

Research Article

Open Access

A Novel ASK Inhibitor AGI-1067 Inhibits TLR-4-Mediated Activation of ASK1 by Preventing Dissociation of Thioredoxin from ASK1

Shuhui Zheng^{1#}, Lingli Long^{1#}, Yonghao Li^{2#}, Yuxia Xu^{1#}, Zhang Jiqin¹, Weidong Ji¹, Wang Min^{1,3*}

¹The Center for Translational Medicine, The First Affiliated Hospital, Sun Yat-sen University, Guangzhou 510080, China;

²State Key Laboratory of Ophthalmology, Zhongshan Ophthalmic Center, Sun Yat-sen University, Guangzhou, China

³Interdepartmental Program in Vascular Biology and Therapeutics, Dept. of Pathology, Yale University School of Medicine, New Haven, CT 06520.

Abstract

The cell type that normally limits the inflammatory and atherosclerotic process is the vascular Endothelial Cell (EC) that can be regulated by pro inflammatory and various stresses. Toll-like Receptor-4 (TLR4) plays an important role in the pathogenesis of atherosclerosis, in part, by activating Apoptosis Signal-regulating Kinase 1 (ASK1) to initiate the activation of MAP kinases pathways and the expression of inflammatory genes. In the present study, we test the hypothesis that AGI-1067 acts as an anti-inflammatory agent by inhibiting the activation of ASK1 in human EC. Pretreatment of human aortic endothelial cells with AGI-1067 inhibits TLR4 ligand (LPS)-induced activation of ASK1 and the downstream p38 and c-Jun N-terminal Kinase (JNK) MAP kinases. LPS dissociates two endogenous inhibitors Thioredoxin-1 (Trx1) and 14-3-3 from ASK1, leading to ASK1 autoactivation. Interestingly, AGI-1067 inhibits the dissociation of Trx1, but not 14-3-3, from ASK1. However, inhibition of Trx1 dissociation from ASK1 by AGI-1067 is sufficient to suppress LPS-mediated phosphorylation of the transcription factors c-Jun and activating transcription factor 2, and inhibit LPS-induced inflammatory genes including vascular cell adhesion molecule 1, E-selectin, IL-6 and monocyte chemo attractant protein 1. Our findings suggest that AGI-1067 as a unique ASK1 inhibitor to inhibit TLR4-mediated ASK1 activation, contributing to its anti-inflammatory properties.

Keywords: AGI-1067; Apoptosis signal-regulating kinase 1; Thioredoxin; Toll-like receptor 4; Mitogen-activated protein kinase

Introduction

Myocardial infarction caused by atherosclerosis of coronary arteries remains the leading cause of death in the developed countries. Atherosclerosis involves plaque formation in the arterial wall and is characterized by inflammation, lipid accumulation, smooth muscle cell proliferation/noon time expansion, cell death and fibrosis [1,2]. Sub endothelial retention and accumulation of lipoproteins could be converted to atherogenic remnant lipoproteins and low-density lipoprotein (LDL) [3,4]. A key early inflammatory response to retained lipoproteins is activation of vascular endothelial cells (EC), which may be enhanced by oxidative modifications of these lipoproteins. Activated EC increase expressions of adhesion molecules and chemokine's which in turn promote infiltration of immune cells, including macrophages, neutrophils, master cells dendritic cells and T cells [2,5-7]. Macrophages take up native and oxidized LDL to become lipid-laden foam cells and together with other cells release atherogenic cytokines and other activators such as Reactive Oxygen Species (ROS) that exacerbate EC dysfunction (characterized by a reduction in amount of bioavailable nitric oxide) and EC apoptosis, and promote atherosclerotic plaque development [8]. Therefore, the phenotypic changes in EC may be an early event during initiation and progression of atherosclerosis [2,6,7,9,10]. TLR4, the primary receptor for lipopolysaccharide (LPS), plays an important role in the pathogenesis of atherosclerosis [3,11]. Epidemiological data suggest that high plasma endotoxin levels constitute a significant risk factor for the development of atherosclerosis [12-17]. TLR4 mRNA and protein levels are dramatically increased in atherosclerotic plaques [18]. Administration of endotoxin increases atherosclerotic lesion formation in apolipoprotein E-deficient (ApoE^{-/-}) mice and hypercholesterolemia rabbits [19,20]. TLR4/ApoE double-null (TLR4^{-/-}ApoE^{-/-}) mice fed with a high cholesterol diet show decreased lesion formation compared to ApoE^{-/-} control mice [21]. Furthermore, in addition to LPS, TLR4 recognizes oxidized variation of low density lipoproteins (LDL) also termed minimally modified

LDL, heat shock proteins, and fibronectin extra domain A; all of which are found in atherosclerotic lesions [22-25].

ASK1, a member of the MAP3K family activating MAP2K-JNK/p38 cascades, can be activated in response to various stress stimuli, including pro-inflammatory cytokines and LPS [26,27]. Studies in overexpression systems and from ASK1 knockout mice have shown that ASK1 is a critical mediator in tumor necrosis factor (TNF), ROS, and LPS-induced inflammatory signaling [28-30]. ASK1 is a 170 kD protein that is composed of an inhibitory N-terminal domain, an internal kinase domain, and a C-terminal regulatory domain. Several intracellular proteins bind to different domains of ASK1, keeping it in an inactive state in resting cells. The redox sensor protein thioredoxin-1 (Trx1) in a reduced form binds to the N-terminal domain of ASK1, while glutaredoxin binds to the C-terminal domain, in each case, inhibiting ASK1 kinase Activity [31,32]. Phosphoserine-binding protein 14-3-3 associates with ASK1 via the pSer967 site of ASK1 and inhibits ASK1-induced apoptosis [33-37]. Stress stimuli activate ASK1, in part by dissociating ASK1 from its inhibitors Trx1 and 14-3-3. Given the critical role of ASK1 in inflammation and cardiovascular diseases, finding of ASK1 inhibitors has been actively investigated in both Pharmaceutical companies and academics labs. We have recently shown that a selective ASK1 inhibitor GS-444217, which

***Corresponding author:** Wang Min, Ph.D., Interdepartmental Program in Vascular Biology and Therapeutics, Department of Pathology, Yale University School of Medicine, 10 Amistad St., New Haven, CT 06520, USA, Tel: 203-785-6047; Fax: 203-737-2293; E-mail: wang.min@yale.edu

Received: February 06, 2015; **Accepted:** February 20, 2015; **Published:** February 26, 2015

Citation: Min W, Zheng S, Long L, Zhou HJ, Ji W, et al. (2015) A Novel ASK Inhibitor AGI-1067 Inhibits TLR-4-Mediated Activation of ASK1 by Preventing Dissociation of Thioredoxin from ASK1. *Cardiol Pharmacol* 4: 132. doi:10.4172/2329-6607.1000132

Copyright: © 2015 Min W, et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

specifically binds to an active form of ASK1 homodimer, can effectively block cardiomyopathy in a mouse model [38]. In the present study, we investigate another type of ASK1 AGI-1067. AGI-1067: [mono[4-[[1-[[3,5-bis(1,1-dimethylethyl)-4-hydroxyphenyl]thio]-1-methylethyl]thio]-2,6-bis(1,1-dimethylethyl)phenyl] ester] (butanedioc acid), is an anti-atherosclerotic agent. Pre-clinical studies establish that AGI-1067 inhibits the development of atherosclerosis in three animal models: hypercholesterolemic cynomolgus monkeys, LDL receptor-deficient mice and ApoE^{-/-} mice [39]. AGI-1067 is a potent lipid peroxide antioxidant, capable of reducing basal levels of ROS in endothelial cells, and inhibiting Tumor Necrosis Factor- α (TNF- α) induced Vascular Cell Adhesion Molecule-1 (VCAM-1) and Monocyte Chemo Attractant Protein-1 (MCP-1) expression without affecting NF- κ B activity [40]. In addition, AGI-1067 inhibits LPS-induced tissue factor production and suppresses the activation of MAP kinases and redox-sensitive ASK1 in both monocytic cells and endothelial cells [41]. However, the mechanism by which AGI-1067 inhibits LPS-induced ASK1 has not been determined. In the present study, we report that AGI-1067 inhibits LPS/TLR4-mediated expression of VCAM-1, E-selectin, IL-6, and MCP-1 in EC. This correlates with AGI-1067 inhibition of LPS/TLR4-induced activation of ASK1 and the downstream p38/JNK1/2 MAP kinase pathway and transcription factors c-Jun and Activating Transcription Factor 2 (ATF2). Mechanistically, we show that AGI-1067 specifically suppresses the dissociation of ASK1 from Trx1, but not 14-3-3, suggesting that AGI is a unique ASK1 inhibitor.

Methods

Materials

LPS (*Escherichia coli* 026:B6) and dimethylsulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO). AGI-1067 was synthesized as previously described [42]. AGI-1067 was dissolved in DMSO and added to cultured cells so that the final DMSO concentration was 0.1%. Antibodies were purchased from a variety of commercial sources: anti-phospho-p38 MAPK (9211), anti-p38 (9217), anti-TLR4 (2246), anti-Phospho-c-Jun (2361), and anti-phospho ASK1 (special order) from Cell Signaling Technology (Beverly, MA); anti-ASK1 (sc-5294) and anti- β -tubulin (sc-5274) and anti-14-3-3 from Santa Cruz Biotechnologies (Santa Cruz, CA); anti-phospho JNK (44-682G) from BioSource International (Camarillo, CA); anti-pan-JNK/SAPK1 (610628) from BD BioScience (San Diego, CA); anti-phospho-ATF2 Thr69/71 (05-891) from Upstate (Lake Placid, NY); and anti-rabbit IRdye700 (611-130-122) and anti-mouse IRdye800 (610-132-121) from Rockland, Inc (Gilbertsville, PA). Anti-Trx1 antibody was described previously [34,43].

Cell culture

Human Aortic Endothelial Cells (HAECs, Cambrex, Walkersville, MD) were cultured in EGM-2 medium with 5% fetal bovine serum (Cambrex) and used between passages 5 and 9. All cells were maintained at 37°C with 5% CO₂. All cells were seeded in plates such that they would reach 90-95% confluence on the day of the experiment.

Measurement of adhesion molecules, cytokines and chemokine's protein expressions

HAEC at 1.0x10⁴ cells per well were plated into a 96-well plate the night before. HAEC were pre-treated for 1 hr with AGI-1067 or control (0.1% DMSO) then co-treatment with 2 μ g/ml LPS. Cell surface expression of VCAM-1 and E-selectin were determined by FACS as described previously [7,44]. The mRNA expression of IL-6 and MCP-1 were determined by qRT-PCR normalized with GAPDH.

Immunoprecipitation and western blots

HAECs were pre-treated for 1 hr with AGI-1067 or control (0.1% DMSO) before co-treatment with 2 μ g/ml LPS for the given time points. Whole cell extracts were collected. Samples were subjected to electrophoresis on SDS-PAGE gels and then transferred to a nitrocellulose membrane. Blots were probed with primary antibodies in Odyssey Blocking Buffer (BB; Licor; Lincoln, NE) +0.1% Tween 20 (BBT) overnight at 4°C or for 2 hr at room temperature. Membranes were washed and incubated for 1 hr with IRdye-labeled secondary antibodies in BBT. Images were obtained using the Odyssey Infrared Imaging System (Licor; Lincoln, NE). For immunoprecipitation to analyze protein interaction *in vivo*, 400 μ g of cell lysate supernatant were pre cleared by incubating with 5 μ g of normal rabbit serum plus protein A/G-agarose beads on rotator at 4°C overnight. The lysates were then incubated with 5 μ g of the first protein-specific antiserum for 2 hr with 50 μ l of protein A/G-agarose beads. The immune complexes were collected after each immunoprecipitation by centrifugation at 14,000 \times g for 10 min followed by four washes with lysis buffer. The immune complexes were subjected to SDS-PAGE followed by immunoblot (Immobilon P, Millipore, Milford, MA) with the second protein (*e.g.* ASK1)-specific antibody (H300, Santa Cruz Biotechnology).

Statistical analysis

Values were expressed as the means \pm standard errors of at least three independent experiments. The data were analyzed using the two sample t-test assuming equal variances and values were considered significantly different at the 95% confidence level.

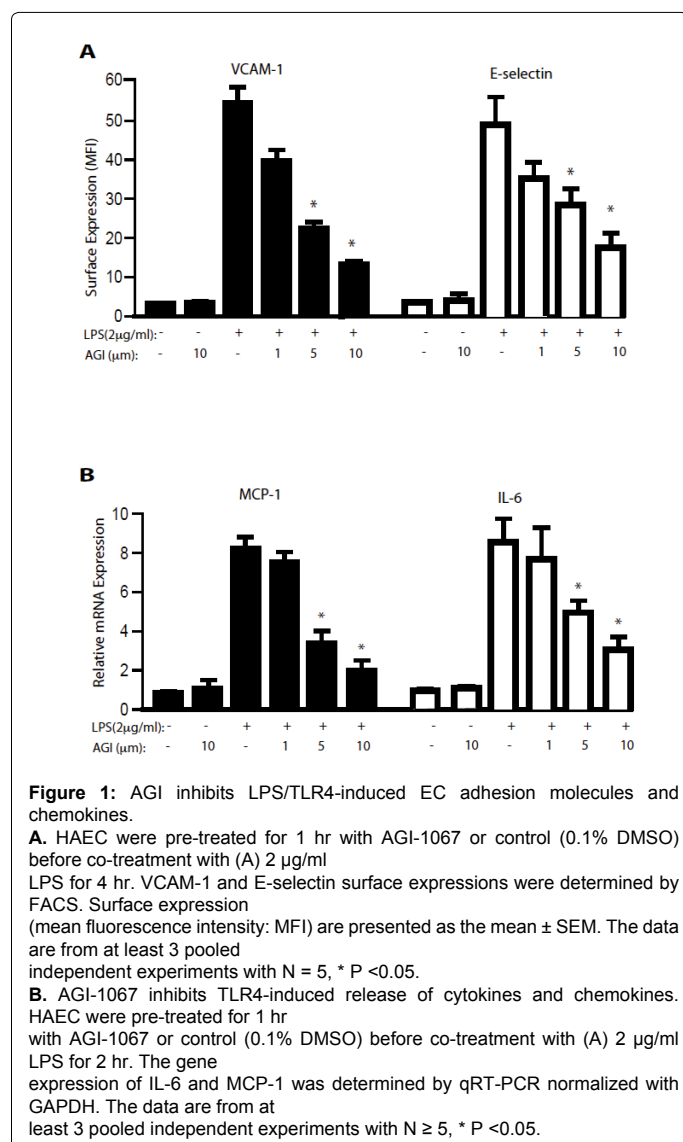
Results

AGI inhibits LPS/TLR4-induced EC adhesion molecules and chemokine's

VCAM-1 and E-selectin are up regulated by a variety of inflammatory stimuli and mediate the attachment of leukocytes to the endothelium [7,45]. We evaluated the ability of AGI-1067 to inhibit the surface expression of VCAM-1 and E-selectin induced by the pro-inflammatory ligands LPS. AGI-1067 inhibits the LPS-induced expression of VCAM-1 and E-selectin in a concentration-dependent manner with IC₅₀ values of approximately 4.5 μ M for both adhesion molecules (Figure 1A). IL-6, IL-8 and MCP-1 are pro-inflammatory cytokines associated with the pathogenesis of atherosclerosis [46]. Pretreatment of HAEC with AGI-1067 inhibits LPS-induced gene expression of IL-6, IL-8 and MCP-1 in a concentration-dependent manner with IC₅₀ values of 3.4 μ M, 3.8 μ M and 4.0 μ M, respectively (Figure 2B).

AGI inhibits LPS/TLR4-induced activation of MAPK and transcriptional factors.

The MAPK pathways regulate the expression of many inflammatory genes including cell surface adhesion molecules, cytokines and chemokines [47]. Treatment of HAEC with LPS activates p38 and JNK1/2. Pretreatment with AGI-1067 at 10 μ M inhibits LPS-induced p38 and JNK activation (Figure 2A with quantifications in 2B). Furthermore, inhibition of both p38 and JNK by AGI-1067 occurred in a concentration-dependent manner with an IC₅₀ value under 5 μ M (Figure 2C with quantifications in 2D). Activated JNK and p38 lead to the phosphorylation of Activating Protein 1 (AP-1) transcription factors c-Jun and ATF2 [48]. AP-1 transcription factors regulate many inflammatory genes [49]. Treatment of HAEC with LPS induces time-dependent phosphorylation of c-Jun and ATF2 within 1 hr of



stimulation (Figure 2E with quantifications in 2F). AGI-1067 at 10 μM inhibits LPS-induced c-JUN and ATF2 phosphorylation.

AGI inhibits LPS/TLR4-induced ASK1 activation by specifically dissociating Trx1 but not 14-3-3 from ASK1

ASK1, a redox regulated MAP3K, is an up stream regulator of p38 and JNK1/2. As expected, treatment of HAEC with LPS activated ASK1 activity as indicated by ASK1 auto phosphorylation at Thr845 (a marker of ASK1 activity) AGI-1067, at 10 μM, inhibits LPS-induced ASK1 phosphorylation at Thr845 (Figure 3A with quantifications in 3B). Trx1 and 14-3-3 bind to ASK1 and inhibit ASK1 activity.

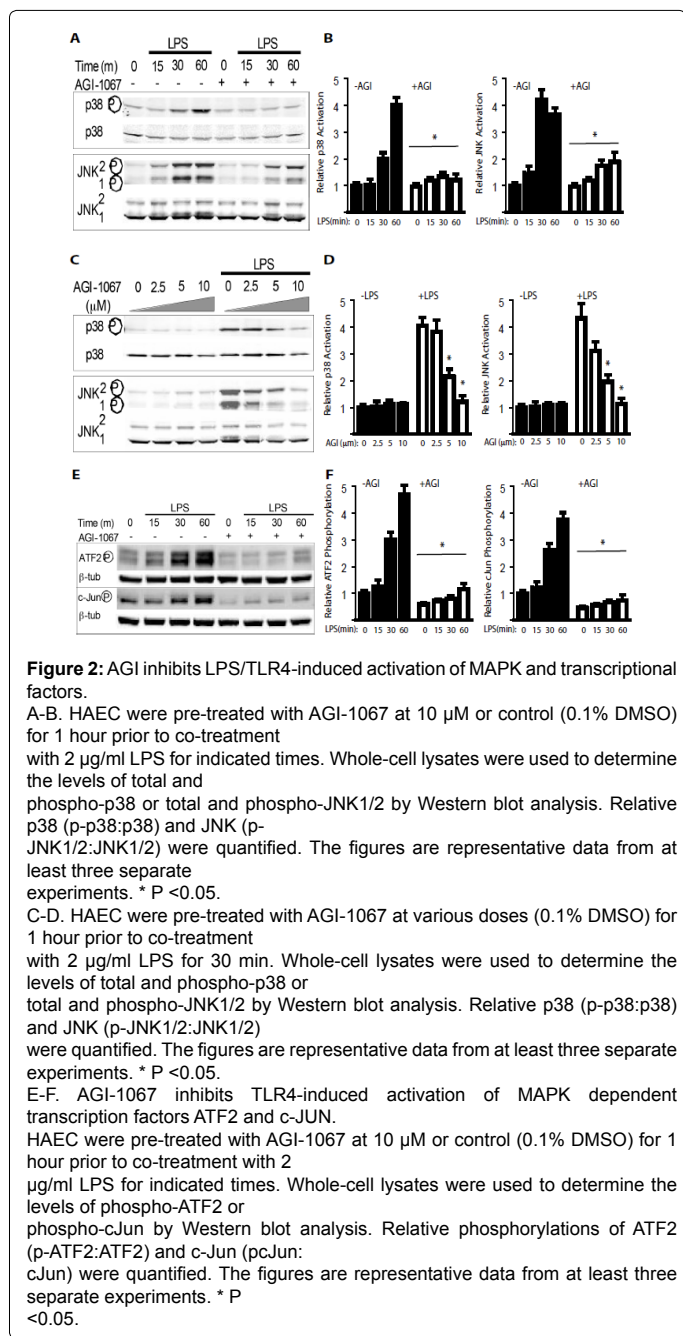
Cytokines such as TNF-α activate ASK1 in part by releasing both Trx1 and 14-3-3 from ASK1. We hypothesize that AGI-1067 may prevent the release of Trx1 or 14-3-3 to inhibit activation of ASK1. As shown in Figure 3C with quantifications in 3D, association of ASK1 with Trx1 and 14-3-3 was detected in untreated HAECs. LPS treatment reduced the interaction of ASK1 with both Trx1 and 14-3-3 in a time dependent manner. Treatment with AGI-1067 prevented the LPS-induced dissociation of ASK1 from thioredoxin, but had no effects on dissociation of 14-3-3 from ASK1. These data are consistent with

the anti-oxidant effects of AGI-1067, suggesting that AGI-1067 may prevent oxidation of Trx1 and as a result prevent dissociation of Trx1 with ASK1 and block ASK1 activation (Figure 4).

Discussion

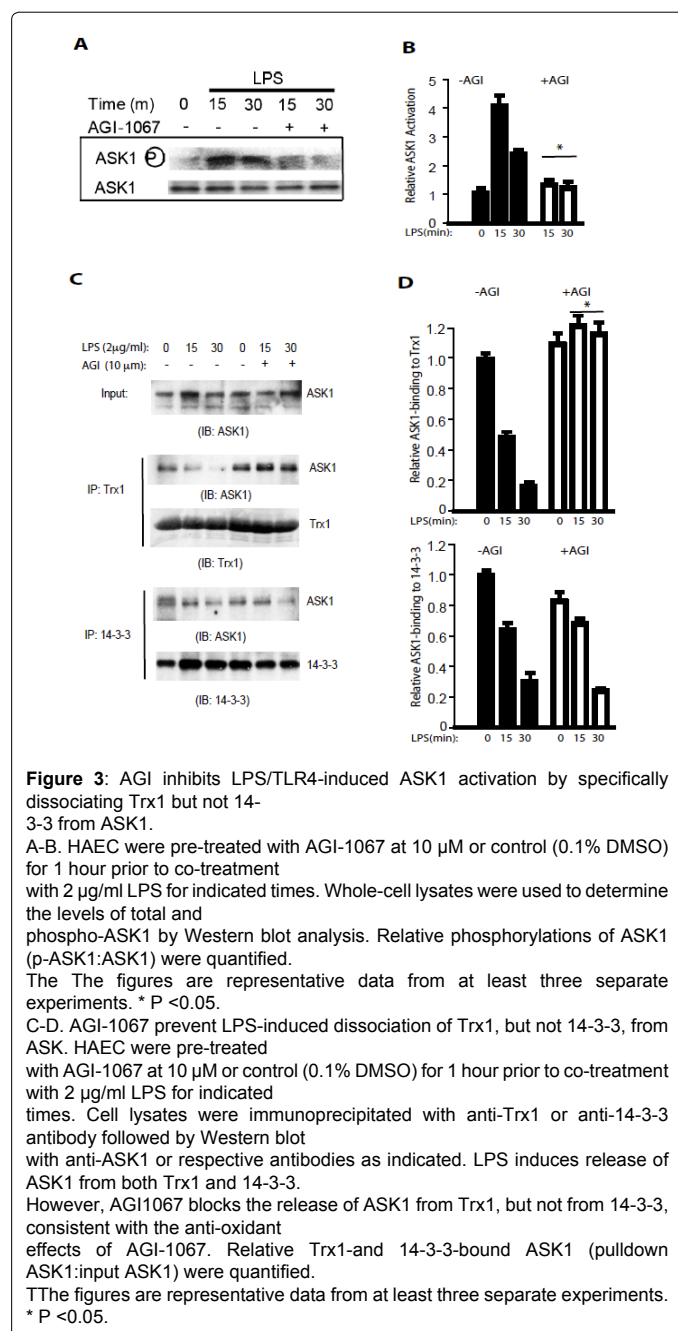
Inflammation is a key initial step in vascular diseases such as atherosclerosis. Inflammation is complicated by the fact that multiple cytokines are involved in the process and each cytokine has pleiotropic functions. One commonality of proinflammatory stimuli is that they activate NF-κB and ASK1-JNK/p38 two parallel signaling cascades, leading to induction of inflammatory molecules. Many anti-inflammatory drugs have been selected to target NF-κB pathway [50]. However, it has been realized that NF-κB triggers survival signal whereas ASK1 induces apoptotic signal in many cell types. Thus, inhibition of ASK1 without disruption of NF-κB survival signal provides a valid approach for anti-inflammatory therapy. This is supported by the findings that proatherosclerotic stimuli (e.g. TNF, IL-1, LPS, oxidized lipids and oxidative stress) activate ASK1 while atheroprotective laminar flow inhibits ASK1 activation without effects on NF-κB [34]. Our current data suggest that AGI-1067 belongs to a new category of potential anti-inflammatory chemicals as it inhibits ASK1 activation without effects on NF-κB. The clinical significance of our work is that AGI-1067 inhibits ASK1-JNK/p38-dependent gene expression of proinflammatory molecules. It has been well documented that both JNK and p38 MAP kinases are downstream of ASK1 and play an important role in the regulation of inflammatory gene expression. Inhibition of p38 activity down-regulates pro-inflammatory gene expressions such as TNF-α, TNF-β, MCP-1, IL-1b, IL-6, iNOS, COX and adhesion molecules [51-53]. While the JNK pathway regulates many inflammatory genes including TNF-α, IL-2, E-selectin, VCAM-1 and MMPs such as collagenase-1 [44,47,54,55]. Downstream of the MAPKs are the members of the AP-1 family of transcription factors. The AP-1 transcription factors are heterodimers between members of the Jun family and the Fos family. AP-1 binding sites are present in many endothelial cell pro-inflammatory genes including VCAM-1, E-selectin, tissue factor and MCP-1 [49]. Adenovirus-mediated over-expression of c-Jun and c-Fos in human endothelial cells induces the expression of adhesion molecules and release of chemokines. Additionally, *in vivo* transfection of dominant negative c-Jun into rats inhibits intimal thickening after balloon injury by preventing vascular smooth muscle cell proliferation [56]. ATF2, another transcription factor activated by p38 and JNK, also forms heterodimers with members of the AP-1 family [57]. ATF2 knock-out mice show decreased LPS-induced inflammation including reduced expression of E-selectin, P-selectin, VCAM-1, IL-6 and KC [58]. ATF2 knock-out mice show decreased LPS-induced inflammation including reduced expression of E-selectin, P-selectin, VCAM-1, IL-6 and KC [58]. Consistent with the inhibition of p38 and JNK1/2, AGI-1067 inhibits the phosphorylation of c-Jun and ATF2 which could explain the reduction in LPS-induced pro-inflammatory gene expression in AGI-1067-treated HAEC. AGI-1067 inhibits IL-6 and MCP-1, which are pro-inflammatory cytokines linked to the pathogenesis of atherosclerosis. IL-6 mRNA is found in atherosclerotic lesions and elevated IL-6 plasma concentrations are associated with an increased risk of myocardial infarction [59,60]. MCP-1, another chemokine present in atherosclerotic lesions, recruits monocytes and plays a role in their adhesion to and infiltration through the endothelium. Additionally, over-expression of MCP-1 in atherosclerotic prone ApoE^{-/-} mice accelerates the disease state, while MCP-1 deficiency reduces the susceptibility of the mice to develop atherosclerosis [61,62].

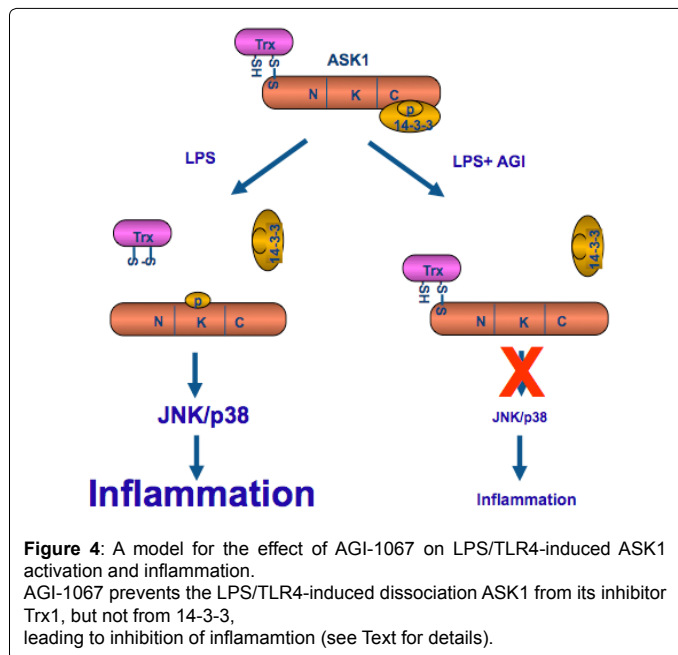
Our results demonstrate that AGI-1067 inhibits endothelial



cell TLR4-mediated activation of p38 and JNK MAP kinases and expression of cytokines and chemokines. These data suggest that modulation of the endothelial intracellular redox state by AGI-1067 inhibits the redox-sensitive ASK1-p38/JNK pathways and expression of pro-inflammatory genes. These findings may provide a mechanistic framework for understanding the anti-inflammatory and anti-atherosclerotic properties of AGI-1067. LPS also activates the interferon (IFN) regulatory factor (IRF)-3 pathway that participates in the transcriptional induction of IFN- α , IFN- β , and a subset of IFN-stimulated genes (ISGs) as a result of viral infection. Interestingly, it has been reported that LPS activates IRF3 in an ASK1-p38-dependent manner [30]. We are investigating if AGI-1067 inhibits the IRF3-mediated innate immune responses. One important finding of our study is that AGI-1067 compound prevents the LPS/TLR4-induced

dissociation ASK1 from its inhibitor Trx1, but not from 14-3-3, leading to inhibition of inflammation (Figure 4 for a model). The mechanism for ASK1 activation has been extensively investigated. The reduced form of Trx1 directly associates with ASK1 in the N-terminal domain of ASK1, inhibiting its kinase activity [63] and blocking activation of ASK1 by TNF [43,63]. Inflammatory signals activate ASK1 in part by oxidizing Trx to release it from ASK1 [43,63]. We have previously shown that a single Cys residue in the catalytic site of Trx1 (C32 or C35) and C250 of ASK1 are critical for the formation of the complex. Furthermore, Trx-C32S and Trx-C35S constitutively bind to ASK1, even in the presence of hydrogen peroxide *in vitro* or of TNF *in vivo*, most likely because they cannot be oxidized to form a disulfide bond between the two catalytic cysteine's leading to its release from ASK1 [43,64]. These results suggest that the binding of Trx1 to ASK1 is critical





for the regulation of ASK1 activity. Our current data demonstrate that AGI-1067 retains Trx1-ASK1 complex to lock ASK1 in an inactive state even in the presence of inflammatory stimuli LPS. Therefore, AGI-1067 could be an effective therapeutics to treat inflammatory diseases.

Our results further provide novel insights into the mechanism by which ASK1 activity is regulated by its two inhibitors Trx1 and 14-3-3. Previously, we have shown that apoptotic stimuli such as TNF together with protein synthesis inhibitor cycloheximide (CHX) specifically induce dissociation of ASK1 from both Trx and 14-3-3 leading to EC apoptosis [10, 35-37, 43, 64]. TNF induced reactive oxygen species (ROS) likely contributes to Trx1 release by oxidizing Trx1. TNF-activated AIP1 recruits PP2A, a phosphatase that dephosphorylates the 14-3-3 binding site pSer-967 on ASK1, to facilitate the 14-3-3 release [10, 35-37, 43, 64]. Therefore, Trx1 and 14-3-3 are dissociated at different steps of ASK1 activation through distinct mechanisms. Interestingly, our recent finding suggests that an anti-cancer prodrug, Laromustine derivative methyl isocyanate dissociates ASK1 from Trx1 but not from 14-3-3 and leads to the non-apoptotic death of EC [65]. These data indicate that 14-3-3-bound ASK1 is partially active with a unique function. Our current study demonstrates another scenario, i.e. Trx1-bound (14-3-3 free) ASK1 is sufficient to block LPS/TLR4-induced ASK1 autoactivation, and downstream JNK/p38 signaling as well as gene expression of inflammatory molecules. It will be interesting to determine if other inflammatory stimuli have similar effects on Trx1-ASK1 and 14-3-3-ASK1 complexes. Of note, laminar flow, the most potent physiological anti-atherosclerotic factor, can prevent the release of both inhibitors Trx1 and 14-3-3 from ASK1. Impotently, our data may provide insights why AGI-1067 failed to meet primary endpoint in late-stage (Phase III) trial as an anti-atherosclerotic drug, but did achieve other predefined endpoints (<http://www.firstwordpharma.com/node/111149>). Therefore, it needs to screen more potent anti-atherosclerotic drugs that can prevent both Trx1 and 14-3-3 release from ASK1. On the other hand, AGI-1067 may have beneficiary effects for other inflammatory diseases such as myocardial infarction and retinopathy as we demonstrated with ASK1 inhibitor GS-44421738 and other drugs [66].

References

- Hansson GK, Libby P (2006) The immune response in atherosclerosis: a double-edged sword. *Nat Rev Immunol* 6:508-19.
- Libby P (2002) Inflammation in atherosclerosis. *Nature* 420:868-74.
- Tabas I, Williams KJ, Boren J (2007) Sub endothelial lipoprotein retention as the initiating process in atherosclerosis: update and therapeutic implications. *Circulation* 116:1832-44.
- Hartvigsen K, Chou MY, Hansen LF, Shaw PX, Tsimikas S et al. (2009) The role of innate immunity in atherogenesis. *J Lipid Res* 50:5388-93.
- Hermansson A, Ketelhuth DF, Strothoff D, Wurm M, Hansson EM et al. (2010) Inhibition of T cell response to native low-density lipoprotein reduces atherosclerosis. *J Exp Med* 207: 1081-93.
- Huang Q, Qin L, Dai S, Zhang H, Pasula S et al. (2013) AIP1 suppresses atherosclerosis by limiting hyperlipidemia-induced inflammation and vascular endothelial dysfunction. *Arterioscler Thromb Vasc Biol* 33:795-804.
- L, Huang Q, Zhang H, Liu R, Tellides G (2014) SOCS1 prevents graft arteriosclerosis by preserving endothelial cell function. *J Am Coll Cardiol* 63: 21-9.
- Moore KJ, Tabas I (2011) Macrophages in the pathogenesis of atherosclerosis. *Cell* 145: 341-55.
- Pober JS, Sessa WC (2007) Evolving functions of endothelial cells in inflammation. *Nat Rev Immunol* 7: 803-15.
- Pober JS, Min W, Bradley JR (2009) Mechanisms of Endothelial Dysfunction, Injury, and Death. *Annu Rev Pathol* 4: 71-95.
- Wan T, Liu T, Zhang H, Tang S, Min W (2010) AIP1 functions as Arf6-GAP to negatively regulate TLR4 signaling. *J Biol Chem* 285: 3750-7.
- Blasi F, Denti F, Erba M, Cosentini R, Raccanelli R (1996) Detection of Chlamydia pneumoniae but not Helicobacter pylori in atherosclerotic plaques of aortic aneurysms. *J Clin Microbiol* 34: 2766-9.
- Chiu B, Viira E, Tucker W, Fong IW (1997) Chlamydia pneumoniae, cytomegalovirus, and herpes simplex virus in atherosclerosis of the carotid artery. *Circulation* 96: 2144-8.
- Kiechl S, Egger G, Mayr M, Wiedermann CJ, Bonora E et al. (2001) J. Chronic infections and the risk of carotid atherosclerosis: prospective results from a large population study. *Circulation* 103: 1064-70.
- Kuo CC, Shor A, Campbell LA, Fukushi H, Patton DL et al. (1993) Demonstration of Chlamydia pneumoniae in atherosclerotic lesions of coronary arteries. *J Infect Dis* 167: 841-9.
- Shor A, Kuo CC, Patton DL (1992) Detection of Chlamydia pneumoniae in coronary arterial fatty streaks and atheromatous plaques. *S Afr Med J* 82: 158-61.
- Wiedermann CJ, Kiechl S, Dunzendorfer S, Schratzberger P, Egger G et al. (1999) Association of endotoxemia with carotid atherosclerosis and cardiovascular disease: prospective results from the Bruneck Study. *J Am Coll Cardiol* 34:1975-81.
- Edfeldt K, Swedenborg J, Hansson GK, Yan ZQ (2002) Expression of toll-like receptors in human atherosclerotic lesions: a possible pathway for plaque activation. *Circulation* 105: 1158-61.
- Engelmann MG, Redl CV, Nikol S (2004) Recurrent perivascular inflammation induced by lipopolysaccharide (endotoxin) results in the formation of atheromatous lesions in vivo. *Lab Invest* 84: 425-32.
- Hollestelle SC, De Vries MR, Van Keulen JK, Schoneveld AH, Vink A (2004) Toll-like receptor 4 is involved in outward arterial remodeling. *Circulation* 109: 393-8.
- Michelsen KS, Wong MH, Shah PK, Zhang W, Yano J et al. (2004) Lack of Toll-like receptor 4 or myeloid differentiation factor 88 reduces atherosclerosis and alters plaque phenotype in mice deficient in Apo lipoprotein E. *Proc Natl Acad Sci U S A* 101:10679-84.
- Kol A, Sukhova GK, Lichtman AH, Libby P (1998) Chlamydial heat shock protein 60 localizes in human atheroma and regulates macrophage tumor necrosis factor-alpha and matrix metalloproteinase expression. *Circulation* 98: 300-7.

23. Miller YI, Viriyakosol S, Worrall DS, Boullier A, Butler S et al. (2005) Toll-like receptor 4-dependent and -independent cytokine secretion induced by minimally oxidized low-density lipoprotein in macrophages. *Arterioscler Thromb Vasc Biol* 25: 1213-9.
24. Okamura Y, Watari M, Jerud ES, Young DW, Ishizaka ST et al. (2001) The extra domain A of fibronectin activates Toll-like receptor 4. *J Biol Chem* 276: 10229-33.
25. Walton KA, Hsieh X, Gharavi N, Wang S, Wang G et al. (2003) Receptors involved in the oxidized 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphorylcholine-mediated synthesis of interleukin-8 A role for Toll-like receptor 4 and a glycosylphosphatidylinositol-anchored protein *J Biol Chem* 278: 29661-6.
26. Ichijo H, Nishida E, Irie K, ten Dijke P, Saitoh M et al. (1997) Induction of apoptosis by ASK1, a mammalian MAPKKK that activates SAPK/JNK and p38 signaling pathways. *Science* 275: 90-4.
27. Hattori K, Naguro I, Runchel C, Ichijo H (2009) The roles of ASK family proteins in stress responses and diseases. *Cell communication and signaling: CCS* 7:9.
28. Tobiume K, Matsuzawa A, Takahashi T, Nishitoh H, Morita K et al. (2001) ASK1 is required for sustained activations of JNK/p38 MAP kinases and apoptosis. *EMBO Rep* 2: 222-8.
29. Matsuzawa A, Saegusa K, Noguchi T, Sadamitsu C, Nishitoh H et al. (2005) ROS-dependent activation of the TRAF6-ASK1-p38 pathway is selectively required for TLR4-mediated innate immunity. *Nat Immunol* 6: 587-92.
30. Chiang E, Dang O, Anderson K, Matsuzawa A, Ichijo H et al. (2006) Cutting edge: apoptosis- regulating signal kinase 1 is required for reactive oxygen species-mediated activation of IFN regulatory factor 3 by lipopolysaccharide. *J Immunol* 176: 5720-4.
31. Liu H, Nishitoh H, Ichijo H, Kyriakis JM (2000) Activation of apoptosis signal-regulating kinase 1 (ASK1) by tumor necrosis factor receptor-associated factor 2 requires prior dissociation of the ASK1 inhibitor thioredoxin. *Mol Cell Biol* 20: 2198-208.
32. Song JJ, Lee YJ (2005) Dissociation of Akt1 from its negative regulator JIP1 is mediated through the ASK1-MEK-JNK signal transduction pathway during metabolic oxidative stress: a negative feedback loop. *J Cell Biol* 170: 61-72.
33. Zhang L, Chen J, Fu H (1999) Suppression of apoptosis signal-regulating kinase 1-induced cell death by 14-3-3 proteins. *Proc Natl Acad Sci U S A*. 96: 8511-5.
34. Liu Y, Yin G, Surapisitchat J, Berk BC, Min W (2001) Laminar flow inhibits TNF-induced ASK1 activation by preventing dissociation of ASK1 from its inhibitor 14-3-3. *J Clin Invest* 107: 917-23.
35. Zhang R, He X, Liu W, Lu M, Hsieh JT et al. (2003) AIP1 mediates TNF-alpha-induced ASK1 activation by facilitating dissociation of ASK1 from its inhibitor 14-3-3. *J Clin Invest* 111: 1933-43.
36. Zhang H, Zhang H, Lin Y, Li J, Pober JS et al. (2007) RIP1-mediated AIP1 phosphorylation at a 14-3-3- binding site is critical for tumor necrosis factor-induced ASK1-JNK/p38 activation. *J Biol Chem* 282: 14788-96.
37. Min W, Lin Y, Tang S, Yu L, Zhang H et al. (2008) AIP1 recruits phosphatase PP2A to ASK1 in tumor necrosis factor-induced ASK1-JNK activation. *Circ Res* 102: 840-8.
38. Huang Q, Zhou HJ, Zhang H, Huang Y, Hinojosa-Kirschenbaum F et al. (2015) Thioredoxin-2 Inhibits Mitochondrial ROS Generation and ASK1 Activity to Maintain Cardiac Function. *Circulation*.
39. Sundell CL, Somers PK, Meng CQ, Hoong LK, Suen KL et al. (2003) AGI-1067: a multifunctional phenolic antioxidant, lipid modulator, anti-inflammatory and antiatherosclerotic agent. *J Pharmacol Exp Ther* 305:1116-23.
40. Kunsch C, Luchoomun J, Grey JY, Olliff LK, Saint LB et al. (2004) Selective inhibition of endothelial and monocyte redox-sensitive genes by AGI-1067: a novel antioxidant and anti-inflammatory agent. *J Pharmacol Exp Ther* 308: 820-9.
41. Luyendyk JP, Piper JD, Tencati M, Reddy KV, Holscher T et al. (2007) A Novel Class of Antioxidants Inhibit LPS Induction of Tissue Factor by Selective Inhibition of the Activation of ASK1 and MAP Kinases. *Arterioscler Thromb Vasc Biol*
42. Meng CQ, Somers PK, Rachita CL, Holt LA, Hoong LK et al. (2002) Novel phenolic antioxidants as multifunctional inhibitors of inducible VCAM-1 expression for use in atherosclerosis. *Bioorg Med Chem Lett* 12: 2545-8.
43. Liu Y, Min W (2002) Thioredoxin promotes ASK1 ubiquitination and degradation to inhibit ASK1-mediated apoptosis in a redox activity-independent manner. *Circ Res* 90: 1259-1266.
44. Min W, Pober JS (1997) TNF initiates E-selectin transcription in human endothelial cells through parallel TRAF-NF-kappa B and TRAF-RAC/CDC42-JNK-c-Jun/ATF2 pathways. *J Immunol* 159: 3508-18.
45. Carlos T, Kovach N, Schwartz B, Rosa M, Newman B et al. (1991) Human monocytes bind to two cytokine-induced adhesive ligands on cultured human endothelial cells: endothelial-leukocyte adhesion molecule-1 and vascular cell adhesion molecule-1. *Blood* 77: 2266-71.
46. Ito T, Ikeda U (2003) Inflammatory cytokines and cardiovascular disease. *Curr Drug Targets Inflamm Allergy* 2:257-65.
47. Berk BC, Abe JI, Min W, Surapisitchat J, Yan C (2001) Endothelial atheroprotective and anti-inflammatory mechanisms. *Ann N Y Acad Sci* 947: 93-109.
48. Karin M, Liu Z, Zandi E (1997) AP-1 function and regulation. *Curr Opin Cell Biol* 9: 240-6.
49. Collins T, Read MA, Neish AS, Whitley MZ, Thanos D et al. (1995) Transcriptional regulation of endothelial cell adhesion molecules: NF-kappa B and cytokine-inducible enhancers. *Faseb J* 9: 899-909.
50. Karin M (2009) NF-kappaB as a critical link between inflammation and cancer. *Cold Spring Harb Perspect Biol* 1: a000141.
51. Pietersma A, Tilly BC, Gaestel M, de Jong N, Lee JC et al. (1997) p38 mitogen activated protein kinase regulates endothelial VCAM-1 expression at the post-transcriptional level. *Biochem Biophys Res Commun* 230: 44-8.
52. Rovin BH, Wilmer WA, Danne M, Dickerson JA, Dixon CL et al. (1999) The mitogen-activated protein kinase p38 is necessary for interleukin 1beta-induced monocyte chemoattractant protein 1 expression by human mesangial cells. *Cytokine* 11: 118-26.
53. Xu W, Yan M, Lu L, Sun L, Theze J et al. (2001) The p38 MAPK pathway is involved in the IL-2 induction of TNF-beta gene via the EBS element. *Biochem Biophys Res Commun* 289: 979-86.
54. Conze D, Krahl T, Kennedy N, Weiss L, Lumsden J et al. (2002) c-Jun NH(2)-terminal kinase (JNK)1 and JNK2 have distinct roles in CD8(+) T cell activation. *J Exp Med* 195: 811-23.
55. Manning AM, Davis RJ (2003) Targeting JNK for therapeutic benefit: from junk to gold? *Nat Rev Drug Discov* 2: 554-65.
56. Yasumoto H, Kim S, Zhan Y, Miyazaki H, Hoshiga M et al. (2001) Dominant transfer inhibits vascular smooth muscle cell proliferation and neointimal hyperplasia in rats. *Gene Ther* 8: 1682-9.
57. Gupta S, Campbell D, D'Erijard B, Davis RJ (1995) Transcription factor ATF2 regulation by the JNK signal transduction pathway. *Science* 267: 389-93.
58. Reimold AM, Kim J, Finberg R, Glimcher LH (2001) Decreased immediate inflammatory gene induction in activating transcription factor-2 mutant mice. *Int Immunol* 13: 241-8.
59. Seino Y, Ikeda U, Ikeda M, Yamamoto K, Misawa Y et al. (1994) Interleukin 6 gene transcripts are expressed in human atherosclerotic lesions. *Cytokine* 6: 87-91.
60. Ridker PM, Rifai N, Stampfer MJ, Hennekens CH (2000) Plasma concentration of interleukin-6 and the risk of future myocardial infarction among apparently healthy men. *Circulation* 101: 1767-72.
61. Aiello RJ, Bourassa PA, Lindsey S, Weng W, Natoli E et al. (1999) Monocyte chemoattractant protein-1 accelerates atherosclerosis in apolipoprotein E-deficient mice. *Arterioscler Thromb Vasc Biol* 19: 1518-25.
62. Gosling J, Slaymaker S, Gu L, Tseng S, Zlot CH et al. (1999) MCP-1 deficiency reduces susceptibility to atherosclerosis in mice that overexpress human apolipoprotein B. *J Clin Invest* 103: 773-8.
63. Saitoh M, Nishitoh H, Fujii M, Takeda K, Tobiume K (1998) Mammalian thioredoxin is a direct inhibitor of apoptosis signal- regulating kinase (ASK) 1. *Embo J* 17: 2596-606.
64. Zhang R, Al-Lamki R, Bai L, Streb JW, Miano JM et al. (2004) Thioredoxin-2 inhibits mitochondria-located ASK1-mediated apoptosis in a JNK-independent manner. *Circ Res* 94: 1483-91.
65. Ji W, Yang M, Praggastis A, Li Y, He Y et al. (2014) Carbamoylating activity associated with the activation of the antitumor agent laromustine inhibits angiogenesis by inducing ASK1-dependent endothelial cell death *PLoS One* 9: e103224.
66. Liang X, Zhou H, Ding Y, Li J, Yang C et al. (2012) TMP Prevents Retinal Neovascularization and Imparts Neuroprotection in an Oxygen-Induced Retinopathy Model. *Invest Ophthalmol Vis Sci*. 53: 2157-69.