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Fasudil inhibits ER stress-induced VCAM-1 expression by modulating unfolded protein response in endothelial cells

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ABSTRACT

The process of atherosclerosis is affected by interactions among numerous biological pathways. Accumulating evidence shows that endoplasmic reticulum (ER) stress plays a crucial role in the development of atherosclerosis. Rho-kinase is an effector of small GTP-binding protein Rho, and has been implicated as an atherogenic factor. Previous studies demonstrated that fasudil, a specific Rho-kinase inhibitor, exerts a cardioprotective effect by downregulating ER stress signaling. However, the molecular link between ER stress and Rho-kinase in endothelial cells has not been elucidated. In this study, we investigated the mechanisms by which fasudil regulates endothelial inflammation during ER stress. Tunicamycin, an established ER stress inducer, increased vascular cellular adhesion molecule (VCAM)-1 expression in endothelial cells. Intriguingly, fasudil inhibited VCAM-1 induction. From a mechanistic stand point, fasudil inhibited expression of activating transcription factor (ATF)4 and subsequent C/EBP homologous protein (CHOP) induction by tunicamycin. Furthermore, fasudil attenuated tunicamycin-induced phophorylation of p38MAPK that is crucial for the atherogenic response during ER stress. These findings indicate that Rho-kinase regulates ER stress-mediated VCAM-1 induction by ATF4- and p38MAPK-dependent signaling pathways. Rho-kinase inhibition by fasudil would be an important therapeutic approach against atherosclerosis, in particular, under conditions of ER stress.

1. Introduction

Atherosclerosis is the underlying disorder in the majority of patients with coronary heart disease and a better understanding of the molecular mechanisms that underlie its pathogenesis is crucial for both scientific and therapeutic reasons. Although the development of atherosclerosis is dependent upon a complex interplay between many factors and processes, accumulating evidence demonstrates that endoplasmic reticulum (ER) stress clearly contributes to the pathogenesis of atherosclerosis [1,2].

ER stress occurs in response to various cellular stressors, which results in the accumulation of unfolded proteins in the ER [3]. Various factors including ischemia, hypoxia, oxidative stress and infections, trigger protein unfolding in the ER, leading to unfolded protein responses (UPR) [1]. In the development of atherosclerotic plaque, oxidized lipids may induce ER stress and subsequent UPR. In addition, a previous study also showed that ER stress is markedly increased in endothelial cells exposed to atherosclerosis-prone shear stress [4]. C/EBP homologous protein (CHOP), which induces cellular apoptosis in response to severe ER stress is upregulated in plaque lesions [5]. Indeed, a recent study demonstrated that cuff injury-induced neointimal formation was markedly inhibited in CHOP-deficient mice with suppression of inflammatory gene expression and proliferation-related proteins in aorta and smooth muscle cells [6]. CHOP deficiency also inhibited aortic plaque formation in hypercholesterolemic ApoE-deficient mice with decreased aortic expression of inflammatory factors and oxidative stress markers [7]. CHOP is regulated by activating transcription factor (ATF)4, a transcription factor that requires phosphorylation of eukaryotic initiation factor (eIF)2α for its translocation [1]. In common with CHOP, ATF4 has been shown to play an important role in endothelial inflammation [8,9]. As such, it is recognized that serial activation of eIF2α, ATF4 and CHOP mediates ER stress with generation of UPR. Chemical agents that pharmacologically activate or deactivate UPR components will be potentially useful in treating cardiovascular diseases.

Rho-associated coiled-coil containing protein kinase (Rho-kinase) is an effector of small GTP-binding protein Rho [10]. Rho-kinase has been shown to regulate various cellular functions such as cellular contraction, proliferation, migration and apoptosis. A significant body of evidence shows that increased Rho-kinase activity in vascular cells leads to the development of cardiovascular disease [11,12]. Furthermore, recent studies including ours, have

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demonstrated that Rho-kinase plays a crucial role in diabetes-associated endothelial dysfunction [12,13]. Importantly, a more recent study has demonstrated that fasudil, a specific Rho-kinase inhibitor, is cardioprotective in animal models against ischemia-reperfusion injury by attenuating aspects of ER stress such as CHOP induction [14]. This finding raises the possibility that modulation of ER stress by Rho-kinase inhibition could attenuate atherosclerosis. However, the role played by Rho-kinase in ER stress-mediated atherosclerosis still remains undefined. In this study, we examined the possibility that Rho-kinase may be involved in ER stress-mediated induction of VCAM-1, an established endothelial marker of inflammation [15].

2. Materials and methods

2.1. Materials

Tunicamycin was purchased from Sigma–Aldrich (St. Louis, MO, USA). The Rho-kinase inhibitor fasudil was kindly provided by Asahikasei Pharma (Tokyo, Japan). SP600125 (a JNK inhibitor) was purchased from Sigma–Aldrich (St. Louis, MO, USA). GGTI-286, a selective inhibitor of geranylgeranyl transferase I, U0126, an inhibitor of MEK and SB203580, an inhibitor of p38MAPK, were purchased from Calbiochem-Novabiochem (La Jolla, CA, USA). Anti-myosin phosphatase target subunit 1 (MYPT1) antibody, anti-phospho-p38MAPK (Thr180/Try182) antibody, and anti-p38MAPK antibody were purchased from Cell Signaling (Beverly, MA, USA). Anti-ATF4 antibody and anti-VCAM-1 antibody were purchased from Santa Cruz Biotechnology (Dallas, TX, USA).

2.2. Cell culture

Human umbilical vein endothelial cells (HUVECs) were purchased from Lonza. HUVECs were maintained in Endothelial Cell Growth Medium containing Phenol Red (Cell Applications, Inc., San Diego, CA, USA). For experiments, cells were cultured in growth factor-starved medium and were then exposed to tunicamycin (10 g/ml) for the indicated time. HUVECs of passage 3–5 were used in all experiments. Cells were cultured at 37 °C in humidified air containing 5% CO_2.

2.3. Rho pull-down assay and Rho activation assay

Rho pull-down assay was performed with the Active Rho Pull-Down and Detection kit (Thermo, Rockford, IL, USA) according to the manufacturer's instructions. Briefly, cell lysates were incubated with GST-fusion protein of the Rhotekin-binding domain (RBD) along with glutathione agarose resin to specifically pull down active Rho. Active (GTP-bound) Rho was detected by Western blot analysis. Rho activation was also measured with the Rho G-LISA Activation Assay Kit (Cytoskeleton, Denver, CO, USA) according to the manufacturer's instructions. Briefly, cell lysates were incubated on a Rho-GTP affinity plate. Bound active Rho protein is then detected by incubation with a specific primary antibody followed by antibody detection reagent.

2.4. Rho-kinase (ROCK) activity assay

Rho-kinase activity was measured using the ROCK activity assay kit (Cell Biolabs, CA, USA) according to the manufacturer's instructions. This kit is an enzyme immunoassay developed for detection of the specific phosphorylation of MYPT1 at Thr^{696} by ROCK. A strip well microtiter plate is precoated with recombinant MYPT1. After incubating the substrate wells with cell lysates, the phosphorylated MYPT1 is detected by an anti-phospho-MYPT1 (Thr^{696}) antibody followed by addition of antibody detection reagent.

2.5. RNA isolation and real-time RT-PCR

Cellular total RNA was isolated with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) followed by chloroform–isopropanol extraction and ethanol precipitation, and 1 μg of total RNA was reverse-transcribed using the Prime Script RT reagent kit (Takara Bio, Otsu, Japan). RT-PCR was performed with the Thermal Cycler Dice Real Time System TP800 (Takara Bio, Otsu, Japan) using SYBR Green fluorescence signals. Relative levels of gene expression were normalized to GAPDH.

2.6. Immunoblot analysis

Cells grown in a 60 mm culture dish were washed twice with ice-cold PBS and lysed at 4 °C in RIPA buffer (Sigma–Aldrich, St. Louis, MO, USA) containing protease inhibitor and phosphatase inhibitor (Roche, Mannheim, Germany). Lysates were clarified by centrifugation at 15,000 × *g* for 10 min. Protein content of the supernatants was quantitated using the BCA protein assay reagent kit (Pierce, Rockford, IL, USA). An equal amount of protein was dissolved in sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) sample buffer, electrophoresed on a 4–20% gradient SDS–polyacrylamide gel for 2 h and transferred onto nitrocellulose membrane at 25 V overnight at 4 °C. Membranes were blocked with 5% dry nonfat milk for 1 h at room temperature and blotted with relevant primary antibodies overnight at 4 °C. Primary antibodies were revealed with anti-rabbit or anti-mouse IgG secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) coupled to horseradish peroxidase. Blots were detected with a chemiluminescence reagent system (Amersham Biosciences, Buckinghamshire, UK).

2.7. Statistical analysis

Data are expressed as mean ± SD. Differences between groups were examined for statistical significance using one-way analysis of variance (ANOVA) with Bonferroni adjustment. Two-tailed, unpaired Student's *t* test was used for comparison of parameters when appropriate. Differences were considered significant at $p < 0.05$.

3. Results

3.1. Fasudil, a specific Rho-kinase inhibitor, attenuates tunicamycin-mediated VCAM-1 expression

To examine whether Rho-kinase may be involved in ER stress-mediated VCAM-1 expression, we stimulated HUVECs with 10 μg/ml tunicamycin, a chemical ER stress inducer, in the presence or absence of fasudil (10 μM), a specific Rho-kinase inhibitor. As shown in Fig. 1A and B, tunicamycin induced VCAM-1 mRNA and protein expression. Interestingly, fasudil inhibited VCAM-1 induction at both mRNA and protein levels (Fig. 1A and B), suggesting that Rho-kinase is involved in tunicamycin-mediated VCAM-1 induction.

Furthermore, we examined the effect of fasudil on CHOP mRNA expression. As shown in Fig. 1C, fasudil inhibited tunicamycin-mediated CHOP induction. These data suggest that fasudil exerts its anti-inflammatory effect by modulating UPR in endothelial cells.

3.2. Rho/Rho-kinase signaling is activated by ER stress

We next examined whether Rho/Rho-kinase signaling may be enhanced under ER stress. To determine if active Rho is involved

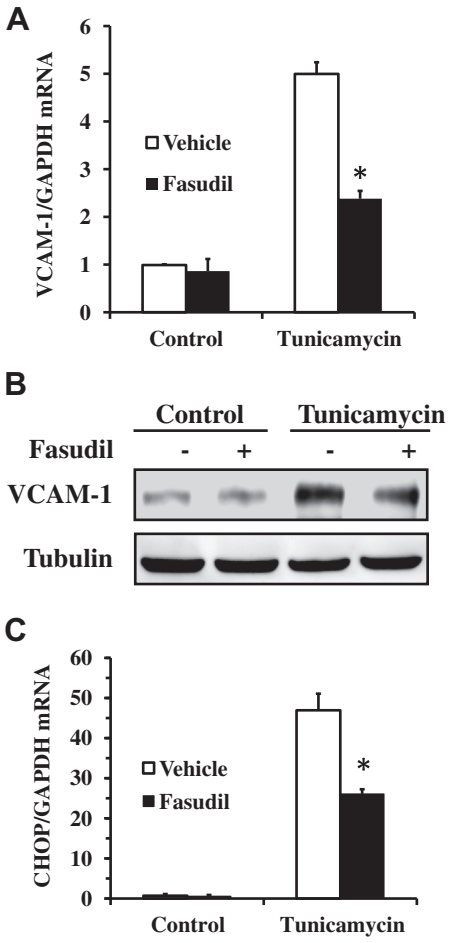

Fig. 1. Effect of fasudil on ER stress-mediated endothelial inflammation. (A) HUVECs were treated with fasudil (10 μM) for 30 min and were then stimulated with tunicamycin (10 μg/ml) for 4 h. RNA was extracted, and VCAM-1 mRNA was analyzed by RT-PCR, with GAPDH mRNA as internal standard. Data are mean ± SD; $n = 3$ per group. $^*p < 0.01$. (B) HUVECs were treated with fasudil (10 μM) for 30 min and were then stimulated with tunicamycin (10 μg/ml) for 8 h. Cells lysates were extracted followed by Western blot analysis. A representative blot of three independent experiments is shown. (C) HUVECs were treated as described above, cells were harvested and RNA was extracted. CHOP mRNA was analyzed by RT-PCR, with GAPDH mRNA as internal standard. Data are mean ± SD; $n = 3$ per group. $^*p < 0.01$.

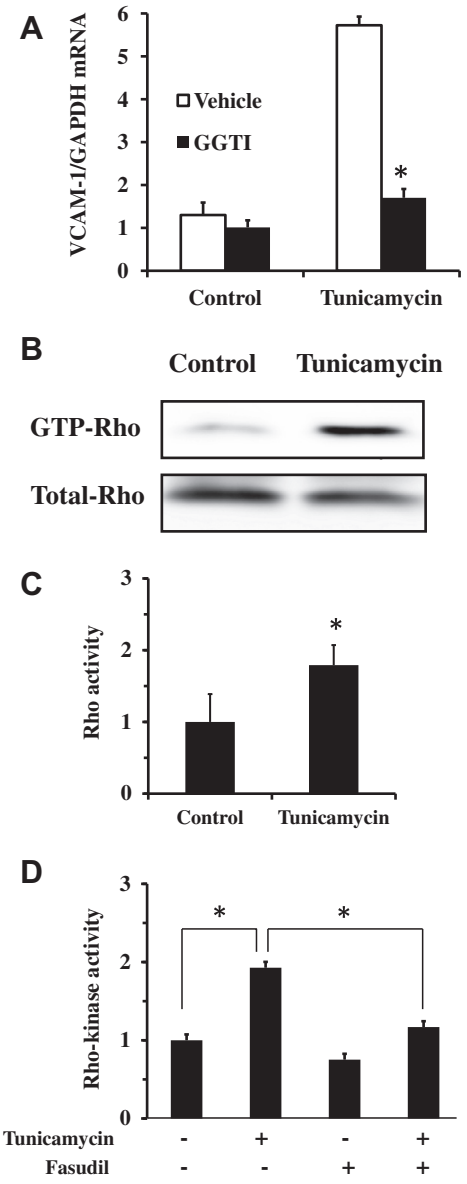

Fig. 2. ER stress induces Rho and Rho-kinase activation in HUVECs. (A) HUVECs were stimulated with tunicamycin (10 μg/ml) in the presence or absence of GGTI-286 (10 μM) for 4 h. VCAM-1 mRNA levels were analyzed by RT-PCR ($n = 3$). Data are mean ± SD; $n = 3$ per group. $^*p < 0.01$. GGTI: GGTI-286. (B) Tunicamycin induces Rho activation. HUVECs were stimulated with tunicamycin (10 μg/ml) for 15 min. Cell lysates were then subjected to pull-down assay as described in Section 2. (C) HUVECs were treasted as described above and cell lysates were subjected to G-LISA ($n = 4$). Data are mean ± SD $^*p < 0.01$. (D) HUVECs were pretreated with fasudil (10 μM) for 30 min, and were then stimulated with tunicamycin (10 μg/ml) for 30 min. Cellular extracts were subjected to the Rho-kinase activity assay as described in Section 2. Data are mean ± SD ($n = 3$). $^*p < 0.01$.

in tunicamycin-mediated VCAM-1 induction, we examined the effect on VCAM-1 induction of GGTI-286, a selective inhibitor of geranylgeranyl transferase-I (GGTase-I) which inhibits the activation of Rho by preventing the prenylation of Rho and its translocation to the membrane. As shown in Fig. 2A, GGTI-286 inhibited VCAM-1 induction, suggesting that active Rho is involved in tunicamycin-mediated VCAM-1 induction. Accordingly, we assessed whether tunicamycin induces Rho activation. The pull-down assay revealed that tunicamycin stimulation increased the amount of GTP-bound Rho (Fig. 2B), which was confirmed by G-LISA (Fig. 2C). Consistent with these observations, Rho-kinase activity (Fig. 2D) was induced by tunicamycin, indicating that tunicamycin induces VCAM-1 expression through Rho and Rho-kinase activation. We confirmed the attenuation of Rho-kinase activity by fasudil (Fig. 2D), suggesting that fasudil-mediated inhibition of VCAM-1 is regulated via downregulation of Rho-kinase activity.

3.3. Fasudil inhibits tunicamycin-induced ER stress signals

Phosphorylation of eIF2α and subsequent ATF4 induction is required for CHOP induction by ER stress. We therefore examined whether Rho-kinase may be involved in this pathway. As shown in Fig. 3A, fasudil inhibited tunicamycin-induced phosphorylation of eIF2α. Consistent with this, fasudil inhibited ATF4 protein expression which was upregulated by tunicamycin (Fig. 3B). These findings indicate that fasudil inhibits UPR, thereby attenuating endothelial inflammation.

3.4. Fasudil inhibits ER stress-mediated p38MAPK activation

It has been shown that MAPK signaling plays an important role in ER stress signaling. To identify the signaling pathway that underlies tunicamycin-mediated VCAM-1 induction, we used

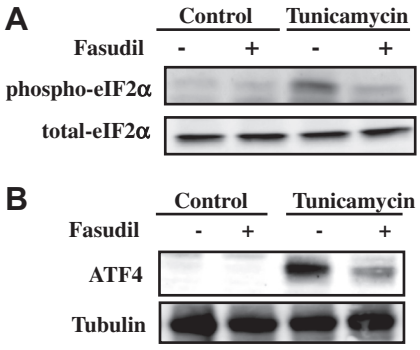

Fig. 3. Fasudil modulates UPR in endothelial cells. (A) Effect of fasudil on tunicamycin-mediated phosphorylation of eIF2α. HUVECs were treated with fasudil (10 μM) for 30 min, then stimulated with tunicamycin (10 μg/ml) for 1 h. Cell lysates were subjected to Western blot analysis. A representative blot of three independent experiments is shown. (B) Effect of fasudil on ATF4 expression. HUVECs were treated with fasudil (10 μM) for 30 min, then stimulated with tunicamycin (10 μg/ml) for 2 h. Cell lysates were subjected to Western blot analysis. A representative blot of three independent experiments is shown.

several MAPK inhibitors and examined the effect of each agent on VCAM-1 induction. As shown in Fig. 4A, neither PD98059 (MEK inhibitor) nor SP600125 (a specific inhibitor of JNK) had any discernible effect on tunicamycin-mediated induction of VCAM-1 mRNA. In contrast, SB203580, a p38MAPK inhibitor, significantly attenuated tunicamycin-mediated VCAM-1 mRNA expression (Fig. 4A), demonstrating that the p38MAPK signaling pathway is involved in tunicamycin-mediated VCAM-1 induction. We therefore sought to examine the effect of fasudil on phosphorylation of p38MAPK. Western blot analysis revealed that fasudil suppressed phosphorylation of p38MAPK by tunicamycin (Fig. 4B). Taken together, these data suggest that fasudil inhibits p38 MAPK-dependent VCAM-1 induction by tunicamycin.

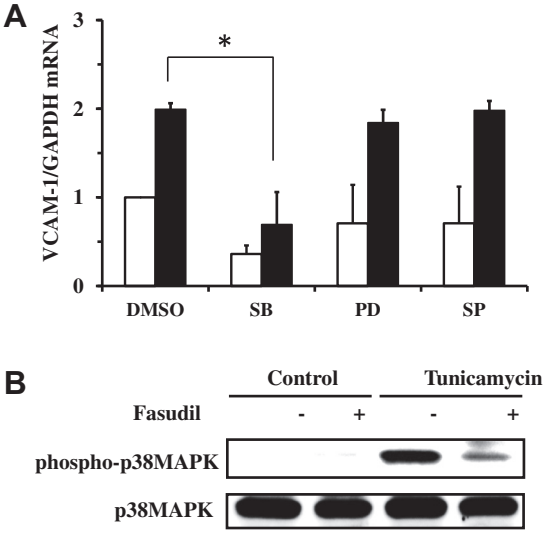

Fig. 4. Effects of fasudil on tunicamycin-induced p38MAPK activation. (A) HUVECs were stimulated with tunicamycin (10 μg/ml) for 2 h with or without pretreatment with PD98059, SB203580 and SP600125 (50 μM). RNA was extracted, and VCAM-1 mRNA was analyzed by Real time-PCR, with GAPDH mRNA as internal standard. (B) HUVECs were pretreated with fasudil (10 μM) for 30 min and then stimulated with tunicamycin (10 μg/ml) for 10 min. Equal amounts of cell lysates were subjected to Western blot analysis with anti-phosho-p38MAPK antibody. Membranes were reprobed with anti-p38MAPK. A representaive blot of three independent experiments is shown.

4. Discussion

Rho/Rho-kinase signaling has been recognized to be involved in the pathogenesis of atherosclerosis, causing dysfunction of endothelial cells and vascular smooth muscle cells and infiltration of macrophages. We have previously reported that activation of Rho-kinase contributes to proliferation of vascular smooth muscle cells exposed to oxidized LDL [16]. Pharmacological inhibition of Rho-kinase by fasudil has been shown to inhibit the development of atherosclerosis in ApoE-deficient mice and diabetic mice [17]. Fasudil has also been shown to exert anti-inflammatory effects on endothelial cells by reducing oxidative stress and NF-κB activation [18]. However, the molecular link between Rho-kinase and ER stress is not fully understood. Here, we provide evidence that Rho-kinase blockade by fasudil can inhibit ER stress-mediated endothelial dysfunction by modulating UPR.

Accumulating evidence shows that UPR plays an important role in endothelial inflammation. In particular, recent studies have paid much attention to the role of ATF4-CHOP signaling in this process. ATF4 is induced by phosphorylated eIF2α under ER stress. ATF4 is a key transcriptional factor for UPR and plays an important role in ER stress-associated endothelial inflammation. It has been reported that overexpression of ATF4 induces expression of intercellular adhesion molecule (ICAM)-1 in endothelial cells [8]. Consistent with this, ATF4-deficient mice showed amelioration of inflammation in the retina with reduction in ICAM-1 expression [8]. In addition, Gao et al. reported that CHOP knockdown in endothelial cells results in decreased expression of endothelial inflammation markers such as VCAM-1, ICAM-1 and MCP-1 [6], demonstrating that ATF4-CHOP regulates these proinflammatory genes in the upstream portion.

We confirmed that expression of CHOP was enhanced in endothelial cells under stimulation with tunicamycin in parallel with expression of VCAM-1, both of which were significantly suppressed by Rho-kinase inhibitor (Fig. 1A, B, and C). Active Rho was involved in tunicamycin-mediated VCAM-1 induction (Fig. 2A). Indeed, activities of Rho and Rho-kinase were upregulated by tunicamycin (Fig. 2B and C). Tunicamycin stimulated phosphorylation of eIF2α and ATF4 expression, which were also suppressed by Rho-kinase inhibitor (Fig. 3A, B). These results clearly indicate that the Rho/Rho-kinase pathway is involved in ER stress-mediated VCAM-1 induction by modulation of the ATF4-CHOP pathway. It has been reported that ER stress induces NF-κB activation which plays an important role in VCAM-1 induction [1,19,20]. Future study is required to assess whether the NF-κB-dependent signaling pathway may contribute to an inhibitory effect of fasudil on VCAM-1 expression under ER stress. So far, there are few studies investigating linkage between the Rho/Rho-kinase pathway and ER stress; however, several studies have indicated that pleiotropic effects of statins may be mediated through suppression of ER stress. A significant body of evidence shows that statins exert pleiotropic effects by inhibiting Rho-kinase [18]. It is known that pressure overload in the heart triggers ER stress. Recent study showed that pravastatin inhibits cardiac remodeling in transverse aortic constriction by ameliorating phosphorylation of eIF2α and subsequent CHOP induction [21]. Consistent with findings *in vivo*, pravastatin was found to attenuate TNF-α-mediated phosphorylation of eIF2α and CHOP expression in cultured cardiomyocytes [21]. Similarly, another study demonstrated that atorvastatin inhibited apoptosis of myocardial cells in a rat model of post-myocardial heart failure with suppression of ER stress molecules such as CHOP [22]. Although target molecules of statins are not fully identified, given fasudil's ability to modulate UPR, these studies with statin may suggest contribution of Rho/Rho-kinase pathway to generation of ER stress in cardiomyocytes.

Recent work has demonstrated activation of the p38-branch of the MAPK pathways in the atherosclerotic lesion under conditions of ER stress [23–25]. For instance, in endothelial cells, p38MAPK activation is involved in IL-8 induction by ER stress [25]. Consistent with this, the present study confirmed that p38MAPK plays an important role in upregulation of VCAM-1 by ER stress (Fig. 4A). Indeed, fasudil attenuated p38MAPK phosphorylation induced by ER stress (Fig. 4B). In the present study, inhibition of tunicamycin-mediated VCAM-1 induction by SB203580 was more potent compared to inhibition by fasudil. This finding raises the possibility that pathways other than Rho-kinase are also involved in p38MAPK activation and subsequent VCAM-1 induction by ER stress. Further studies will be required to elucidate the precise mechanism by which ER stress induces p38MAPK activation. We previously reported that Rho-kinase inhibition attenuates activation of p38MAPK by thrombin in endothelial cells [12]. These findings indicate that p38MAPK is an important player downstream of Rho-kinase-mediated inflammatory responses in endothelial cells. Consistent with a previous study [20], we observed that SB203580 did not inhibit CHOP induction during ER stress (data not shown), indicating that p38MAPK does not regulate the ATF4-CHOP signaling pathway. Taken together, we speculate that the ATF4-dependent and p38MAPK-dependent pathways are regulated by Rho-kinase via different mechanisms during ER stress.

ER stress is reportedly induced by various stimuli such as hyperglycemia, oxidative stress, inflammatory cytokines and hypoxia, all of which are involved in the pathogenesis of atherosclerosis. The mechanism by which tunicamycin activates Rho/Rho-kinase signaling is unclear in this study. Wu et al. demonstrated that human immunodeficiency virus 1 (HIV-1) Tat, a virus peptide which is also an ER stress inducer, causes activation of Rho in HUVECs [26]. Although the mechanism by which HIV-1 Tat induces Rho activation remains unknown, it is considered physiologically relevant to tunicamycin because of their functional similarities [27]. Precise mechanisms underlying Rho/Rho-kinase activation by ER stress should be elucidated in future studies.

Our findings provide evidence that Rho-kinase mediates ER stress-induced VCAM-1 expression by modulating UPR in endothelial cells. We conclude that Rho-kinase could be an important therapeutic target against atherosclerosis under ER stress conditions such as diabetes.

Acknowledgments

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