A Novel Class of Antioxidants Inhibit LPS Induction of Tissue Factor by Selective Inhibition of the Activation of ASK1 and MAP Kinases

James P. Luyendyk, J. Daniel Piper, Michael Tencati, K. Veera Reddy, Todd Holscher, Rong Zhang, Jayraz Luchoomun, Xilin Chen, Wang Min, Charles Kunsch, Nigel Mackman

Objective—Oxidative stress contributes to the pathogenesis of many diseases, including atherosclerosis and sepsis. We have previously described a novel class of therapeutic compounds with antioxidant and antiinflammatory properties. However, at present, the intracellular targets of these compounds have not been identified. The purpose of this study was to elucidate the mechanism by which 2 structurally-related antioxidants (AGI-1067 and AGI-1095) inhibit LPS induction of tissue factor (TF) expression in human mononcytic cells and endothelial cells.

Methods and Results—We found that succinobucol (AGI-1067) and AGI-1095 inhibited LPS induction of TF expression in both mononcytic cells and endothelial cells. These compounds also reduced LPS induction of nuclear AP-1 and expression of Egr-1 without affecting nuclear translocation of NF-κB. Importantly, these antioxidants inhibited LPS activation of the redox-sensitive kinase, apoptosis signal-regulating kinase-1 (ASK1) and the mitogen-activated protein kinases (MAPKs) p38, ERK1/2, and JNK1/2.

Conclusions—AGI-1067 and AGI-1095 inhibit TF gene expression in both mononcytic cells and endothelial cells through a mechanism that involves the inhibition of the redox-sensitive MAP3K, ASK1. These compounds selectively reduce the activation/induction of MAPK, AP-1, and Egr-1 without affecting NF-κB nuclear translocation. (Arterioscler Thromb Vasc Biol. 2007;27:1857-1863.)

Key Words: tissue factor ▪ LPS ▪ oxidative stress ▪ ASK1 ▪ AGI-1067

Oxidative stress contributes to the pathogenesis of many inflammatory diseases, including atherosclerosis and sepsis.1,2 Reactive oxygen species (ROS) are critical regulators of cellular homeostasis. However, disruption of the intracellular prooxidant/antioxidant balance causes excessive ROS generation, modulation of intracellular signaling, and oxidative stress.3 The potential benefit of antioxidant vitamins such as vitamins C and E as therapeutic agents for oxidative stress.3 The potential benefit of antioxidant vitamins such as vitamins C and E as therapeutic agents for cardiovascular disease has been aggressively debated. Although several studies have indicated benefits of antioxidant therapy in the treatment of various cardiovascular diseases, it has not been effective in all cases.4

Probucol is an antioxidant drug that exhibits antiatherosclerotic and antirestenotic activity in patients with cardiovascular disease.5 However, treatment with probucol was associated with several side effects, including prolongation of the cardiac QT interval and decreased circulating HDL levels.5 Recently, a new class of chemically and metabolically-stable probucol derivatives has been synthesized that retain antioxidant activity and display enhanced cellular antioxidant and antiinflammatory activity compared with probucol, which may be attributable to enhanced cellular uptake.6,7 One of these compounds, succinobucol (AGI-1067), was found to reduce atherosclerosis in several animal models including hypercholesterolemic primates.8 In addition, clinical studies with AGI-1067 have demonstrated its ability to decrease the rate of restenosis and regress coronary atherosclerosis.9–11 AGI-1067 is currently being evaluated in a large phase III clinical trial called The Aggressive Reduction of Inflammation Stops Events (ARISE) trial that will determine the effect of AGI-1067 on major cardiovascular events, including stroke, myocardial infarction, and death.

AGI-1067 and related compounds also possess antiinflammatory activity.6,7,12 We have shown that both AGI-1067 and a related compound, AGIX-4207, inhibited tumor necrosis factor (TNF)-α induction of monocyte chemoattractant protein-1 (MCP-1) and vascular cell adhesion molecule (VCAM)-1 expression in endothelial cells and LPS induction of TNF-α and interleukin (IL)-6 expression in peripheral blood mononuclear cells (PBMCs).6,7 Importantly, nuclear
translocation of NF-κB was not affected by these compounds, suggesting that the antiinflammatory effects of these compounds are mediated by inhibition of intracellular pathways other than the NF-κB pathway. At present, the precise mechanism by which these compounds reduce inflammation has not been elucidated.

Bacterial LPS, inflammatory cytokines, and oxidized lipids all induce oxidative stress in cells and increase ROS. This leads to the activation of many intracellular signaling pathways, such as the mitogen activated protein kinase (MAPK) and 1×kβ kinase pathways, and ultimately activation of transcription factors such as AP-1, Egr-1, and NF-κB. The MAPK pathways include ERK1/2, p38, and JNK1/2. A key redox-regulated kinase that controls the activation of MAPK pathways is ASK1. The inactive form of ASK1 is bound to thioredoxin and to 14-3-3 proteins. Oxidation of thioredoxin and the release of 14-3-3 results in the activation of ASK1 and the subsequent activation of p38. Recently, it was shown that LPS-mediated ROS production leads to the activation of ASK1. Moreover, the ROS-dependent TRAF6-ASK1-p38 axis plays a crucial role in TLR4-mediated mammalian innate immunity.

LPS stimulation of monocytes and endothelial cells induces the expression of the procoagulant protein tissue factor (TF). Oxidized low-density lipoproteins (ox-LDL) also induce TF expression in endothelial cells and pathologic expression of TF within the vasculature leads to disseminated intravascular coagulation. In addition, high levels of TF are present in atherosclerotic plaques and likely contribute to thrombosis after plaque rupture. Importantly, several studies have shown that antioxidants inhibit LPS induction of TF expression in monocytes, macrophages, and endothelial cells. These results suggest that inhibition of ROS-dependent intracellular signaling may be an effective strategy for reducing TF expression and thrombotic complications associated with inflammatory diseases, such as atherosclerosis and sepsis.

We and others have characterized the intracellular signaling pathways and transcription factors that mediate LPS induction of TF gene expression in monocytes and endothelial cells. Activation/inhibition of the transcription factors AP-1, NF-κB, and Egr-1 was required for maximal induction of TF expression. In addition, inhibition of the MAPK pathways, ERK1/2 and p38, reduced LPS-induced TF expression in monocytes and endothelial cells.

In this study, we demonstrate that 2 novel antioxidant compounds, AGI-1067 and AGI-1095, inhibit LPS induction of TF expression in human monocytes and endothelial cells. Importantly, these compounds inhibited LPS activation of the redox-sensitive kinase ASK1, as well as the downstream MAPK pathways ERK1/2, JNK1/2, and p38, and the transcription factors AP-1 and Egr-1 without affecting the nuclear translocation of NF-κB.

**Methods**

**Materials**

LPS (E coli serotype 0111:B4 or 026:B6), dimethylsulfoxide (DMSO), and pyrrolidine dithiocarbamate (PDTC) were purchased from Sigma-Aldrich. SP600125 were purchased from EMD Biosciences Inc. The antioxidant compounds AGI-1067 and AGI-1095 were synthesized by AtheroGenics Inc, and their chemical structures have been described previously. Compounds were dissolved in DMSO.

**Cell Culture**

The human monocyte THP-1 cells were obtained from American Type Culture Collection (Manassas, Va), and human aortic endothelial cells (HAECs) were obtained from Cambrex (Walkersville, Md). PBMCs were isolated from citrated blood from healthy volunteers by buoyant density centrifugation on low endotoxin Ficoll-Paque Plus (GE Healthcare). Cells were pretreated with compounds for either 30 or 60 minutes before the addition of LPS.

**Tissue Factor Activity**

TF activity in cell lysates was measured using a 1-stage clotting assay.

**Western Blotting**

Levels of IκBα and Egr-1 were determined using antibodies from Santa Cruz Biotechnology. Activation of ERK1/2, p38, and JNK1/2 in THP-1 cells was assessed using antiphosphospecific antibodies (New England Biolabs). Activation of p38 and JNK1/2 in HAECs was evaluated with an antiphosphospecific p38 antibody (Cell Signaling Technology) and an antiphosphospecific JNK1/2 antibody (Biosource International), respectively. Activation of ASK1 was assessed by measuring phosphorylation of Thr845 using a rabbit polyclonal anti-ASK1 Thr845 antibody (Cell Signaling Technology). Nonphosphospecific forms of each protein were used to monitor loading.

**Northern Blotting**

The level of TF mRNA was determined by Northern blotting. Blots were rehybridized with the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) for monitoring loading.

**Electrophoretic Mobility Shift Assay**

Nuclear extracts were incubated with a radiolabeled double-stranded oligonucleotide probe (Operon Technologies) containing a prototypic AP-1 site, a kβ site from the murine IgG gene, or the human TF kβ site. Protein-DNA complexes were separated from free probe by electrophoresis through 6% nondenaturing acrylamide gels (Invitrogen) using 0.5X Tris borate EDTA (TBE) buffer and visualized by autoradiography.

**Evaluation of Cytotoxicity**

Cell viability was evaluated by Hoescht staining and by trypan blue exclusion.

**Statistics**

All experiments were performed at least 3 independent times. Statistical analyses were performed using SigmaStat version 3.1 (SPSS Inc). Student t test was used when only 2 groups were compared. For comparisons of more than 2 groups, data were analyzed by ANOVA with Tukey post-hoc test. The criterion for significance for all studies was P<0.05.

**Results**

AGI-1067 and AGI-1095 Inhibit LPS-Induced TF Activity in Monocytic Cells and Endothelial Cells

In this study, we investigated the effect of AGI-1067 and AGI-1095 on LPS induction of TF expression in human monocytes and endothelial cells. Both AGI-1067 and AGI-1095 reduced LPS induction of TF activity in THP-1 monocytic cells and PBMCs in a concentration-dependent manner (Figure 1). In addition, both compounds inhibited...
LPS-induced TF activity in HAECs (Figure 1). These compounds did not cause cytotoxicity (data not shown). These results indicate that AGI-1067 and AGI-1095 inhibit LPS induction of TF activity in monocytic cells and endothelial cells.

Inhibition of LPS-Induced TF mRNA Expression in THP-1 Monocytic Cells by AGI-1067 and AGI-1095

To determine whether AGI-1067 and AGI-1095 inhibited LPS induction of TF mRNA expression in THP-1 monocytic cells, we measured levels of TF mRNA. LPS induced TF mRNA expression in THP-1 cells (Figure 2A). The larger band is an alternatively spliced transcript that contains the majority of intron 1. We found that both compounds inhibited the increase in TF mRNA expression in LPS-stimulated THP-1 cells (Figure 2A). AGI-1067 and AGI-1095 in monocytic cells reduced LPS-induced TF mRNA expression by 59±7% and 68±18% (mean±SD, n=3), respectively. These results suggest that these antioxidant compounds inhibit TF expression at the level of gene transcription in monocytic cells.

Effect of AGI-1067 and AGI-1095 on the LPS-Induced Increase in Nuclear AP-1, Induction of Egr-1 Expression, and Nuclear Translocation of NF-κB

The transcription factors AP-1, NF-κB, and Egr-1 are required for the induction of TF gene expression in monocytic cells. Therefore, we analyzed the effect of AGI-1067 and AGI-1095 on LPS induction of Egr-1 expression, nuclear AP-1, and nuclear translocation of NF-κB. LPS stimulation induced a time-dependent increase in Egr-1 protein expression, which was inhibited by pretreatment of the cells with...
either of the antioxidant compounds (Figure 2B). AGI-1067 and AGI-1095 reduced LPS-induced Egr-1 expression by 83±8% and 74±9% (mean±SD, n=3), respectively. AGI-1067 and AGI-1095 also significantly reduced LPS induction of nuclear AP-1 (Figure 3A). LPS-induced nuclear translocation of NF-κB requires degradation of the cytoplasmic inhibitor IκBα. Therefore, we first analyzed the effect of AGI-1067 and AGI-1095 on LPS-induced degradation of IκBα. The antioxidants did not affect degradation of IκBα in THP-1 cells (Figure 3B). Similar results were observed using HAECs (data not shown). Next, we analyzed nuclear translocation of NF-κB by EMSA. AGI-1067 and AGI-1095 did not affect LPS-induced nuclear translocation of NF-κB(p50/p65) or c-Rel/p65 in THP-1 cells (Figure 3C). The c-Rel/p65 heterodimer binds to the TF κB site.24 In contrast, and consistent with a previous study,31 the antioxidant PDTC (100 μmol/L) significantly reduced LPS-induced nuclear translocation of NF-κB (data not shown). Taken together, these data indicate that AGI-1067 and AGI-1095 inhibit LPS induction of Egr-1 expression and nuclear levels of AP-1 without affecting the nuclear translocation of NF-κB.

Inhibition of LPS Activation of MAPKs in Monocytic Cells and Endothelial Cells by AGI-1067 and AGI-1095

The MAPKs ERK1/2 and p38 regulate LPS induction of TF gene expression in monocytic and endothelial cells by activating various transcription factors. We found that inhibition of JNK1/2 with the inhibitor SP600125 (45 μmol/L) significantly reduced LPS-induced TF expression in THP-1 cells (data not shown), indicating that activation of JNK1/2 is required for LPS induction of TF expression. JNK1/2 and p38 regulate the activation and expression of AP-1, whereas ERK1/2 regulates Egr-1 expression.32 Therefore, we determined the effect of AGI-1067 and AGI-1095 on LPS activation of various MAPK pathways in monocytic cells and
endothelial cells. The activation of all 3 MAPK pathways was strongly inhibited by AGI-1067 and AGI-1095 (Figure 4). Similarly, AGI-1067 and AGI-1095 reduced LPS activation of both p38 and JNK1/2 in HAECs (supplemental Figure I, available online at http://atvb.ahajournals.org). LPS activation of ERK1/2 could not be evaluated in LPS-treated HAECs because of a high basal ERK1/2 phosphorylation in these cells (data not shown). The levels of inhibition of the different MAPK pathways by AGI-1067 and AGI-1095 are shown in supplemental Table I. AGI-1067 (5 μmol/L) and AGI-1095 (5 μmol/L) also inhibited LPS activation of p38 in PBMCs (data not shown). These results demonstrate that these antioxidants inhibit LPS activation of MAPK pathways in both monocytic and endothelial cells.

LPS Activation of ASK1 Is Inhibited by AGI-1067 and AGI-1095 in Monocytic and Endothelial Cells

Because ASK1 is a redox-regulated MAP3K that is activated in cells exposed to LPS and regulates various MAPK pathways, we determined whether LPS activation of ASK1 was inhibited by AGI-1067 and AGI-1095. LPS treatment increased the phosphorylation of ASK1 in THP-1 cells within 15 minutes, and this activation was inhibited by pretreatment with either AGI-1067 or AGI-1095 (Figure 5A). Similarly, LPS activated ASK1 in HAECs, and this activation was inhibited by treatment with AGI-1067 (Figure 5B). These data indicate that these antioxidant compounds inhibit LPS activation of ASK1 in monocytic and endothelial cells.

Discussion

In this study, we determined a mechanism by which a new class of antioxidant compounds derived from probucol inhibits LPS induction of TF expression in human monocytic cells and endothelial cells. LPS stimulation leads to the activation

**Figure 4.** AGI-1067 and AGI-1095 inhibit activation of MAPKs in LPS-stimulated THP-1 cells. THP-1 cells were pretreated with AGI-1067 (5 μmol/L), AGI-1095 (5 μmol/L), or vehicle for 30 minutes before stimulation with LPS (10 μg/mL). At various times after LPS treatment (0 to 60 minutes), whole cell lysates were prepared, and phospho-ERK1/2, phospho-p38, and phospho-JNK1/2 levels were analyzed by Western blotting. Results from a representative experiment of 3 independent experiments are shown. Normalized levels of the different phosphorylated proteins are shown below the blots.

**Figure 5.** AGI-1067 and AGI-1095 inhibit LPS activation of ASK1 in monocytic and endothelial cells. A, THP-1 cells were pretreated with AGI-1067 (5 μmol/L), AGI-1095 (5 μmol/L), or vehicle for 30 minutes before stimulation with LPS (10 μg/mL). B, HAECs were pretreated with AGI-1067 (10 μmol/L), or vehicle for 60 minutes before stimulation with LPS (2 μg/mL). At various times after LPS treatment (0 to 60 minutes), whole cell lysates were prepared and phospho-ASK1 (T845) levels were analyzed by Western blotting. Each blot was stripped and reprobed for the nonphosphorylated form of ASK1. Results from a representative experiment of 3 independent experiments are shown.
of various ROS-sensitive intracellular signaling pathways (eg, MAPKs) and transcription factors (NF-κB, AP-1, and Egr-1) that mediate the induction of TF expression.\textsuperscript{3,23,26,32–34} We found that the antioxidants inhibited LPS induction of Egr-1, AP-1, and the activation of MAPK pathways. However, these compounds did not reduce the nuclear translocation of NF-κB. In contrast, the antioxidants PDTC and N-acetyl cysteine (NAC) inhibit LPS induction of inflammatory genes and TF in monocytes, macrophages, and endothelial cells by reducing NF-κB activation.\textsuperscript{6,21,22,31,35,36} These results indicate that AGI-1067 and AGI-1095 inhibit LPS induction of gene expression via a mechanism that is distinct from other antioxidant compounds, such as PDTC and NAC (Figure 6). Other antioxidant compounds (ie, flavonoids) also do not inhibit inducible NF-κB activation\textsuperscript{37,38} despite their ability to inhibit inflammatory gene expression in endothelial cells. Currently, it is not clear what governs the ability of some antioxidants, but not others, to inhibit inducible NF-κB activation.

How do AGI-1067 and AGI-1095 inhibit LPS activation of the MAPK signaling pathways? Interestingly, a recent article showed that activation of the redox-sensitive MAP3K ASK1 is critical for LPS induction of inflammatory cytokines, but not for the activation of the NF-κB pathway.\textsuperscript{16} We found that LPS activated ASK1 in monocytes and endothelial cells, and that ASK1 activation was inhibited by both AGI-1067 and AGI-1095. Thus, the inhibition of ASK1 by these compounds may account for the selective inhibition of the MAPK pathways without affecting the NF-κB pathway (Figure 6). Importantly, we showed that these antioxidants inhibited LPS activation of the 3 major MAPK pathways, ERK1/2, JNK1/2, and p38, which control the activation of AP-1 and Egr-1. A recent study found that LPS activation of ASK1 was required for p38, but not JNK1/2 signaling.\textsuperscript{16} However, other studies have found that ASK1 was required for the activation of JNK1/2 signaling in cells stimulated by TNFα.\textsuperscript{39} Thus, consistent with the inhibition of ASK1 by AGI-1067 and AGI-1095, both compounds inhibited activation of JNK1/2 and p38. Although a role for ASK1 in the LPS activation of ERK1/2 has not yet been established, our data suggest that activation of ASK1 may be required for the activation of ERK1/2. Alternatively, these compounds may inhibit ERK1/2 by affecting another upstream activator of this pathway. Taken together, our results suggest that unlike other antioxidants, such as PDTC and NAC, AGI-1067 and AGI-1095 selectively inhibit LPS activation of ASK1 and MAPK signaling pathways without affecting the nuclear translocation of NF-κB (Figure 6).

Oxidative stress and inflammation contribute to the initiation and progression of atherosclerosis. One mechanism by which the generation of ROS, such as superoxide, can contribute to the development of atherosclerotic lesions is through the formation of oxidized proteins and lipoproteins such as LDL.\textsuperscript{40,41} In addition, intracellular ROS have been shown to modulate intracellular signaling pathways and inflammatory gene expression in the vasculature.\textsuperscript{42} We have previously found that this class of antioxidants inhibited expression of inflammatory cytokines and adhesion molecules, such as VCAM-1 and MCP-1, in monocytes and endothelial cells both in vitro and in vivo.\textsuperscript{6,8} Inhibition of the expression of these inflammatory mediators may reduce the accumulation of inflammatory cells, such as macrophages, into the atherosclerotic lesion. Indeed, AGI-1067 reduced the size of atherosclerotic lesions in hypercholesterolemic rabbits, LDLR\textsuperscript{−/−} mice, ApoE\textsuperscript{−/−} mice, hypercholesterolemic primates, and coronary atherosclerosis in humans.\textsuperscript{6,8,9} Here, we show that both AGI-1067 and AGI-1095 reduced MAPK activity and TF expression in monocyteic cells and endothelial cells. Importantly, the thrombogenicity of atherosclerotic plaques is associated with an increase in TF expression.\textsuperscript{20} Plaque rupture results in exposure of TF to circulating coagulation factors, which can lead to myocardial infarction and stroke.\textsuperscript{20} Thus, a reduction in TF expression is one potential mechanism by which these antioxidants may reduce the risk of myocardial infarction.

Taken together, these findings indicate that this novel class of antioxidants may inhibit the development of atherosclerotic lesions, in part, by reducing ROS and inhibiting the activity of the redox-sensitive kinase ASK1 in both monocytes and endothelial cells, resulting in the inhibition of inflammatory mediators and TF expression. This study therefore provides a molecular mechanism for how this class of antioxidants may target redox-sensitive signaling pathways that modulate inflammatory and prothrombotic processes.\textsuperscript{7}

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Disclosures
None.

References
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Antioxidant Inhibition of TF Expression


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Figure I. AGI-1067 and AGI-1095 inhibit LPS induction of TF protein expression in HAECs. HAECs were pretreated with AGI-1067 (10 µM), AGI-1095 (5 µM) or vehicle for 60 minutes prior to stimulation with LPS (2 µg/ml). At various times after LPS treatment (0-60 minutes), whole cell lysates were prepared and phospho-p38 and phospho-JNK1/2 levels were analyzed by western blotting. Each blot was stripped and reprobed for the non-phosphorylated form of each MAPK. For B, western blots were developed using Licor Odyssey technology.

Table I: Inhibition of LPS activation of MAPK pathways by AGI-1067 and AGI-1095

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<td>THP-1</td>
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Numbers show percentage inhibition of LPS activation of MAPK pathways using either AGI-1067 or AGI-1095 (mean ± SD) at 60 minutes for 3 independent experiments. For THP-1 cells 5µM of each compound was used, whereas for HAEC we used 10µM of AGI-1067 and 5µM of AGI-1095. *p<0.05