Cardio Protective Effects of Lumbrokinase and Dilong on Second-Hand Smoke-Induced Apoptotic Signaling in the Heart of a Rat Model

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Abstract

Exposure to second-hand tobacco smoke (SHS) has been epidemiologically linked to heart disease among non-smokers. However, the molecular mechanism behind SHS-induced cardiac disease is not well known. This study found that SD rats exposed to cigarette smoke at a dose of 10 cigarettes for 30 min twice a day for 1 month had a reduced left ventricle-to-tibia length ratio (mg/mm), increased cardiomyocyte apoptosis by TUNEL assay and a wider interstitial space by H&E staining. However, lumbrokinase and dilong both reversed the effects of SHS. Western blotting demonstrated significantly increased expression of the pro-apoptotic protein caspase-3 in the hearts of the rats exposed to SHS. Elevated protein expression levels of Fas, FADD and the apoptotic initiator activated caspase-8, a molecule in the death-receptor-dependent pathway, coupled with increased t-Bid and apoptotic initiator activated caspase-9 were found. Molecules in the mitochondria-dependent pathway, which disrupts mitochondrial membrane potential, were also found in rats exposed to SHS. These factors indicate myocardial apoptosis. However, treatment with lumbrokinase and dilong inhibited SHS-induced apop-

tosis. Regarding regulation of the survival pathway, we found in western blot analysis that cardiac protein expression of pAkt, Bcl2, and Bcl-xL was significantly down-regulated in rats exposed to SHS. These effects were reversed with lumbrokinase and dilong treatment. The effects of SHS on cardiomyocytes were also found to be mediated by the Fas death receptor-dependent apoptotic pathway, an unbalanced mitochondria membrane potential and decreased survival signaling. However, treatment with both lumbrokinase and dilong inhibited the effects of SHS. Our data suggest that lumbrokinase and dilong may prevent heart disease in SHS-exposed non-smokers.

Key Words: cardiac survival signaling, caspases, death-receptor-dependent pathway, dilong, lumbrokinase, mitochondria-dependent pathway, second-hand smoke (SHS)

Introduction

Second-hand smoke (SHS) is a mixture of smog from mainstream smoke exhaled from smokers and side stream smoke emitted from the tip of a burning cigarette. Exposure to SHS, also known as environmental tobacco smoke (ETS), has been epidemiologically linked to coronary heart disease (2, 5, 7-9, 15, 16, 24-26). Cardiovascular disease is the leading global cause of death, accounting for 30% of deaths worldwide. Tobacco use is a major cause of cardiovascular disease (25). Passive smoke exposure is associated with a 25-30% increased risk of coronary heart disease. SHS contains more than 7,000 chemicals. At least 250 are known to be harmful, and 69 are toxic chemicals that cause cancer. Epidemiological studies have indeed shown that SHS increases the incidence of cardiac disease by 4-fold. The mortality from heart failure among passive smokers is 38% higher than non-smokers or those who are not exposed to SHS (1). Pope *et al.* found that a 2-hour exposure to SHS significantly destroyed cardiac autonomic function based on decreased heart rate variability in 16 adult non-smokers (20). SHS also increases platelet activity, accelerates atherosclerotic lesions and increases tissue damage following ischemia or myocardial infarction. Increased platelet activity is associated with the formation of blood clots and atherosclerosis, both of which are predisposing factors for a myocardial infarction. The principal mechanisms by which SHS affects the cardiovascular system include reduced oxygen delivery to the myocardium and increased platelet activity. Further, DNA adducts formed due to excessive stress, generated due to cigarette smoke, is also known to result in mutations that cause abnormalities in tissue proliferation (29).

Apoptosis is a recognized mechanism for eliminating redundant cells, but apoptosis may also inhibit cell proliferation. Apoptosis has been suggested to play a critical role in the pathogenesis of human diseases, including heart disease (7). Apoptosis has been reported to contribute to the loss of cardiomyocytes, after which collagen secretion by fibroblasts replaces the damaged cardiomyocytes, leading to cardiomyopathy. Hence, the fibrosis following apoptosis is a predictor of adverse outcomes in patients with cardiomyopathy (13, 18). Therefore, evaluating the process of apoptosis and/or fibrosis could be an excellent way to predict the development of cardiomyopathy due to SHS; however, the specificity of the signaling pathways related to the development of apoptosis and/or fibrosis also needs to be identified.

The induction of apoptosis is associated with the activation of aspartate-specific cysteine proteases, including caspase-3 (5, 11). Several studies have demonstrated that mitochondria may play an important role in apoptosis by releasing cytochrome c and activating caspase-9, which activates caspase-3, the molecule responsible for DNA cleavage (17, 21). In addition, the death receptor-induced apoptotic pathway has been reported to be involved in the pathogenesis of heart diseases (22). This pathway is initiated by death-receptor agonists, including the Fas ligand. After ligand binding, Fas receptor oligomerization results in the activation of caspase-8, which acts upstream of caspase-3, causing activation of apoptosis (7). Therefore, caspase-3, as a common component of apoptotic signaling, mediates both mitochondria-dependent and death receptor-dependent apoptosis pathways. The down-regulation of a survival pathway is another possible factor that promotes cell apoptosis. In cardiomyocytes, insulin-like growth factor-1 (IGF-1), the survival factor through which IGF-1 receptor (IGF-1R) activates phosphatidylinositol-3 kinase/protein kinase B (19), should be considered for preventing cardiomyocyte apoptosis (10). In particular, activated PI3K enhances Akt phosphorylation (23), which regulates Bad and Bcl_2 activity to control cardiomyocyte apoptosis (3).

To understand whether the SHS effects on the heart are mediated through the activation of apoptotic pathways, including the mitochondria-dependent and Fas death-receptor-dependent signaling, or through suppressing survival pathways, we examined in a rat model in this study the myocardial expression of signaling proteins in these pathways using western blotting. These results were used to explore the

molecular pathogenesis of heart disease induced by cigarette smoke.

The earthworm (Lumbricus rubellus), in the form of a raw animal ''herb,'' has been used as traditional medicine for several thousand years. The extract from earthworms has been used empirically in Asia to treat vascular disorders. Recent clinical trials have confirmed its clinical efficacy in treating coronary artery disease and thrombotic cerebral infarcts, albeit publications related to the former have been largely confined to the Chinese literature. Lumbrokinase, a term given to the group of enzymes extracted from the earthworm, possesses plasminogen-activating and direct fibrinolytic properties (12). Various concentrations of earthworm extracts ranging between 80 to 300 mg/kg have been successfully used for various treatments. High amounts of earthworm extracts were found to be non-toxic and have shown higher therapeutic potentials (4). A dilong extract showed significant therapeutic potential at a relatively low dosages used in treatment. The anti-fibrotic effects of 100 mg/kg of dilong extract show that it is one of the highly efficient earthworm extracts. The chemical structures and amino acid sequences of each of the six isoenzymes from lumbrokinase with their respective fibrinolytic activities were previously identified and characterized (12, 28). Oral lumbrokinase improves regional myocardial perfusion in patients with stable angina (12). This study was designed to investigate the effects of earthworms and lumbrokinase on SHS-induced cardiomyocyte apoptosis.

Materials and Methods

Dilong Extraction

Powdered earthworm was extracted with 70% absolute ethanol at room temperature for 24 h, and the extract was collected after centrifugation for 5 min at 2,000 g, and was maintained in a water bath at 37°C for 4 h to remove the alcohol.

Animal Model and Exposure to Cigarette Smoke

We purchased male SD rats (6 weeks of age; body weight, 300 ± 30 g) from the National Science Council Animal Center, Taipei, Taiwan. The animals were housed three per cage in an environment-controlled animal room with water provided *ad libitum*. All animals were handled according to the guidelines of the Taiwan Society for Laboratory Animals Sciences for the care and use of laboratory animals (Institute of Laboratory Animal Resources 1996). Twenty-four rats were divided into four groups, six rats per group. The rats were placed in whole-body exposure chambers and exposed to 0 or 10 cigarettes

(Gentle, Taiwan), representing the control and smoke groups, respectively. The smoke group was further subdivided into the smoke group with lumbrokinase (1.2 mg/kg/day, twice weekly) treatment, or smoke group with dilong (100 mg/kg/day, twice weekly) treatment. Filtered air was introduced into the chamber at a flow rate of 200 L/min. The rats were exposed to the cigarette smoke for 30 min, twice a day, 6 days/week for 1 month. The room temperature was maintained at 22-25°C, and the relative humidity was approximately 40%. The lumbrokinase and dilong were administered by intraperitoneal injection. After 1 month, the rats were weighed and sacrificed. After removing the thorax, the hearts were cleaned with double-distilled water and dried before weighing. The left and right atria and the right ventricle were then removed, and the left ventricle was weighed. The ratios of the total heart weight and left ventricular weight to the tibia length (mg/mm) were calculated.

Hematoxylin-Eosin Staining

The hearts were fixed in formalin, embedded in paraffin and sectioned. The slides were hydrated through a series of ethanol concentrations (100, 95 and 75%) for 15 min each. The slides were then stained with hematoxylin and eosin (H&E). After gently rinsing with water, the slides were dehydrated through serial ethanol concentrations for 15 min each, cleaned with xylene, and cover slipped. Photomicrographs were obtained using a Zeiss Axiophot microscope (Zeiss, Oberkochen, Germany).

Tissue Extraction

The heart tissue extracts were obtained by homogenizing the left ventricle samples in phosphate-buffered saline (PBS; 0.14 M NaCl, 3 mM KCl, 1.4 mM KH_2PO_4, 14 mM K_2HPO_4) at a concentration of 1 mg tissue/10 μl PBS for 5 min. The homogenates were placed on ice for 10 min and then centrifuged at 12,000 rpm for 30 min. The supernatant was collected and stored at -80°C for future experiments.

Protein Content

The protein content of heart tissue extracts was determined using the Bradford protein assay and a protein-dye kit (Bio-Rad, Richmond, CA, USA). Bovine serum albumin (Sigma Chemical, St. Louis, MO, USA) was used as a standard. Changes in optical density were measured at 595 nm.

Electrophoresis and Western Blot

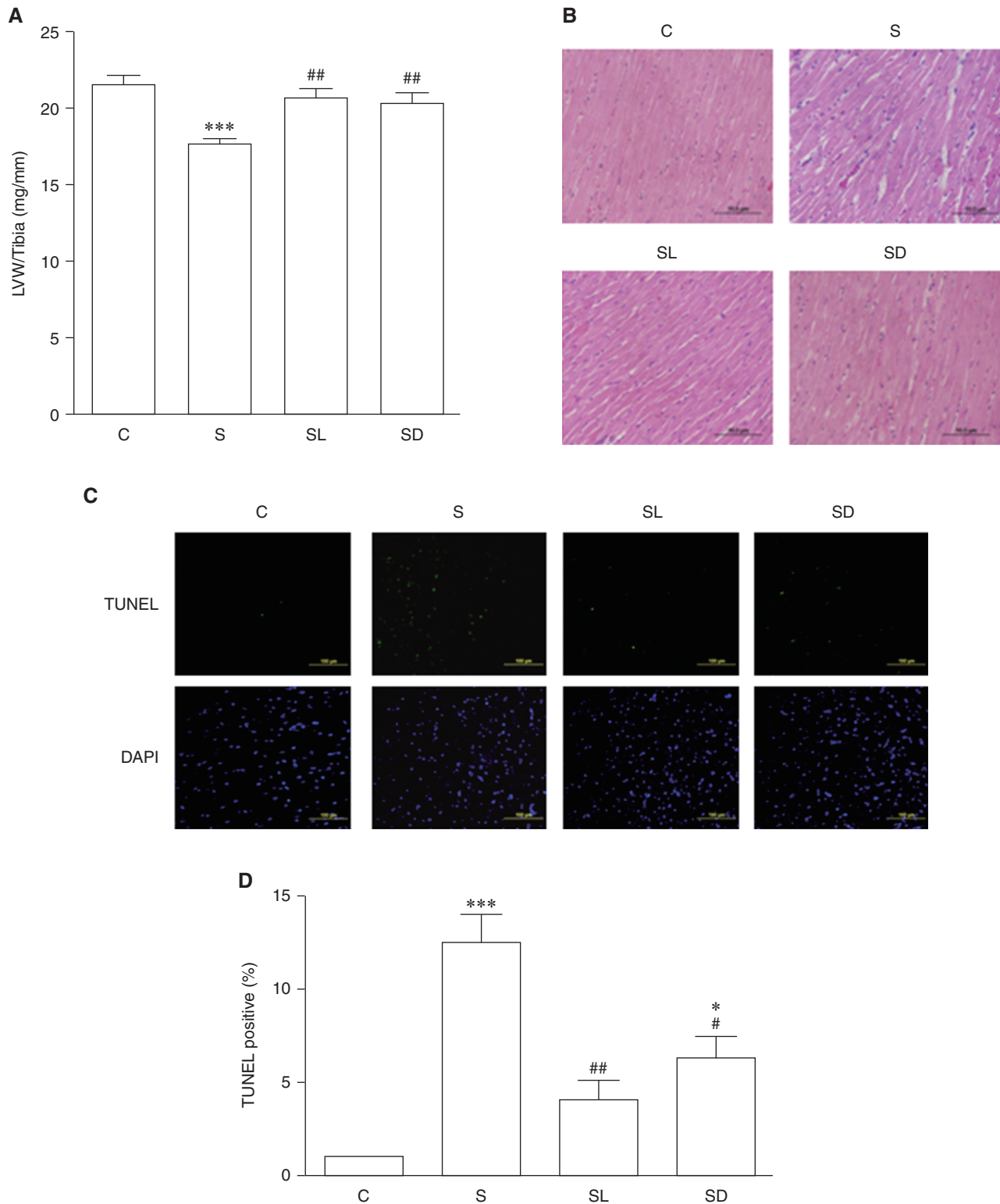Tissue extract samples were prepared as described

Fig. 1. Lumbrokinase and dilong improved SHS-induced cardiomyopathic changes in rats. C, control; S, second-hand smoke; SL, SHS rats treated with lumbrokinase; SD, SHS rats treated with dilong. The numbers represent the sample sizes. (A) The left ventricular weight to tibia length ratio (mg/mm) (mean ± SD) was lower in the rats exposed to SHS (at least six rats per group). (B) Histopathologic analysis of heart tissue sections stained with H&E. Magnification: 400×; bars = 100 μm. An enlarged interstitium was observed in the SHS-administered animal hearts, and the arrows indicate the myocardial interstitium. (C) Histopathologic analysis of heart tissue sections stained with TUNEL. Magnification: 400×; bars =100 μm. *$P < 0.05$ and ***$P < 0.001$ indicate significant differences when compared to the control group. #$P < 0.05$ and ##$P < 0.01$ indicate significant differences when compared with second hand smoking group.
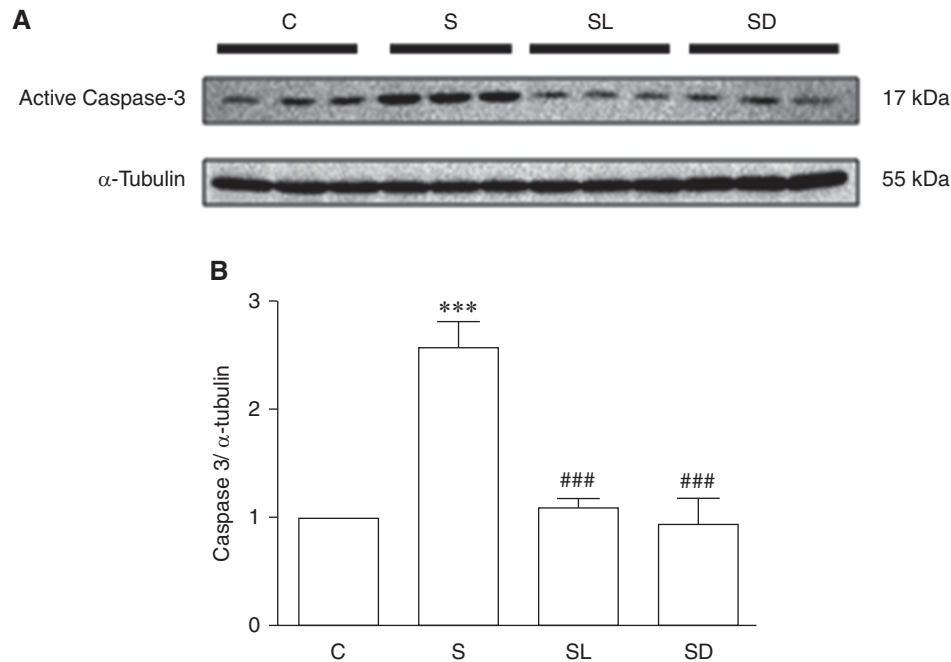
A

B

Fig. 2. Lumbrokinase and dilong reduced SHS-induced activation of caspase-3 in rat hearts. Abbreviations as in Fig. 1. (A) Western blot analysis of activated caspase-3 in the left ventricle. (B) Caspase-3 activity shown as a percent of the control (mean ± SD of three independent experiments). Significant differences compared to the control group: ***$P < 0.001$ indicate significant difference when compared to the control group. ###$P < 0.001$) indicate significant difference when compared with second hand smoking group.

above. SDS-PAGE was performed with 10% polyacrylamide gels. The samples were electrophoresed at 100 V for 3.5 h and equilibrated for 15 min in 25 mM Tris-HCl, pH 8.3, containing 192 mM glycine and 20% (vol/vol) methanol. The electrophoresed proteins were transferred to a polyvinylidene difluoride (PVDF) membrane at 100 V for 70 min. The PVDF membrane was incubated in a blocking buffer for 1 h at room temperature. Monoclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were diluted 1:1,000 in an antibody binding buffer containing 100 mM Tris-HCl, pH 7.5, 0.9% NaCl, and 0.1% (vol/vol) Tween 20. The membranes were incubated at 4°C overnight. The immunoblots were washed three times in 10 mL blotting buffer for 10 min and then immersed in the secondary antibody solution containing horseradish peroxidase goat anti-rabbit IgG (Santa Cruz Biotechnology) diluted 2,000-fold in binding buffer for 1 h. The filters were then washed three times in blotting buffer for 10 min. The immunoblotted proteins were visualized using an ECL western blot analysis luminal reagent and quantified using a Fujifilm LAS-3000 chemiluminescence detection system (Fujifilm, Tokyo, Japan).

Statistical Analysis

Data between groups of animals were compared using one-way analysis of variance. The Scheffe test was used to determine differences. *P*-values < 0.05 were considered to be significant.

Results

Lumbrokinase and Dilong Reversed SHS-Induced Cardiomyopathic Changes in Rats

After 1-month of SHS, all of the rats generally appeared healthy. The rats were then weighed and sacrificed, and the hearts were removed and weighed. The ratio of the left ventricular weight to the tibia length (mg/mm) was significantly reduced in the SHS group. Feeding the rats with lumbrokinase or dilong reversed the effect of SHS (Fig. 1A). To understand the mechanism of decreased heart weight, ventricular tissues were stained with H&E. The ventricular myocardium from the healthy controls had a normal architecture and an organized myocyte alignment with minimal interstitial spacing at 400× magnification. In contrast, disarray with markedly enlarged interstitium was evident in the cardiomyocytes from the SHS group. The SHS effect was attenuated in the lumbrokinase and dilong groups (Fig. 1B). Additionally, lumbrokinase and dilong inhibited SHS-induced cardiomyocyte apoptosis based on TUNEL assays (Fig. 1C).
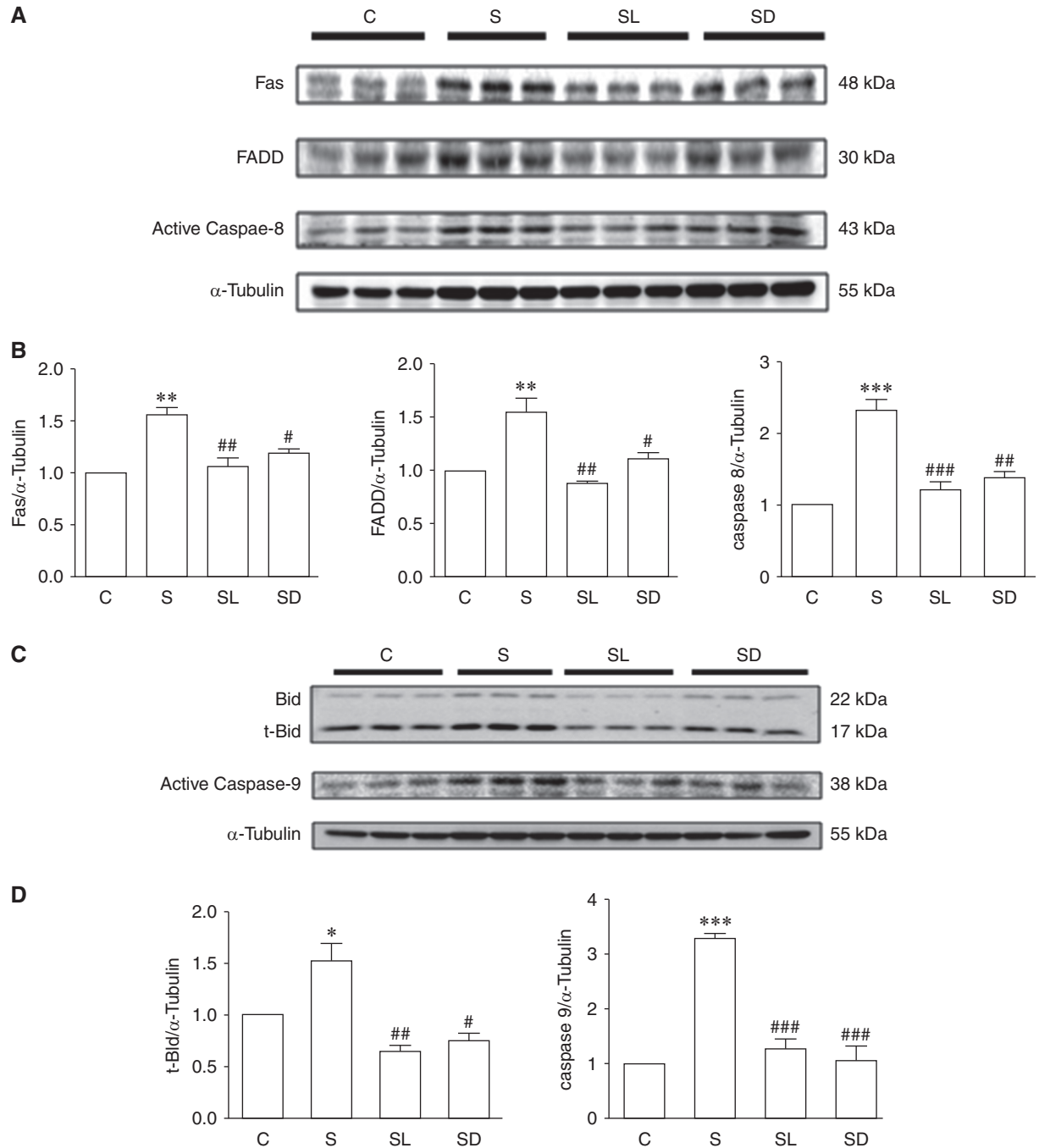
Fig. 3. Lumbrokinase and dilong attenuated SHS-induced activation of Fas, FADD, caspase-8, tBid and caspase-9 in rat hearts. Abbreviations as in Fig. 1. Fas, FADD, and caspase-8 activation in the left ventricles shown by (A) western blot analysis and (B) quantification of signal intensity for Fas, FADD, and caspase-8 (mean ± SD of three independent experiments, using α-tubulin as a loading control). Activation of tBid and caspase-9 in the left ventricles shown by (C) western blot analysis and (D) quantification of the signal intensity for tBid and activated caspase-9 (mean ± SD of three independent experiments). Significant differences compared to the control group: *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ indicate significant differences when compared to the control group. #$P < 0.05$, ##$P < 0.01$ and ###$P < 0.001$ indicate significant differences when compared with second hand smoking group.

Lumbrokinase and Dilong Decreased Caspase-3 Activation in Rats Exposed to SHS

SHS can lead to cardiomyocyte apoptosis-related heart failure. To assess the effects on apoptosis, expression levels of the apoptotic protein caspase-3 was measured, and was found to be higher in the SHS groups than the controls, and was lower in the lum-
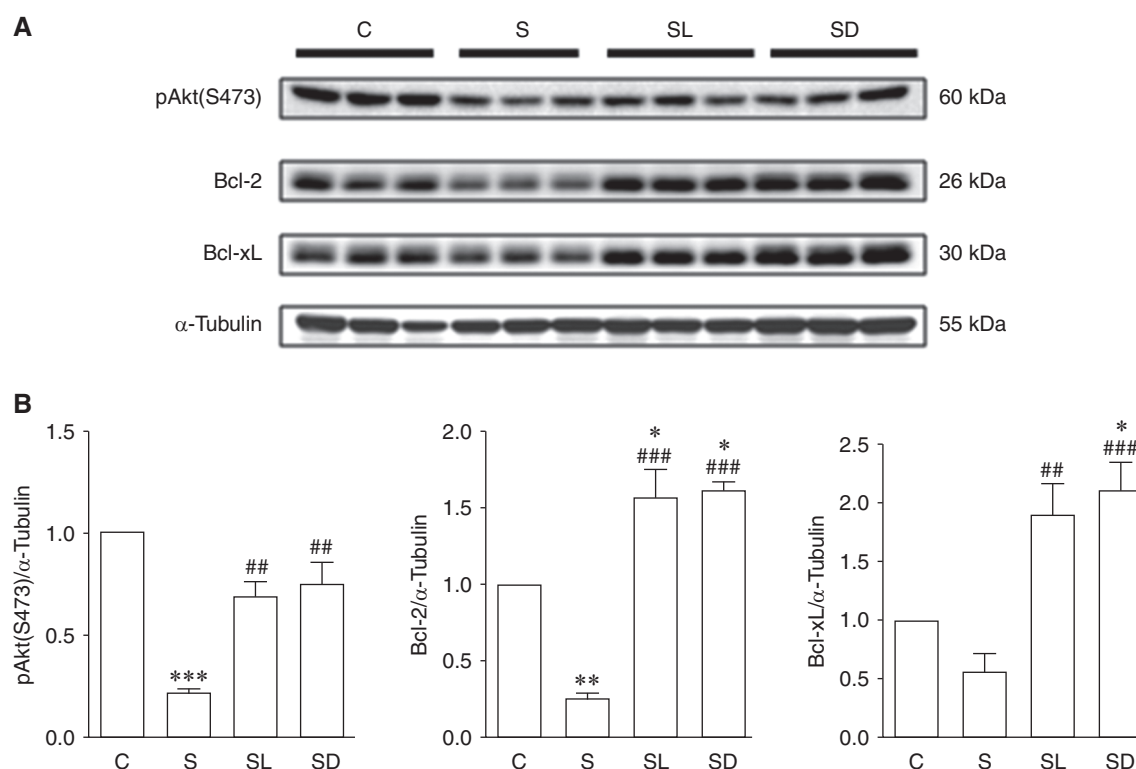
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Fig. 4. Lumbrokinase and dilong restored expression of pAkt, Bcl-2 and Bcl-xL in the rat hearts exposed to SHS. Bcl-xL, pAkt, and Bcl-xL in the left ventricles shown by (A) western blot analysis and (B) quantification of the signal intensity for pAkt, Bcl2, and Bcl-xL (mean ± SD of three independent experiments). *Significant differences compared to the control group: *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ indicate significant differences when compared to the control group. ##$P < 0.01$ and ###$P < 0.001$ indicate significant differences when compared with second hand smoking group.

brokinase and dilong groups than the SHS untreated groups (Fig. 2).

Lumbrokinase and Dilong Inhibit Components of the Fas-Receptor-Dependent Apoptotic Pathway in the Hearts of Rats Exposed to SHS

To further understand the upstream signaling pathways associated with caspase-3 activation, we examined expression of components of the Fas death receptor-dependent (FADD) apoptotic pathway. Compared to the control animals, Fas and FADD protein expression was up-regulated in the SHS group. In addition, the SHS group had significantly higher expression levels of active caspase-8 than the control group; however, the lumbrokinase and dilong inhibited the apoptotic signaling induced by SHS (Fig. 3, A and B). There was a significant increase in t-Bid and active caspase-9 protein expression in the SHS group. However, there was a significant decrease in the expression of these proteins in the lumbrokinase and dilong groups, which maintained the mitochondrial membrane potential (Fig. 3, C and D).

Lumbrokinase and Dilong Up-Regulated the Expression

of pAkt, Bcl-2 and Bcl-xL in Hearts of Rats Exposed to SHS

We also examined the expression of the cell survival-related proteins pAkt, Bcl-2 and Bcl-xL. In the SHS group, pAkt, Bcl-2 and Bcl-xL were more significantly decreased than in the control group. The lumbrokinase and dilong groups exhibited greater increases than in the SHS group (Fig. 4).

Discussion

SHS increases the mortality risk of ischemic heart disease or myocardial infarction by 30%. Ischemia induces adverse pathological events including reperfusion that deteriorates in time. Cigarette smoke triggers platelets through numerous mechanisms such as endothelial dysfunction and oxidative stress by reducing the production of platelet-derived nitric oxide, accelerating the levels of fibrinogen and thromboxane. The effect on platelet activation when exposed to SHS is 96% to that of active smokers. Activation of platelets potentially leads to thrombosis, which is a major risk factor of myocardial infarction and sudden death (6, 14, 27). After ex-
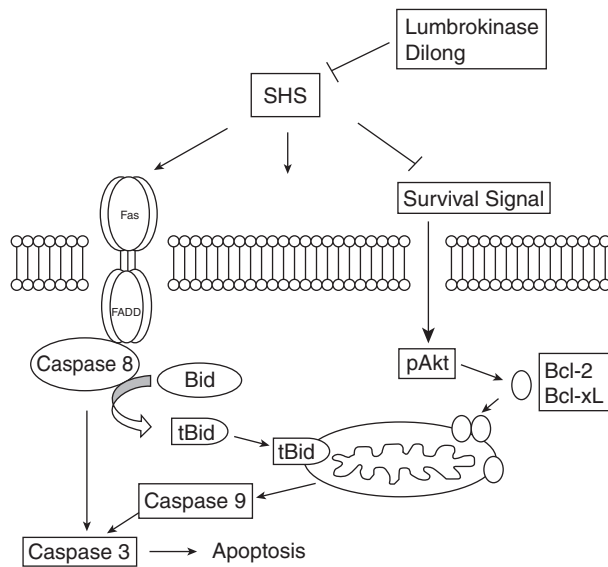
Fig. 5. Proposed mechanism of lumbrokinase and dilong inhibition of SHS-induced cardiomyocyte apoptosis. SHS activates Fas, and the receptor recruits the cytosolic adaptor protein Fad-associated death domain (FADD). The adaptor protein transmits a signal from the activated receptor Fas to the initiator caspase-8. Caspase recruitment to the plasma membrane increases the local concentration of the protease and induces autocleavage and caspase activation. Activated initiator caspases cleave and activate the effector caspase-3. Once activated, the effector caspases are responsible for the proteolytic cleavage of cellular targets, which ultimately leads to cell death. The extrinsic apoptotic pathway, induced by Fas crosstalk to the intrinsic pathway through caspase-8-mediated cleavage of BID (a BH3-only member of the BCL2 family of proteins), produces the pro-apoptotic tBID fragment. In the mitochondria, tBID disrupts mitochondrial membrane integrity and activates caspase-9, which cleaves and activates caspase-3. In addition, SHS inhibits myocardial survival signaling, including pAkt, Bcl-2, and Bcl-xL. However, both lumbrokinase and dilong reversed SHS-induced cardiomyocyte apoptosis.

posure to 10 cigarettes for 30 min, twice per day for 1 month, the hearts of the experimental rats in this study showed weight loss and a wider interstitial space. However, the lumbrokinase and dilong inhibited SHS-induced apoptosis. We demonstrated that myocardial expression of pAkt, Bcl-2 and Bcl-xL was lower in rats exposed to SHS. However, treatment with the lumbrokinase and dilong produced an increased pAkt, Bcl-2 and Bcl-xl expression (Fig. 5). The normal myocardial architecture became disorganized after SHS in this study. H&E staining showed myofibril disarray in the SHS-exposed heart and wider interstitial spaces (Fig. 1). These results suggest that the development of premature cardiomyocyte death is characterized by distorted myocardial architecture.

Cardiomyocyte apoptosis has important implications in cardiac dysfunction because of the reduced number of cardiomyocytes per functional units. The weight loss we found in the ventricles of the SHS rats may be associated with the progression of heart failure similar to that found in diabetes mellitus rats (30) (Fig. 1). The increased caspase-3 expression and activity shown by western blotting raise the possibility of cardiomyocyte apoptosis (31) (Fig. 2). Caspase-3 is shown to be an important molecular marker of apoptotic signaling that plays a key role in mitochondria-dependent and Fas death receptor-dependent apoptotic pathways (32). Findings of elevated Fas, FADD and caspase-8 levels, coupled with increased t-Bid and caspase-9 activities in the ventricles of the SHS rats further confirm the pathway involved (Fig. 3). These results demonstrate that the Fas-signaling pathway is associated with increased caspase-3 expression, which may indicate apoptosis. Myocardial pAkt, Bcl-2 and Bcl-xL survival signaling was also decreased in the hearts of the SHS-exposed rats (Fig. 4). Notably, SHS led to decreased heart weight and wider interstitial spaces.

SHS activates blood platelets and thereby increases the risk of thrombus formation and damage the lining of arteries facilitating the development of atherosclerosis. The inflammatory responses that follow would induce cardiomyocyte apoptosis. Lumbrokinase and dilong, with their thombolytic activities, would potentially ameliorate such effects of SHS and provide cardio-protection. Our findings on the SHS effects in heart tissues include weight loss, altered morphology and apoptosis-related effects due to caspase-3 activation and Fas death receptor-pathway-dependent signaling. These molecular mechanisms (Fig. 5) may explain how SHS leads to the increased risk of cardiac events reported in epidemiological studies. Because cardiomyocyte apoptosis is typically an end-stage condition, earlier intervention is warranted. Therefore, our results suggest that the lumbrokinase and dilong could be used as appropriate treatments to block cardiac Fas signaling, which might help to prevent the development of apoptosis-related SHS-induced heart disease.

Acknowledgments

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