for its preventive action on cardiovascular diseases. The observed apoptotic effects on endothelial cells at high concentrations sheds new light on its clinical use as a preventive drug for the carcinogenesis of digestive tract cancers

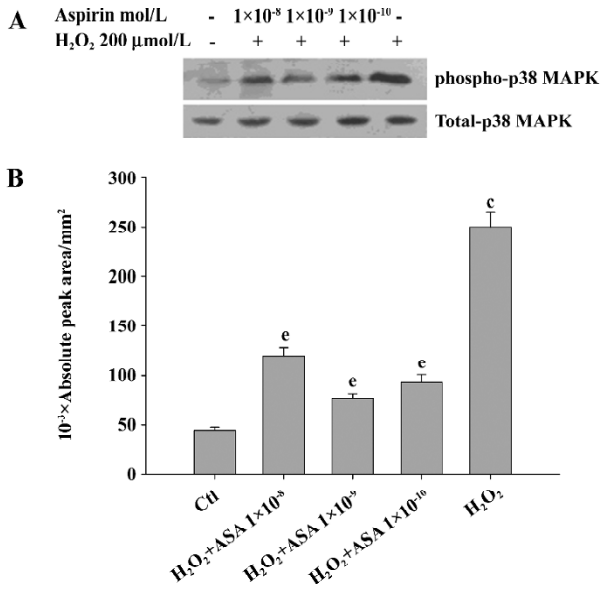

Figure 6. Aspirin inhibited H_2O_2-induced phosphorylation of p38 MAPK. (A) a representative Western blot photograph. (B) densitometry assessment of the effects of aspirin on H_2O_2-induced phospho-p38 MAPK activation. n=3 experiments. Mean±SD. ^cP<0.01 *vs* control. ^eP<0.05 *vs* H_2O_2 (200 μmol/L).

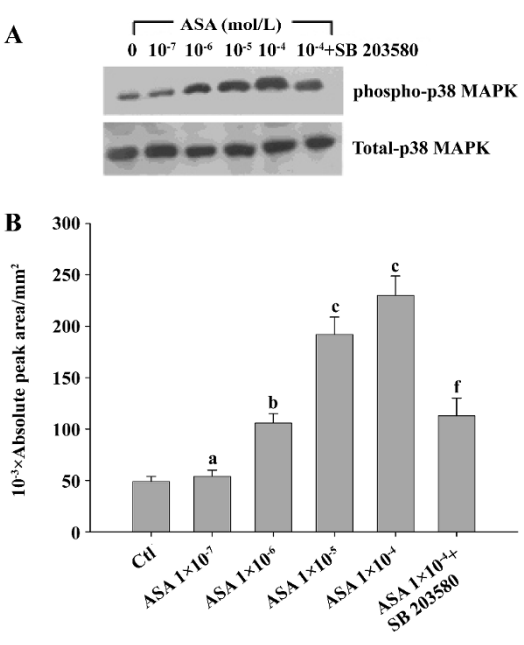

Figure 7. Aspirin induced phosphorylation of p38 MAPK in a concentration-dependent manner and SB203580 (10 μmol/L) significantly antagonized such effects. (A) a representative Western blot photograph. (B) densitometric assessment of the effect of aspirin on phosphorylation of p38 MAPK. n=3 experiments. Mean±SD. ^aP>0.05, ^bP<0.05, ^cP<0.01 *vs* control. ^fP<0.01 *vs* aspirin 1×10^{-4} mol/L.

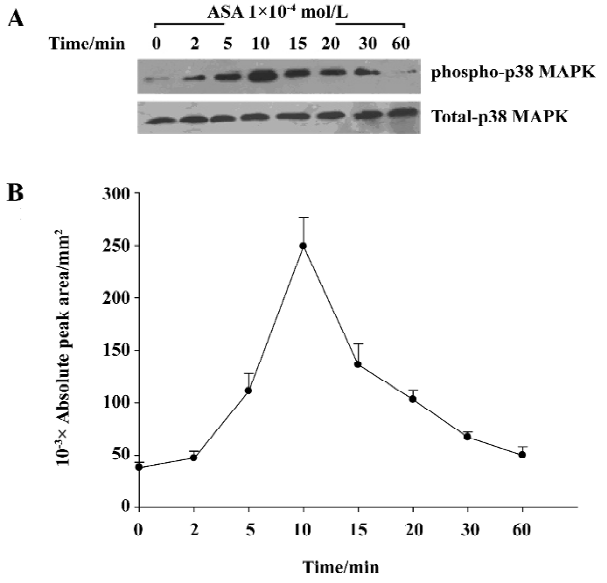

Figure 8. Time course of aspirin-induced phosphorylation of p38 MAPK. Treatment with aspirin for the indicated time induced phosphorylation of p38 MAPK, which peaked at 10 min. (A) a representative Western blot photograph. (B) densitometric analysis of the effect of aspirin on phosphorylation of p38 MAPK. n=3 experiments. Mean±SD.

because apoptosis of endothelial cells is a very effective way of blocking angiogenesis, a pivotal biological aspect in tumor development[10]. Our findings may also explain that the preventive effect of aspirin on carcinogenesis is mostly limited in the digestive tract, because a relatively high local concentration of aspirin may only be reached in the digestive tract.

We next investigated the mechanisms mediating the biphasic effect of aspirin on BAEC apoptosis. Aspirin has been shown to induce apoptosis of oesophageal cancer cells and MCF-7 cells in a COX-dependent and independent manner, respectively[5,19]. However, aspirin has also been shown to inhibit endothelial cell apoptosis via its antioxidant effects[20]. MAPK signal cascades have been extensively studied in the regulation of cell proliferation and apoptosis. There are mainly 3 subfamilies, of which, p44/42 MAPK is activated by growth factors and considered to be related to cell growth and survival. In contrast, p38 MAPK and stress-activated protein kinase (SAPK)/JNK are usually activated by stress and pro-inflammatory cytokines, and are closely associated with apoptosis. Recent studies indicate that MAPK signal cascades mediate the effect of aspirin on the proliferation or apoptosis of different cell types[12,21,22]. In this study, we found that aspirin at low concentrations inhibited H_2O_2-induced apoptosis as well as the phosphory-

lation of p38 MAPK in BAEC. In contrast, at relatively high concentrations, aspirin directly triggered apoptosis and p38 MAPK activation in BAEC. By using a specific p38 MAPK inhibitor, SB203580, we provide convincing evidence that p38 MAPK is involved in mediating the biphasic effect of aspirin on endothelial cells. Previous studies reported in our lab or by others have shown that SB203580 blocks p38 MAPK-mediated apoptosis in a stimulus-dependent manner[16,23]. Recent studies have shown that there are at least 4 different subfamily members of p38 MAPK: α, β, γ, and δ; SB203580 can only inhibit member α and β[24]. Our data suggest that the member α and/or β of p38 MAPK are involved in mediating the effects of aspirin on the apoptosis of BAEC. However, we can not exclude the involvement of the other 2 p38 MAPK sub-family members. Further investigation is required to clarify this.

In conclusion, aspirin has a biphasic effect on the apoptosis of BAEC. It can induce apoptosis at high concentrations and inhibit apoptosis at low concentrations. p38 MAPK is an important signal molecule mediating the effects of aspirin on endothelial cell apoptosis.

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