Original Research

Histone deacetylase-high mobility group box-1 pathway targeted by hypaconitine suppresses the apoptosis of endothelial cells

Ye Bai[1], Shaohui Du[2], Fei Li[1], Fengyuan Huang[1], Rudong Deng[1], Jianhong Zhou[1] and Dongfeng Chen[1]

[1]Department of Anatomy, Guangzhou University of Chinese Medicine, Guangzhou 510405, China; [2]Department of Internal Medicine, Affiliated Shenzhen Hospital, Guangzhou University of Chinese Medicine, Shenzhen 518033, China
Corresponding authors: Shaohui Du. Email: dushaohui354@sina.com; Dongfeng Chen. Email: cdf27212@21cn.com

Impact statement

First, our study shows the antiapoptosis effect of *Aconitum carmichaelii* and its active component hypaconitine on endothelial cells. It may provide new strategies for the treatment of diseases involving endothelium damage. Second, this finding indicates the function of hypaconitine in regulating HDAC3-HMGB1 pathway, which suggests a new anti-inflammatory therapy. Third, due to its poisonousness, *A. carmichaelii* is always used with caution in clinics. Thus, the identification of hypaconitine as an active component of *A. carmichaelii* could contribute to the development of toxicity-decreasing procedure for *A. carmichaelii*.

Abstract

Hypaconitine is an active component of *Aconitum carmichaelii* Debx, a Chinese medicinal herb for the treatment of cardiovascular diseases, but the mechanism underlying its effect remains elusive. In this study, we found that hypaconitine, rather than aconitum alkaloids in *A. carmichaelii* (e.g. aconitine, mesaconitine and benzoylaconitine), prevented endothelial cells from damage due to oxidized low-density lipoprotein (oxLDL) challenge. Cleaved caspase 3 expression in endothelial cells was up-regulated by oxLDL and markedly attenuated by hypaconitine, suggesting that hypaconitine inhibited the oxLDL-induced cell apoptosis. Microarray analysis revealed that histone deacetylase 3 (HDAC3) was significantly increased by hypaconitine. The cytoplasmic relocation and extracellular release of high-mobility group box 1 (HMGB1, an HDAC3 downstream effector) in endothelial cells were significantly increased by oxLDL and markedly decreased by hypaconitine. The effect of hypaconitine on the oxLDL-induced apoptosis and HMGB1 release in endothelial cells was significantly reduced by the suppression of HDAC3 by siRNA or a specific inhibitor. Thus, this study proves that the histone deacetylase-HMGB1 pathway targeted by hypaconitine suppresses the apoptosis of endothelial cells. Our findings are of therapeutic significance and provide the potential of hypaconitine exploitation.

Keywords: Hypaconitine, endothelial cells, HDAC3, HMGB1, OXLDL, apoptosis

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Introduction

Apoptosis is a programmed cell death by which the body tissues remove the unwanted, damaged or aged cells.[1,2] It is often referred to as a cell suicide event that contributes to disease pathogenesis and progression.[2] The apoptosis of endothelial cells is reportedly a cause of cardiovascular diseases (CVDs), such as atherosclerosis and myocardial infarction.[2,3] These apoptosis events are always considered to be induced by oxidized low-density lipoproteins (oxLDL) in serum.[4,5] Nowadays, the common strategies for CVDs are to improve hypertension and reduce blood fat and antioxidant treatments, which are implemented through preventing endothelial cells from damages. However, the antiapoptotic effects on endothelial cells of these strategies are still pessimistic.[6] Therefore, it is very significant to develop a pharmacotherapy that protects endothelial cells from apoptosis in CVD patients.

Histone deacetylase 3 (HDAC3) is considered as an antiapoptotic regulator in endothelial cells.[7] This enzyme is responsible for altering the chromosome structure and affecting transcription factor to bind DNA through deacetylation. The occurrence of cell damages would induce cytoplasmic relocation and HMGB1 extracellular release, which play pivotal roles in cell apoptosis.[8,9] HDAC3 can suppress the activity of apoptotic pathway through HMGB1 deacetylation and preventing its relocation and release in cells.[10,11] Therefore, the HDAC3-HMGB1 pathway may be the antiapoptosis target for the treatment in both endothelial cells as well as CVDs.

Aconitum carmichaelii Debx (Ranunculaceae), a Chinese medicinal herb, is always used to treat CVDs as an ingredient of formulae (e.g. Shenfu injection and Sini Decoction) that possess protective effects on endothelial cells in mammals.[12,13] The main ingredients of *A. carmichaelii* are

aconitum alkaloids, including hypaconitine, aconitine, mesaconitine and benzoylaconitine, but its active component(s) is(are) still unclear. Hypaconitine in *A. carmichaelii* is able to protect mammalian myocardial cells from apoptosis induced by oxidative damage. Thus, we ask whether *A. carmichaelii* could protect endothelial cells from oxidative damage, and whether hypaconitine has the antiapoptotic effect in endothelial cells like in myocardial cells. For these questions, we developed an oxLDL-induced damage model in endothelial cells. We evaluated the protective effects of hypaconitine and other aconitum alkaloids in *A. carmichaelii* on endothelial cells *in vivo* and explored the molecular mechanisms of the antiapoptotic effect.

Materials and methods

Materials and antibodies

Human umbilical vein endothelial cells were purchased from the Institute of Biomedical Sciences in Fudan University (Shanghai, China). OxLDL (high oxidized, YB-002-1) was purchased from Yiyuan Biotechnology Co., Ltd (Guangzhou, China). Hypaconitine (no. 110798-201106), aconitine (no. 110720-200410), mesaconitine (no. 110799-201106) and benzoylaconitine (no. 111794-201102) (all with purity >99%) were purchased from National Institute for Control of Pharmaceutical and Biological Products (Beijing, China). Dulbecco's Modified Eagle Media: Nutrient Mixture F-12 (DMEM/F12), fetal bovine serum (FBS), Trizol reagent and Lipofectamine 2000 were purchased from Invitrogen (Carlsbad, USA). Methyl thiazolyl tetrazolium (MTT) assay kit, dimethyl sulphoxide (DMSO) and bovine serum albumin (BSA) were obtained from Sigma (Saint Louis, USA). HDAC3 small interfering RNA (siRNA) (human) and control siRNA were purchased from Santa Cruz (Texas, USA). Other materials included microporous polyvinylidene di-fluoride (PVDF) membranes (0.45 mm), enhanced chemiluminescence (ECL) Plus detection kit (both Merck Millipore, Darmstadt, Germany); rabbit monoclonal antibodies against HMGB1, HDAC3 and cleaved caspase3 (all Epitomics, Burlingame, USA); mouse polyclonal antibodies against β-actin, rabbit polyclonal antibodies against Lamin B (Boster Bio-Engineering Ltd Co., Wuhan, China); and horseradish peroxidase (HRP)-labeled secondary antibody (Boster Bio-Engineering Ltd Co.). Also, 96-well and 6-well cell culture plates were obtained from Corning, USA.

Cell cultures

Endothelial cells were cultured in DMEM/F12 containing 10% FBS at 37°C in 5% CO_2. Cells were passaged by trypsinization with 0.25% trypsin, seeded onto cell culture plates at 5000 cells/cm^2 and cultured overnight. Endothelial cells of low passages (3–8) were used in all experiments.

MTT assay

After treatment, each well of the plates was incubated with 10% MTT diluted with the basic medium for 4 h. Then, DMSO, instead of MTT, was used to dissolve the formazan crystals in the plates. After that, the amount of formazan in wells was determined by measuring the optical density (OD) at 492 nm under a microplate reader (RT2100C, Rayto Ltd Co., Shenzhen, China).

Western blot

Whole-cell proteins were extracted by radio immunoprecipitation assay (RIPA) lysis buffer (Beyotime, Beijing, China), and nuclear and cytoplasmic proteins were isolated using the corresponding extraction kit (KeyGEN BioTECH, Guangzhou, China). Protein extracts were separated by 12.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretically transferred to PVDF membranes. Then the membranes were incubated first with a blocking buffer containing 5% BSA and 0.1% Tween-20 in phosphate-buffered saline solution (PBS) at room temperature for 1 h, followed by incubation at 4°C overnight with specific primary antibodies against HMGB1 (1:10,000), HDAC3 (1:10,000), cleaved caspase3 (1:5000), β-actin (1:500) or Lamin B (1:250). The final incubation was carried out with an HRP-labeled secondary antibody at room temperature for 1 h. Membranes were detected using the ECL system according to the manufacturer's protocol.

Microarray analysis

After cell treatment, total mRNA was extracted using the Trizol method,[14] and RNA quality was determined on Agilent Bioanalyzer 2200 (Agilent Technologies, Santa Clara, USA). Total RNAs were profiled on self-designed oligo microarray of human transcription factors (Ribobio Ltd Co., Guangzhou, China). Raw data from GeneChips were loaded into the GenePix 4000B (Molecular Device, San Francisco, USA), normalized using the median of raw data from each array as a reference, and then were analyzed.

The antiapoptotic regulator targeted by active component of *A. carmichaelii* was identified using the information of UCSC genome browser (http://genome.ucsc.edu).

Quantitative real-time polymerase chain reaction

Total cell mRNA was extracted using Trizol reagent. The purified mRNA (1.5 μg) was reverse transcribed (Reverse Transcriptase M-MLV (RNase H-), TAKARA, JPN) following the manufacturer's protocol. PCR was performed on the ViiA RT-PCR system (ABI, Hercules, USA). Conditions of fluorescent quantity PCR were: pre-denature at 95°C for 30 s; 45 cycles at 95°C for 3 s and 60°C for 34 s. The relative mRNA expression levels were normalized to β-actin. The sense and antisense primers are as follows: 5′-GCCTGTCCATTGGTGATGTTGC-3′ and 5′-CCTTCAG CTTCGCAGCCTTCT-3 (HMGB1); 5′-CCTGCCTGACCTC TTTGC-3′ and 5′-TGGGGAGGAGGGCAACAT-3′ (HDAC3); 5′-ATGTGGCCGAGGACTTTGAT-3′ and 5′-TGGCTTTAGG ATGGCAAGG -3′ (β-actin).

Endothelial cells in transfection with HDAC3 siRNA

In each well, 10 μL of Lipo2000 and 0.1 nM HDAC3 siRNA or 0.1 nM control vector were diluted to 250 μL with

Opti-mem, and then transfected to endothelial cells in 6-well plates using Lipofectamine 2000 (Lipo2000). After 5-minincubation at room temperature, these two reagents were mixed and incubated at room temperature for 20 min to form an HDAC3 siRNA-lipo 2000 complex. Then, 500 μL of complex and 500 μL of basic medium were mixed and used to incubate the cells at 37°C in 5% CO_2 for 8 h. Thereafter, the medium in wells was replaced with DMEM/F12, and the cells were incubated at 37°C in 5% CO_2 for another 24 h. After that, the silencing efficiency of HDAC3 siRNA was detected using Western blot against HDAC3.

Enzyme linked immunosorbent assay

After cell treatment, the culture supernatants were analyzed for human HMGB1 using an enzyme linked immunosorbent assay (ELISA) kit (Cloud-Clone Corp., Houston, USA) following the manufacturer's protocol. Briefly, the diluted standard and test samples were applied to antibody-coated microtiter plates and incubated at 37°C for 2 h. After the liquid was fully removed from each plate, the cells were incubated first with a biotin-labeled antibody solution for 1 h and then with an HRP-conjugated secondary antibody working solution at 37°C for 1 h. Thereafter, the plates were washed with a wash solution and incubated with a substrate solution in the dark for 20 min. Then stop solution was added and the absorbance at 450 nm was measured on a microplate reader (RT2100C). The HMGB1 concentrations of each culture supernatant were expressed in pg/mL.

Data analysis

All data were expressed as mean ± standard error (SE) for each group. Analysis of variance was performed on SPSS 14.0 with significant level at $P < 0.05$.

Results

Hypaconitine prevents oxLDL-induced endothelial cell damage

The report that *A. carmichaelii* protects endothelial cells from damages encouraged us to explore its active component(s) for endothelial cells. We selected four of its main components (hypaconitine, aconitine, mesaconitine and benzoylaconitine) and evaluated their separate effects on oxLDL-induced endothelial cell damage. We first confirmed the reported cytotoxicity of oxLDL on endothelial cells. Endothelial cells were treated without or with oxLDL at indicated concentrations. The relative cell viability was determined 21 h later by MTT analysis, and the dose-response effect of oxLDL was presented on cell viability reduction (Figure 1(a)). After that the endothelial cells were treated without or with oxLDL for 21 h in the absence or presence of an active component at indicated concentrations, followed by MTT assay. Hypaconitine, aconitine, mesaconitine, and benzoylaconitine are structurally related aconitum alkaloids in *A. carmichaelii* (Figure 1(c)). Hypaconitine, which has no –OH at the 3-position, markedly increased the cell viability in a dose-dependent manner, while the other three components, possessing the –OH at the 3-position, showed no such effect (Figure 1(b)). These results suggest that –OH at 3-position inhibited the protective effect of aconitum alkaloids, indicating that the –H at 3-position of hypaconitine is responsible for its protective effect on endothelial cells. Taken together, hypaconitine is an active component of *A. carmichaelii* that prevents oxLDL-induced damage in endothelial cells.

Hypaconitine attenuates oxLDL-induced cleaved caspase 3 expression in endothelial cells

To determine whether hypaconitine protected endothelial cells from oxLDL via antiapoptosis, we investigated the separate effects of oxLDL and hypaconitine on the expression of cleaved caspase 3, a pro-apoptotic protein. Endothelial cells were treated without or with oxLDL and used to prepare whole-cell protein. Western blot showed that the expression of cleaved caspase 3 was up-regulated by oxLDL in a dose-dependent manner (Figure 2(a)). After that the endothelial cells were treated without or with oxLDL in the absence or presence of hypaconitine for 21 h, and used to prepare whole-cell protein for Western blot. Figure 2(b) reveals the dose-dependent effect of hypaconitine on caspase3 expression attenuation. Therefore, hypaconitine suppresses the oxLDL-induced apoptosis of endothelial cells.

Hypaconitine up-regulates HDAC3 expression in endothelial cells

To explore the antiapoptotic mechanism of hypaconitine, we treated endothelial cells with oxLDL in the absence or presence of hypaconitine, and prepared the total mRNA for microarray analysis of transcription factors. HDAC3 mRNA was significantly up-regulated by hypaconitine (Figure 3(a)). To confirm the effect of hypaconitine on promoting HDAC3 expression, we treated endothelial cells without or with oxLDL for 21 h in the absence or presence of hypaconitine, and prepared total mRNA for quantitative real-time PCR and whole-cell protein for Western blot. We found hypaconitine up-regulated HDAC3 mRNA and protein expressions in oxLDL-stimulated endothelial cells (Figure 3(b) and (c)). All these results suggest that hypaconitine promoted the HDAC3 expression in oxLDL-damaged endothelial cells.

Hypaconitine prevents oxLDL-induced HMGB1 cytoplasmic relocation and extracellular release in endothelial cells

As is well known, the cytoplasmic relocation and extracellular release of HMGB1, a HDAC3 downstream effector, play crucial roles in cell apoptosis. Thus, to explore the inactivation capability of hypaconitine in the HDAC3-HMGB1 pathway of endothelial cells, we observed the effect of hypaconitine on HMGB1 protein levels in cell culture supernatant, cytoplasm and nucleus separately. At first, we transfected the endothelial cells with control siRNA vector or HDAC3 siRNA plasmid and checked the silencing efficiency of HDAC3 siRNA by Western blot.

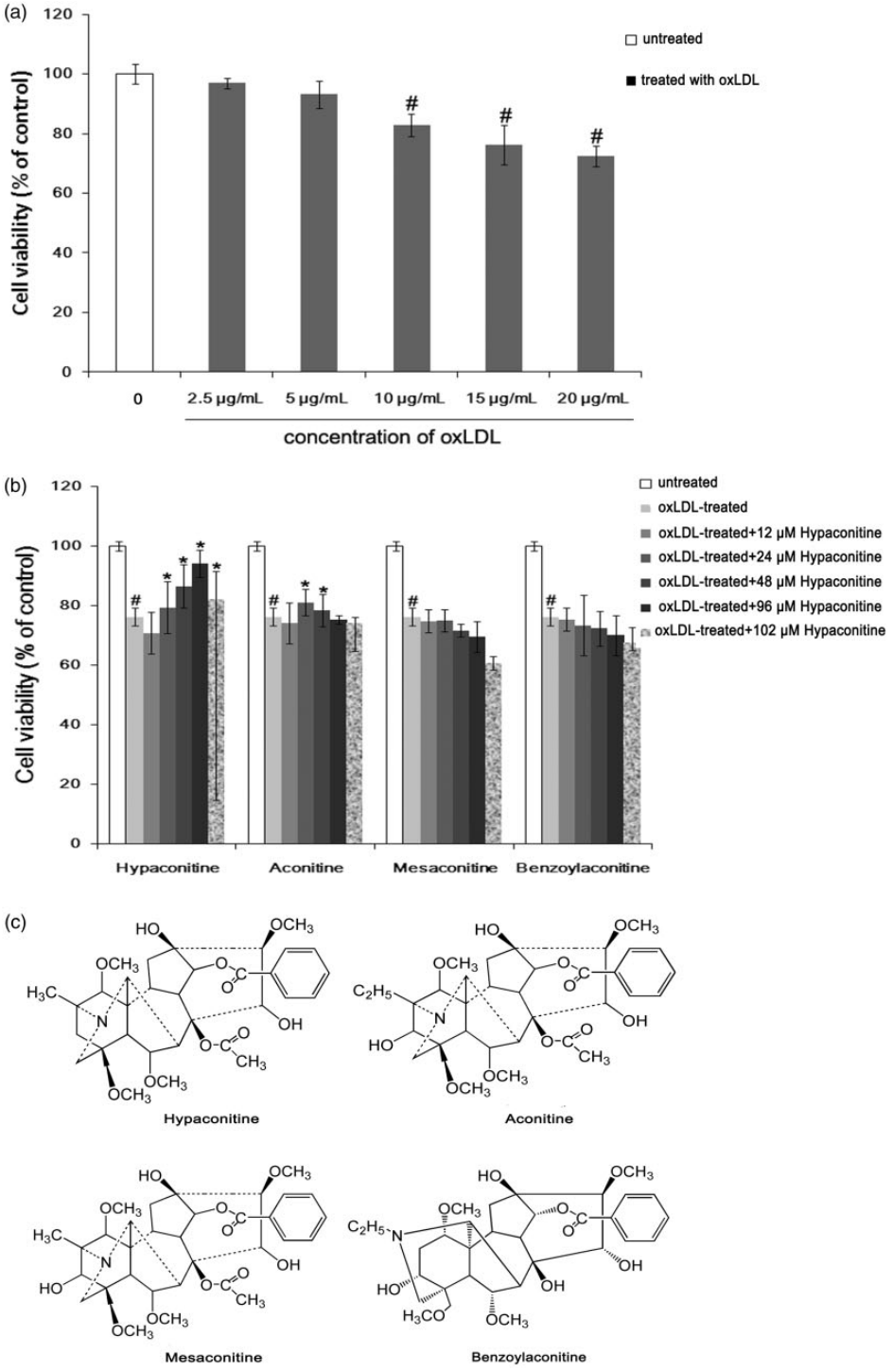

Figure 1 Hypaconitine prevents oxLDL-induced endothelial cell damage. (a) oxLDL induced the cell viability loss in endothelial cells. Endothelial cells were treated without or with oxLDL for 21 h. (b) Comparative effects of hypaconitine, aconitine, mesaconitine and benzoylaconitine on the cell viability loss in endothelial cells treated with oxLDL. Endothelial cells were treated with oxLDL for 21 h in the absence or presence of hypaconitine, aconitine, mesaconitine, benzoylaconitine. The relative cell viability was assessed by t MTT analysis. Data are mean ± S.E. values ($n = 5$). (#)$P < 0.05$, the cell viability was significantly different from cell viability in untreated group; (*)$P < 0.05$, the cell viability was significantly higher compared with cell viability in oxLDL-treated group. (c) Structures of hypaconitine, mesaconitine, aconitine and benzoylaconitine

Clearly, transfection of HDAC3 siRNA significantly decreased the HDAC3 protein expression in endothelial cells (Figure 4(a)). One day after the transfection, cells were treated without or with oxLDL for 21 h in the absence or presence of hypaconitine. Then cell culture supernatants were collected for ELISA, and whole-cell, cytoplasmic and nuclear proteins were extracted for Western blot. Clearly, the HMGB1 protein expressions in culture supernatants were induced by oxLDL, but markedly decreased in the presence of hypaconitine (Figure 4(b)). However, with the

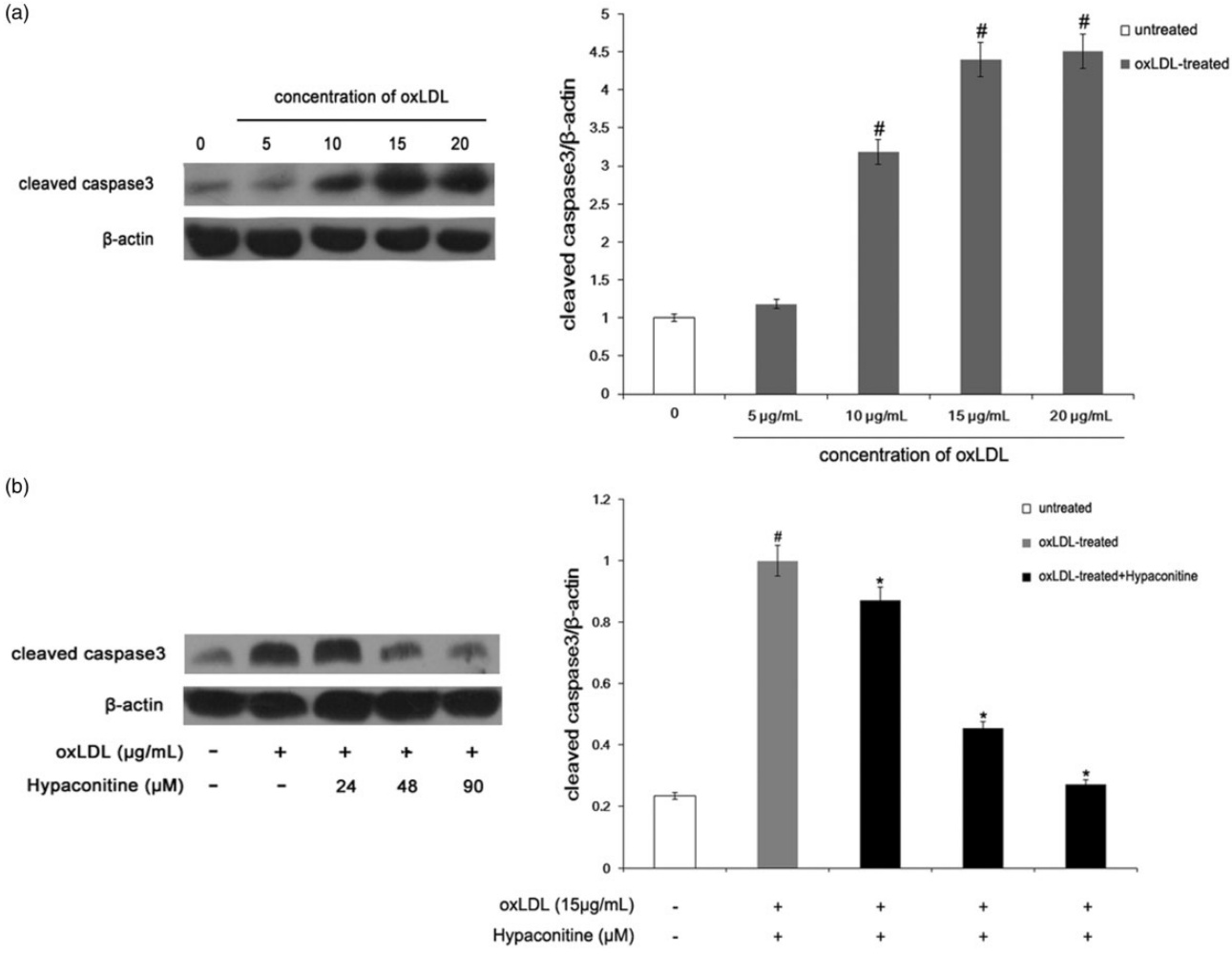

Figure 2 Hypaconitine attenuates oxLDL-induced cleaved caspase3 expression in endothelial cells. (a) oxLDL induced increase of cleaved caspase 3 in endothelial cells. Endothelial cells were treated without or with oxLDL for 21 h. B, cleaved caspase 3 expression was down-regulated by hypaconitine in oxLDL-exposed endothelial cells. Endothelial cells were treated without or with oxLDL in the absence or presence of hypaconitine for 21 h, and used to prepare whole-cell protein for Western blot (left panel); density comparison of cleaved caspase 3/β-actin bands among the experimental groups was established (right panel). Data are mean ± S.E. values obtained from three independent experiments. (#)$P < 0.05$, showed a significant difference compared with untreated group; (*)$P < 0.05$, showed a significant difference compared with oxLDL-treated group

suppression of HDAC3 expression, the effect of hypaconitine on HMGB1 protein down-regulation was significantly suppressed. The cytoplasmic HMGB1 level was significantly up-regulated by oxLDL, but was reduced in the presence of 90 μM hypaconitine. The effect of hypaconitine was significantly reduced when HDAC3 expression was suppressed (Figure 4(c)). Moreover, the nuclear HMGB1 level was down-regulated obviously by oxLDL and up-regulated in the presence of 24–90 μM hypaconitine. These results suggest that hypaconitine prevented the oxLDL-induced cytoplasmic relocation and extracellular HMGB1 release in endothelial cells, and silencing HDAC3 gene suppressed the effect of hypaconitine.

HDAC3 antagonist attenuates the effect of hypaconitine on oxLDL-induced apoptosis in endothelial cells

To further detect the involvement of hypaconitine in the relocation and release of HMGB1, we used siRNA or sodium butyrate (SB), a specific inhibitor of HMGB1 relocation and release, to suppress HDAC3 expression. Then cells were treated with oxLDL in the absence or presence of hypaconitine. Western blot showed that both HDAC3 siRNA and SB attenuated the antiapoptosis function of hypaconitine and reduced its preventive effect on the relocation and release of HMGB1 (Figure 5). These results strongly indicate that hypaconitine prevented oxLDL-induced apoptosis of endothelial cells through up-regulating HDAC3 expression and preventing cytoplasmic relocation and extracellular release of HMGB1.

Discussion

Our study demonstrated that hypaconitine is an active component of the *A. carmichaelii* for preventing endothelial cells from apoptosis due to oxLDL challenge, and its antiapoptotic mechanism is to promote HDAC3 expression and decrease HMGB1 cytoplasmic relocation and extracellular release.

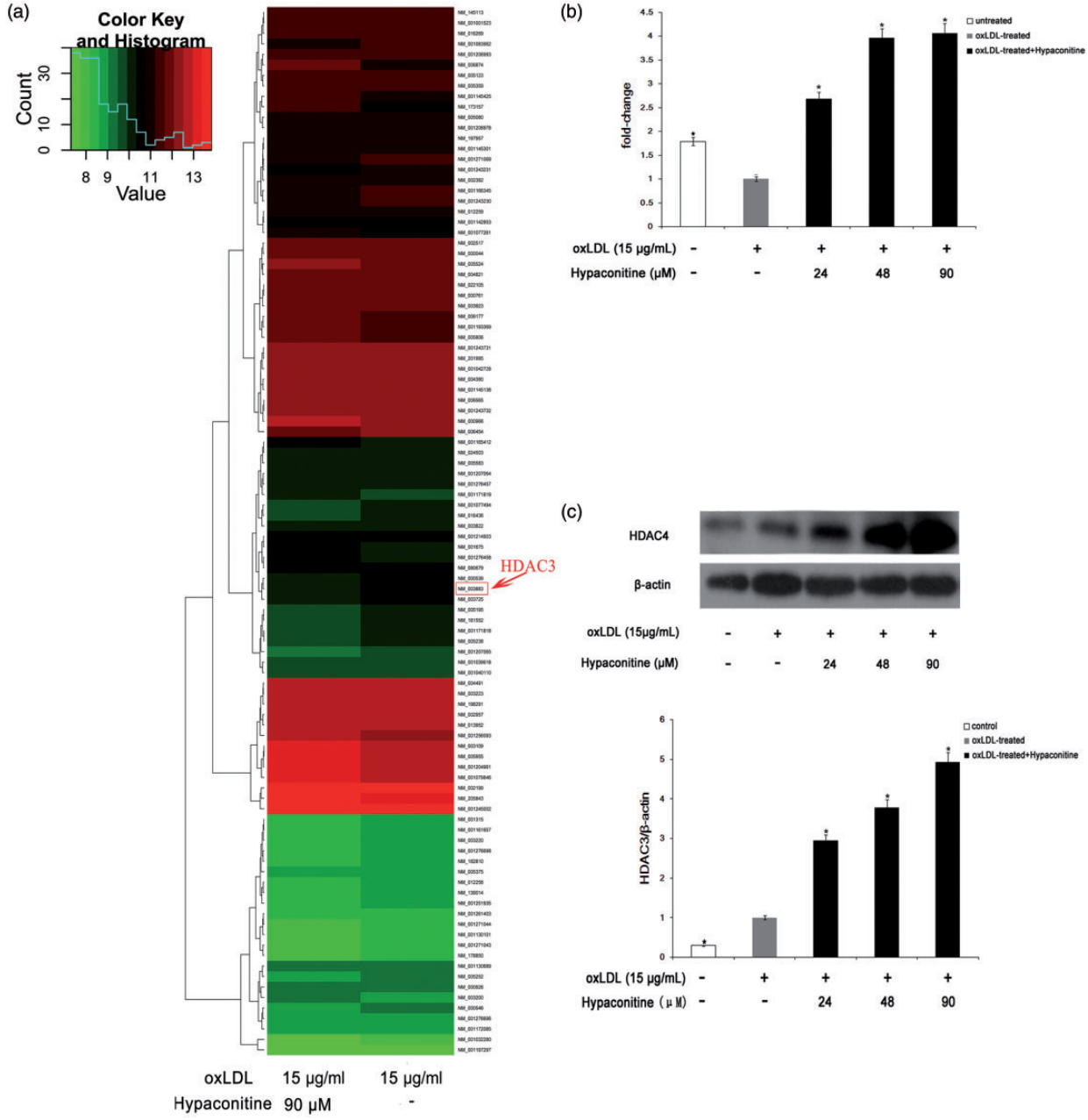

Figure 3 Hypaconitine increases HDAC3 expression in oxLDL-treated endothelial cells. (a) Heat map of differentially expressed transcription factor genes targeted by hypaconitine, and HDAC3 mRNA was upregulated by hypaconitine. Endothelial cells were treated with oxLDL for 21 h in the absence or presence of hypaconitine as indicated concentrations. The levels of mRNAs were analyzed by microarray analysis. (b) HDAC3 mRNA was up-regulated by hypaconitine. Endothelial cells were treated without or with oxLDL for 21 h in the absence or presence of hypaconitine. The HDAC3 mRNA levels were analyzed by qRT-PCR. MRNA expression results were expressed as fold-change compared to oxLDL-treated group. (*) fold-change >2.0, $P < 0.05$, compared with oxLDL-treated group. (c) HDAC3 protein was up-regulated by hypaconitine. Endothelial cells were treated without or with oxLDL for 21 h in the absence or presence of hypaconitine, and were used to prepare whole-cell protein for Western blot (upper panel); density comparison of HDAC3/β-actin bands among the experimental groups was established (lower panel). Data are mean ± S.E. values obtained from three independent experiments. (#)$P < 0.05$, indicated a remarked difference with untreated group; (*)$P < 0.05$, showed a significant difference compared with oxLDL-treated group

A. carmichaelii, a medicinal herb in China, is commonly used to treat CVDs.[10] Hypaconitine, one stable aconitum alkaloid in *A. carmichaelii*, is found to be an active component for treatment of heart disease.[15,16] However, little is known about its effect on endothelial cells. Our data indicate that hypaconitine protects endothelial cells from oxidative damage, and its function is exerted through HDAC3-HMGB1 pathway. It may reveal the mechanism of hypaconitine in treatment of CVDs involved in oxidative damage of endothelial cells.

The results we obtained from this study have several important implications in clinic. First, many diseases besides CVDs are associated with oxLDL-induced apoptosis of endothelial cells, such as cerebrovascular disease and type 2 diabetes.[17,18] Thus, these diseases can be treated by the antiapoptotic hypaconitine or *A. carmichaelii*. Second,

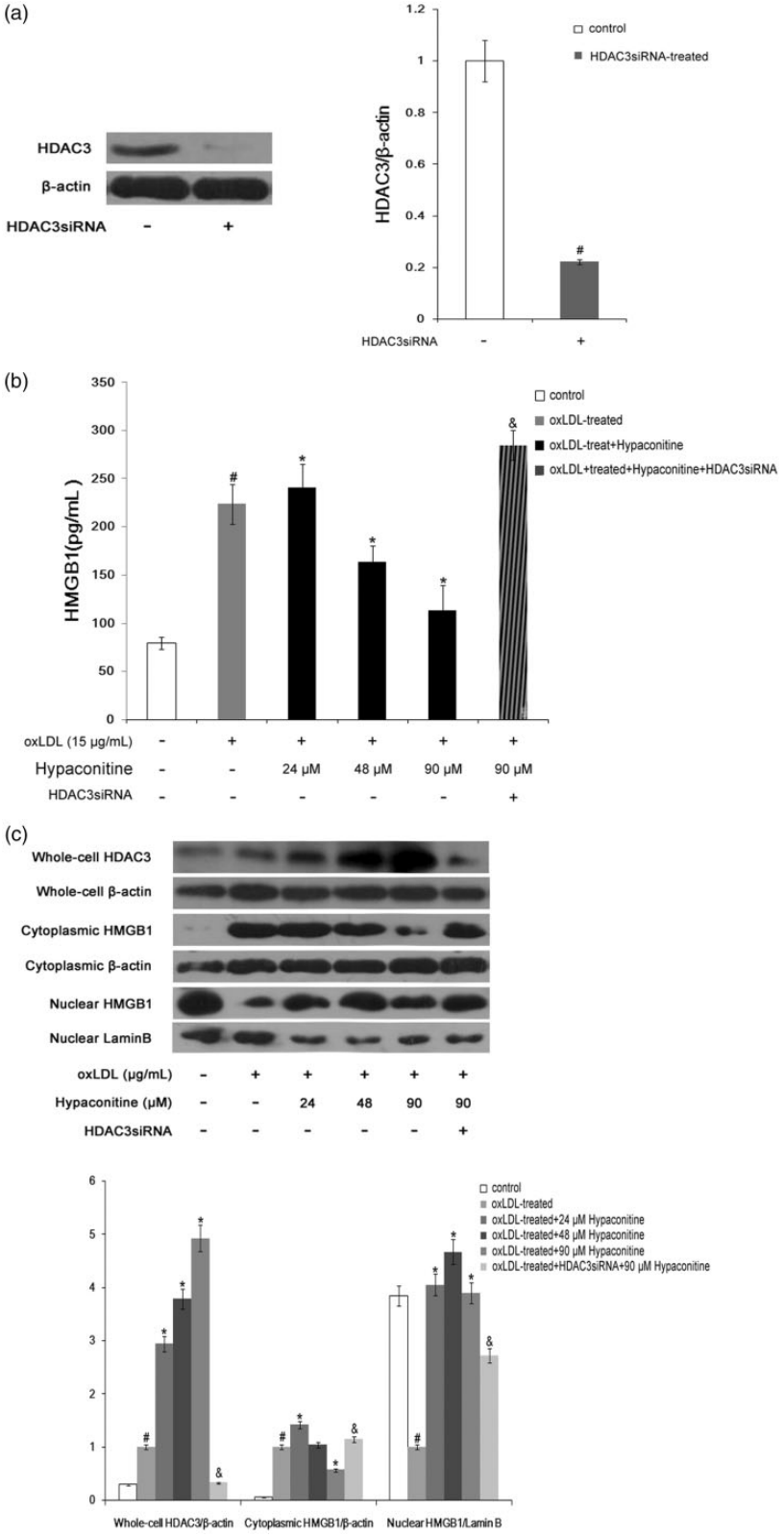

Figure 4 Hypaconitine prevents oxLDL-induced HMGB1 cytoplasmic relocation and extracellular release in endothelial cells. (a) Silencing efficiency of HDAC3 siRNA was checked by Western blot analysis. Endothelial cells were treated with control siRNA vector or HDAC3 siRNA, and the cells were used to prepare whole-cell protein for Western blot analysis (left panel); density comparison of HDAC3/β-actin bands among the experimental groups was established (right panel). (b) The presence of HMGB1 in cell culture supernatants was decreased by hypaconitine. One day after transfection of control siRNA or HDAC3 siRNA plasmid, endothelial cells were treated without or with oxLDL for 21 h in the absence or presence of hypaconitine, and the cell culture supernatants were collected for HMGB1 ELISA analysis. (c) The relocation of HMGB1 was prevented by hypaconitine. The treatments of cells were the same as Panel B, and cytoplasmic protein and nuclear protein were prepared for Western blot analysis (upper panel); density comparisons of Whole-cell HDAC3/β-actin, Cytoplasmic HMGB1/β-actin and Nuclear HMGB1/LaminB bands among the experimental groups were up built (lower panel). Data are mean ± S.E. values obtained from three independent experiments. (#)$P < 0.05$, showed a significant difference compared with corresponding siRNA control group. (*)$P < 0.05$, showed a significant difference compared with corresponding oxLDL-treated group; (&)$P < 0.05$, showed a significant difference compared with corresponding oxLDL-treated +90 μM hypaconitine group

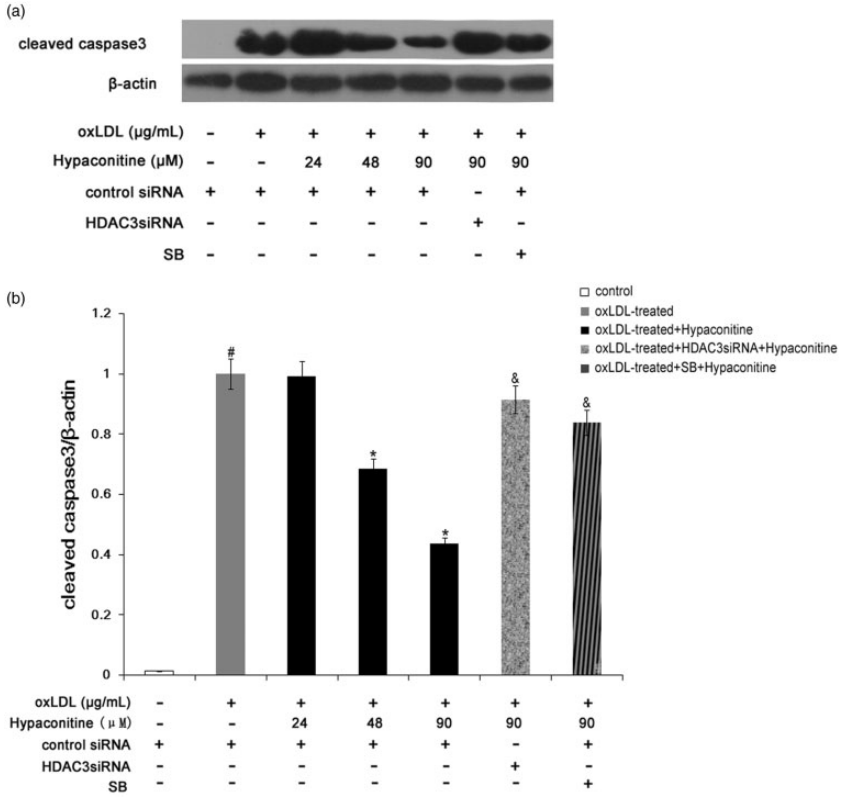

Figure 5 Promotion of HMGB1 relocation and release and silencing of HDAC3 attenuate antiapoptosis effect of hypaconitine in endothelial cells. After transfection of control siRNA or HDAC3 siRNA plasmid, endothelial cells were exposed to oxLDL, and treated without or with hypaconitine for 21 h in the absence or presence of sodium butyrate (SB). Cells were used to prepare whole-cell protein for Western blot analysis (upper panel); density comparison of cleaved caspase 3/β-actin bands among the experimental groups was built (lower panel). Data are mean ± S.E. values obtained from three independent experiments. (#)$P < 0.05$, showed a significant difference compared with control siRNA group. (*)$P < 0.05$, showed a significant difference compared with oxLDL-treated group; (&)$P < 0.05$, showed a significant difference compared with oxLDL-treated + 90 μM group

besides vascular disease, many inflammatory diseases are also induced by the relocation and release of HMGB1,[19,20] so the hypaconitine in regulation of the HDAC3-HMGB1 pathway may provide a new anti-inflammatory therapy. Third, *A. carmichaelii* is a commonly used Chinese herb in clinic. Due to its poisonousness, however, toxicity-decreasing procedure (e.g. heating, steaming and salt-water processing) is required before its application. Hence, identification of hypaconitine as an active component of *A. carmichaelii* contributes to the development of toxicity-decreasing procedure.

Although our data showed the effect of hypaconitine that contributes to the protection of oxLDL-damaged endothelial cells, it remains to be further investigated in animal and clinical experiments. Nevertheless, our study makes an important contribution in revealing the antiapoptosis function of Chinese medicinal herb *A. carmichaelii* and its active component hypaconitine. Clarity of their antiapoptosis mechanism in endothelial cells may promote the establishment of new therapeutic strategies in diseases involved in endothelial cell damage.

Authors' contribution: YB, SD and DC conceived the project, interpretation of the studies and analysis of the data; YB and SD planned the experiments; YB, FL, FH, RD and JZ conducted the experiments; BY and SD wrote the manuscript, and DC provided valuable suggestions on the first manuscript and helped to revise the manuscript.

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DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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