Cantharidin attenuates the proliferation and migration of vascular smooth muscle cells through suppressing inflammatory response

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Abstract

Abnormal proliferation and migration of vascular smooth muscle cells (VSMCs) and the chronic inflammation regulated by various inflammatory factors are the major pathological processes in the development of neointimal hyperplasia and in-stent restenosis after angioplasty. Cantharidin is a potent and selective inhibitor of protein phosphatase 2A, which plays pivotal roles in cell cycle progression, cell fate, and inflammation. This study was to explore whether Cantharidin could inhibit VSMCs proliferation, migration and inflammation. Transwell migration assay, Cell Counting Kit 8 and flow cytometry were performed. Western blot, Quantitative real-time PCR, and ELISA were used to detect the expression of the markers. Results showed that Cantharidin remarkably suppressed VSMCs proliferation and migration induced by PDGF-BB. Meanwhile, Cantharidin could significantly inhibit the phosphorylation of Akt (P-AKT) and p38 MAPK (P-p38), the expression of p38 MAPK (p38), and also the phosphorylation level of NF-κB p65 (p65). Cantharidin obviously inhibited the expression of IL-6 and TNF-α, and also the level of IL-6 and TNF-α in culture supernatants. Inhibitors for p38 MAPK, PI3K/AKT and NF-κB signaling pathways didn’t affect the inhibition of Cantharidin on VSMCs proliferation, migration and inflammation. These findings indicated that Cantharidin could significantly inhibit the proliferation, migration and inflammatory response of VSMCs, which suggested that Cantharidin may be a potential inhibitor for neointimal hyperplasia and restenosis after angioplasty.

Keywords Cantharidin; Vascular Smooth Muscle Cell; Neointimal Hyperplasia; Inflammation
Introduction

Cardiovascular and cerebrovascular disease is the main cause of death worldwide\(^1\). Minimally invasive interventional therapies, including percutaneous transluminal angioplasty and stent implantation, have become the main methods for treating obstructive cardiovascular diseases. Although the wide use of drug eluting stent decreases the cardiovascular events, in-stent restenosis remain one of the most common complications after percutaneous coronary intervention (PCI). The occurrence of restenosis is related to the local vascular inflammation, excessive proliferation and migration of VSMCs after endothelial injury during PCI. Activated platelets, leukocytes and lipid particles deployed at the injury site resulting in locally severe inflammatory reaction. Meanwhile, cytokines and growth factors released by macrophages could induce migration and proliferation of VSMCs, enhance extracellular matrix synthesis, and ultimately result in neointimal hyperplasia\(^2, 3\). Thus, simultaneously inhibition of VSMCs migration and proliferation and mitigation of inflammatory response could be promising for prevention and treatment of restenosis.

Cantharidin is a chemical compound secreted by blister beetles and an active constituent of mylabris, a traditional Chinese medicine. Cantharidin exhibits selective inhibition of serine/threonine protein phosphatase 2A (PP2A), which plays a key role in regulating multiple cellular processes, including proliferation and signal transduction pathways\(^4, 5\). Studies showed that Cantharidin held effective anticancer activity in many tumor cells through regulation of MAPK and PI3K/AKT signaling pathways\(^6-8\). In addition, Cantharidin could inhibit the migration and invasion of tumor cells, suppress the expression of MMP-2/9, and reduce the level of NF-κB p65 in human lung cancer NCI-H460 cells\(^9\). Recent research
demonstrated that Cantharidin suppressed the proliferation and migration of human umbilical vascular endothelial cells and tube formation in vitro\(^{(10)}\). As far as we know, the effect of Cantharidin on proliferation and migration of VSMCs remains unknown. Therefore, this study was to investigate the effect of Cantharidin on VSMCs and the underlying mechanisms.

**Materials and methods**

**Materials and agents**

Cantharidin, Lipopolysaccharide (LPS) and DMSO were purchased from Sigma-Aldrich (USA). Wortmannin, Ammonium pyrrolidinedithiocarbamate (PDTC) and SB203580 were purchased from Solarbio (China). PDGF-BB was obtained from R&D systems (USA). Trypsin-EDTA, BCA kit, Penicillin and streptomycin and primary antibodies against glyceraldehyde-phosphate dehydrogenase (GAPDH), NF-κB p65 and α-Smooth Muscle Actin (α-SM actin) were purchased from Beyotime (China). Antibodies against AKT, P-AKT, p38 MAPK, P-p38 MAPK, P-(NF-κB p65) and matrix metalloproteinase-9 (MMP-9) were obtained from Cell Signaling Technology (USA). Propidium iodide (PI) Staining Kit was obtained from Sangon Biotech (China). Cell Counting Kit 8 (CCK-8) was obtained from Dojindo Molecular Technologies (Japan). Enzyme-linked immunosorbent assay (ELISA) kits were obtained from ImmunoWay Biotechnology Company (USA). TRIzol was purchased from AIDLAB (China). IRDye 800CW Secondary Antibody and Odyssey family of imagers were obtained from LI-COR (USA).
Cell culture

This study was approved by the Ethic Committee of Renmin Hospital of Wuhan University and was in accordance with the Guidelines for the Care and Use of Laboratory Animals published by the US National Institutes of Health. Thoracic aortas were resected from 6-8 week old male Sprague-Dawley rats purchased from the Institute of Laboratory Animal of Hubei Provincial Academy of Preventive Medicine. VSMCs were isolated from the thoracic aortas and cultured in DMEM/F12 medium added with 20% FBS, 1% penicillin and streptomycin at 37°C under air with 5% carbon dioxide. The third to fifth passage of VSMCs were used for the following experiments.

Cell viability assay

The cell viability was determined by CCK-8 assay. VSMCs were seeded at a density of 8000 cell/well in 96-well plates. As the cells reached about 70% confluence, the medium was replaced with serum-free DMEM (SFM). VSMCs were serum-starved before co-incubated with Cantharidin in different concentration (0.625, 1.25, 2.5, 5 and 10 µM) or DMSO alone for 24 h. Then the medium was removed and cells were incubated with 10% CCK-8 reagent at 37°C for 2 h as instructed by the manufacturer. The optical density was detected at a wavelength of 450 nm using a microplate reader (Tecan, Switzerland).

Cell proliferation assay

Cell proliferation was explored with CCK-8 assay. Initially, cells were seeded into 96-well cell culture plates. As the cells reached about 70% confluence, the medium was replaced with SFM. After being serum-starved for 24 h, VSMCs were stimulated with 2% FBS and 20 ng/mL PDGF-BB in the absence or presence of Cantharidin at varied
concentrations for 24 h. After that, the medium was removed and cells in each well were incubated with 10% CCK-8 reagent at 37°C for 2 h. Then the optical density was measured at a wavelength of 450 nm using a microplate reader.

**Cell migration assays**

Cell migration was investigated by wound-healing assay and Transwell migration assay. In the wound-healing assay, VSMCs were seeded into 6-well cell culture plates. As the cells reached full confluence, the medium was replaced with SFM. The cells were serum-starved for 24 h and then wounded with 1-mL sterile pipette tips. After washing with PBS for three times, VSMCs were stimulated by 2% FBS and 20 ng/mL PDGF-BB in the absence or presence of Cantharidin (5 and 10 µM) for 36 h. The migrated cell areas were measured and analyzed with the Image J software (NIH, USA). For the Transwell migration assay, VSMCs were seeded at a density of \(5 \times 10^5\) cells/mL in 200 µL DMEM with or without Cantharidin in the upper chamber. Then the cells were immersed with 600µL of DMEM/F12 with 10% FBS, in the absence or presence of 20 ng/mL PDGF-BB, in the lower chamber. Ten hours later, cells remain in the upper chamber were removed with a cotton swab, and the migrated cells on the back face of the chamber were fixed with 4% paraformaldehyde and then stained with 1% crystal violet. After washing the membranes, and the migrated cells were photographed by light microscope at the magnification of 200× (IX71-F22FL/PH, Olympus, Japan). Then the migrated cells were stained with 1 µg/mL DAPI for 2 min and images were captured in each sample. The migrated cell numbers were counted with Image J 1.51w software.
**Western blot**

Total cellular proteins were extracted using a lysis buffer following the manufacturer’s instruction. After quantification using a BCA protein assay kit, an equal amount of 30 µg protein was loaded to a 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to polyvinylidene fluoride membranes for 2 h at 200 mA using a transfer system. Membranes were thereafter blocked with 5% non-fat milk in tris buffered solution containing 1% tween 20 for 2 h. After washing with PBS containing 1% tween 20 (PBST) for three times, the membranes were incubated with corresponding primary antibodies overnight at 4°C. After washing with PBST for three times, The blots were developed with secondary antibodies at room temperature for 1 h. Signal of target proteins was detected with Odyssey infrared imaging system (Li-Cor Biosciences, USA). The density of the bands in the images was quantified with Odyssey application software.

**Quantitative real-time PCR (q-PCR)**

Total RNA was extracted using TRIzol reagent according to the manufacturer’s instruction. First-strand cDNA was synthesized from 500 ng to 1,000 ng RNA by using oligo dT-Adaptor primers and Hiscript reverse transcriptase (VAZYME, China). Then cDNA was amplified with gene-specific primers (Shanghai DNA biotechnologies, China) and SYBR Green dye kit (VAZYME, China). The primers used in q-PCR are listed as follows:

Rat GAPDH forward primer, 5’-ACAGCAACAGGGTGTTGGAC-3’
and reverse primer, 5’-TTTGAGGTTGACGCGGACTT-3’;

Rat IL-6 forward primer, 5’-GTTGCCTTCTTGGGACTGATG-3’
and reverse primer, 5’-TACTGGTCTGTTGTTGGTG-3’;
Rat TNF-a forward primer, 5’-GAAACAGTCTGCGAGGTGTG-3’
and reverse primer, 5’-TTCTTTCTTGAGGTCAGG-3’.

The analysis of relative mRNA expression was carried out using 2^ΔΔCt method^{11}, and GAPDH was used as an endogenous housekeeping gene to normalize the mRNA level.

**ELISA of culture supernatants**

VSMCs were seeded into 6-well cell culture plates. Cells were serum-starved for 24 h before being stimulated by 1 µg/mL LPS in the absence or presence of Cantharidin (5 and 10 µM) for 24 h. The concentration of IL-6 and TNF-α in the culture supernatants was determined by double-antibody sandwich ELISA as specified by the manufacturer. The optical density was measured at 450 nm by microplate reader.

**Cell cycle analysis**

The cell cycle progression was analyzed by flow cytometry. VSMCs were serum-starved and pretreated with 5 µM Cantharidin for 1 h. The cells were subsequently stimulated with 20 ng/mL PDGF-BB for 24 h. The trypsin-harvested cells were fixed with 70% ethanol and stored at 4°C overnight, then washed with PBS and incubated with 0.4 mL PI solution (50 µg/mL PI in buffer containing 100 µg/mL RNase A) at room temperature for 30 min. The fluorescence was measured and analyzed with the FACSCalibur flow cytometer (Becton Dickinson, USA). The respective percentages of cells in the G0/G1, S and G2/M phases of the cell cycle peak were calculated.

**Statistical analysis**

Data are presented as the Means±SEM. One-way or two-way analysis of variance (ANOVA) was used for multiple group comparisons. A value of p<0.05 was considered to
indicate a statistically significant difference. GraphPad Prism 6 software (GraphPad Software, San Diego, CA) was used for all of the statistical analysis.

Results

Cantharidin suppresses PDGF-BB-induced VSMCs proliferation

As shown in Fig 1. A, Cantharidin at concentrations between 0-10 µM did not affect VSMCs viability. Therefore, the concentration of Cantharidin was limited to 10 µM or less in the following experiments. PDGF-BB is a potent factor that promotes the proliferation and migration of VSMCs\textsuperscript{12,13}. CCK-8 assay showed that, compared to the non-stimulated group, the proliferation of VSMCs was significantly increased following treatment with PDGF-BB. However, Cantharidin significantly abrogated PDGF-BB-induced VSMCs proliferation in a dose-dependent manner as presented in Fig 1. B (\(p<0.05\)).

Cantharidin inhibits PDGF-BB-stimulated VSMCs migration

To test whether Cantharidin inhibits PDGF-BB-stimulated VSMCs migration, the wound-healing assay and Transwell assay were performed simultaneously. The wound-healing assay showed that PDGF-BB stimulation dramatically increased the rate of migration by 33% compared to SFM group (\(p<0.05\)). However, Cantharidin significantly inhibited PDGF-BB-induced VSMCs migration in a dose-dependent manner (\(p<0.05\)) (Fig 2. A, B). Consistently, Transwell assay showed that the VSMCs migration was notably increased after treatment with PDGF-BB, and expectedly, pretreatment of Cantharidin concentration-dependently inhibited PDGF-BB-stimulated VSMCs migration (\(p<0.05\)) (Fig 2.
Meanwhile, Cantharidin inhibited the baseline of the rate of migration levels compared to normal group in both assays.

**Cantharidin suppresses LPS-stimulated expression of pro-inflammatory cytokines in VSMCs**

LPS has been shown to induce the expression of many pro-inflammatory cytokines, including IL-6, TNF-α, MCP-1\(^1\)\(^4\),\(^1\)\(^5\). Results showed that the mRNA levels of IL-6 and TNF-α were significantly increased by 44% and 37% upon LPS stimulation, respectively. Cantharidin inhibited LPS-induced mRNA expression of IL-6 and TNF-α in a dose-dependent manner (Fig 3. A, B). ELISA assay revealed that Cantharidin remarkably inhibited the concentration of IL-6 and TNF-α in the culture supernatants in LPS-stimulated VSMCs in a concentration-dependent manner (both \(p<0.05\)) (Fig 3. C, D).

**Cantharidin retards PDGF-BB-induced cell cycle progression**

As shown in Fig 4, compared with the unstimulated cells, there was a remarkable increase in the percentage of VSMCs in the S phase in PDGF-BB-stimulated group. However, this increase in the S phase cell population was mitigated by Cantharidin (\(p<0.05\)). The percentage of cells in the G0/G1 phase was increased in the cells pre-treated with cantharidin, suggesting that cantharidin could significantly inhibit the proliferation of the VSMCs by retarding the progression of the cell cycle from the G0/G1 to the S phase.

**Effects of Cantharidin on the signaling pathways**

PI3K/AKT and p38 MAPK signaling pathways play important roles in the regulation of proliferation, migration and survival of mammalian cells\(^1\)\(^6\)\(^–\)\(^1\)\(^9\). Results demonstrated that phosphorylation levels of AKT (P-AKT) at the serine 473 site, the phosphorylation of p38
MAPK (P-p38) and total p38 MAPK (T-p38) were remarkably enhanced upon PDGF-BB stimulation, while their levels were gradually inhibited by 5 and 10 µM Cantharidin (both \(p<0.05\)) (Fig 5. A, B). Meanwhile, Cantharidin inhibited the baseline P-AKT, P-p38 and T-p38 MAPK levels compared to normal group. Moreover, the expression level of MMP-9 and the decreased α-SMA level in PDGF-BB-induced VSMCs were gradually inhibited by Cantharidin in a dose-dependent manner (Fig 5. C, D). Furthermore, pretreatment with Cantharidin significantly inhibited the degradation of IκBα and the phosphorylation of NF-κB p65 in LPS-induced VSMCs (Fig 5. E, F).

**Involvement of p38 MAPK, PI3K/AKT and NF-κB signaling pathways in the inhibitory effect of Cantharidin on VSMCs**

VSMCs were treated with the p38 MAPK inhibitor SB203580 or the PI3K/AKT inhibitor Wortmannin with or without Cantharidin. Results showed that both inhibitors significantly abrogated the proliferation and migration of VSMCs induced by PDGF-BB (Fig 6. A-C, Fig S1). However, addition of SB203580 or Wortmannin didn’t further inhibit cell proliferation and migration induced by PDGF-BB in Cantharidin-pretreated VSMCs (\(p>0.05\)). Moreover, the NF-κB pathway inhibitor PDTC significantly reduce the levels of IL-6 and TNF-α in the culture supernatants in LPS-stimulated VSMCs (both \(p<0.05\)) (Fig 6. D-E). Expectedly, compared to those treated with Cantharidin only, addition of PDTC failed to reduce the levels of IL-6 and TNF-α in the culture supernatants in LPS-stimulated VSMCs (both \(p>0.05\)).
Discussion

The proliferation and migration of VSMCs are widely recognized as the basic pathological process for the development of atherosclerosis and restenosis after angioplasty\(^{20}\). Activation of PI3K/AKT, p38 MAPK, and NF-κB signaling pathway promotes proliferation, migration, and inflammation in various cell types\(^{21,22}\). This study, for the first time, proved that Cantharidin could significantly attenuate the proliferation, migration and inflammatory response of VSMCs through regulation of PI3K/AKT, p38 MAPK, and NF-κB signaling pathway.

Cantharidin is a strong inhibitor of protein phosphatases types 1 (PP1) and 2A (PP2A). Previous studies have shown that the concentration of Cantharidin for the 50% inhibitory concentration (IC\(_{50}\)) of PP2A and PP1 are 0.16 μM and 1.7 μM, respectively\(^{23}\). However, in this study, we found that treatment of 2 μM Cantharidin didn’t reduced the proliferation and migration of VSMCs induced by PDGF-BB (Fig S2). Meanwhile, addition of signaling pathway inhibitors demonstrated that Cantharidin regulates p38 MAPK, PI3K/AKT and NF-κB signaling pathways. Thus, we speculate that Cantharidin inhibits the proliferation and migration of VSMCs not by inhibiting PP1 and PP2A, but in a non-specific manner.

PDGF-BB, a potent mitogen, promotes proliferation and migration of VSMCs through activation of intracellular signal transduction pathways\(^{24,25}\). Our data showed that VSMCs proliferation and migration, the number of cells entering S phase and the expression of total p38 MAPK, phosphorylated p38 MAPK, phosphorylated AKT and MMP-9 in VSMCs were all increased significantly after PDGF-BB stimulation. While Cantharidin dose-dependently
attenuated the above-mentioned effects of PDGF-BB and inhibited proliferation and migration of VSMCs.

Studies have demonstrated that LPS promotes the expression of proinflammatory cytokines and enhances inflammatory responses in VSMCs\cite{26}. LPS also participates in the pathological process of atherosclerosis by stimulating the NF-κB signaling pathway\cite{14}. Our data showed the phosphorylation of NF-κB p65 in nucleus and levels of pro-inflammatory cytokines IL-6 and TNF-α both in cells and culture supernatants were increased in LPS-induced VSMCs, while Cantharidin profoundly inhibited the pro-inflammatory effect of LPS in a dose-dependent manner.

Kim et al. found that Cantharidin could inhibit cell migration and proliferation by inhibiting PI3K/AKT signaling pathway in A549 human lung cancer cells\cite{27}. Previous studies have demonstrated that activation of PI3K/AKT signaling pathway induced by PDGF-BB increased proliferation and migration of VSMCs\cite{28,29}. Similarly, in this study, our results showed that Cantharidin dramatically attenuated PDGF-BB stimulated PI3K/AKT signaling pathway activation dose-dependently. Meanwhile, the inhibitor for PI3K/AKT didn’t affect the inhibition of Cantharidin on VSMCs. These results suggest that Cantharidin inhibits the proliferation and migration of VSMCs through suppression of PI3K/AKT signaling pathway.

The mitogen-activated protein kinases (MAPKs) family includes ERK, p38 MAPK and c-Jun NH2-terminal kinase (JNK). Previous studies have demonstrated that p38 MAPK signaling pathway is involved in the progress of vascular remodeling in blood vessel-proliferating diseases\cite{30,31}. In addition, the signaling is activated in response to treatment with PDGF-BB in VSMCs\cite{32}. Therefore, we further examined the activity of the
p38 MAPK signaling pathway. The data showed that Cantharidin significantly inhibited the expression of total p38 MAPK and phosphorylation of p38 MAPK induced by PDGF-BB. In addition, Cantharidin also reduced their baseline levels in VSMCs. These results indicated that Cantharidin inhibits proliferation and migration of VSMCs, at least in part, by inhibiting the activation of p38 MAPK signaling pathway. Whereas, a recent study showed that Cantharidin suppressed proliferation and migration of tumor cell through inhibiting the phosphorylation of p38 MAPK without affecting the expression of total p38 MAPK. This difference may be attributed to the reduction of stimulants, such as TNF-α, in Cantharidin-treated VSMCs and the cell types used in the studies. Further research is necessary to elucidate this difference.

Migration of VSMCs contributes to intimal hyperplasia and other vascular diseases. Matrix metalloproteinases (MMPs) play key roles to regulate the migration activity of VSMCs. MMP-9, a pivotal member of MMPs, plays an important role in cell growth, migration and angiogenesis. Previous reports have showed that MMP-9 promoted neointima formation in animal models. Huang et al. found that Cantharidin can exert an anti-metastasis effect by inhibiting MMP-9 in TSGH-8301 human bladder cancer cells. Similarly, our results (Fig 5. C) showed that Cantharidin dose-dependently inhibited the expression of MMP-9 in VSMCs and cell migration induced by PDGF-BB.

NF-κB plays an indispensable role in inflammation-related diseases. Previous research have indicated that NF-κB, especially p65, plays an important role in the phenotypic transformation of VSMCs and the formation of neointima after vascular injury. Landry et al. found nuclear NF-κB was activated in VSMCs after balloon injury to rat carotid arteries.
and in the VSMCs of human atherosclerotic lesions \(^{41}\). And inhibition of NF-κB pathway can reduce the formation of neointima after vascular injury \(^{42}\). In this study we found that Cantharidin inhibited LPS-mediated IκBα/NF-κB-p65 signaling in VSMCs. In addition, the expression of pro-inflammatory cytokines IL-6 and TNF-α were remarkably reduced in Cantharidin-treated VSMCs. These pro-inflammatory cytokines, which play pivotal roles in the pathological processes of vascular restenosis, are also regulated by NF-κB signaling pathway.

In conclusion, this work demonstrated that Cantharidin could inhibit the proliferation, migration and inflammatory response of VSMCs by inhibiting the activities of PI3K/AKT, p38 MAPK, and NF-κB pathways. Therefore, Cantharidin may be a promising therapeutic agent for the prevention and treatment of vascular intimal hyperplasia, including atherosclerosis and vascular restenosis.

Acknowledgments

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Conflict of interests

The authors declare no conflict of interest.
Supplementary Materials

The online version of this article contains supplementary materials.
References


Figure legends

**Fig 1. Cantharidin suppresses PDGF-BB-induced VSMCs proliferation.** A. Treatment with Cantharidin (0-10 µM) didn’t affect the viability of rat aortic VSMCs. B. PDGF-BB remarkably increased the proliferation of VSMCs and Cantharidin could significantly reduce the proliferation of VSMCs induced by PDGF-BB. *p<0.05, **p<0.01 vs. SFM group, #p<0.05, ##p<0.01 vs. PDGF-BB group.

**Fig 2. Cantharidin inhibits PDGF-BB-induced VSMCs migration.** A. Representative images of wound healing assays were obtained from each group. B. Cantharidin inhibited the rate of migration in PDGF-BB-induced VSMCs. C. Representative images of Transwell migration assays were obtained from each group (Magnification: 200×). D. Cantharidin reduced the migrated cell number in PDGF-BB-induced VSMCs chamber. *p<0.05, **p<0.01 vs. control group, #p<0.05, ##p<0.01 vs. PDGF-BB group.

**Fig 3. Cantharidin suppresses LPS-stimulated expression of pro-inflammatory cytokines in VSMCs.** LPS increased the mRNA levels of IL-6 and TNF-α, and Cantharidin inhibited LPS-induced expression of IL-6 and TNF-α in a dose-dependent manner (A, B). In addition, treatment with Cantharidin could remarkably inhibited the concentration of IL-6 and TNF-α in the culture supernatants in LPS-stimulated VSMCs in a concentration-dependent manner (C, D). *p<0.05, **p<0.01 vs. SFM group, #p<0.05, ##p<0.01 vs. LPS group.

**Fig 4. Cantharidin retards PDGF-BB-induced cell cycle progression.** Representative images of cell cycle distribution were obtained from each group (A, B, C). There was a marked increase in the percentage of PDGF-BB-stimulated cells in the S phase compared
with the unstimulated cells, which could be mitigated by Cantharidin (D). *p<0.05, **p<0.01 vs. control group, #p<0.05, ##p<0.01 vs. PDGF-BB group.

**Fig 5. Effects of Cantharidin on the signaling pathways.** P-AKT at the serine 473 site, P-p38 MAPK and T-p38 MAPK levels were remarkably enhanced upon PDGF-BB stimulation, while their levels were gradually inhibited by increasing concentration of Cantharidin (A, B). Meanwhile, Cantharidin inhibited the baseline P-Akt, P-p38 MAPK and T-p38 MAPK levels compared to normal group. In addition, the expression level of MMP-9 and the decreased α-SMA level in PDGF-BB-induced VSMCs were gradually inhibited by Cantharidin in a dose-dependent manner (C, D). Moreover, treatment with Cantharidin significantly inhibited the degradation of IkBα and the phosphorylation of NF-κB p65 in LPS-induced VSMCs (E, F). *p<0.05, **p<0.01 vs. SFM group, #p<0.05, ##p<0.01 vs. PDGF-BB or LPS group.

**Fig 6. Involvement of p38 MAPK, PI3K/AKT and NF-κB signaling pathways in the inhibitory effect of Cantharidin on VSMCs.** Cells were pretreated with SB203580 (25 μM), Wortmannin (10 nM), and PDTC (80 μM) for 1 h before adding Cantharidin (10 μM), and then incubated with 20 ng/mL PDGF-BB or 1 μg/mL LPS. Migration was measured by wound healing (A-B), and the proliferation was measured by CCK-8 (C). The production of IL-6 and TNF-α was measured by ELISA kits (D-E). *p<0.05, **p<0.01 vs. control group, #p<0.05, ##p<0.01 vs. PDGF-BB or LPS group. &p < 0.05, &&p < 0.01 vs. PDGF-BB+Cantharidin or LPS+Cantharidin group.
Fig. 1

(A) CCK-8 Activity (OD at 450nm) for different concentrations of cantharidin.

(B) CCK-8 Activity (OD at 450nm) with PDGF-BB (20 ng/ml) treatment.
Fig. 2
Fig. 3

(A) IL-6 mRNA expression
(B) TNF-α mRNA expression
(C) IL-6 (pg/ml)
(D) TNF-α (pg/ml)
Fig. 4

A. Control

B. PDGF-BB

C. PDGF-BB+Cantharidin

D. Percentage of cells (%)

- control
- PDGF-BB
- PDGF-BB+Cantharidin

Channels (FL2-AFL3-Area)
Fig. 5

A) P-AKT/T-AKT protein relative expression

B) P-p38/T-p38 protein relative expression

C) MMP-9/GAPDH protein relative expression

D) α-SMA/GAPDH protein relative expression

E) iκBα/GAPDH protein relative expression

F) P-p65/p65 protein relative expression

Biological and Pharmaceutical Bulletin Advance Publication
Fig. 6

A

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B

Rate of Migration (%)

C

CCK-8 Activity (OD at 450nm)

D

IL-6 (Pg/mL)

E

TNF-α (Pg/mL)