

Induction of lactadherin mediates the apoptosis of endothelial cells in response to advanced glycation end products and protective effects of grape seed procyanidin B2 and resveratrol

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Abstract One of characteristics of diabetes mellitus (DM) is endothelial cell (EC) dysfunction and apoptosis which contributes to the development of vasculopathy. Advanced glycation end products (AGEs) continuously produced in the setting of DM play an important role in causing EC dysfunction and apoptosis. However, the underlying molecular mechanism remains largely elusive. Lactadherin, a secreted glycoprotein of milk-fat globule, is expressed by multiple cell types of arterial wall including ECs. Our previous proteomic studies showed that the expression of lactadherin was significantly increased in the aorta of diabetic rats as compared with control rats and treatment with grape seed procyanidin extracts significantly inhibited the lactadherin expression in diabetic rats. We hypothesized that lactadherin plays a critical role in AGEs-induced EC apoptosis; grape seed procyanidin B2 (GSPB2) and resveratrol protect against AGEs-induced EC apoptosis through lactadherin regulation. Our results showed that AGEs upregulated lactadherin expression and lactadherin RNA interference significantly attenuated AGEs-induced EC apoptosis. Overexpression of lactadherin increased EC apoptosis with up-regulation of Bax/Bcl-2 ratio, cytochrome *c* release, caspase-9 and caspase-3

activation suggesting the involvement of mitochondria apoptosis pathway. Mechanistically, overexpression of lactadherin reduced the phosphorylation of GSK3beta at baseline. Our study also revealed nine proteins interacting with lactadherin in HUVEC and study of these candidate proteins could unveil further underlying molecular mechanisms. In summary, our study identified lactadherin as a key player responsible for AGEs-induced EC apoptosis and antioxidants GSPB2 and resveratrol protect against AGEs-induced EC apoptosis by inhibiting lactadherin. Targeting lactadherin with antioxidant could be translated into clinical application in the fighting against DM complications.

Keywords Lactadherin · Advanced glycation end products · Cell apoptosis · Grape seed proanthocyanidin B2 · Resveratrol

Introduction

Premature development of microvascular and macrovascular disease is the leading cause of morbidity and mortality associated with diabetes mellitus (DM) [1, 2]. Among the many metabolic abnormalities of DM, advanced glycation end products (AGEs) have been most consistently and significantly correlated with diabetic vascular complications by epidemiological studies. AGEs lead to endothelial dysfunction and apoptosis, which plays a critical role in the pathophysiology of vascular complications in DM [3–5].

In endothelial cells, AGEs induce mitochondrial dysfunction thus increased oxidative stress leading to cellular dysfunction and cell death [6, 7]. However, the molecular mechanism underlying AGEs-induced tissue damage and DM complications remains largely elusive, which has been

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the bottle neck in the development of effective therapeutic approaches to the prevention and treatment of DM and its complications. Protection of endothelial injury from AGEs in DM could be the first step in preventing the cardiovascular complications of DM.

In this study we provided strong evidence that lactadherin is an important player in the pathogenesis of endothelial dysfunction in AGE-induced endothelial dysfunction. Lactadherin is a secreted glycoprotein of milk-fat globule that shares structural domain homology with Del-1. Mouse lactadherin is also known as milk-fat globule-EGF factor 8 (MFG-E8). It consists of two C domains with homology to the C1 and C2 domains of coagulation factor V and factor VIII and one (in human) or two (in mouse) epidermal growth factor-like domains with an Arg-Gly-Asp (RGD) integrin-binding sequence [8, 9]. Lactadherin is produced in and around blood vessels. In arteries, lactadherin is mainly expressed by adventitial microvessels, medial smooth muscle cells and some luminal endothelial cells [9]. Our previous proteomic studies showed that the expression of lactadherin in the aorta of diabetic rats was significantly higher than those of control rats. We also found that treatment with grape seed procyanidin extracts (GSPE) significantly inhibited the expression of lactadherin in diabetic rats [10]. Among the signaling molecules in cell apoptosis, GSK3 β is a proapoptotic kinase, as its overexpression sensitizes cells to apoptosis and pharmacological inhibition of GSK3 β attenuates cytochrome *c* release from mitochondria thus inhibits apoptosis. Phosphorylation of GSK3 β on residue serine-9 results in GSK3 β inactivation [11].

Polyphenols are secondary metabolites of plants that are classified according to their structures. Some polyphenols are ubiquitous in plants, whereas others are restricted to particular families or species. Procyanidins and resveratrol are a complex family of polyphenol polymers widespread in nature and occur in processed products such as grape wines, a few fruits and vegetables [12, 13]. GSPE derived from grape seeds have been reported to possess anti-oxidant, anti-nonenzymatic glycosylation, anti-inflammation, and anti-tumor effects, among others [14–16]. Dimeric procyanidin B2 is one of the main components of GSPE, composed of two molecules of the flavan-3-ol (–)-epicatechin linked by a 4b \rightarrow 8 bonds. Several studies have shown that procyanidin B2 and resveratrol have a variety of anti-inflammatory, anti-tumor effects and cardiovascular protective properties [17–19].

In our present study, we unveiled a novel molecular mechanism underlying AGE-induced endothelial cell apoptosis. Our data showed that lactadherin was a key mediator in endothelial cell apoptosis in response to AGE treatment and grape seed procyanidin B2 (GSPB2) and

resveratrol were protective against AGE-induced endothelial apoptosis by regulating lactadherin. We also further investigated the role of lactadherin in the signaling cascade implicated in apoptotic process.

Materials and methods

Materials

GSPB2 (>95% purity, Lot No: 20080915) and resveratrol (>98% purity, Lot No: 0810018-22) were provided by Jianfeng Inc (Tianjin, China). Bovine serum albumin (BSA), D-glucose, collagenase, trypsin/EDTA solution, dimethyl sulfoxide (DMSO), and 3-(4,5-dimethylthiazol)-2,5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma (St. Louis, USA). Fetal bovine serum (FBS) and RPMI 1640 were obtained from GIBCO (Grand Island, USA). Cell counting kit-8 (CCK-8) was obtained from Dojindo (Kumamoto, Japan). HUVEC were obtained from American Type Culture Collection (Rockville, USA). Terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling (TUNEL) in situ apoptosis detection kit (Roche Diagnostic, Indianapolis, IN). Cytochrome *c* enzyme-linked immunosorbent assay kit were purchased from eBioscience (San Diego, USA). Caspase-9 activity kit was purchased from R&D Systems (Minneapolis, USA). The antibody of lactadherin and prohibitin2 (PHB2) were purchased from Abcam (Cambridge, USA). The antibodies of GSK3 β , phospho-GSK3 β , cleaved caspase 3, caspase 3, Bax and Bcl-2 were purchased from Cell Signaling Technology (Beverly, MA). All other reagents were standard commercial high-purity materials.

AGE-BSA preparation

AGEs were prepared by incubating 50 mg/ml BSA with 0.5 mol/l glucose in 0.2 mol/l phosphate buffered saline (PBS, pH 7.4) in the dark at 37°C for 12 weeks [20, 21]. Before incubation, the solution was sterile filtered by passing it through a 0.2 μ m filter. Unmodified BSA was incubated under the same conditions in the absence of glucose for control. After incubation, unincorporated sugars were removed by extensive dialysis. The AGEs concentration was determined by fluorescence spectrophotometry (Hitachi V-2001, Japan; 390 nm excitation wavelength, 450 nm emission wavelength). As a result, the AGEs concentration in AGE-modified BSA was 94.5 U/mg proteins, whereas that in control group was 0.5 U/mg proteins. Endotoxin concentrations were measured by the limulus amoebocyte lysate assay (Endos), which revealed negligible values (<0.2 μ g/l).

Cell cultures

HUVEC were cultured in complete medium RPMI 1640 containing 10% FBS at 37°C in a humidified atmosphere containing 50 ml/l CO₂. GSPB2 and resveratrol were dissolved in DMSO and diluted so that the final concentration of DMSO was <0.1%.

Knockdown of lactadherin by siRNA, construction of lactadherin overexpression plasmids and transfection

Short interfering RNAs (siRNAs) for lactadherin and negative control siRNAs were designed and chemically synthesized from Shanghai GenePharma Co., Ltd (Shanghai, China). The siRNA sequence targeting lactadherin (GenBank accession no: NM_001114614) including: sense 5'-CGAGGAGAUUCCCAAGAATT-3', antisense 5'-UUCUUGGGAAAUCUCCUCGTT-3'. The sequence of negative control siRNA is: sense 5'-UUCUCCGAACGUGUCACGUTT-3', antisense 5'-ACGUGACACGUUCGGAGAATT-3'. For transient transfection, HUVEC were cultured in six-well plates overnight and transfected with siRNA against lactadherin using Lipofectamine2000 (Invitrogen Life Technologies, Carlsbad, CA) according to the instructions of the manufacturer. The expression of lactadherin was assayed 48 h after transfection by real-time PCR and western blotting.

Overexpression plasmids pGC-FU-lactadherin were constructed as follows from Shanghai GeneChem Co., Ltd (Shanghai, China). Briefly, full-length cDNA of lactadherin (GenBank accession no: NM_001114614) was amplified from a human cDNA library (open biosystem, USA) by PCR using forward primer (5'-CATTCTCCAGGCCA GTGGCTCGACACAT-3') and reverse primer (5'-GCC ACTGGCCTGGAGAATGGGAACATTGCC-3'). The sequences of lactadherin were confirmed by sequencing. The PCR products were then subcloned into pGC-FU expression vectors. The overexpression lentiviral vectors with lactadherin (LV-lactadherin) or green fluorescent protein (LV-GFP) were made. HUVEC (1×10^6) were transduced with lentiviral vectors at a multiplicity of infection (MOI = 10) according to the manufacturer's instructions. Stably transfectant clones were characterized using real-time PCR and western blotting for the expression levels of lactadherin at 5 days.

Cell viability analysis

Cell viability was measured by MTT or CCK-8 colorimetric assay. HUVEC with overexpression or siRNA knockdown of lactadherin (1×10^6 cells/ml) were incubated for 48 h at 37°C in the presence or absence of GSPB2 and resveratrol (10 µmol/l). Moreover, HUVEC

transfected with lactadherin siRNA were treated with 200 µg/ml of AGEs. MTT (0.5 mg/ml) or CCK-8 (20 µl) was added at 37°C for 4 h and cell viability was measured per instruction.

Detection of apoptosis by TUNEL assay

HUVEC with overexpression or siRNA knockdown of lactadherin were treated with or without GSPB2 and resveratrol (10 µmol/l) for 48 h. The apoptotic cells were determined by TUNEL assay using an in situ apoptosis detection kit according to the manufacturer's instructions. The number of apoptotic cells was counted in ten randomly selected fields.

Cell morphology

Logarithmic phase cells were trypsinized and plated to the subconfluent density of 10^6 /ml in 75 cm² Petri dish and harvested after incubated with or without GSPB2 and resveratrol (10 µmol/l) for 48 h. The cells were fixed in 2.5% glutaraldehyde (v/v) and 2% osmium tetroxide (w/v), washed in PBS, post-fixed in osmium tetroxide, dehydrated in an ethanol series, embedded in epoxy resin, and examined with a HITACHI H-800 electron microscope as described [22].

Cytochrome *c* release and caspase-9 activity assay

After subcellular fractionation, the cytochrome *c* concentration in the cytosol was measured using the ELISA kit following the manufacturer's instructions. The caspase-9 activity in cell lysates was measured using colorimetric assay kit, per the manufacturer's instructions. The data are expressed as the mean optical density of the samples normalized to a percentage of the control value.

Quantitative real-time PCR

Total RNA was extracted from cells and underwent quantitative analysis of mRNA expression of lactadherin by real-time PCR with an ABI Prism 7500 sequence detection system (Applied Biosystems, UK). The reaction reagent SYBR Green I Master Mix Kit (Stratagene) was used according to the manufacturer's instructions. First strand cDNA was synthesized from total RNA per instruction (RT kit from MBI, Canada). The real-time PCR reaction components consisted of 10 µl of 2 × SYBR Green I Master Mix, 0.5 µl up-stream primers (10 µmol/l), 0.5 µl down-stream primers (10 µmol/l), 1 µl cDNA and 8 µl double distilled water. Sequences of the primer sets used were as follows: lactadherin (forward) 5'-CGTAC ACCTGCACGTGCCTTA-3', (reverse) 5'-GGACCCA

ATGCTGCAAAC-3'; β -actin (forward) 5'-TGGCACC-CAGCA CAATGAA-3', (reverse) 5'-CTAAGTCATAGT CCGCCTAGAAG CA-3'. The PCR conditions for all genes were as follows: 50°C for 2 min; preheating, 95°C for 10 min; followed by 40 cycles of denaturation (95°C for 15 s) and annealing/elongation (62°C for 50 s). Each sample was run in triplicate. Gene-specific mRNA was normalized to β -actin mRNA as an internal control. The amounts of lactadherin mRNA were expressed as fold change relative to those of untreated cells set as 1.

Western blotting

Equal amount of proteins were separated by SDS-PAGE (12%) and transferred onto a polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). Proteins of interest were revealed by specific antibodies followed by secondary antibody. Signal detection was performed via exposing of the blots to enhanced DAB color reagent following the manufacturer's recommendations. Densitometry was obtained for quantification of each identified protein band (Digital Protein DNA Imagineware, Huntington Station, NY, USA).

Co-immunoprecipitation

Sample preparation and LTQ-ESI-MS/MS analysis

HUVEC overexpressing lactadherin were lysed and the cell lysates (600 μ l) were equally divided. The lysates were incubated with either anti-lactadherin antibody or IgG (2 μ g/ml) overnight at 4°C. The samples were then treated with 30 μ l of protein A/G-agarose beads for 2 h at 4°C. The beads were centrifuged and washed for 5 times. The beads were resuspended in loading buffer and boiled before subjected to 12% SDS-PAGE followed by LTQ-ESI-MS/MS analysis and database searching. The SDS-PAGE gels were scanned using GS-800 Calibrated Densitometer (Bio-Rad) and image analysis. Comparative studies was performed using the PDQuest Image Analysis software (Bio-Rad) per instruction. Different bands were identified when at least 100% of volume difference between matching spots consistently in all the triplicate experiments. These gels were fixed and stained with colloidal Coomassie brilliant blue (CBB). The specific bands of lactadherin immunoprecipitation (L-IP) group, as defined by the PDQuest Image analysis, were excised from the cCBB-stained gels for a modified in-gel trypsin digestion procedure. The peptides mixture was solubilized with 1% TFA for mass spectrometry analysis.

The peptide mixtures were measured using on a LTQ-Orbitrap Velos MS/MS (ThermoFinnigan, San Jose, CA, U.S.A.), using a surveyor high performance liquid

chromatography (HPLC) system. The system was fitted with a C18 RP column (0.15 mm \times 150 mm, Thermo Hypersil-Keystone). Mobile phase A (0.1% formic acid in water) and the mobile phase B (0.1% formic acid in ACN) were selected. The tryptic peptide mixtures were eluted using a gradient of 2–98% B over 60 min. The temperature of the heated capillary was set at 170°C. A voltage of 3.0 kV applied to the ESI needle resulted in a distinct signal. The normalized collision energy was 35.0. The number of ions stored in the ion trap was regulated by the automatic gain control. Voltages across the capillary and the quadrupole lenses were tuned by an automated procedure to maximize the signal for the ion of interest. The LTQ mass spectrometer was set so that one full MS scan was followed by ten MS/MS scans on the ten most intense ions from the MS spectrum. Dynamic Exclusion was set at repeat count 2; repeat duration 30 s, exclusion duration 90 s.

For protein identification and statistical validation, the acquired MS/MS spectra were automatically searched against the International Protein Index (IPI) HUMAN version 3.53 database using the Turbo SEQUEST program in the BioWorks™ 3.1 software suite. An accepted SEQUEST result had to have a DelCN score of at least 0.1 (regardless of charge state). Peptides with a +1 charge state were accepted if they were fully tryptic and had a cross correlation (X_{corr}) of at least 1.9. Peptides with a +2 charge state were accepted if they had an $X_{\text{corr}} \geq 2.2$. Peptides with a +3 charge state were accepted if they had an $X_{\text{corr}} \geq 3.75$. Identifications were validated when considering at least two peptide sequences per protein or one peptide repeated 4 times. To sort out a single protein member from a protein group, we chose the protein with the highest sequence coverage.

Confirmation of lactadherin–PHB2 interaction

The proteins to be analyzed by immunoblotting were removed from the beads and separated by 1D gel electrophoresis as described above (Sample preparation). The gel was blotted and incubated with a polyclonal antibody to PHB2 overnight followed by incubation with the HRP-conjugated secondary antibody. Signal detection was performed via exposing of the blots to enhanced DAB color reagent.

Statistical analysis

Data are expressed as mean \pm standard deviation. Statistical analysis between groups was made using one-way analysis of variance (ANOVA) followed by Tukey's HSD test for multiple comparisons. *P* value < 0.05 was considered statistically significant. All analyses were

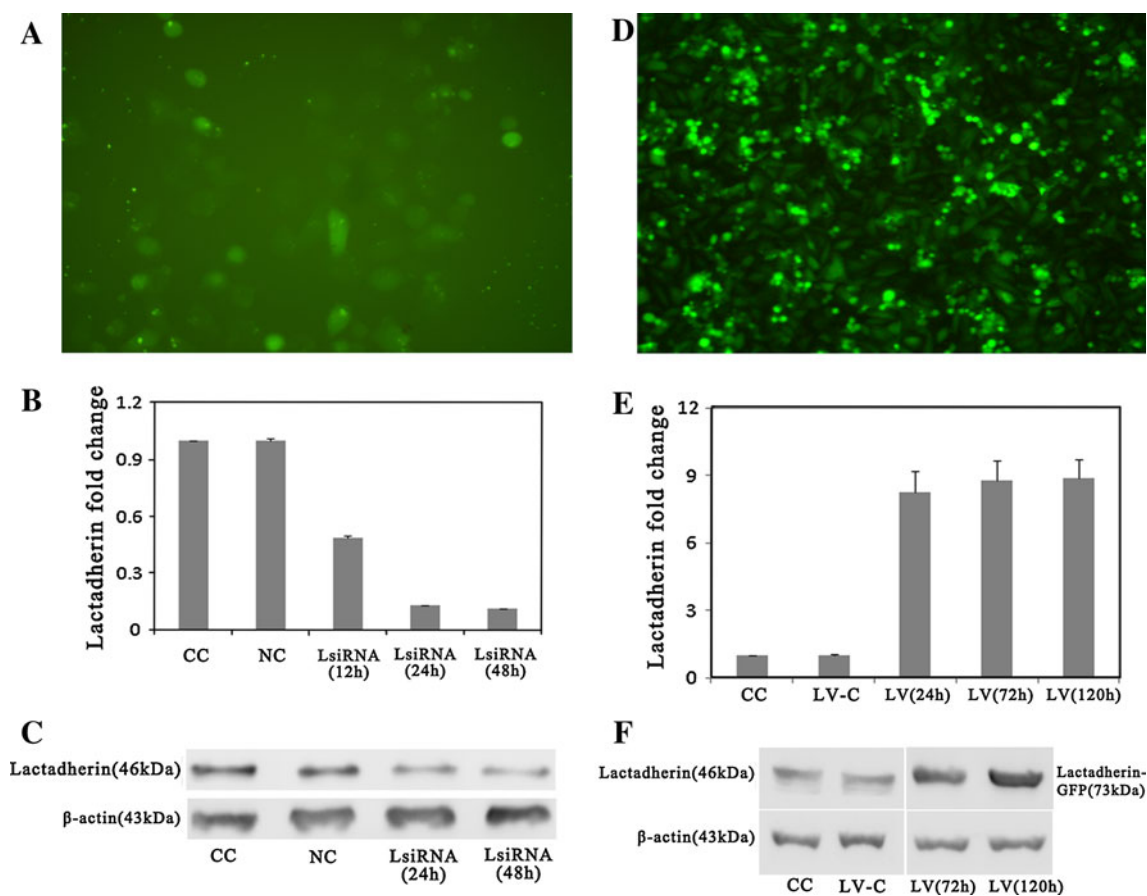


Fig. 1 Lactadherin siRNA and overexpression plasmids transfection HUVEC. **a** Fluorescence micrograph displays lactadherin siRNA in HUVEC ($\times 200$). **b** RT-PCR analysis demonstrates lactadherin mRNA expression in HUVEC at 12, 24, and 48 h after transfection. **c** Western blot analysis demonstrates lactadherin protein expression in HUVEC at 24 and 48 h after transfection. **d** Fluorescence micrograph displays lactadherin-GFP overexpression in HUVEC ($\times 200$). **e** RT-PCR analysis demonstrates lactadherin mRNA

expression in HUVEC at 24, 72, and 120 h after transfection. **f** Western blot analysis demonstrates lactadherin protein expression in HUVEC at 72 and 120 h after transfection. *CC* group normal control cells, *NC* group negative control siRNA cells, *LsiRNA* group siRNA against lactadherin cells, *LV-C* group HUVEC carrying GFP, *LV* group HUVEC carrying both GFP and lactadherin, *HUVEC* human umbilical vein endothelial cells

performed with SPSS for Windows software version 10.0 (SPSS, Chicago, USA).

Results

Transduction efficiency with lactadherin siRNA and overexpression plasmids

We transduced the HUVEC with siRNA or lentivirus vector. Transduction conditions were optimized by using different MOI and the transduction efficiency was assessed by fluorescence microscopy, real-time PCR and western blotting. HUVEC carrying negative control siRNA (NC), HUVEC carrying siRNA against lactadherin (LsiRNA) and HUVEC carrying GFP (LV-C), HUVEC carrying both

GFP and lactadherin genes (LV) were harvested. The transduction efficiency was about 95% or higher at day 5 or longer. Transgene expression was confirmed by fluorescent microscopic imaging for lactadherin protein expression (Fig. 1a, d). The protein expression of lactadherin in LsiRNA group decreased to more than 60% the level of the NC group at 48 h after transfection (Fig. 1b, c). Lactadherin mRNA and protein expression reached its highest level at day 5 after virus removal (Fig. 1e, f).

Effects of lactadherin on viability in HUVEC treated by AGEs

To investigate the role of lactadherin in HUVEC viability, we transduced HUVEC with lactadherin overexpression lentiviral vectors and siRNA, and estimated the cell

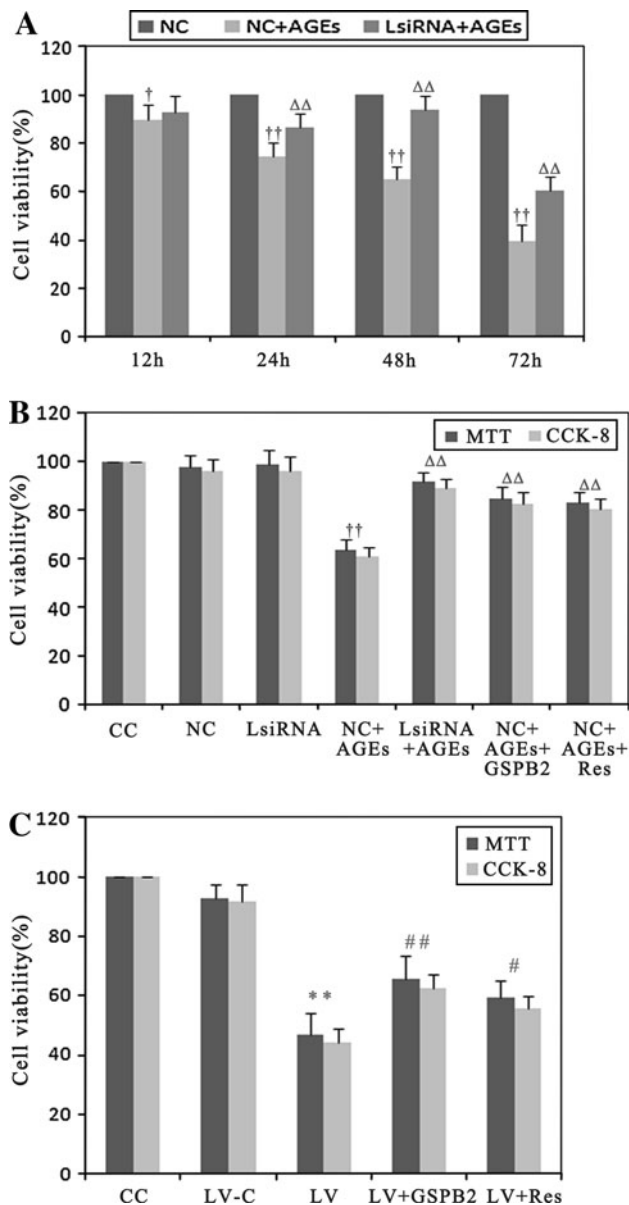


Fig. 2 Effects of lactadherin on cell viability treated by AGEs with MTT and CCK-8. **a** Effects of lactadherin siRNA on viability in HUVEC treated by AGEs at 12, 24, 48, and 72 h after transfection with MTT. **b** Effects of lactadherin siRNA, GSPB2 and resveratrol on viability in HUVEC treated by AGEs. **c** Effects of lactadherin overexpression, GSPB2 and resveratrol on viability in HUVEC treated by AGEs. Results are expressed as percent of untreated cells (100%) and are given as mean \pm SD from five independent experiments. [†] $P < 0.05$, ^{††} $P < 0.01$ compared with NC group; ^Δ $P < 0.05$, ^{ΔΔ} $P < 0.01$ compared with NC + AGEs group. * $P < 0.05$, ** $P < 0.01$ compared with LV-C group; # $P < 0.05$, ## $P < 0.01$ compared with LV group. NC + AGEs group NC group with AGEs (200 μ g/ml), LsiRNA + AGEs group LsiRNA group with AGEs (200 μ g/ml), NC + AGEs + GSPB2 group NC + AGEs group treatment with GSPB2 (10 μ mol/l), NC + AGEs + Res group NC + AGEs group treatment with resveratrol (10 μ mol/l), LV + GSPB2 group LV group treatment with GSPB2 (10 μ mol/l), LV + Res group LV group treatment with resveratrol (10 μ mol/l), AGEs advanced glycation end products, GSPB2 grape seed procyanidin B2, CCK-8 cell counting kit-8

viability using MTT and CCK-8 assay. The cell viability was decreased when HUVEC were exposed to AGEs (200 μ g/ml) at 12, 24, 48, and 72 h. Meanwhile, siRNA against lactadherin significantly increased the cell viability compared with NC + AGEs group at 24, 48, and 72 ($P < 0.05$) (Fig. 2a). GFP or negative control siRNA did not affect cell viability. There were no differences in the cell viability between LsiRNA group and NC group. Lactadherin siRNA significantly attenuated AGEs treated the decrease of cell viability compared with NC + AGEs group ($P < 0.05$). The treatment of NC group with GSPB2 and resveratrol (10 μ mol/l) significantly improved the AGEs treated cell viability for 48 h ($P < 0.05$) (Fig. 2b). Moreover, the overexpression of lactadherin significantly decreased the cell viability ($P < 0.05$). The cell viability was increased when LV group was exposed to GSPB2 and resveratrol (10 μ mol/l) for 48 h ($P < 0.05$) (Fig. 2c).

Role of lactadherin in apoptosis of HUVEC treated by AGEs and the anti-apoptotic effect of GSPB2 and resveratrol

To examine whether lactadherin plays a role in AGEs-mediated apoptosis, LsiRNA group and NC group were treated with or without AGEs (200 μ g/ml) and treated with GSPB2 and resveratrol (10 μ mol/l) for 48 h. Induction of NC group with AGEs (200 μ g/ml) resulted in a significant increase in the cell apoptosis, whereas siRNA against lactadherin significantly attenuated AGEs treated the cell apoptosis compared with NC + AGEs group ($P < 0.05$). The treatment of NC group with GSPB2 and resveratrol (10 μ mol/l) also significantly decreased the AGEs treated cell apoptosis for 48 h ($P < 0.05$) (Fig. 3a). HUVEC overexpressing lactadherin was susceptible to cell apoptosis, while GSPB2 and resveratrol (10 μ mol/l) significantly attenuated the cell apoptosis for 48 h (Fig. 3b). These data clearly show that lactadherin promote AGEs-mediated cell apoptosis.

The morphological changes of HUVEC overexpressing lactadherin

To support these results, we performed electronic microscope analysis of HUVEC overexpressing lactadherin treated and not treated with GSPB2 and resveratrol (10 μ mol/l). Under electronic microscope, typical apoptosis phenomena were found in the HUVEC overexpressing lactadherin: apoptotic body, assemblage of heterochromatin and edema of mitochondria were found in many apoptotic cells. While GSPB2 and resveratrol (10 μ mol/l) significantly attenuated the apoptotic phenomena and organelle necrosis for 48 h (Fig. 4).

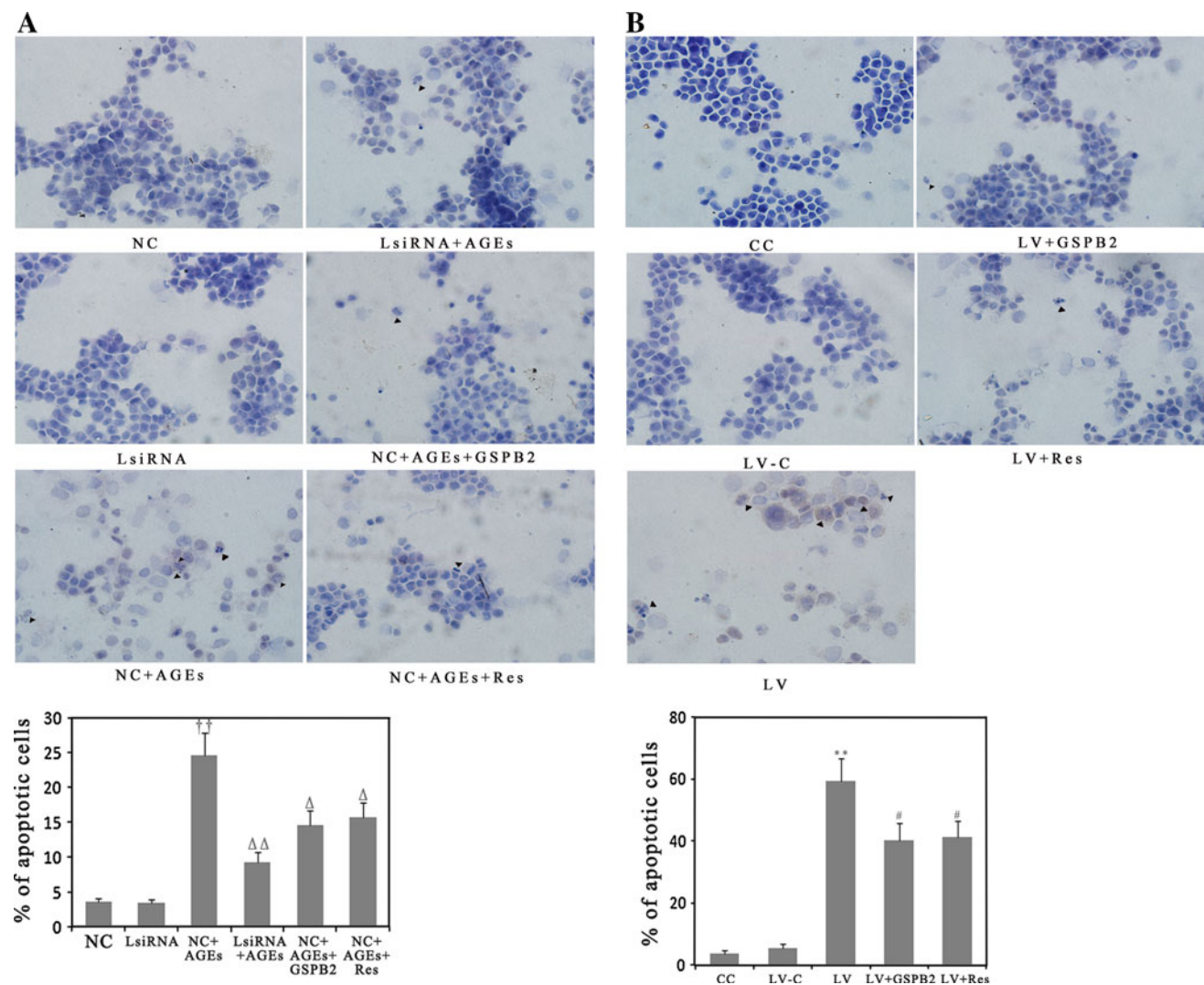


Fig. 3 Effects of lactadherin on cell apoptosis treated by AGEs with TUNEL assay ($\times 400$). **a** Effects of lactadherin siRNA, GSPB2 and resveratrol on apoptosis in HUVEC treated by AGEs. **b** Effects of lactadherin overexpression, GSPB2 and resveratrol on apoptosis in HUVEC treated by AGEs. *Black arrows* showed TUNEL positive cells. The *bar graph at the bottom* shows the percentage of apoptotic

cells. Results represent mean \pm SD of five independent experiments. $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$ compared with NC group; $^{\Delta}P < 0.05$, $^{\Delta\Delta}P < 0.01$ compared with NC + AGEs group. $*P < 0.05$, $^{**}P < 0.01$ compared with LV-C group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared with LV group

Effects of lactadherin on cytosol cytochrome *c* concentration, caspases and Bax/Bcl-2

To further examine the molecule mechanism of the apoptosis pathway, we examined the cytosol cytochrome *c* concentration, caspase-9 activity, caspase-3 cleavage and Bax/Bcl-2 induced by lactadherin in HUVEC by western blotting. The uncleaved caspase-3 levels were unchanged in LV-C group, LV group, LsiRNA group and NC group. Stimulation of NC group with AGEs (200 $\mu\text{g}/\text{ml}$) resulted in a significant increase in the cytosol cytochrome *c* concentration, caspase-9 activity, cleaved caspase-3 and Bax/Bcl-2 ratio, whereas siRNA against lactadherin significantly attenuated AGEs treated the cytosol cytochrome

c concentration, caspase-9 activity, cleaved caspase-3 and Bax/Bcl-2 ratio compared with NC + AGEs group ($P < 0.05$). The treatment of NC group with GSPB2 and resveratrol (10 $\mu\text{mol}/\text{l}$) significantly improved the AGEs treated the cytosol cytochrome *c* concentration, caspase-9 activity, caspase-3 cleavage and Bax/Bcl-2 ratio for 48 h ($P < 0.05$) (Fig. 5a–f). The cytosol cytochrome *c* concentration, caspase-9 activity, caspase-3 cleavage and Bax/Bcl-2 ratio significantly increased in LV group compared with those in LV-C group, while GSPB2 and resveratrol (10 $\mu\text{mol}/\text{l}$) significantly inhibited the cytosol cytochrome *c* concentration, caspase-9 activity, caspase-3 cleavage and Bax/Bcl-2 ratio in HUVEC overexpressing lactadherin for 48 h (Fig. 5g–l). These results suggest that mitochondria

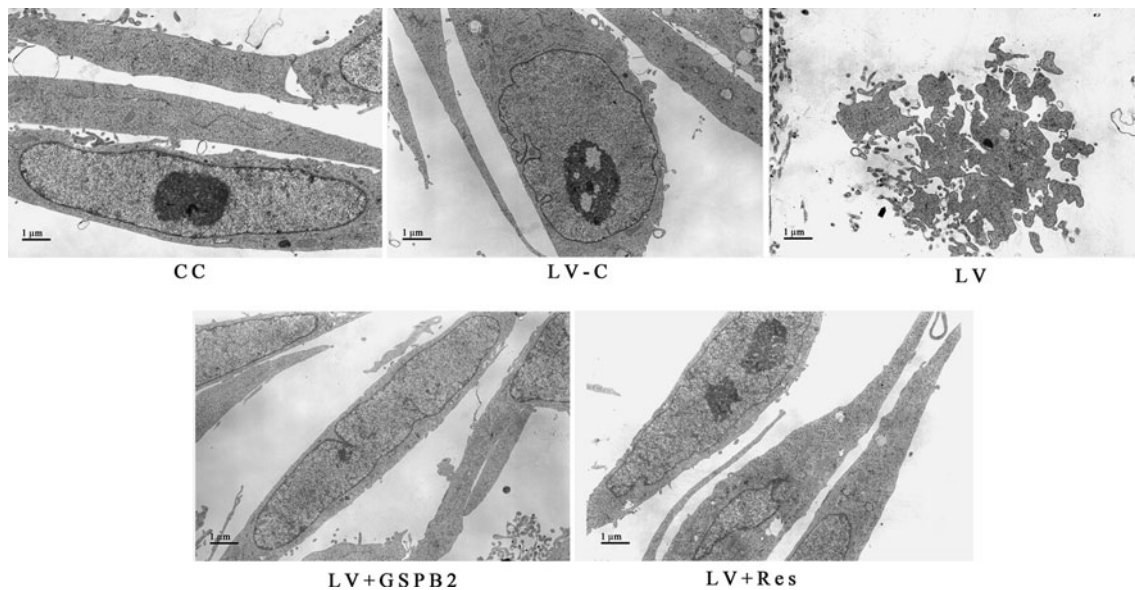


Fig. 4 Representative electron micrographs of HUVEC overexpressing lactadherin (scale bar, 1 μ m). Black arrows showed apoptotic bodies

pathway is involved in lactadherin-mediated endothelial cell apoptosis in response to AGEs treatment.

Effect of lactadherin on the levels of phospho-GSK3 β

Given the well-established role of GSK3 β in cell apoptosis, we determined the effect of lactadherin on phosphorylation of GSK-3 β (phospho-GSK3 β) in HUVEC. Treatment of NC group with AGEs (200 μ g/ml) resulted in a significant decrease in the levels of phospho-GSK3 β , whereas siRNA against lactadherin significantly reversed the decreased levels of phospho-GSK3 β in response to AGEs. Treatment with GSPB2 and resveratrol (10 μ mol/l) significantly attenuated the AGEs-induced decrease of phospho-GSK3 β level for 48 h ($P < 0.05$) (Fig. 6a, b). The level of phospho-GSK3 β significantly decreased in LV group compared with LV-C group, while GSPB2 and resveratrol (10 μ mol/l) significantly reversed the decreased phospho-GSK3 β in HUVEC overexpressing lactadherin (Fig. 6c, d). Since GSK3 β phosphorylation inhibits GSK3 β activity, these results suggested that AGEs increases GSK3 β activity in a lactadherin-dependent manner.

Identification of proteins interacting with lactadherin

SDS-PAGE was performed and the bands of proteins with differential expression between L-IP groups and control groups were revealed (Fig. 7a). In total, five bands were detected on the three gels as determined by the PDQuest Image Analysis Software. Nine protein spots were successfully identified by LTQ-ESI-MS/MS, including Swiss-Prot accession number, protein description, theoretical

Mr/PI, unique peptides and sequence coverage, etc (Table 1). The interaction proteins were prohibitin-2, prohibitin, annexin A2, glyceraldehyde-3-phosphate dehydrogenase, serpin B3, elongation factor 1-alpha 2, ADP/ATP translocase 2, isoform M1 of pyruvate kinase isozymes M1/M2, and myoferlin. We also used co-immunoprecipitation to specifically test for the presence of these proteins. Figure 7b showed that the PHB2 was detected in the lactadherin immunoprecipitate, which was undetectable in the control samples.

Discussion

Although classical paradigms defining the pathophysiology of vascular disease have focused on the abnormal regulation of cell growth in response to growth factors, it has been recently postulated that the regulation of cell death by apoptosis may be another determinant of vascular structure and lesion formation [23]. It has become increasingly clear that the process of cell death by apoptosis is a relatively ubiquitous phenomenon observed in a variety of cell types, including endothelial cells. Disruption or dysfunction of endothelial cells induced by AGEs has been hypothesized to play a pivotal role in the progression and/or development of vascular disease in diabetes [5, 24, 25]. In this study, we demonstrated that AGEs induced endothelial cell death through the induction of apoptosis. However, little is known about the molecular mechanisms on AGEs induced endothelial cell death.

The present study demonstrated that elevated AGEs induces apoptosis of HUVEC and up-regulates lactadherin

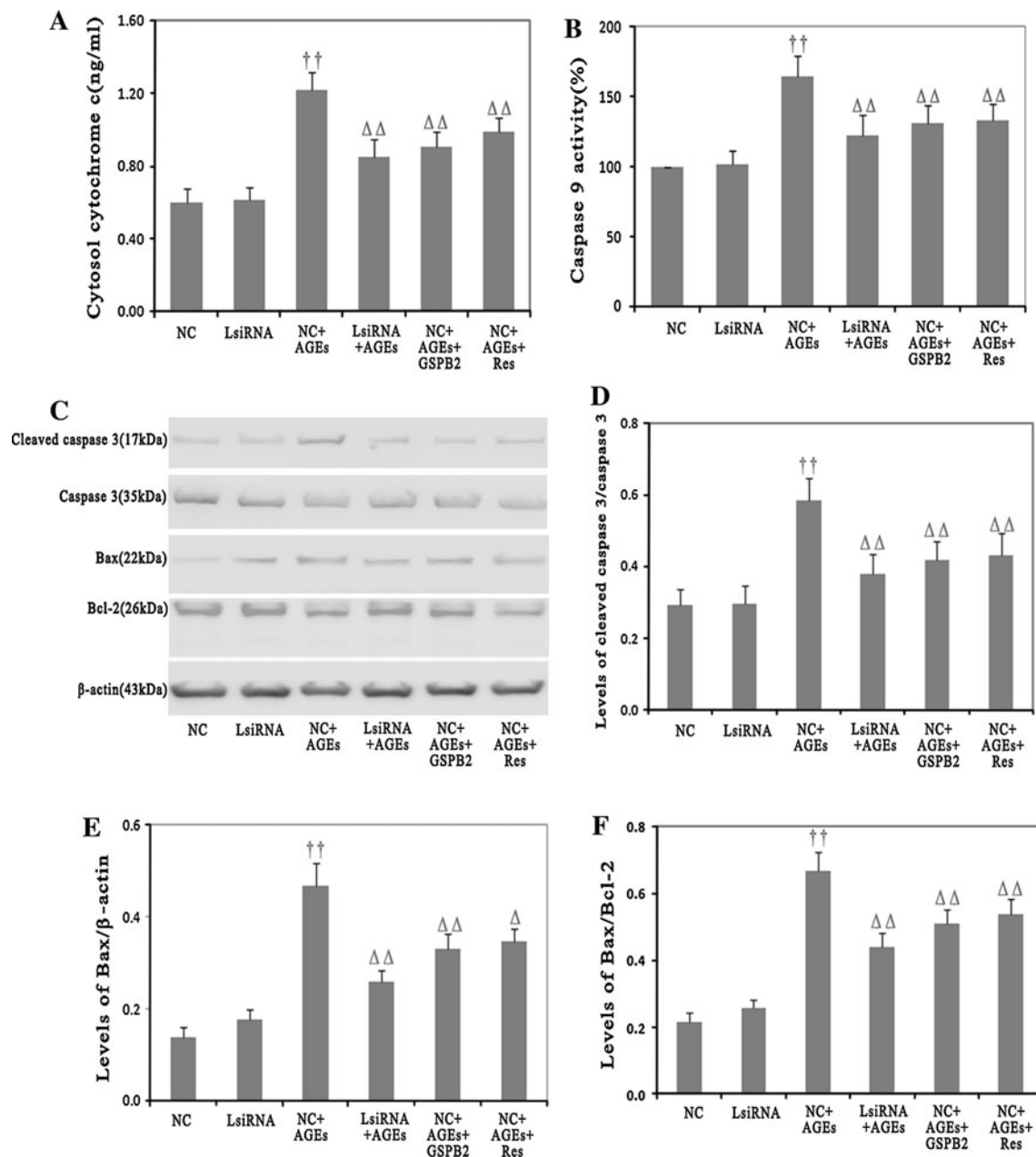


Fig. 5 Effects of lactadherin on cytosol cytochrome *c* concentration, caspase-9 activity, cleaved caspase-3 and Bax/Bcl-2 ratio in HUVEC treated by AGEs. **a–c** Effects of lactadherin siRNA, GSPB2 and resveratrol on cytosol cytochrome *c* concentration, caspase-9 activity, cleaved caspase-3 and Bax/Bcl-2 ratio in HUVEC treated by AGEs. **g–i** Effects of lactadherin overexpression, GSPB2 and resveratrol on cytosol cytochrome *c* concentration, caspase-9 activity, cleaved

caspase-3 and Bax/Bcl-2 ratio in HUVEC treated by AGEs. **d–f, j–l** Data were expressed as the expression ratio of cleaved caspase 3/caspase 3, Bax/β-actin, Bax/Bcl-2. Each data point represents mean ± SD of five independent experiments. [†]*P* < 0.05, ^{††}*P* < 0.01 compared with NC group; ^Δ*P* < 0.05, ^{ΔΔ}*P* < 0.01 compared with NC + AGEs group. **P* < 0.05, ***P* < 0.01 compared with LV-C group; [#]*P* < 0.05, ^{##}*P* < 0.01 compared with LV group

in HUVEC. Several pathways have been demonstrated in mediating cell apoptosis, such as death receptors, mitochondria and endoplasmic reticulum pathway. Our study shows that EC apoptosis induced by AGEs is at least in part through the mitochondrial pathway. The lactadherin siRNA inhibited HUVEC apoptosis from AGEs exposure by inhibition of Bax/Bcl-2 ratio and cleaved caspase-3. These

results suggest that upregulation of lactadherin plays a critical role in apoptosis of endothelial cells induced by AGEs and inhibition of lactadherin might prevent endothelial dysfunction in the early stage of diabetes.

Previous studies have shown that lactadherin is abundantly expressed in atherosclerotic plaques. Our results showed for the first time to the best of our knowledge that

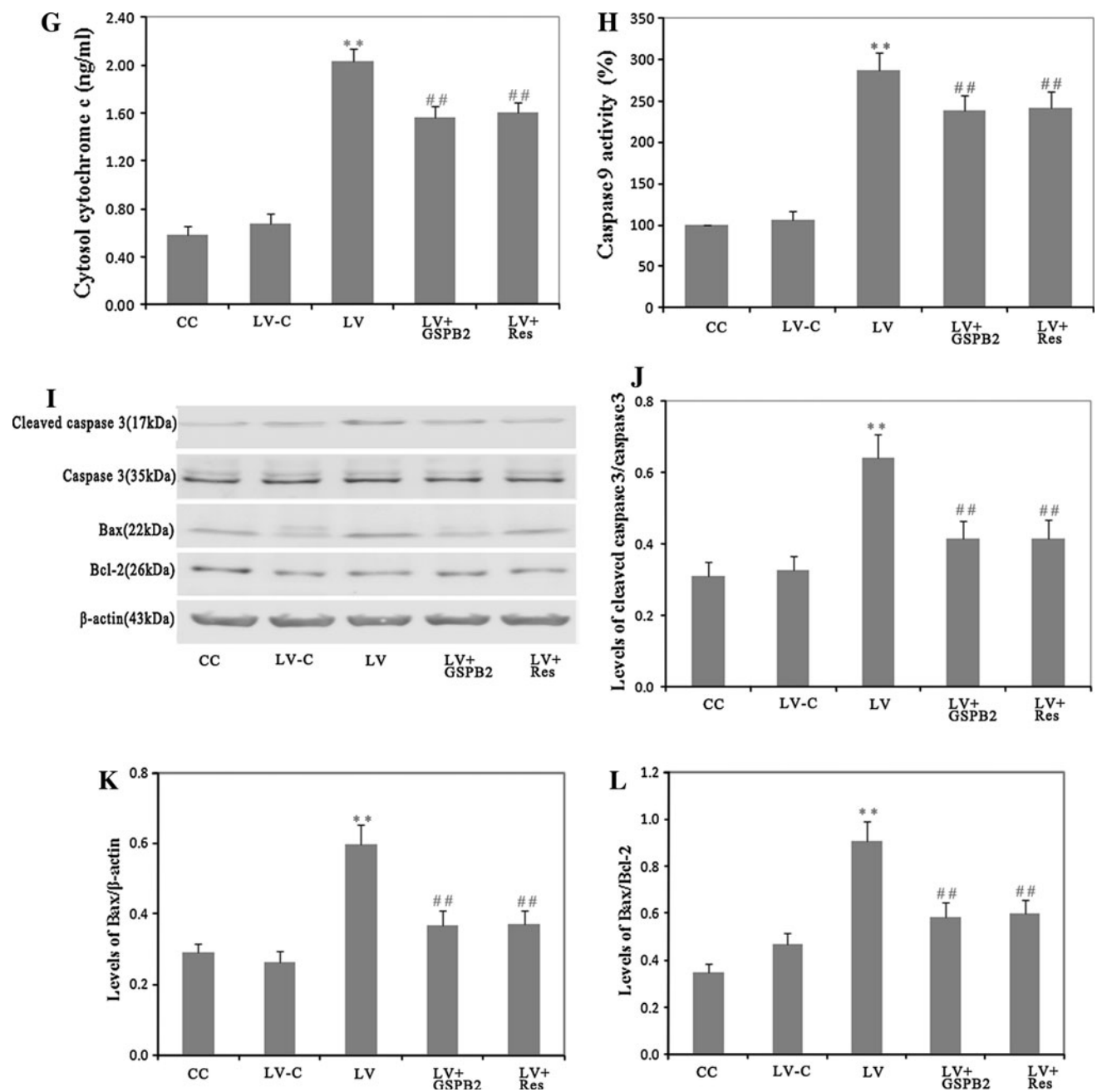


Fig. 5 continued

lactadherin plays a critical role in apoptosis of endothelial cells induced by AGEs. Some studies had reported that MFG-E8 helped phagocytosis of apoptotic cells by thioglycollate-elicited peritoneal macrophages and by tangible body macrophages [26, 27]. It has recently been reported that glucose of high concentration upregulated lactadherin in the adiposomes. ROS production was increased when cultured in the high-glucose medium, and ROS scavenger N-acetyl cysteine decreased lactadherin in the adiposomes. Expression of lactadherin was up-regulated in epididymal adipose tissues of diet-induced obese C57BL/6 mice as

well as genetically obese *ob/ob* and *db/db* mice [28]. Endothelial cell apoptosis is critically involved in vascular remodeling in normal development and in pathological neovascularization [9, 29].

It is well known that the mitochondria play a pivotal role in cell death and cell survival, and that mitochondrial dysfunction constitutes a critical event in the apoptotic process [30]. Here, we demonstrated that the overexpression lactadherin may be attributable to an inappropriate increase in the cytosol cytochrome *c* concentration, caspase-9 activity, ratio of bax to bcl-2 and cleaved caspase-3.

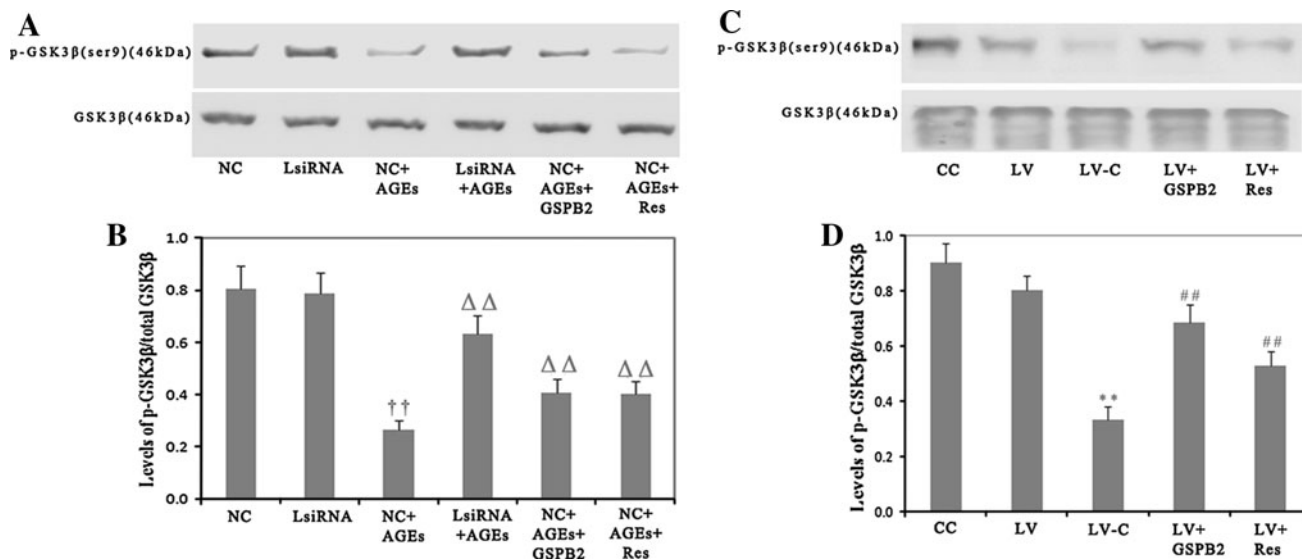


Fig. 6 Effects of lactadherin on phospho-GSK3 β in HUVEC treated by AGEs. **a** Effects of lactadherin siRNA, GSPB2 and resveratrol on phospho-GSK3 β in HUVEC treated by AGEs. **c** Effects of lactadherin overexpression, GSPB2 and resveratrol on phospho-GSK3 β in HUVEC treated by AGEs. **b, d** Data were expressed as the expression

ratio of phospho-GSK3 β /total-GSK3 β and given as mean \pm SD from five independent experiments. $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$ compared with NC group; $^{\Delta}P < 0.05$, $^{\Delta\Delta}P < 0.01$ compared with NC + AGEs group. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with LV-C group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared with LV group

Cytochrome *c* release is a key step in activation of caspase cascade for initiation of apoptosis. This leads to caspase-9 oligomerization and activation and to caspase-3 cleavage. Caspase-3 is one of the key executioners of apoptosis, and its activation is a good marker for apoptosis. Moreover, apoptotic death signals converge on the mitochondria through the activation of pro-apoptotic members of the Bcl-2 family, such as Bax, while Bcl-2 serves as an anti-apoptotic protein. The apoptosis-related proteins Bax and Bcl-2 and caspases appear to be important for the progression of apoptotic cell death in mitochondrial pathway.

The homo and heterodimerization of Bcl-2 family proteins is important for transduction and integration of apoptotic signals and control of the permeability of mitochondria and endoplasmic reticulum membranes. Bcl-2 interacts with activated Bax during apoptosis in an effective manner to neutralize the proapoptotic activity of Bax, auto-activation of the apoptosis protein Bax increases mitochondrial membrane permeability and is inhibited by Bcl-2. The formation of a Bax/Bcl-2 heterodimer can inhibit Bcl-2 homodimerization, once Bax/Bcl-2 ratio elevated can trigger apoptotic cell death. The elevated Bax/Bcl-2 ratio

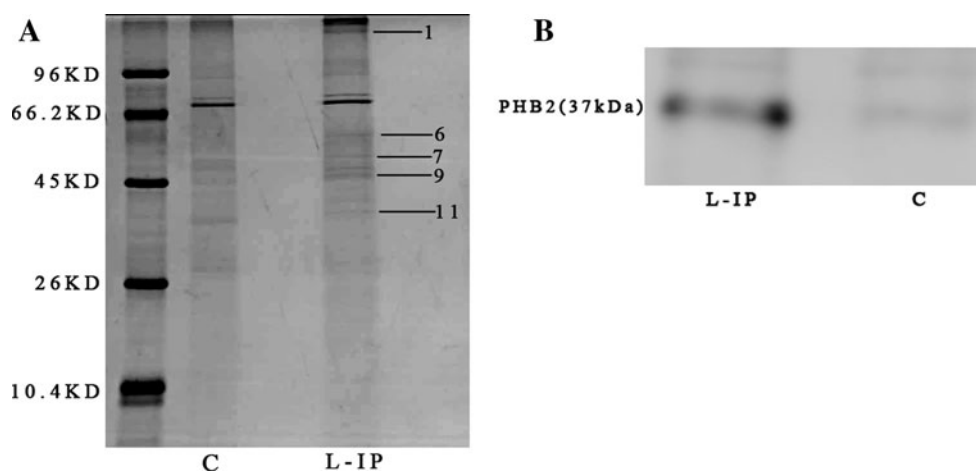


Fig. 7 Interaction proteins of lactadherin. **a** SDS-PAGE map of the lactadherin protein complex from the overexpressing lactadherin cell extracts. **b** Confirmation of novel, high confidence lactadherin-PHB2 interactions by co-immunoprecipitation. *L-IP* immunoprecipitation of

lactadherin groups were incubated overnight at 4°C with primary antibody (anti-lactadherin, 2 μ g/ml). *C* control groups were incubated overnight at 4°C without the antibody. Bands 1, 6, 7, 9, 11 are the different expression of bands between *L-IP* group and *C* group

Table 1 Differentially expressed proteins identified by LTQ-ESI-MS/MS

Band no.	Accession number	Protein name	Mr (Da)	PI	Sequence coverage (%)	Peptide sequence
11	Q99623	Prohibitin-2	33296	9.83	23	K.FNASQLITQR.A K.LLLGAGAVAYGVR.E R.IGGVQQDTILAEGLHFR.I R.IYLTADNLVNLQDESFTFR.G
11	P35232	Prohibitin	29804	5.57	20	R.FDAGELITQR.E R.IFTSIGEDYDER.V R.KLEAAEDIAYQLSR.S R.NITYLPAGQSULLQLPQ.-
11	P07355	Annexin A2	40411	8.53	26	K.ALLYLCCGGDD.- K.GLGTDEDSLIEIICSR.T K.GVDEVTIVNILTNR.S R.TNQELQEINR.V
11	P04406	Glyceraldehyde-3-phosphate dehydrogenase	36053	8.57	12	K.IISNASCCTTNCLAPLAK.V R.GALQNIIPASTGAAK.A
9	P29508	Serpin B3	44565	6.35	9	K.VLHFDQVTENTTGK.A
7	Q05639	Elongation factor 1-alpha 2	50470	9.11	5	K.IGGIGTVPVGR.V
6	P05141	ADP/ATP translocase 2	32895	9.76	17	R.YFAGNLASGGAAGATSLCFVYPLDFAR.T
1	P14618	Isoform M1 of Pyruvate kinase isozymes M1/M2	65930	7.96	7	R.LDIDSPITAR.N R.NTGIICITGPASR.S
1	Q9NZM1	Myoferlin	179551	5.94	12	K.ANVTVLDTQIR.K K.NLVDPFVEVSFAGK.M

Band no: band numbers are unique numbers assigned to different expression of bands between L-IP group and C group. Theoretical Mr (Da)/pI are based on the amino acid sequence of the identified proteins. L-IP: immunoprecipitation of lactadherin groups were incubated overnight at 4°C with primary antibody (anti-lactadherin, 2 µg/ml). C: control groups were incubated overnight at 4°C without the antibody

could activate cleavage of caspase-3 and then trigger the apoptosis of endothelial cells [31, 32]. Glycogen synthase kinase-3 β (GSK3 β) is a constitutively active enzyme. GSK3 β is an important downstream target of the Akt signaling pathway. Phosphorylation of GSK3 β on the inactivating residue serine-9 by Akt results in GSK3 β inactivation [33]. Pro-apoptotic functions of GSK3 β have been shown in apoptosis by withdrawal of growth factors, DNA damage, mitochondrial toxins, oxidant stress and other conditions that trigger apoptosis via the mitochondrial pathway [34–37]. In our study, we showed that AGEs increases GSK3 β activity in HUVEC through a lactadherin dependent manner.

Moreover, GSPB2 and resveratrol significantly attenuated the AGEs-mediated cell apoptosis by inhibition of lactadherin. Antioxidant therapy is therefore considered to be a promising strategy to prevent the glycosylated damage to the vascular endothelium in the early stage of diabetic vascular complications [38–40]. Our previous studies showed that GSPE possesses potent anti-nonglycosylated glycation and anti-inflammation effects, inhibiting expression of high level vascular cell adhesion molecule 1 induced by AGEs in endothelial cells [20, 41, 42].

Our study also demonstrates that there are nine interaction proteins of lactadherin, such as prohibitin-2, prohibitin, etc. Prohibitin2, also designated as repressor of estrogen receptor activity is a 37 kDa protein exhibiting high homology to the putative tumor suppressor protein prohibitin [43]. PHB1 and 2 are evolutionarily conserved membrane proteins that are localized in the mitochondrial membrane. PHB2 are structurally related and assemble into large, ring-like complexes. Recent studies suggested that PHBs have been showed to be involved not only in the regulation of transcription but also cellular senescence, apoptosis, and mitochondrial respiratory activity and maintenance of mitochondrial morphology [44–46]. Prohibitins bind to several mitochondrial proteins such as voltage-dependent anion channel 2, adenine nucleotide translocator 2, and anti-apoptotic factor HS-associated protein X-1, and regulate their functions [47]. The results reconfirmed that the mitochondria apoptosis pathway played a pivotal role in the cell apoptosis by lactadherin.

Taken together, our study suggests that up-regulation of lactadherin plays a critical role in endothelial cells apoptosis induced by AGEs. The down-regulation of lactadherin inhibits cell apoptosis from AGEs exposure. Moreover, GSPB2 or resveratrol might have benefits in the early stage

of diabetic endothelial dysfunction by inhibition of lactadherin. The present study provides the first evidence that lactadherin can modulate cell apoptosis and is a molecular target of GSPB2 and resveratrol.

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Conflict of interest The authors declare that they have no conflict of interest.

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