EXPERIMENTAL STUDY

Novel ASK1 Inhibitor AGI-1067 Attenuates AGE-Induced Fibrotic Response by Suppressing the MKKs/p38 MAPK Pathway in Human Coronary Arterial Smooth Muscle Cells

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Summary

The phenotype shifting of vascular smooth muscle cells (VSMCs) was indicated to play a role during the initial stage of atherosclerotic plaque formation by facilitating extracellular matrix deposition. This study was aimed at investigating the involvement of the apoptosis signal-regulating kinase 1 (ASK1)/mitogen-activated protein kinase (MAPK) kinases (MKKs)/p38 MAPK pathway in the advanced glycation end product (AGE)-induced fibrotic response of VSMCs. The effect of the novel ASK1 inhibitor AGI-1067 was also studied.

Cultured human coronary smooth muscle cells (HCSMCs) were exposed to AGEs. AGI-1067 and siRNAs silencing mkk3, mkk6, and p38 mapk were used to treat the cells. The activation of MKK3, MKK6, and p38 MAPK was assessed by immunoblotting. Fibrotic response was assessed by the fluorescence immunohistochemistry staining of collagen I and collagen VIII. Activation of immunoprecipitation determined the association of ASK1 and its inhibitor thioredoxin. A kinase assay was used to determine ASK1 activity.

AGE incubation significantly activated ASK1, MKK3, and MKK6, which led to activation of p38 MAPK, resulting in upregulated fibrotic response in HCSMCs. However, siRNAs knocking down mkk3, mkk6, and p38 mapk impaired this fibrotic response. AGI-1067 administration not only dramatically inhibited the activation of ASK1/MKKs/p38 MAPK but also suppressed the expression of the downstream proteins, including transforming growth factor-β1, connective tissue growth factor, collagen I, and collagen VIII in HCSMCs exposed to AGES.

The ASK1/MKKs/p38 MAPK pathway was activated by AGEs, leading to the fibrotic response in VSMCs. AGI-1067 reversed this process by maintaining the inactive state of ASK1.

Key words: Vascular smooth muscle cells, Advanced glycation end products

The formation and progression of atherosclerotic plaques are characteristic pathological features of diabetic vascular complications.6) Evidence from clinical observations indicated that uncontrolled sustained hyperglycemia facilitated the adverse outcomes of atherosclerosis.7,8) Excessive glucose in circulation participates in the nonenzymatic glycation reactions with nucleic acids and certain amino groups of proteins and lipids, resulting in sets of high-molecular-weight protein and fluorescent entities referred to as advanced glycation end products (AGEs).9) It is accepted that AGEs were highly correlated with diabetic vascular complications.9)

Vascular smooth muscle cells (VSMCs) play an important role in the occurrence and progression of atherosclerosis.6) VSMCs exhibit various phenotypes.7) Under normal physiological conditions, VSMCs are quiescent and participate in the regulation of arterial contractions. However, under certain pathological conditions, arteriosclerosis for instance, the phenotype of VSMCs is converted to the synthetic status.8,9) During the phenotype shifting, synthetic VSMCs migrate from the arterial media to the intimal area to synthesize extracellular matrix (ECM) molecules and inflammatory cytokines.8,9) The more advanced atherosclerotic plaques are characterized by accumulation of fibrous tissue.11,12)

When encountering pathological stimuli, including oxidative stress, hypoxia, and inflammation, the nuclear factors mitogen-activated protein kinases (MAPKs) are ac-
tivated and translocated to the nucleus to initiate transcription of genes regulating critical cell events such as apoptosis, autophagy, redox balancing, and intercellular communication. 13,14 The fibrotic and inflammatory responses could be conducted by p38 MAPK, which is activated by a set of its upstream MAPK kinases (MKKs), MKK3 and MKK6 specifically. 15 As one of the MKK kinases (MAPKKK), the apoptosis signal-regulating kinase 1 (ASK1) was reported to mediate the activation of MKK3 and MKK6. 16,17 It has been shown that ASK1 was activated by disassociation from its physiological inhibitor thioredoxin (Trx). 18 A few previous reports drew our attention to the fact that AGEs could induce the activation of ASK1. 19 In the current study, the involvement of activation of the ASK1/MKK/p38 signaling pathway in fibrotic and inflammatory responses in AGE-incubated human coronary smooth muscle cell (HCSMC) fibrosis and inflammation was investigated. The antifibrotic and anti-inflammatory effects of the novel ASK1 inhibitor AGI-1067 were also studied in AGE-incubated HCSMCs.

**Methods**

**AGE-bovine serum albumin (BSA) preparation:** AGE-BSA was prepared as described previously. 20,21 Briefly, BSA (Hyclone) was incubated with glyceraldehyde (0.1 mmol/L, Sigma-Aldrich) in NaPO4 buffer (0.2 mmol/L, pH 7.4) under sterile conditions at 37°C for 7 days.

**Cell culture:** HCSMCs purchased from PromoCell (Germany) were cultured in Dulbecco’s modified Eagles medium (Gibco) supplemented with fetal bovine serum (10%, FBS, Hyclone) and antibiotic mix (Invitrogen) at 37°C in a humidified cell incubator providing 5% CO2 and 95% fresh air. When cells reached a confluence of 90%, they were washed and used for the subsequent experiments. AGI-1067 (Santa Cruz) at a final concentration of 10 μmol/L was used to incubate the cells for 4 hours. AGEs at final concentrations of 0, 5, and 10 μmol/L for 24 hours. Several cells were also transfected with small interference RNAs (siRNAs).

**Fibrosis analysis:** The fibrosis in HCSMCs was evaluated by immunofluorescent staining. Cultured HCSMCs were fixed and incubated with primary antibodies against collagen I (Abcam, 1:250) and collagen VIII (Abcam, 1:250) at 4°C for 8 hours. Secondary antibody conjugated with Alexa 488 (Abcam, 1:1000) was used to incubate the cells. Potential fluorescence quenching was alleviated using the SlowFade Light Antifade Kit (Molecular Probes) according to the manufacturer’s instructions. Stains were observed with an inverse fluorescence microscope (Axio Imager 2, Zeiss) at 422 nm after the samples were excited with an inverse fluorescence microscope (Axiom microscope (Axio Imager 1, Zeiss), which was designed and synthesized by GenePharma. Control siRNA (Cell Signaling Technology, Cat#6568S) was used as the negative control. Cultured HCSMCs (at 80%-90% confluence) were transfected with the above siRNAs using Mirus TransIT-TKO reagent (Mirus) for 48 hours according to the manufacturer’s instructions.

**Western blotting:** Whole-cell extract from cultured HCSMCs using the cell lysis buffer system (Santa Cruz). Total protein was extracted with Total Protein Extraction Reagents (Beyotime) according to the manufacturer’s instructions. Protein concentration was determined by the BCA method with a BCA kit (Pierce). Specific primary antibodies against MKK3 (Sigma-Aldrich, 1:500), phosphor-MKK3 (Sigma-Aldrich, 1:500), MKK6 (Sigma-Aldrich, 1:500), phosphor-MKK6 (Sigma-Aldrich, 1:500), p38 (Cell Signaling Tech, 1:250), phospho-p38 (Cell Signaling Tech, 1:250), transforming growth factor-β1 (TGF-β1, Abcam, 1:250), connective tissue growth factor (CTGF, Abcam, 1:250), and GAPDH (Abcam, 1:500) were used to incubate the membranes at 4°C for 10 hours. After washing with Tris-buffered saline-Tween 20 (0.02%, TBST), the membranes were incubated with secondary antibodies conjugated to horse radish peroxidase (Abcam) at room temperature for 2 hours. Membranes were developed with Super Signal West Pico chemiluminescence reagent and visualized on X-ray films. The band intensities were quantified and analyzed by Image J software (version 1.38, NIH).

**Immunoprecipitation:** Immunoprecipitation was used to evaluate the association between ASK1 and Trx. This assay was carried out in accordance with previous descriptions. 19,20 Briefly, 400 μg whole-cell extract was precleared by incubating with 5 μg normal rabbit serum plus protein A/G-agarose beads at 4°C on a rotator for 10 hours. Then, 5 μg first protein (Trx)-specific antiserum was used to incubate the extracts with 50 μL protein A/G-agarose beads for 2 hours. After centrifugation at 14,000 × g for 10 minutes and washing with lysis buffer, the immune complexes were harvested after each immunoprecipitation. SDS-PAGE was carried out to separate the immune complexes, which were then detected by immunoblot methods (Immobilon P, Millipore) with the second protein (ASK1)-specific antibody. The band intensities were quantified and analyzed by Image J software (version 1.38, NIH).

**ASK1 kinase activity assay:** The ASK1 kinase activity assay was carried out according to the method used in several previous studies with some modifications. 21,22 Briefly, the immunocomplex-bonded A/G-agarose beads mentioned above were washed with kinase buffer (20 mmol/L Tris-HCl; 20 mmol/L MgCl2; pH = 7.4) three times. Then, these beads were incubated with GST-MKK6 fusion protein (Millipore) at 30°C for 15 minutes in a final volume of 25 μL kinase buffer containing ATP at 100 μmol/L. Harvested samples were subjected to SDS-PAGE. The expression of phosphorylated MKK6 was detected with primary antibody phosphor-MKK6 (Sigma-Aldrich, 1:500) by following the protocol described in the Western.
Results

AGE exposure induced the fibrosis response of HCSMCs by activating MKKs/p38 MAPK signaling: The results are shown in Figures 1, 2. After being exposed to AGEs, the expression levels of collagen I and collagen VIII increased significantly in HCSMCs in an AGE concentration-dependent manner. The phosphorylation levels of MKK3, MKK6, and p38 MAPK as well as the expression levels of TGFβ1 and CTGF were dramatically increased in HCSMCs receiving treatments of AGEs and/or siRNAs against mkk3, mkk6, and p38 mapk were demonstrated. B, C, and D: Columns indicated the phosphorylation levels of MKK3, MKK6, and p38 MAPK in HCSMCs. E: Columns indicated the expression levels of TGFβ1 and CTGF in HCSMCs. [Differences were significant: *P < 0.05; **P < 0.01.]
Figure 2. The upper panel demonstrated the captured images of fluorescent staining of collagen I and collagen VIII by immunohistochemistry in cultured HCSMCs receiving treatments of AGEs and/or siRNAs against mkk3, mkk6, and p38 mapk. Columns in the lower panel indicated the intensities of fluorescent staining of collagen I (white columns) and collagen VIII (black columns) in cultured HCSMCs. [Differences were significant: *P < 0.05; **P < 0.01.]

cally upregulated, indicating the activation of MKKs/p38 MAPK signaling. The transfections of siRNAs against mkk3, mkk6, and p38 mapk significantly attenuated the expression levels of collagen I, collagen VIII, TGFβ1, and CTGF in AGE-exposed HCSMCs.

AGI-1067 suppressed the AGE-induced fibrosis response of HCSMCs: Figure 3 shows the results. The treatment of AGI-1067 significantly reduced the expression levels of collagen I and collagen VIII in cultured HCSMCs exposed to AGE incubation.

AGI-1067 inhibited the activation of MKKs/p38 MAPK signaling in AGE-exposed HCSMCs: The results are shown in Figure 4. After being treated with AGI-1067, the phosphorylation levels of MKK3, MKK6, and p38 MAPK as well as the expression levels of CTGF and TGFβ1 were dramatically downregulated in HCSMCs exposed to AGE incubation.

AGI-1067 inhibited the activation of ASK1 by enhancing the association of ASK1 and Trx: The results are shown in Figure 5. In this study, the association of ASK1 and Trx was evaluated by immunoprecipitation. In HCSMCs, AGE incubation significantly facilitated the dissociation of Trx from ASK1, which promoted the activation of ASK1. However, the treatment of AGI-1067 dramatically inhibited the dissociation of Trx and ASK1 in cultured HCSMCs exposed to AGE incubation.

activity was also assessed by detection of the Trx1-ASK1 immunocomplex-induced phosphorylation of GST-MKK6. The results indicated that AGE incubation significantly upregulated the Trx1-ASK1 immunocomplex-induced phosphorylation of GST-MKK6. However, AGI-1067 treatment dramatically suppressed the Trx1-ASK1 immunocomplex-induced phosphorylation of GST-MKK6.

Discussion

The mortality and morbidity of diabetes is rapidly increasing in both developing and developed countries. Uncontrolled and sustained hyperglycemia causes the metabolic disorders of diabetes.23 The formation, development, and progression of atherosclerotic plaques are characteristic pathological features of diabetic vascular complications. The plaques consist of cells, lipids, connective tissue, and debris. VSMCs are one of the fundamental cell types of the arterial wall. Pathological stimuli generated by the metabolic disorder of diabetes promote VSMCs to change their state from a quiescent contractile state to an active synthetic one.24 Activated VSMCs proliferate, migrate, and secrete ECM.25 AGEs are fostered during diabetes and further induce diabetic pathological processes. Previous studies suggested that AGEs were highly correlated with fibrosis in several organs, including the heart,
liver, lens, and lung. In this study, we found that AGE incubation induced fibrosis response in cultured HCSMCs in a concentration-dependent manner, which has rarely been reported previously.

Vascular endothelial cells are injured during diabetes, which further recruits platelets and immune cells to secrete inflammatory factors. As a result, VSMCs undergo phenotype shifting from a differentiated, contractile status to an activated, synthetic status. The synthesis of ECM is an important hallmark of activated VSMCs. The deposition of ECM participated in the formation of atheroma, which become the core of plaque with foam cells and extracellular lipid droplets. Collagen I and collagen VIII are believed to be pathogenic ECM components leading to atherosclerosis. Collagen I itself could promote the proliferation and migration of VSMCs. It was reported that in the atherosclerotic plaque, collagen VIII was produced by VSMCs abundantly. Moreover, collagen I and collagen VIII were reported to facilitate the phenotype shifting of VSMCs. In the current study, we found that the AGEs incubated significantly upregulated the expression of collagen I and collagen VIII in HCSMCs.

In the normal physiological status, p38 MAPK binds to its inhibitor Keap1 to maintain its static status. When encountering various stressful challenges, p38 MAPK is activated by phosphorylation, which further regulates its downstream gene transduction directly or through several signaling pathways. Activated p38 MAPK could increase the transcription of TGF-β1 in a Smad signaling-dependent or independent manner. TGF-β1 induces the expression of CTGF, and both are profibrotic factors. In this study, we found that in AGE-incubated HCSMCs, p38 MAPK signaling was activated. As a result, the expression levels of the profibrotic factors TGF-β1 and CTGF were significantly upregulated. However, the transfection of specific siRNA silencing p38 MAPK impaired the activation of p38 MAPK signaling. The activity of p38 MAPK is modulated by its upstream MKKs. Among them, MKK3 and MKK6 were reported to be specifically involved in the activation of p38 MAPK. Indeed, in this study, mkk3 and mkk6 were silenced by specific siRNAs in cultured HCSMCs. The results indicated that the silencing of both MKK3 and MKK6 impaired the activation of p38 MAPK signaling. Trx was initially discovered in bacteria several decades ago.
and is now recognized as a 20 kDa protein molecule participating in several cell biological processes such as apoptosis, inflammation, redox balancing, and proliferation.\textsuperscript{20) Investigations revealed that Trx was one of the critical regulators of ASK1. By interacting with the N-terminus of ASK1 to form the Trx1-ASK complex, the kinase activity of ASK1 was blocked.\textsuperscript{39) ASK1 was recognized as one of the activators of MKKs, also referred to as MAPKKK. In previous studies, AGEs were described as activating ASK1 by increasing the dissociation of Trx from the ASK-Trx complex in myocytes and neurocells.\textsuperscript{16,40) In this study, we confirmed that AGEs also activated ASK1 activation by facilitating the collapse of the ASK-Trx complex in a concentration-dependent manner in VSMCs. As a result, the downstream signaling of ASK1, namely the MKKs/p38 MAPK pathway, was activated to induce the fibrotic response in HCSMCs. Of note, administration of the novel ASK inhibitor AGI-1067 significantly inhibited AGE-induced ASK1 activation by suppressing the dissociation of Trx from ASK1. The Trx1-ASK1 complex was also used to treat its substrate GST-MKK6. As a result, AGI-1067 treatment significantly suppressed the

Figure 4. A: Immunoblots of p-MKK3, MKK3, p-MKK6, MKK6, p-p38 MAPK, p38 MAPK, TGFβ1, CTGF, and GAPDH in HCSMCs receiving treatments of AGEs at different concentrations and/or AGI-1067. B, C, and D: Columns indicated the phosphorylation levels of MKK3, MKK6, and p38 MAPK in HCSMCs. E: Columns indicated the expression levels of TGFβ1 and CTGF in HCSMCs. [Differences were significant: *P < 0.05; **P < 0.01.]
Figure 5. In the upper part, the left panel demonstrated the results of immunoprecipitation that evaluated the association between ASK1 and Trx1. The cell extract was immunoprecipitated with antibodies against Trx1. Relative Trx1-bound ASK1 was quantified by comparing pulldown ASK1/input ASK1. Columns in the right panel indicated the relative levels of ASK1 binding to Trx1 in HCSMCs. In the lower part of this figure, the immunoblot on the left side demonstrated the expression of p-MKK6 resulting from GST-MKK6 incubated with the Trx1-ASK1 immunocomplex. Columns on the right side indicated the band densities of p-MKK6. [Differences were significant: *P < 0.05; **P < 0.01.]

Trx1-ASK1 complex-induced phosphorylation of GST-MKK6 in AGE-incubated VSMCs. These results indicate that downstream MKKs/p38 MAPK signaling activation was shut down. Data in this study suggested that the application of AGI-1067 attenuated the AGE-induced fibrotic responses of VSMCs.

In this study, we provided evidence of the activation of ASK1/MKKs/p38 MAPK in the AGE-induced phenotype shifting of VSMCs. Administration of the ASK inhibitor AGI-1067 attenuated the AGE-mediated fibrotic response in VSMCs by inhibiting the activation of this signaling pathway. Moreover, our data may provide new insight into a possible explanation for why AGI-1067 failed to meet the primary endpoint of relative risk reduction as an antiatherosclerotic agent compared with placebo in the Aggressive Reduction of Inflammation Stops Events (ARISE) study. AGI-1067 interferes with the phenotype shifting of VSMCs that takes place during the very early stage of atherosclerotic plaque formation. However, patients recruited in the ARISE study were diagnosed with acute coronary syndrome. Perhaps earlier AGI-1067 administration in patients would have prevented the occurrence of acute coronary syndrome. Of course, these are just proposed theories based on our data. More rigorous clinical trials are needed to reassess the clinical application potential of AGI-1067.

Limitations: Other important MKKs such as MKK4 and MKK7 are also supposed to be activated by ASK1. It was shown that JNK, the downstream effector of MKK4/7, was associated with fibrosis through crosstalk with Smads. Perhaps MKK4/7-JNK is another MAPK signaling conducting AGE-induced fibrotic responses in VSMCs. ERK1/2 was also described to be involved in inducing fibrosis in several organs such as the heart and kidney, indicating the possible involvement of the MKKs/MAPK/ERK signaling pathway. More studies should be performed to investigate this signaling. Another limitation of this study was that the possible molecular mechanisms concerning how AGEs affected the dissociation of the Trx1-ASK1 complex were not elucidated. Actually, there is hardly any literature describing the correlation between AGEs and ASK1. It would be interesting to perform such investigations in the future. Additionally, in this study, AGES were used to simulate the environment of metabolic disorder conducted by T2DM. Moreover, AGES are generated in vivo under conditions of high blood glucose. The high blood glucose is the basic metabolic disorder for both T1DM and T2DM. Notably, it was reported that high glucose could also affect the activation of ASK1. Thus, it is necessary to implement further investigations on the therapeutic effects of AGI-1067 on the high glucose-induced fibrotic response in vitro and in vivo.
Disclosures

Conflicts of interest: None.

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