Acadesine Inhibits Tissue Factor Induction and Thrombus Formation by Activating the Phosphoinositide 3-Kinase/Akt Signaling Pathway

Weiyu Zhang, Jianguo Wang, Huan Wang, Rong Tang, John D. Belcher, Benoit Viollet, Jian-Guo Geng, Chunxiang Zhang, Chaodong Wu, Arne Slungaard, Chuhong Zhu, Yuqing Huo

Objective—Acadesine, an adenosine-regulating agent and activator of AMP-activated protein kinase, has been shown to possess antiinflammatory activity. This study investigated whether and how acadesine inhibits tissue factor (TF) expression and thrombus formation.

Methods and Results—Human umbilical vein endothelial cells and human peripheral blood monocytes were stimulated with lipopolysaccharide to induce TF expression. Pretreatment with acadesine dramatically suppressed the clotting activity and expression of TF (protein and mRNA). These inhibitory effects of acadesine were unchanged for endothelial cells treated with ZM241385 (a specific adenosine A2A receptor antagonist) or AMP-activated protein kinase inhibitor compound C, and in macrophages lacking adenosine A2A receptor or H9251/AMP-activated protein kinase. In endothelial cells and macrophages, acadesine activated the phosphoinositide 3-kinase/Akt signaling pathway, reduced the activity of mitogen-activated protein kinases, and consequently suppressed TF expression by inhibiting the activator protein-1 and NF-κB pathways. In mice, acadesine suppressed lipopolysaccharide-mediated increases in blood coagulation, decreased TF expression in atherosclerotic lesions, and reduced deep vein thrombus formation.

Conclusion—Acadesine inhibits TF expression and thrombus formation by activating the phosphoinositide 3-kinase/Akt pathway. This novel finding implicates acadesine as a potentially useful treatment for many disorders associated with thrombotic pathology, such as angina pain, deep vein thrombosis, and sepsis. (Arterioscler Thromb Vasc Biol. 2010; 30:1000-1006.)

Key Words: anticoagulants ■ atherosclerosis ■ thrombus ■ tissue factor

Acadesine, also called aminoimidazole-4-carboxamide-1-beta-4-ribofuranoside, is an adenosine-regulating agent that acts to increase the bioavailability of adenosine. In clinical trials, treatment with acadesine was found to decrease the incidence of perioperative myocardial infarction, cardiac death, and a combined outcome of perioperative myocardial infarction, stroke, and cardiac death. The mechanisms underlying these beneficial effects remain ill-defined but may involve the inhibitory effects of acadesine on platelet aggregation, neutrophil activation, and thrombus formation.

Acadesine is phosphorylated by adenosine kinase (ADK) to 5-aminoimidazole-4-carboxamide-1-beta-4-ribonucleotide. The 5-aminoimidazole-4-carboxamide-1-beta-4-ribonucleotide mimics AMP, activating AMP-activated protein kinase (AMPK) without altering the cellular levels of ATP, ADP, or AMP. The activation of AMPK has been associated with many antiinflammatory and antidiabetic effects, and many of the effects of acadesine are thought to occur through AMPK activation. These effects include, for example, the ability of acadesine to suppress the expression of adhesion molecules on endothelial cells and to inhibit the production of proinflammatory factors (tumor necrosis factor-α, IL-1β, IL-6, and nitric oxide) in astrocytes, microglia, and macrophages via inhibition of NF-κB and C/EBP pathways. Consequently, acadesine treatment protects animals from experimental autoimmune encephalomyelitis.

Tissue factor (TF) is a critical initiator of the physiological and pathological coagulation cascade. In the TF-initiated extrinsic coagulation pathway, prothrombin is converted to thrombin, thereby inducing fibrin formation, platelet activation, and thrombus formation. Endothelial cells and monocytes are major cellular origins of TF undergoing pathologi-
In response to various inflammatory stimuli, all of the following are activated: mitogen-activated protein kinase (MAPK) p38, extracellular signal-regulated kinase 1/2 (p44/42), and c-jun terminal NH2-kinase. These, in turn, activate transcription factors such as AP-1, NF-κB, and EGR-1, which signal the TF promoter to induce TF expression in endothelial cells and monocytes. In contrast, the phosphoinositide 3-kinase (PI3K) pathway negatively regulates TF expression. This effect occurs through cross-talk between PI3K and MAPK.

We hypothesized that acadesine inhibited TF expression and thrombus formation. To test this hypothesis, we determined the effect of acadesine on TF expression in endothelial cells and monocytes. We also explored the intracellular signaling pathways involved in this process and examined the effect of acadesine on TF expression in vivo by using mouse models of sepsis, deep vein thrombosis, and atherosclerosis.

**Materials and Methods**

Human umbilical vein endothelial cells (HUVEC) and human monocytes were prepared and assayed for TF activity (1-stage clotting assay) and TF expression at the protein (Western blot assay) and mRNA levels (real-time reverse-transcription polymerase chain reaction), as previously described. Intracellular signaling pathways, including those of PI3K and MAPK, were also studied using Western blotting. To determine the effects of acadesine on lipopolysaccharide (LPS)-induced TF expression and intracellular signaling, cells were first pretreated with acadesine at indicated concentrations for 1 hour and then stimulated with LPS. To determine the effect of acadesine on TF expression and thrombus formation in vivo, mice were injected intraperitoneally with acadesine at a dose reported in previous studies. The detailed Materials and Methods are provided in Supplementary files (available online at https://atvb.ahajournals.org).

**Results**

**Acadesine Suppresses LPS-Induced or Cytokine-Induced TF Expression in HUVEC and Human Monocytes**

To determine whether acadesine regulates TF expression in HUVEC, we first pretreated cells with acadesine at various concentrations for 60 minutes and then stimulated cells with LPS to induce TF expression. Using a 1-stage clotting assay of cell lysates, we found that acadesine downregulated the clotting activity of TF in a dose-dependent manner (Figure 1A). The suppression of clotting activity of TF was further confirmed by an assay that specifically examines cell-surface TF activity (Supplementary Figure I). Consistent with these data, acadesine decreased TF expression at the mRNA and protein levels, as shown by real-time reverse-transcription polymerase chain reaction and Western blots in a dose-dependent manner (Figure 1B,C). In addition, acadesine suppressed IL-1β–induced or tumor necrosis factor-α–induced TF expression in HUEVC, and murine tumor necrosis factor-α–stimulated TF expression in murine bEND.3 endothelial cells (Supplementary Figures II, III). Similar declines in TF clotting activity and expression were found in human blood monocytes that were pretreated with acadesine, followed by stimulation with LPS (Figure 1D–F).

**AMPK Is Not Involved in the Inhibitory Effect of Acadesine on TF Expression**

Acadesine is an activator of AMPK, which is associated with many antiinflammatory and antiadipic effects. Some of the antiinflammatory effects of acadesine appear to be mediated by AMPK. To determine whether AMPK mediates the inhibitory effect of acadesine on TF expression, we pretreated HUVEC with AMPK inhibitor compound C before adding acadesine and LPS to the cell medium. Acadesine was able to inhibit TF expression in compound C–treated cells to the same extent as in vehicle (DMSO)–treated cells; this was demonstrated by the 1-stage clotting assay for TF activity, real-time reverse-transcription polymerase chain reaction for TF mRNA levels, and Western blots for TF protein levels (Figure 2A–C). As another test of the involvement of AMPK in the inhibitory effect of acadesine on TF expression, we isolated macrophages from mice deficient in α1-AMPK, which is the sole AMPK catalytic unit in vascular cells and leukocytes. These mice were obtained from Benoit Viollet Laboratory, Institut Cochin, Paris, France. Compared to
Acadesine increases adenosine levels to regulate a variety of physiological and pathological functions. Adenosine A2A receptor (A2A-R) mediates most of the anti-inflammatory effects of adenosine. To test whether A2A-R is involved in the inhibitory effect of acadesine on TF expression, we pretreated HUVEC with the A2A-R antagonist ZM241385 before adding acadesine and LPS to the cell medium. Pretreatment of HUVEC with ZM241385 did not change the inhibitory effect of acadesine on TF expression, as evidenced by the results of the 1-step clotting assay and by TF mRNA and protein levels (Figure 2D–F). We also used macrophages isolated from A2A-R−/− mice (obtained from Jiang-Fan Chen Laboratory, Boston University School of Medicine, Boston, Mass) to determine the involvement of A2A-R in acadesine-mediated TF suppression. In these A2A-R-deficient cells, LPS induced the same level of TF-mediated clotting as it did in wild-type cells, and acadesine suppressed TF activity to the same extent as in wild-type cells (Supplementary Figure V). These data indicate that A2A-R does not contribute to the inhibitory effect of acadesine on LPS-induced TF expression.

### PI3K/Akt Signaling Is Crucial to the Inhibitory Effect of Acadesine on TF Expression in Endothelial Cells and Monocytes

The PI3K/Akt signaling pathway has been shown to negatively regulate TF expression in vitro and in vivo. We investigated whether acadesine could activate the PI3K/Akt pathway and affect LPS-induced Akt phosphorylation. Under resting conditions, acadesine increased the levels PI3K activity and Akt phosphorylation in a time-dependent manner in both HUVEC and human monocytes (Figure 3A, B; Supplementary Figure VIA). The PI3K inhibitor wortmannin blocked the activation of Akt by acadesine, indicating that Akt phosphorylation in acadesine-treated cells is attributable to acadesine-mediated PI3K activation. Treatment with LPS initiated minor Akt phosphorylation in HUVEC and monocytes. Acadesine pretreatment significantly elevated the levels of phospho-Akt in LPS-treated cells (Figure 3C; Supplementary Figure VIB).

PI3K/Akt can negatively regulate multiple signaling pathways, GS-Δ/β, a crucial molecule for p65 phosphorylation, is 1 downstream phosphorylation target of PI3K. Corresponding to the levels of phospho-Akt, acadesine treatment increased the levels of phospho-GSK-3β in HUVEC and monocytes under both resting and LPS-treated conditions (Figure 3D; Supplementary Figure VIC). Extracellular signal-regulated kinase, an important MAPK for TF expression, is the target of the Raf-1 pathway. The latter is regulated by PI3K/Akt signaling. In HUVEC treated with LPS, extracellular signal-regulated kinase phosphorylation was almost completely blunted by acadesine (Figure 3D). In human monocytes, the phosphorylation of extracellular signal-regulated kinase and p38 that was induced by LPS was also dramatically inhibited by acadesine (Figure VIC). To determine the role of PI3K/Akt activation in the inhibitory effect of acadesine on TF expression, HUVEC were pretreated with the PI3K inhibitor wortmannin and LY294002, followed by the addition of LPS and acadesine. Under these conditions, the suppression of TF expression by acadesine was greatly reduced (Figure 3E). Thus, the PI3K/Akt pathway is critically involved in the inhibitory effect of acadesine on TF expression in HUVEC.

The activation of transcription factors NF-κB and AP-1 is necessary for TF expression. We examined whether...
AP-1 and NF-κB after treatment with LPS and acadesine, the nuclear levels of transcription factors, especially AP-1, to their oligonucleotides (Supplementary Figure VII). Electrophoretic mobility shift assays showed that acadesine directly interfered with the binding of transcription factors, especially AP-1, to their oligonucleotides (Supplementary Figure VII).

PI3K/Akt participated in the inhibitory effect of acadesine on the activation of NF-κB and AP-1 in HUVEC and monocytes. Electrophoretic mobility shift assays showed that 60 minutes after treatment with LPS and acadesine, the nuclear levels of AP-1 and NF-κB p50/p65 bound to their oligonucleotides were much lower than those of cells treated with LPS only (Figure 4A–D). Wortmannin, a PI3K inhibitor, reversed the inhibition of AP-1 and NF-κB activation by acadesine (Figure 4A–D). Additionally, electrophoretic mobility shift assays using nuclear extracts from LPS-treated cells also showed that acadesine directly interfered with the binding of transcription factors, especially AP-1, to their oligonucleotides (Supplementary Figure VII).

Acadesine Inhibits TF Expression in Mouse Models of Sepsis, Atherosclerosis, and Thrombosis

In a mouse LPS-induced sepsis model, TF production is produced at high levels and is a major contributor to the hypercoagulation state. To determine whether acadesine can suppress TF expression in this murine model, we injected acadesine intraperitoneally into C57BL/6J mice (purchased from the Jackson Laboratory, Bar Harbor, Me) 1 hour before LPS administration. LPS induced the expression of TF mRNA in many major organs, including the lung and liver. This expression was dramatically reduced by acadesine treatment (Figure 5A). Consistent with the results for TF mRNA, fibrin levels in the livers of mice treated with LPS and acadesine were much lower than those of mice treated with LPS only (Figure 5B). In immunohistochemistry experiments that used an antibody against fibrin, robust fibrin deposition was found in the endothelium of venules in the livers of LPS-treated mice but not in control mice. Treatment with acadesine almost completely eliminated LPS-induced fibrin deposition (Figure 5C).

Macrophages are a major source of TF in atherosclerotic lesions. On sections of atherosclerotic arteries from apolipoprotein El−/− mice fed a western diet for 3 months, strong TF-positive staining (brown) was observed (Figure 5D). Peritoneal injection of acadesine for 5 days did not significantly reduce the number of macrophages in atherosclerotic lesions.24 On sections of atherosclerotic arteries from apolipoprotein El−/− mice fed a western diet for 3 months, strong TF-positive staining (brown) was observed (Figure 5D). Peritoneal injection of acadesine for 5 days did not significantly reduce the number of macrophages in atherosclerotic lesions.
lesions (data not shown). However, acadesine treatment dramatically decreased TF expression, demonstrated by the weak TF staining on the sections of arteries from mice treated with acadesine (Figure 5D). Additionally, the level of TF mRNA in atherosclerotic arteries of apolipoprotein E–/– mice treated with acadesine was significantly lower than that in arteries from apolipoprotein E–/– mice treated with vehicle (Figure 5E). These results indicate that TF expression by macrophages in atherosclerotic lesions is dramatically inhibited by acadesine treatment.

TF is also important in the formation of deep vein thrombosis. Using a mouse model of deep vein thrombosis, we examined whether acadesine can affect thrombus formation. Mice treated with vehicle had large thrombi in their inferior vena cava, whereas mice treated with acadesine had very small thrombi in their ligated inferior vena cava (Figure 5F).

**Discussion**

Our study demonstrates that acadesine inhibits TF expression in endothelial cells and in monocytes/macrophages, and that this inhibition occurs through the activation of the PI3K/Akt signaling pathway. PI3K/Akt signaling reduces the activity of MAPK, phosphorylates GSK-3β, and suppresses the AP-1 and NF-κB pathways, thereby inhibiting TF expression. Acadesine is also effective in suppressing the expression of TF in mouse models of sepsis, atherosclerosis, and thrombosis.

Our findings indicate that the PI3K/Akt pathway is a focal point for the inhibitory effect of acadesine on TF expression. The PI3K/Akt pathway is a conserved family of signal transduction enzymes that participate in the regulation of cell proliferation and survival. A number of studies have demonstrated that PI3K/Akt is a negative-feedback regulator of TF expression. Acadesine affects this signaling pathway differently in different cells. Acadesine inhibits PI3K/Akt signaling in C6, MCF-7, PC3, CEM, and K562 tumor cells but enhances it in acute lymphoblastic leukemia cells. Acadesine seems to have no effect on PI3K/Akt in vascular smooth muscle cells. However, we found that in endothelial cells and monocytes, the PI3K/Akt pathway is dramatically activated by acadesine under resting and activated conditions. LPS or proinflammatory cytokines, such as tumor necrosis factor-α, also induced Akt phosphorylation in endothelial cells. After incubation with LPS, the levels of phospho-Akt peaked at 15 to 30 minutes (Figure 3D; Supplementary Figure V1D). Thus, the delay in PI3K/Akt activation allows proinflammatory cytokines to activate MAPK, thereby activating the NF-κB pathway. Furthermore, the inhibited phospho-Akt was also able to inhibit the phosphorylation of MAPK, thereby suppressing the NF-κB and AP-1 pathways. The inhibitory effect of acadesine on TF expression in endothelial cells was reversed by the PI3K inhibitor wortmannin and LY294002, indicating that PI3K/Akt activation is required for the suppression of TF expression by acadesine. LY294002 is more specific than wortmannin for inhibition of PI3K activity. In our study, reversal of the inhibitory effect of acadesine on TF expression by LY294002 was not as great as that by wortmannin, although the difference did not reach statistical significance. This may be attributable to more broad effects on pathways other than...
PI3K for wortmannin or incomplete inhibition of PI3K activity by LY294002 at the dose used in our assay.

In addition to the inhibitory effect on translocation of transcription factors, acadesine was able to directly interfere with the binding of transcription factors to their specific DNA sequences, indicating 1 more level at which acadesine could inhibit TF expression (Supplementary Figure VII). This observation is consistent with a previous report.30

Our findings also indicate that acadesine-mediated suppression of TF is not associated with its role in AMPK activation. Endogenous AMPK activity is important in maintaining the resting status of cells, and others have suggested that many effects of acadesine, especially its role in the regulation of inflammation, are linked to its activation of AMPK.9–12 We did find that in mouse macrophages lacking α1AMPK, TF activity was increased more than that of wild-type macrophages, even without any stimulation (Supplementary Figure IV). Additionally, acadesine was able to activate AMPK in endothelial cells and macrophages. However, neither the suppression of AMPK activity in human endothelial cells with compound C nor the depletion of α1AMPK in mouse macrophages abrogated the inhibitory effect of acadesine on LPS-induced TF expression. Furthermore, it has been previously reported that acadesine activates endothelial Akt in an AMPK-independent manner.31 In this study, we observed that the inhibitory role of acadesine in TF expression is PI3K/Akt-dependent. These results provide convincing evidence that the observed inhibitory effect of acadesine in TF expression is not related to its role in AMPK activation.

Acadesine increases the bioavailability of adenosine; however, the exact mechanism for this has not been elucidated.32 It is known that after entrance into the intracellular compartment, adenosine is converted by ADK to 5-aminoimidazole-4-carboxamide-1-beta-4-ribofuranoside. ADK is a crucial adenosine-removing enzyme.33 Thus, it may be that the metabolism of acadesine by ADK interferes with the adenosine-removal function of this enzyme, thus leading to an increase in intracellular adenosine. It is plausible that the suppression of TF expression by acadesine is related to its role in adenosine regulation. Adenosine exerts many of its anti-inflammatory effects through the receptor A2AR. In our study, however, blockade or deficiency of A2AR did not influence the inhibitory effect of adenosine on TF expression. This suggests that the mechanism of inhibition is A2AR-independent. Adenosine can activate PI3K/Akt in either an A1AR-dependent or an A1AR-independent manner.34–36 Because HUVEC do not express A1AR, it is likely that our observed activation of PI3K/Akt by acadesine occurs via an adenosine receptor-independent pathway. To mimic the role of acadesine in PI3K/Akt activation and TF expression, we used lentiviral RNAi to knock-down ADK in HUVEC.40 In these cells, PI3K/Akt was activated and LPS-induced TF expression was suppressed (Supplementary Figure VIII). Thus, it is very likely that acadesine suppresses TF expression through its regulation of ADK activity.

Given its inhibitory effect on TF expression, acadesine shows promise as a treatment for diseases associated with pathological thrombosis. Individuals with sepsis have a high level of TF and elevated coagulation states in their blood.37,38 Elevated TF has been found in the local coronary circulation and systemic blood flow of patients with acute coronary syndromes, such as unstable angina and myocardial infarction.39 Deep vein thrombosis is also linked to TF production.39 In our study, acadesine dramatically inhibited TF expression in models of mouse sepsis and deep vein thrombosis, and it also decreased the production of TF by lesional macrophages. Because clinical trials have demonstrated the safety of acadesine for the prevention of perioperative myocardial infarction, its potential extension to other diseases is a realistic option for improving clinical outcomes through the inhibition of TF expression.

Acknowledgments

The authors thank Dr Anne Marie Weber-Main for her critical review and editing of manuscript drafts.

Sources of Funding

This work was supported by AHA 0430151N, NIH HL78679, and HL080569 (to Y. Huo).

Disclosures

None.

References

12. Nath N, Giri S, Prasad R, Salem ML, Singh AK, Singh I. 5-aminimidazole-4-carboxamide ribonucleoside: a novel immuno-


