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

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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 academicals labs. We have recently shown that a selective ASK1 inhibitor GS-444217, which

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specifically binds to an active form of ASK1 homodimer, can effecively block cardiomyopathy in a mouse model [38]. In the present study, we investigate another type of ASK1 AGI-1067. AG-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-alpha (TNF-α) induced Vascular Cell Adhesion Molecule-1 (VCAM-1) and Monocyte Chemo Attractant Protein-1 (MCP-1) expression without affecting NF-κB 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 mechansim by which AGI-1067 inhibts 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 μg/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 μg/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 μg of cell lysate supernatant were pre cleared by incubating with 5 μg of normal rabbit serum plus protein A/G-agarose beads on rotator at 4°C overnight. The lysates were then incubated with 5 μg of the first protein- specific antiserum for 2 hr with 50 μl of protein A/G-agarose beads. The immune complexes were collected after each immunoprecipitation by centrifugation at 14,000 × *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 ± 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-selection induced by the pro-inflammatory ligands LPS. AGI-1067 inhibits the LPS-induced expression of VCAM-1 and E-selection in a concentration-dependent manner with IC50 values of approximately 4.5 μM 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 μM, 3.8 μM and 4.0 μM, 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 μM 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 μM (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

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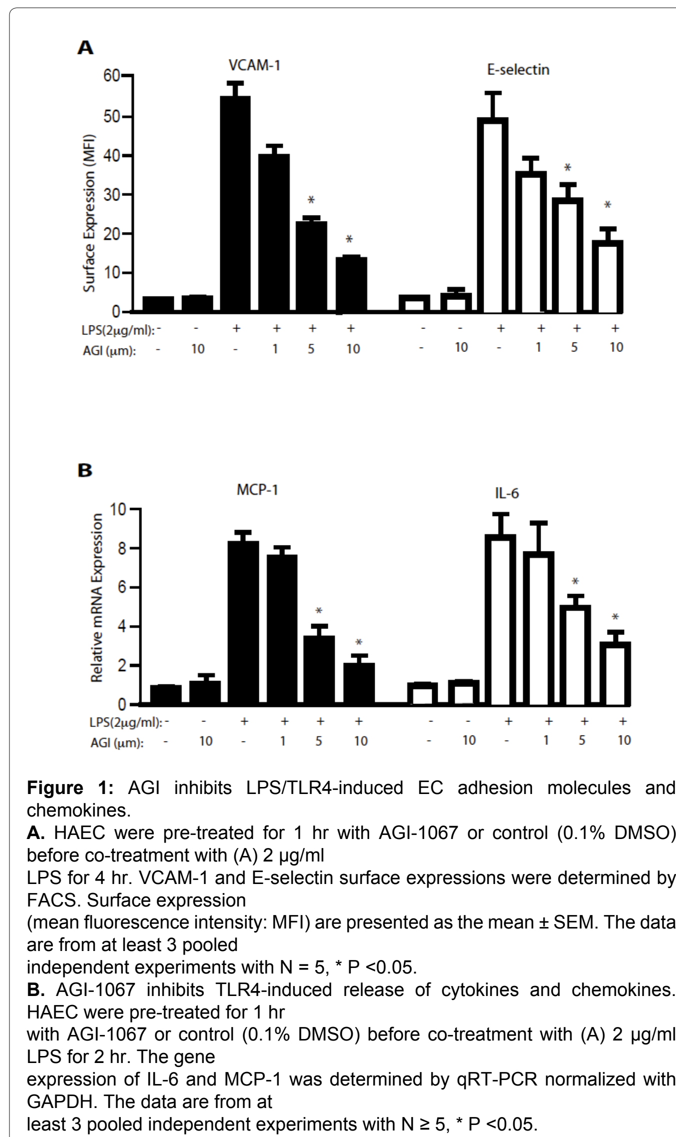

Figure 1: AGI inhibits LPS/TLR4-induced EC adhesion molecules and chemokines.
A. HAEC were pre-treated for 1 hr with AGI-1067 or control (0.1% DMSO) before co-treatment with (A) 2 μg/ml
LPS for 4 hr. VCAM-1 and E-selectin surface expressions were determined by FACS. Surface expression
(mean fluorescence intensity: MFI) are presented as the mean ± SEM. The data are from at least 3 pooled
independent experiments with N = 5, * P <0.05.
B. AGI-1067 inhibits TLR4-induced release of cytokines and chemokines. HAEC were pre-treated for 1 hr
with AGI-1067 or control (0.1% DMSO) before co-treatment with (A) 2 μg/ml LPS for 2 hr. The gene
expression of IL-6 and MCP-1 was determined by qRT-PCR normalized with GAPDH. The data are from at
least 3 pooled independent experiments with N ≥ 5, * P <0.05.

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-a 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 proinflamamtory 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-a, 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-a, IL-2, E-selectin, VCAM-1 and MMPs such as collagease-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

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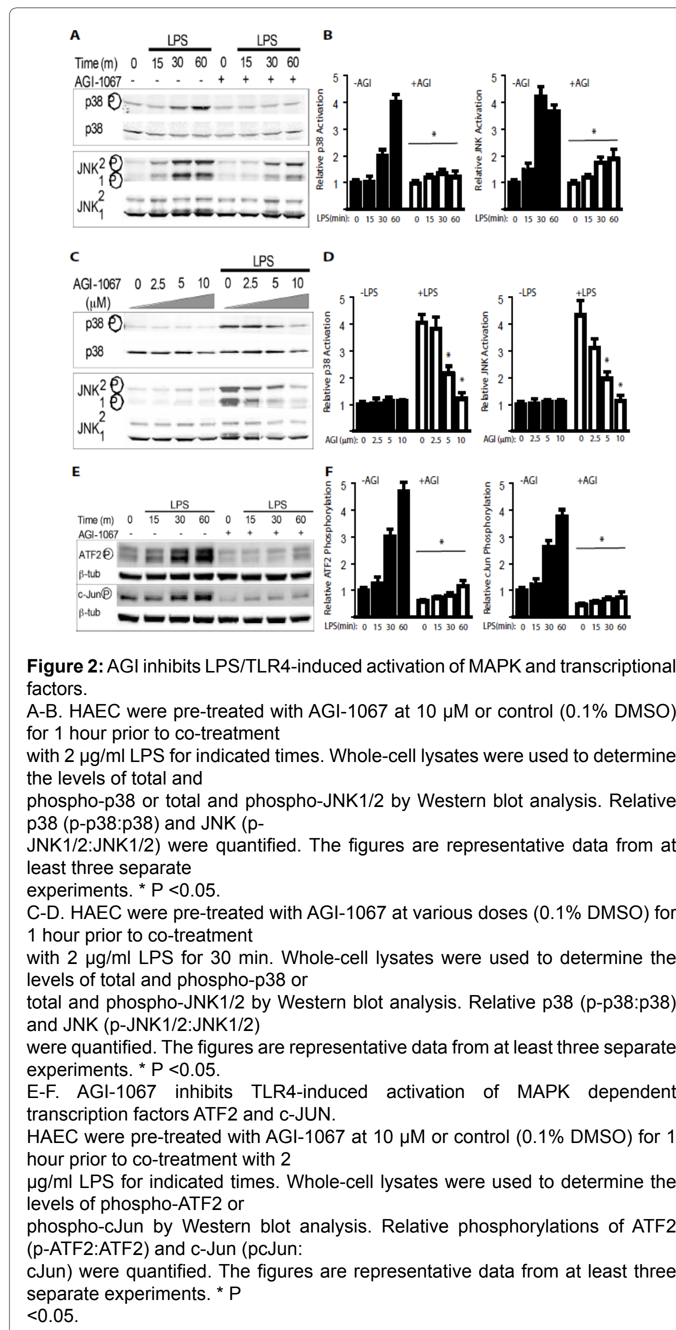

Figure 2: AGI inhibits LPS/TLR4-induced activation of MAPK and transcriptional factors.
A-B. HAEC were pre-treated with AGI-1067 at 10 μM or control (0.1% DMSO) for 1 hour prior to co-treatment
with 2 μg/ml LPS for indicated times. Whole-cell lysates were used to determine the levels of total and
phospho-p38 or total and phospho-JNK1/2 by Western blot analysis. Relative p38 (p-p38:p38) and JNK (p-
JNK1/2:JNK1/2) were quantified. The figures are representative data from at least three separate
experiments. * P <0.05.
C-D. HAEC were pre-treated with AGI-1067 at various doses (0.1% DMSO) for 1 hour prior to co-treatment
with 2 μg/ml LPS for 30 min. Whole-cell lysates were used to determine the levels of total and phospho-p38 or
total and phospho-JNK1/2 by Western blot analysis. Relative p38 (p-p38:p38) and JNK (p-JNK1/2:JNK1/2)
were quantified. The figures are representative data from at least three separate experiments. * P <0.05.
E-F. AGI-1067 inhibits TLR4-induced activation of MAPK dependent transcription factors ATF2 and c-JUN.
HAEC were pre-treated with AGI-1067 at 10 μM or control (0.1% DMSO) for 1 hour prior to co-treatment with 2
μg/ml LPS for indicated times. Whole-cell lysates were used to determine the levels of phospho-ATF2 or
phospho-cJun by Western blot analysis. Relative phosphorylations of ATF2 (p-ATF2:ATF2) and c-Jun (pcJun:
cJun) were quantified. The figures are representative data from at least three separate experiments. * P
<0.05.

cell TLR4-mediated activation of p38 and JNK MAP kinases and expression of cytokines and chemokine's. 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

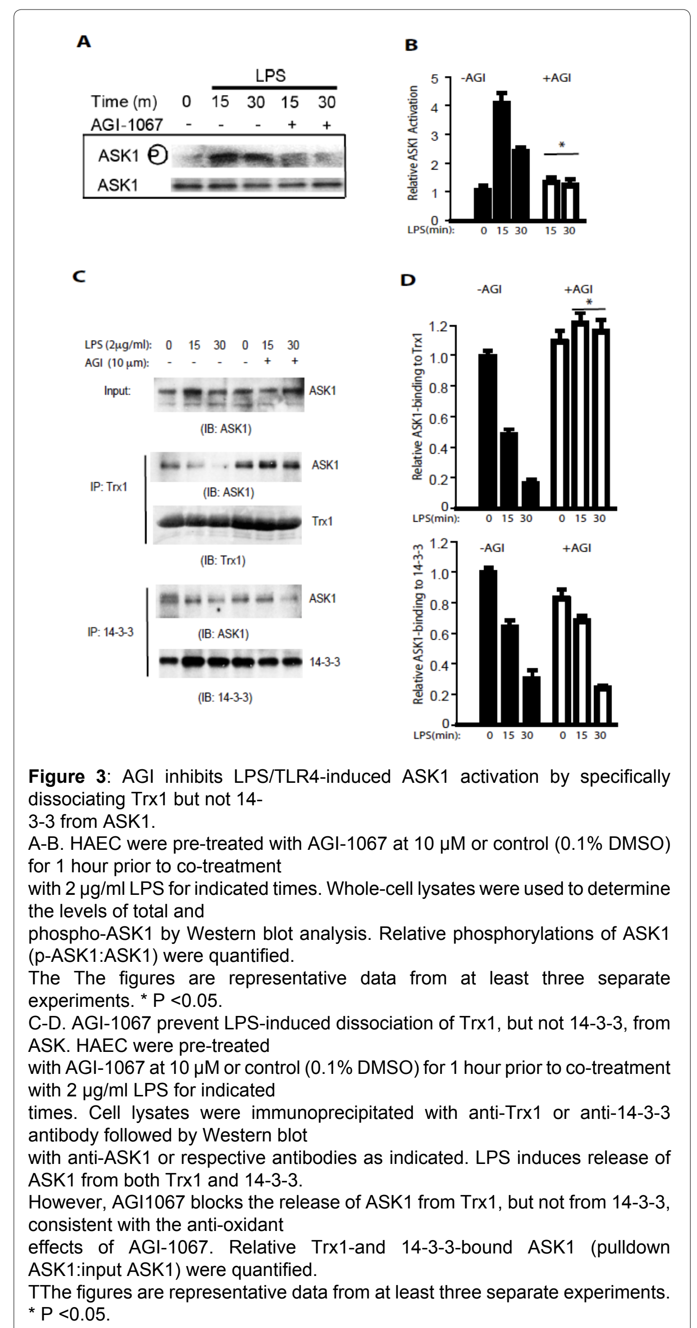

Figure 3: AGI inhibits LPS/TLR4-induced ASK1 activation by specifically dissociating Trx1 but not 14-
3-3 from ASK1.
A-B. HAEC were pre-treated with AGI-1067 at 10 μM or control (0.1% DMSO) for 1 hour prior to co-treatment
with 2 μg/ml LPS for indicated times. Whole-cell lysates were used to determine the levels of total and
phospho-ASK1 by Western blot analysis. Relative phosphorylations of ASK1 (p-ASK1:ASK1) were quantified.
The The figures are representative data from at least three separate experiments. * P <0.05.
C-D. AGI-1067 prevent LPS-induced dissociation of Trx1, but not 14-3-3, from ASK. HAEC were pre-treated
with AGI-1067 at 10 μM or control (0.1% DMSO) for 1 hour prior to co-treatment with 2 μg/ml LPS for indicated
times. Cell lysates were immunoprecipitated with anti-Trx1 or anti-14-3-3 antibody followed by Western blot
with anti-ASK1 or respective antibodies as indicated. LPS induces release of ASK1 from both Trx1 and 14-3-3.
However, AGI1067 blocks the release of ASK1 from Trx1, but not from 14-3-3, consistent with the anti-oxidant
effects of AGI-1067. Relative Trx1-and 14-3-3-bound ASK1 (pulldown ASK1:input ASK1) were quantified.
TThe figures are representative data from at least three separate experiments. * P <0.05.

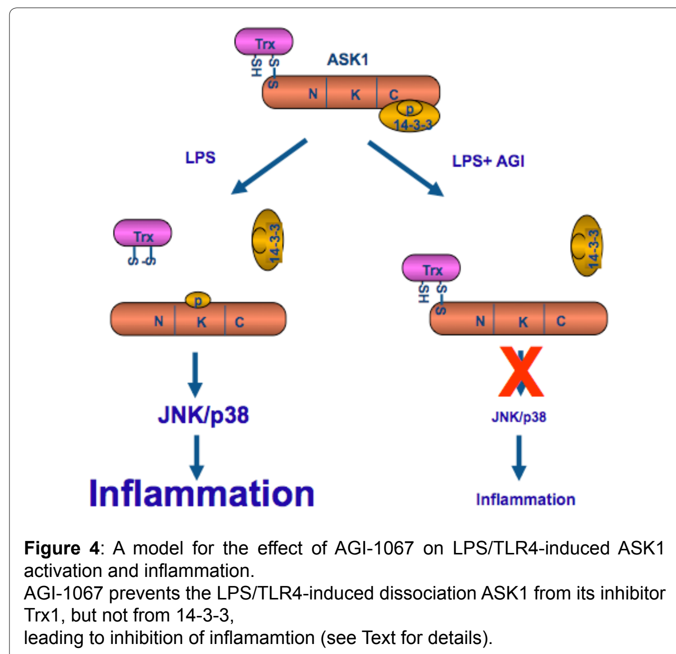

Figure 4: A model for the effect of AGI-1067 on LPS/TLR4-induced ASK1 activation and inflammation.
AGI-1067 prevents the LPS/TLR4-induced dissociation ASK1 from its inhibitor Trx1, but not from 14-3-3,
leading to inhibition of inflamamtion (see Text for details).

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].

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