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FSD-C10 Shows Therapeutic Effects in Suppressing oxidized low-density lipoprotein (ox-LDL)-Induced Human Brain Microvascular Endothelial Cells Apoptosis via Rho-Associated Coiled-Coil Kinase (ROCK)/Mitogen-Activated Protein Kinase (MAPK) Signaling

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
Funds Collection G

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Background: ox-LDL-induced injury of brain microvascular endothelial cells (BMECs) is strongly associated with cerebral vascular diseases such as cerebral arterial atherosclerosis. ROCK inhibitor was proved to be anti-apoptotic and has been used in treating cerebral vascular diseases. Research on the neuroprotective effects of a novel ROCK inhibitor, FSD-C10, is still limited. The present study investigated the anti-apoptotic effect and underlying molecular mechanism of FSD-C10 in ox-LDL-mediated apoptosis of BMECs.

Material/Methods: ox-LDL and/or FSD-C10 were used to incubate immortalized human BMECs. MTT assay was used to assess cell viability. Cell apoptosis was evaluated by TUNEL assay. A colorimetric method was used to assess ROCK activity. Western blot analysis was used to examine the expression and phosphorylation levels of proteins.

Results: ox-LDL incubation reduced the viability of BMECs by inducing cell apoptosis in a concentration-dependent manner. ROCK activity was also elevated by ox-LDL incubation in BMECs in a concentration-dependent manner. Expression level of Bcl2 was reduced while expression levels of Bax and active caspase3 were increased by ox-LDL treatment in a concentration-dependent manner. ox-LDL also increased the phosphorylation levels of p38, JNK, and ERK1/2 in a concentration-dependent manner. FSD-C10 treatment increased the cell viability by reducing apoptosis of BMECs exposed to ox-LDL. Moreover, FSD-C10 was found to suppress the phosphorylation levels of p38, JNK, and ERK1/2 and the expression levels of Bax and active caspase3 in ox-LDL treated BMECs.

Conclusions: FSD-C10 increases cell viability in ox-LDL-treated BMECs by reducing cell apoptosis. ROCK/MAPKs-mediated apoptosis appears to be the underlying molecular mechanism.

MeSH Keywords: Apoptosis • Receptors, Oxidized LDL • rho-Associated Kinases

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Background

Patients with metabolic disorders such as hyperglycemia and hyperlipidemia exhibit a higher risk of cerebrovascular and cardiovascular diseases [1]. It has been recognized that homeostasis of the blood-brain barrier (BBB) is essential for maintenance of brain functions. Brain microvascular endothelial cells (BMECs) are one of the major components of the BBB [2]. It is accepted that dysfunction and damage of the microvascular endothelium are pathological features of several cerebrovascular diseases at the early stage [3]. Accumulating evidence indicates that by inducing endothelial injuries, oxidized low-density lipoprotein (ox-LDL) is both the trigger and the hallmark of atherosclerosis [4]. ox-LDL is taken up by macrophages and arterial endothelial cells and facilitates formation of foam cells and endothelial cell damage. Previous studies reported that ox-LDL suppressed endothelial cell proliferation and caused DNA damage [5]. Earlier investigations found that ox-LDL induced apoptosis of endothelial cells by activating a caspase cascade [6]. However, the molecular mechanisms are still not clearly delineated and the effects of ox-LDL on BMECs has received little research attention.

RhoA is a small GTPase which participates in the regulation of contraction of vascular smooth muscle cells [7]. The RhoA-mediated kinases were reported to be involved in cardiovascular diseases such as heart failure, hypertension, and atherosclerosis [8–10]. Rho kinase (ROCK) is a family of serine/threonine kinases that participate in several vital cellular biological processes, including proliferation, migration, differentiation, and cell death [11]. Increasing evidence from both *in vivo* and *in vitro* studies proved that ROCK is closely associated with regulation of apoptosis [12]. Particularly, previous investigations suggested that several MAPKs – ERK1/2, JNK, and p38 – are the downstream molecular targets for Rho/ROCK signaling [13, 14]. This mechanism was thought to be crosstalk between Rho/ROCK and MAPKs pathways. It has been well established that the activation of MAPKs pathways can lead to cell apoptosis via a mitochondria-dependent mechanism. Thus, agents inhibiting Rho/ROCK activity are anti-apoptotic and could ameliorate ox-LDL-induced endothelial damage.

Indeed, ROCK inhibition was proved to be a protective therapeutic strategy against inflammation and apoptosis in several vascular diseases such as reperfusion injury, pulmonary arterial hypertension, and cerebral ischemia [15]. Fasudil is a ROCK inhibitor which has been widely used since 1995 in clinical treatment of cerebral vasospasm and symptoms of cerebral ischemia after subarachnoid hemorrhage [16]. Fasudil was also proved to be neuroprotective in an experimental cerebral ischemic model by reducing apoptosis [17]. However, several features, including low oral bioavailability, short-course treatment, and cell toxicity, limited the clinical application of Fasudil.

A novel ROCK inhibitor which is also one of the derivatives of Fasudil – FSD-C10 – was developed in 2015 [18]. This novel agent showed low cell toxicity and longer- course treatment and exhibited therapeutic potential in treating central nervous system (CNS) inflammation [19]. In the present study, apoptosis was induced by ox-LDL exposure in cultured BMECs. The novel Fasudil derivative FSD-C10 was used to treat the cells. We investigated the anti-apoptotic effect of FSD-C10 as well as the possible mechanisms involved. We believe that results from this study will not only add to the understanding of ox-LDL-induced cerebral endothelial injury, but also provide a theoretical basis for clinical application of FSD-C10 in the future.

Material and Methods

Immortalized human BMECs were provided by the Cell Bank of the Chinese Academy of Sciences Committee on Type Culture Collections (China). This cell line has been used and reported in several previous investigations [20]. The cells were maintained in M199 (Sigma-Aldrich, USA) medium supplemented with 10% fetal bovine serum (FBS, Gibco, USA) and 1% penicillin streptomycin mix (Invitrogen) at 37°C in a humidified incubator providing atmosphere containing 95% fresh air and 5% CO_2. Cells were exposed to ox-LDL (Sigma-Aldrich, USA) at dosages of 0, 10, 20, and 50 μg/ml for 24 h. Several cells were co-treated with FSD-C10 at dosages of 0, 15, 30, and 60 μg/ml for 24 h.

Cell viability determination

The cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenylterazolium bromide (MTT) assay. Briefly, cultured cells were seeded into a 96-well culture plate at a cell density of 5×10^3/well. Cells were incubated with ox-LDL and/or FSD-C10 as described in the above paragraph. Then, 20μl MTT (5 mg/ml, Sigma-Aldrich, USA) was added into each well. After incubation at 37°C for 4 h, 100 μL dimethyl sulfoxide (DMSO, Sigma-Aldrich, USA) was added into each well to dissolve the formazan crystals. A plate reader (Bio-Rad, USA) was used to determine the absorbance at 490 nm (A490). The formula (A490 treatment/A490 control)×100% was used to calculate cell viability. Six independent assays were carried out.

Cell apoptosis evaluation

The *in situ* apoptosis detection was carried out by terminal transferase UTP nick-end labeling (TUNEL) assay. Cultured BMECs were treated with proteinase K (20 μmol/l, Sigma) and then fixed with iced acetone. A TUNEL assay kit (Roche, Germany) was used to detect the apoptosis per the instructions provided by the manufacturer. The cell nuclei were stained using 4',6-diamidino-2-phenylindole (DAPI, Invitrogen, USA).

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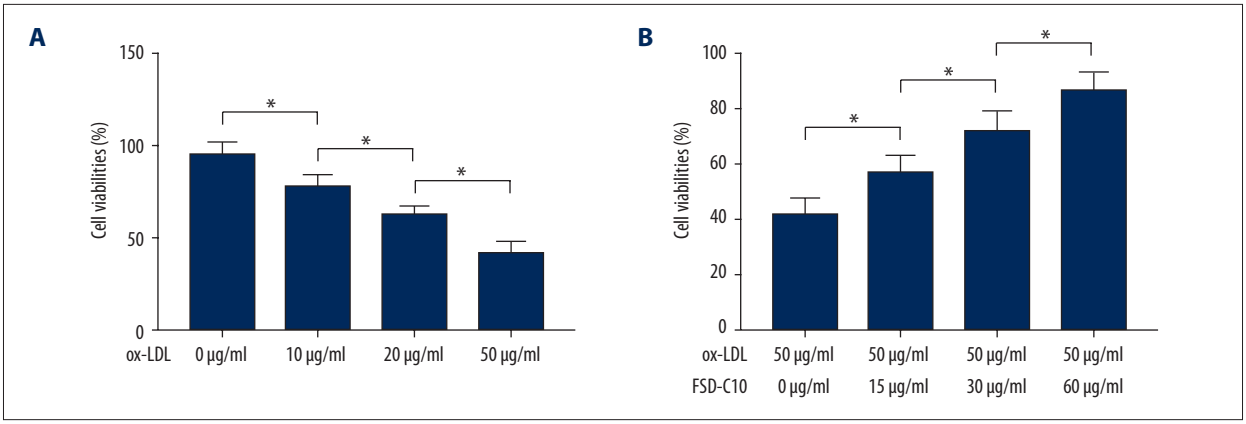

Figure 1. (**A**) Columns indicate the cell viabilities of cultured BMECs treated with ox-LDL at 0, 10, 20, and 50 μg/ml. (**B**) Columns indicate the cell viabilities of cultured BMECs treated with ox-LDL at 50 μg/ml and FSD-C10 at 0, 15, 30, and 60 μg/ml.

An inverted fluorescence microscope was used to observe the cells. Six independent experiments were carried out. Five images were quantified in each independent replicate.

Western blot analysis

Cultured BMECs were subjected to the Cell Lysis Buffer System (Santa Cruz, USA) supplemented with phenylmethylsulfonyl fluoride (PMSF, Santa Cruz, USA). A Cytoplasmic Extraction Kit (Beyotime, China) was used to extract the protein according to the manufacturer's instructions. A BCA kit (Pierce, USA) was used to determine the protein concentrations of the samples, which were then subjected to the vertical SDS-PAGE. The separated proteins were then electronically transferred to PVDF membranes. Primary antibodies against Bax (Abcam, USA), Bcl-2 (Abcam, USA), activated caspase3 (Abcam, USA), p38 (Abcam, USA), phosphorylated p38 (p-p38, Abcam, USA), JNK (Abcam, USA), phosphorylated JNK (p-JNK, Abcam, USA), ERK1/2 (Abcam, USA), phosphorylated ERK1/2 (p-ERK1/2, Abcam, USA), and GAPDH (Abcam, USA) were used to incubate the membranes at 4°C for 8 h. The membranes were washed with TBST and then incubated with HRP-conjugated secondary antibodies. The membranes were developed by using an ECL kit (Pierce, USA). The densities of the immunoblots were determined and analyzed by Gene Genius (Syngene, England) and Image J (VER1.28, NIH, USA). Six independent experiments were carried out for immunoblots density quantification.

ROCK activity assay

The ROCK activity was measured using a ROCK assay kit (CycLex, USA) with cell extracts. Cells were homogenized on ice with extraction buffer (50 mmol/l Tris-HCl, 0.1% Triton X-100, 1 mmol/l EGTA, 1 mmol/l EDTA, 10 mmol/l NaF, 10 mmol/l β-mercaptoethanol, and 0.5 mmol/l PMSF). The supernatant was collected after centrifugation at 12 000 g for 30 min. ROCK activity in supernatant was detected with the ROCK activity kit following the protocol provided by the manufacturer. OD at 450 nm (A450) detected by a plate reader (Bio-Rad, USA) was used to express the results. Six independent assays were carried out.

Statistical analysis

Data collected are presented as mean ±SD and were analyzed with SPSS software (ver16.0, SPSS, USA). One-way ANOVA was used to analyze differences between groups. NSK tests were carried out as post hoc tests. The differences were considered statistically significant when $p<0.05$. Six independent experiments were carried out.

Results

FSD-C10 treatment increased cell viabilities of ox-LDL-incubated BMECs

The results are demonstrated in Figure 1. The cell viability was significantly reduced in ox-LDL-incubated BMECs in a concentration-dependent manner. However, FSD-C10 treatment increased cell viability in ox-LDL-incubated BMECs in a concentration-dependent manner.

FSD-C10 treatment inhibited ox-LDL-induced cell apoptosis in BMECs

The results are shown in Figure 2. ox-LDL incubation significantly increased the apoptotic rate of BMECs in a concentration-dependent manner. The apoptotic rate of BMECs was dramatically reduced by FSD-C10 treatment in a concentration-dependent manner.

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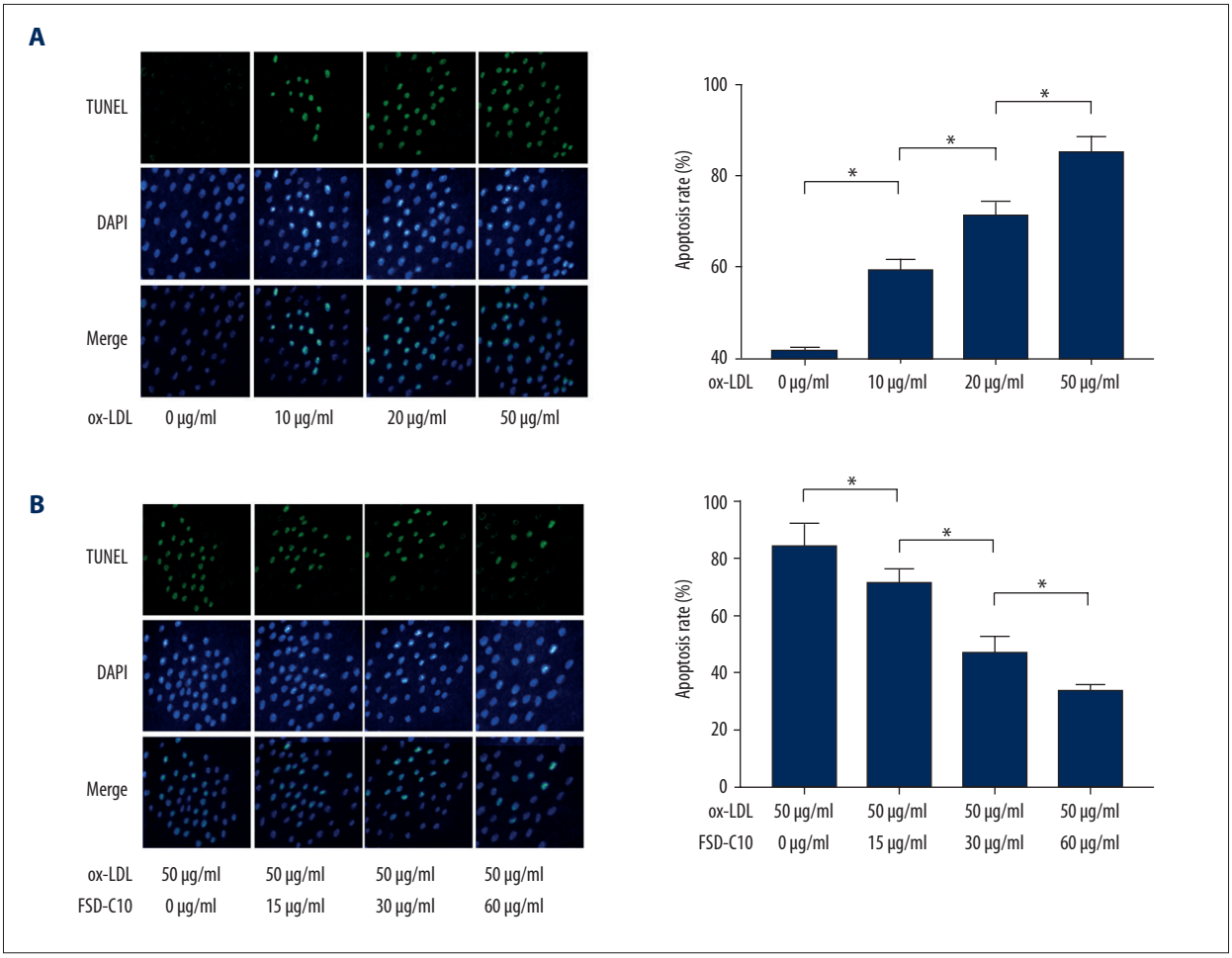

Figure 2. (**A**) **Left panel** demonstrates the captured fluorescent images of TUNEL and DAPI and their merged images. Columns on the **right panel** indicate the apoptosis rate of BMECs treated with ox-LDL at 0, 10, 20, and 50 μg/ml. (**B**) **Left panel** demonstrates the captured fluorescent images of TUNEL and DAPI and their merged images. Columns on the **right panel** indicate the apoptosis rates of BMECs treated with ox-LDL at 50 μg/ml and FSD-C10 at 0, 15, 30, and 60 μg/ml.

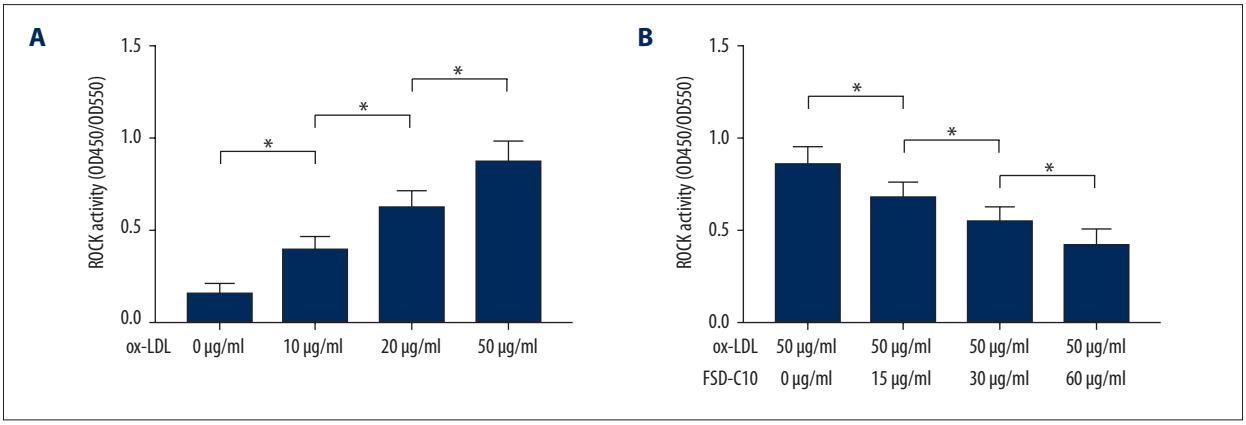

Figure 3. (**A**) Columns indicate the detected ROCK activities in cultured BMECs treated with ox-LDL at 0, 10, 20, and 50 μg/ml. (**B**) Columns indicate the ROCK activities in cultured BMECs treated with ox-LDL at 50 μg/ml and FSD-C10 at 0, 15, 30, and 60 μg/ml.

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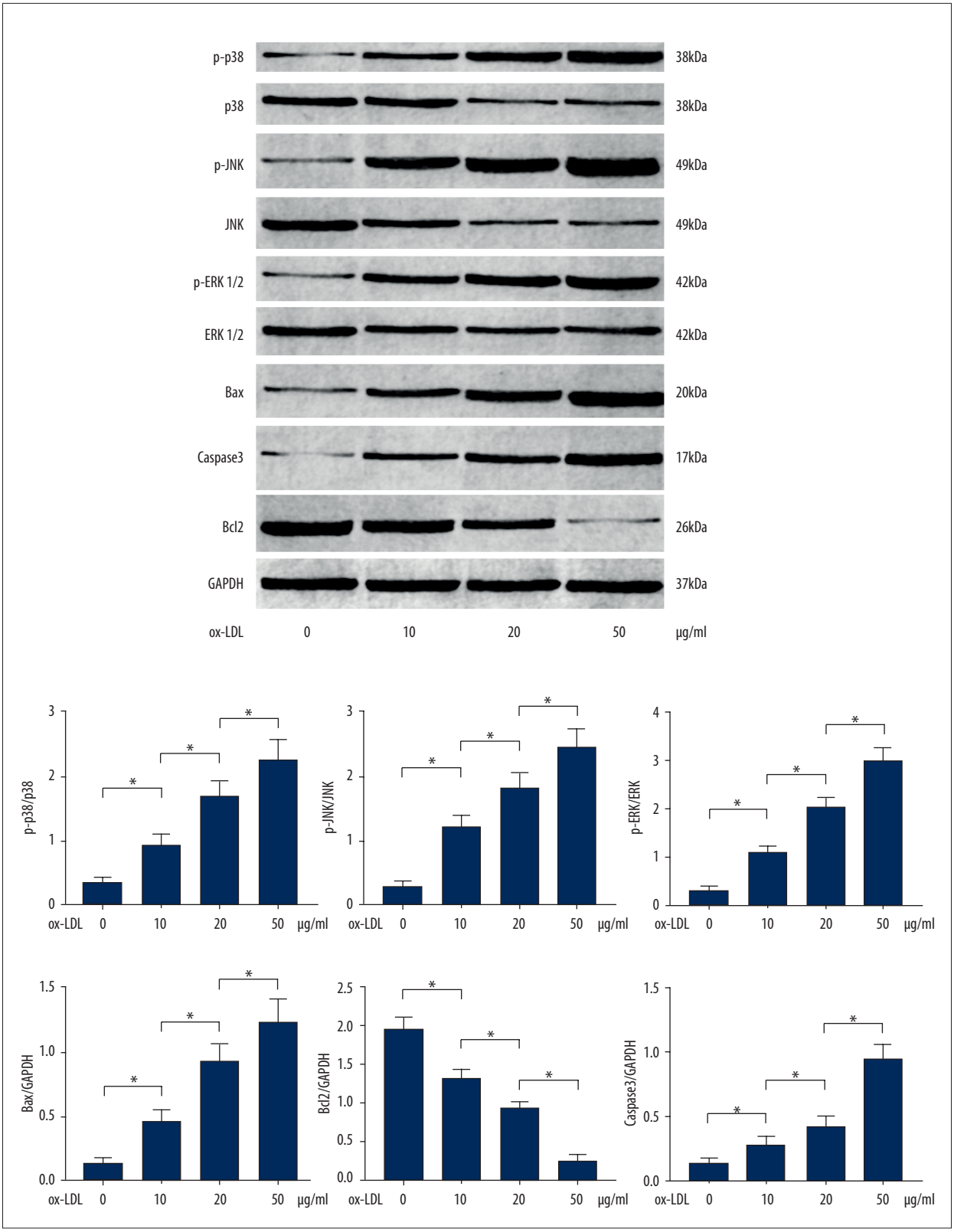

Figure 4A. A Left panel demonstrates the immunoblots of p-p38, p38, p-JNK, JNK, p-ERK1/2, ERK1/2, Bax, caspase3, Bcl2, and GAPDH. Bar graphs on the **right panel** indicate the ratio of p-p38/p38, p-JNK/JNK, p-ERK/ERK, Bax/GAPDH, Bcl2/GAPDH, and caspase3/GAPDH in cultured BMECs treated with ox-LDL at 0, 10, 20, and 50 μg/ml.

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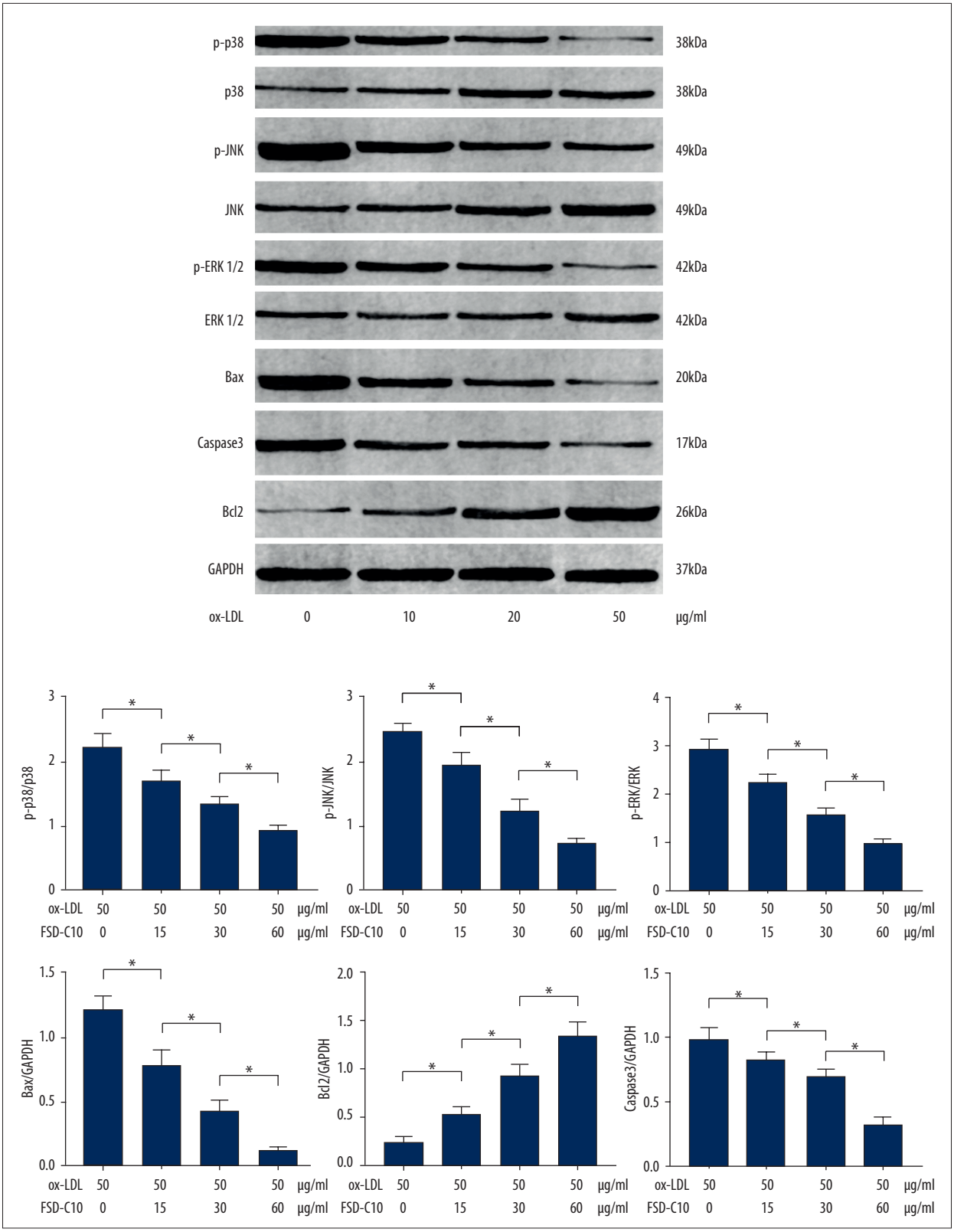

Figure 4B. **Left panel** demonstrates the immunoblots of p-p38, p38, p-JNK, JNK, p-ERK1/2, ERK1/2, Bax, caspase3, Bcl2, and GAPDH. Bar graphs on the **right panel** indicate the ratio of p-p38/p38, p-JNK/JNK, p-ERK/ERK, Bax/GAPDH, Bcl2/GAPDH, and caspase3/GAPDH in cultured BMECs treated with ox-LDL at 50 μg/ml and FSD-C10 at 0, 15, 30, and 60 μg/ml.

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FSD-C10 suppressed the increased ox-LDL-induced ROCK activity in BMECs

The results of ROCK activity assay are shown in Figure 3. The ox-LDL incubation significantly increased ROCK activity in BMECs in a concentration-dependent manner. However, the FSD-C10 treatment dramatically reduced ROCK activity in BMECs exposed to ox-LDL.

FSD-C10 inhibited activation of MAPK apoptotic signaling in ox-LDL-incubated BMECs

The results are shown in Figure 4. The phosphorylation levels of the MAPKs p38, ERK1/2, and JNK were increased in ox-LDL-treated BMECs in a concentration-dependent manner. Moreover, the expression level of Bcl2 was decreased while the expression levels of Bax and active caspase3 were increased in ox-LDL-treated BMECs in a concentration-dependent manner. However, the FSD-C10 treatment dramatically reduced the phosphorylation levels of MAPKs. The elevated expression levels of Bax and active capsase3 and the reduced expression levels of Bcl-2 were reversed with FSD-C10 administration in BMECs exposed to ox-LDL.

Discussion

ox-LDL is known as one of the major proatherogenic lipoproteins that participate in the formation and development of atherosclerosis [21]. Elevated ox-LDL level is highly associated with endothelial dysfunction and damage, leading to abnormity of endothelial biomechanical properties such as integrity, permeability, and continuance [22]. Although the underlying molecular mechanisms are very complicated and still unclear, accumulating evidence indicates that apoptosis of microcirculatory endothelial cells is critical to ox-LDL-associated endothelial injury in multiple organs. In the present study, ox-LDL was used to incubate cultured BMECs. The ox-LDL treatment reduced the viability of BMECs in a concentration-dependent manner. Suggested by the TUNEL assay results, we found that ox-LDL treatment induced apoptosis of BMECs in a concentration-dependent manner. We also found that BMEC apoptosis was correlated with increased ROCK activity, indicating possible involvement of the ROCK signaling pathway.

ROCK has been recognized as one of the regulators of apoptosis [23]. Results from several previous investigations suggested that Rho/ROCK activation is required for endothelial cell apoptosis induced by certain agents or pathogens [24]. Several pathways were identified as the downstream targets. Particularly, the MAPKs p38, JNK, and ERK1/2 were found to be activated after activation of ROCK [13,25,26]. Various harmful stimuli can activate these MAPKs by facilitating their phosphorylation [27]. It has been accepted that activation of these MAPKs is associated with cell apoptosis [28]. Several direct or indirect mechanisms are involved in this process. It is believed that MAPKs can induce apoptosis by facilitating caspase cleavage. Moreover, cell apoptosis can also be triggered through several downstream pathways, such as inflammatory pathways [29]. In the present study, we found that the phosphorylation levels of p38, JNK, and ERK1/2 were significantly increased after ox-LDL treatment. As a result, the expression level of anti-apoptotic protein bcl-2 was decreased while the expression levels of pro-apoptotic protein Bax and active caspase3 were significantly increased in BMECs exposed to ox-LDL. Thus, it is reasonable to propose that inhibiting ROCK activity could be an optimized strategy to reduce ox-LDL-induced endothelial damage.

Indeed, accumulating evidence indicates the therapeutic effects of ROCK inhibitors on vascular disease by suppressing endothelial inflammation and apoptosis [30]. Fasudil, also referred to as [hexahydro-1-(5-isoquinolinylsulfonyl)-1H-1,4-diazepine monohydrochloride (HA 1077)], is the most frequently used ROCK inhibitor in clinical treatment since it was first used in 1995 in Japan [31]. Previous investigations have suggested the neuroprotective effects of Fasudil both *in vivo* and i*n vitro* [32]. Notably, Fasudil produces its neuroprotective effects by preventing apoptosis [33]. There are several limitations to use of Fasudil in clinical practice due to its actions of bioavailability, cell toxicity, and blood pressure fluctuation. Recently, a novel ROCK inhibitor derived from Fasudil, named FSD-C10, has been developed [18]. Information on the curative effects of FSC-C10 has been quite limited. In the present study, our results provide evidence supporting the anti-apoptotic effects of FSD-C10 on the ox-LDL-induced apoptosis of endothelial cells. Rho/ROCK activation was significantly inhibited by FSD-C10 treatment in BMECs exposed to BMECs. The activation of the pro-apoptotic nuclear factor MAPKs, including p38, JNK, and ERK1/2, were dramatically suppressed. As a result, as shown by the altered expressions of Bcl2, Bax, and active caspase3, MAPKs-mediated apoptosis was reduced by FSD-C10 treatment.

Conclusions

We found that FSD-C10 reduced the ox-LDL-induced apoptosis of cultured BMECs. ROCK/MAPKs apoptotic signaling appears to be the underlying molecular mechanism. Although we demonstrated that FSD-C10 was effective, the evidence is still very preliminary and limited. Further *in vivo* and pre-clinical studies warrant are needed to provide a more solid theoretical basis for the potential clinical application of FSD-C10 in cerebral vascular diseases.

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