Antiinflammatory and Antiatherogenic Effects of the NF-κB Inhibitor Acetyl-11-Keto-β-Boswellic Acid in LPS-Challenged ApoE−/− Mice

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Objective—In this article, we studied the effect of acetyl-11-keto-β-boswellic acid (AKβBA), a natural inhibitor of the proinflammatory transcription factor NF-κB on the development of atherosclerotic lesions in apolipoprotein E–deficient (apoE−/−) mice.

Methods and Results—Atherosclerotic lesions were induced by weekly LPS injection in apoE−/− mice. LPS alone increased atherosclerotic lesion size by ≈100%, and treatment with AKβBA significantly reduced it by ≈50%. Moreover, the activity of NF-κB was also reduced in the atherosclerotic plaques of LPS-injected apoE−/− mice treated with AKβBA. As a consequence, AKβBA treatment led to a significant downregulation of several NF-κB–dependent genes such as MCP-1, MCP-3, IL-1α, MIP-2, VEGF, and TF. By contrast, AKβBA did not affect the plasma concentrations of triglycerides, total cholesterol, antioxidized LDL antibodies, and various subsets of lymphocyte-derived cytokines. Moreover, AKβBA potently inhibited the IκB kinase (IKK) activity immunoprecipitated from LPS-stimulated mouse macrophages and mononuclear cells leading to decreased phosphorylation of IκBα and inhibition of p65/NF-κB activation. Comparable AKβBA-mediated inhibition was also observed in LPS-stimulated human macrophages.

Conclusion—The inhibition of NF-κB activity by plant resins from species of the Boswellia family might represent an alternative for classical medicine treatments for chronic inflammatory diseases such as atherosclerosis. (Arterioscler Thromb Vasc Biol. 2008;28:272-277)

Key Words: boswellic acid ▪ inflammation ▪ cytokines ▪ atherosclerosis ▪ NF-κB

Atherosclerosis is the main cause of coronary heart disease. The classical risk factors for atherosclerosis do not fully explain the incidence of the disease, and there is increasing recognition of the link between inflammation and atherosclerosis.1 Although markers of chronic inflammation, such as C-reactive protein, are clearly predictive of clinical atherosclerosis,2 the sources of inflammatory responses, and the mechanisms by which inflammation leads to vascular disease, are not fully understood. Common bacteria and viruses might contribute to the development of atherosclerosis, probably by triggering inflammation. Thus, epidemiological studies have shown the presence of Chlamydia pneumoniae and cytomegalovirus in plasma and in atherosclerotic plaques from heart disease patients.3 In addition, an increased incidence of coronary artery disease also occurs in patients with Helicobacter pylori, chronic dental, and chronic bronchitis infections in which microorganisms are not localized in the vessel wall.3–6 However, it is not certain whether or not microorganisms play a causal role in atherosclerosis and its complications.

Chronic infection can be mimicked by infusion of endotoxins such as LPS, which is a component of the Gram-negative bacterial wall. LPS interacts mainly with monocytes and macrophages via the toll-like receptor 4 (TLR4)7 and activates a number of intracellular signal pathways leading to the release of proinflammatory cytokines and reactive oxygen metabolites.7 It has been suggested that increased low-density lipoprotein (LDL) oxidation could be one of the mechanisms by which infection and inflammation may promote atherosclerotic lesions.8

Mice with targeted disruption of the apolipoprotein E gene (apoE−/−) develop severe atherosclerosis that progresses from fatty streaks to fibrofatty plaques and advanced lesions. In these animals, antisera specific for malondialdehyde (MAD)-lysine and 4-hydroxynonenal (4-HNE)-lysine have revealed the existence of oxidation-specific epitopes in atherosclerotic...
lesions. In addition, plasma from such animals contained autoantibodies directed against MAD-lysine. Immunohistochemical analysis of aortic lesions of these mice revealed a prominent involvement of macrophages and T lymphocytes. It was recently demonstrated that apoE−/− mice were highly susceptible to endotoxemia. This is probably attributable to the absence of apoE, because this apolipoprotein appears to be involved in the detoxification of LPS. Thus, LPS-treated apoE−/− mice seem to be a model of special interest to study inflammation and anti-inflammatory compounds in atherogenesis.

The nuclear transcription factor NF-κB is the key player in the development and progression of chronic inflammatory diseases, such as rheumatoid arthritis, asthma, and atherosclerosis. NF-κB is therefore believed to be a good target for anti-inflammatory intervention.

Apart from synthetic substances, a number of natural compounds including resveratrol, a polyphenolic phytoalexin present in red grapes, and several other secondary products from plants were found to interfere with NF-κB signaling leading to inhibition of NF-κB activation. Some preparations from the oleogum resin of Boswellia species, commonly known as frankincense, have been used in traditional medicine as anti-inflammatory remedies and clinical pilot trials with extracts from Boswellia oleogum resins in patients with rheumatoid arthritis or inflammatory bowel diseases have yielded promising results. Only recently we have shown that chemically pure acetyl-boswellic acids such as acetyl-11-keto-β-boswellic acid (AKβBA) inhibit the cytokine production in human monocytes in vitro. Moreover, acetyl-boswellic acids were found to exert cytotoxic activity in treatment-resistant, androgen-independent, prostate cancer cells, an effect that was apparently attributable to inhibition of NF-κB signaling and the subsequent reduction of NF-κB-dependent antiapoptotic gene expression. Interestingly, apoptosis induction was not observed in normal nontumor cells such as MRC-5 fibroblasts suggesting specificity for tumor cells. Consistent with this observation, the traditional remedies from frankincense oleogum resins were never observed to induce any nonspecific cytotoxicity.

In our present in vivo study, we tested whether the natural NF-κB inhibitor, AKβBA, administered as a special water-soluble γ-cyclodextrin complex, would affect the development of atherosclerosis in apoE−/− mice treated with LPS to mimic a systemic infection.

**Materials and Methods**

AKβBA was isolated from African frankincense and purified to chemical homogeneity (>99.9% purity) by reversed phase high performance liquid chromatography as previously described. The compound was further characterized by mass spectrometry and 1- and 2-dimensional nuclear magnetic resonance spectroscopy. For the application of AKβBA in animals we developed a hydrophilic derivative by generating γ-cyclodextrin complexes that allowed administration of the lipophilic compound in aqueous solutions in vivo.

**Immunohistochemistry**

Immunohistochemistry was performed in aortic sinus sections. Tissue sections were pretreated with acetone-methanol (50/50 vol/vol) for 2 minutes and then rehydrated with PBS for 5 minutes. After washing with 0.1% Triton X-100 in PBS, sections were incubated with the appropriate antibodies. All antibodies were used at optimal dilutions. The following antibodies were used to detect the phosphorylated form of IKKα (Ser180)/IKKβ (Ser181), phospho-IκBα (Cell Signaling Technology), and p65 (Abcam, Cambridge) and were incubated at 4°C overnight. The sections were counterstained with hematoxinil and digitally recorded with an Axioskop microscope (Carl Zeiss) and a Sony MC-3249 charge-coupled device (CCD) camera.

**Cell Preparation and Culture**

**Macrophages and Mononuclear Cells**

Monocyte-derived human macrophages were differentiated from buffy coats for 7 days with 15 ng/mL M-colony stimulating factor (CSF; R&D Systems) in RPMI 1640, 10% FCS (Invitrogen). Mouse peripheral blood mononuclear cells (PBMCs) were isolated from blood obtained by cardiac puncture and anticogulated with EDTA 5 mmol/L by density gradient centrifugation using Nycoprep 1.077A (Axis Shield). Mouse peritoneal macrophages were collected by peritoneal lavage with 10 mL PBS containing 10 U/mL of heparin. Cells were resuspended in RPMI 1640, 10% FCS, and let for 1 hour before stimulation. For the ex vivo analysis of AKβBA effects, mice were challenged with LPS (50 μg) and treated for 7 days daily either with control-complex or AKβBA. Peritoneal macrophages were isolated 6 hours after the last injection of AKβBA and used for the biochemical analysis of the NF-κB inhibition.

**Western Blotting**

The cells (1 to 2×106 cells per sample) were treated with the indicated concentrations of AKβBA for 30 minutes and subsequently stimulated with LPS (100 ng/mL) for additional 30 minutes. Smooth muscle cells were treated with 10 μg/mL LPS. Whole cell lysates were prepared and phosphorylation of IκBα was analyzed as described. For the control of equal protein loading, blots were reprobed with IκBα antibody (Santa Cruz).

**Immunoprecipitation and Kinase Assay**

Human or mouse macrophages (5×106 cells per assay) were stimulated with LPS (1 μg/mL) for 30 minutes or left untreated. Cells were lysed with buffer containing 1% Triton X100. Lysates were precleared with protein A agarose beads. The IKK complex was immunoprecipitated from the precleared cell lysates with anti-IKKα/β antibodies (H-470, Santa Cruz Biotechnology) and protein A agarose beads. After extensive washing of immunoprecipitated IKKs, equal amounts of kinases in terms of protein were pretreated with different concentrations of AKβBA at 30°C for 30 minutes and used for kinase assays with recombinant IκBα (Santa Cruz) in the presence of 32P-labeled ATP, at 30°C for 20 minutes. Samples were separated by SDS polyacrylamide gel electrophoresis and blotted onto polyvinylidene fluoride (PVDF) membranes. Phosphorylated IκBα was visualized using a PhosphorImager (Molecular Dynamics). Membranes were immunostained with anti-IKK antibody to ensure equal loading. Alternatively, mouse macrophages were pretreated with AKβBA for 30 minutes, stimulated with LPS (100 ng/mL), followed by IKK immunoprecipitation and in vitro kinase assay.

**Activation of NF-κB**

DNA-binding activity of p65 was measured using TransAM NF-κB family transcription assay kit (Active Motif). Mouse PBMCs or peritoneal macrophages were pretreated with AKβBA (10 μmol/L) or DMSO (control) for 30 minutes followed by stimulation with LPS (100 ng/mL) for additional 60 minutes. DNA binding activity of p65 was analyzed in nuclear extracts. Results are normalized for the protein contents and expressed as fold activation of p65 compared with control.
Results

This study was designed to evaluate the impact of NF-κB inhibition by AKβBA on atherosclerotic lesion size and inflammatory responses in apoE−/− mice after LPS administration.

AKβBA Reduces Atherosclerotic Lesion Size and Inhibits NF-κB in Atherosclerotic Lesions of LPS-Challenged ApoE−/− Mice

Consistent with the findings of Ostos et al20 we observed significantly increased lesion size in LPS-challenged apoE−/− mice in comparison to PBS-treated animals. Similarly, LPS-injected apoE−/− mice treated additionally with the control complex have similar lesion size as LPS-injected mice indicating that the control complex did not affect the lesion size. However, LPS-injected apoE−/− mice treated additionally with AKβBA have a significantly reduced lesion size in comparison to the control group (2.42±0.88 arbitrary unit (=50%), n=8, P<0.05 versus control group (4.74±1.70 arbitrary unit [100%] n=8; Figure 1). Because AKβBA has been reported to inhibit NF-κB signaling in human monocytes15 and cancer cells,16 we investigated the effect of AKβBA treatment on the NF-κB activity in atherosclerotic lesions in LPS-challenged apoE−/− mice.

Activated IKK phosphorylates the endogenous NF-κB inhibitor, IκBα, leading to its degradation and subsequent nuclear translocation of NF-κB proteins such as p65. We therefore analyzed the phosphorylation of IKK and IκBα as parameters of NF-κB activity. Indeed, our results showed that phospho-IKK and -IκBα staining intensities in atherosclerotic lesions from AKβBA-treated animals were clearly reduced in comparison to lesion from the control mice (Figure 2B, supplemental Figure IA, available online at http://atvb.ahajournals.org). Moreover, dark nuclear staining shows nuclear localization of p65 protein in lesions of the control group. By contrast, low nuclear staining for this protein was observed in lesions of the AKβBA group (supplemental Figure IB).

Next, we characterized the molecular mechanism of the AKβBA-mediated NF-κB inhibition. We have previously shown that AKβBA directly binds and inhibits human IKK.15 To ensure that AKβBA is able to inhibit murine IKK as well, we performed additional in vitro experiments. Western blot analysis of macrophages and peripheral blood mononuclear cells showed that AKβBA inhibits the LPS-induced NF-κB activity in human and murine cells as analyzed by decreased phosphorylation of IκBα (Figure 3A). In vitro kinase assays demonstrated efficient inhibition of human and murine IKK immunoprecipitated from LPS-stimulated macrophages (Figure 3B). Inhibition was even achieved when IKK was immunoprecipitated from pretreated macrophages, although this certainly results in dissociation of the reversible inhibitor AKβBA during the isolation procedure as evidenced by the somewhat weaker efficacy (supplemental Figure II A). As expected, inhibition of IKK resulted in decreased nuclear translocation of active p65 in peritoneal macrophages and PBMC (Figure 3C) indicating inhibition of NFκB transcriptional activation.

To analyze how prolonged exposure of mice to LPS and AKβBA might influence the ability of AKβBA to inhibit

Figure 1. AKβBA reduces atherosclerotic lesions size in LPS-challenged apoE−/− mice. Mice were challenged for 5 weeks once a week with LPS and were daily treated or not with AKβBA (see Methods). The mean lesion area per animal was quantified by video microscopy. Data are mean±SD. *P<0.05.

Figure 2. AKβBA inhibits NF-κB activity in atherosclerotic plaques of LPS-challenged apoE−/− mice. Mice were challenged with LPS and were daily treated or not with AKβBA (see Methods). Sections of the aortic roots were analyzed immunohistochemically. A, Negative control. B, Immunohistochemistry of the phosphorylated form of IKK.
NF-κB, we isolated peritoneal macrophages from mice challenged with LPS and treated daily either with control complex or AKβBA (100 μmol/kg) for 1 week. Macrophages from control animals responded well to the LPS challenge with increased phosphorylation of IkBα, which was inhibited by in vitro treatment with 10 μmol/L AKβBA. By contrast, macrophages from animals treated with daily injections of AKβBA (100 μmol/kg) showed a clearly reduced phosphorylation of IkBα, which was completely abolished when 10 μmol/L AKβBA was added in vitro (Figure 3D). These data demonstrate that no tolerance was acquired neither to LPS nor to AKβBA and that AKβBA applied in vivo inhibits the NF-κB activation.

In analogy to macrophages, addition of 10 μmol/L AKβBA in vitro was also able to inhibit the LPS-induced phosphorylation of IkBα in human endothelial and vascular smooth muscle cells (supplemental Figure II B).

AKβBA Has No Effect on Plasma Levels of Triglycerides and Cholesterol in LPS-Challenged ApoE−/− Mice

Because infection and inflammation are known to be accompanied by an increase in serum triglyceride (TG) levels in all species including mice and in serum total cholesterol levels in rodents,21 we have evaluated these parameters in apoE−/− mice, in LPS-injected apoE−/− mice, and those treated with either AKβBA or the control complex. The results showed a dramatic increase in both cholesterol and TG levels in all animals treated with LPS and that neither AKβBA nor the control complex affected these plasma parameters (supplemental Table I), suggesting that the beneficial effect of AKβBA on atherosclerotic lesions cannot be explained by the reduction of lipid levels. In addition, lipoprotein profiles were analyzed by fast-protein liquid chromatography (FPLC) using pooled plasma from LPS-challenged mice and treated with either AKβBA or the control complex. The results indicated no difference in the lipoprotein profiles (data not shown).

AKβBA Has No Effect on Plasma Levels of Autoantibodies Directed Against oxLDL and on Cytokines Produced by Lymphocyte Subsets in Blood, Liver, and Spleen in LPS-Challenged ApoE−/− Mice

The titer of antibodies directed against oxLDL was found to be significantly increased in the sera of LPS-treated mice compared with that of the solvent counterpart. Neither the treatment with AKβBA nor the control complex was able to affect the plasma concentration of anti-oxLDL antibodies (data not shown). In addition, neither AKβBA nor the control complex affected the expression of IFNγ, IL-4, or tumor necrosis factor (TNF-α) in blood isolated CD4+, CD8+, and NKT cells, respectively (supplemental Figure III). Moreover, we did not observe any change in IL-6 and IFNγ expression in NKT cells and CD4+ lymphocytes, respectively, that were isolated from the liver of control mice or mice treated with AKβBA (data not shown). Finally, expressions of IFNγ and IL-6 in CD4+ and NKT cells, respectively, isolated from the spleen of control mice or AKβBA-treated animals, were similar (data not shown).

AKβBA Inhibits Plasma Levels of Prothrombotic and Proinflammatory Factors in LPS-Treated ApoE−/− Mice

Plasma samples from LPS-injected apoE−/− mice treated with AKβBA or the control complex were analyzed using the Test multi-analyte murine MAP1.4, which was developed by Rules-Based Medicine, USA. Among 74 parameters, monocyte chemotactrant protein-1 (MCP-1), MCP-3, IL-1α, macrophage inflammatory protein-2 (MIP-2), lymphotoxin (Lphn), vascular endothelial growth factor (VEGF), and tissue factor (TF) were found to be significantly decreased in the AKβBA-treated mice in comparison to control mice (Table I).

Discussion

Frankincense, the oleogum resins from various Boswellia species, has been used in traditional medicine for the treatment of various inflammatory diseases. In fact, frankincense was listed in several European Pharmacopoeias until the middle of the last century. Specifically in complementary and alternative medicine, frankincense extracts are still used as a remedy for chronic inflammatory diseases. Several clinic pilot studies provided evidence for some therapeutic efficacy, for example in rheumatoid arthritis and chronic inflammatory bowel diseases. Such treatment was generally associated with only minor side effects.14 Only recently we showed that boswellic acids, the major pharmacologically active compounds of frankincense, act as inhibitors of NF-κB signaling by intercepting IKK activity, thereby inhibiting expression of NF-κB–dependent genes including cytokines.15,16

Several naturally occurring NF-κB inhibitors such as the flavonoids quercetin and resveratrol are believed to afford protection from vascular diseases possibly by inhibition of NF-κB signaling pathways.22 Antiatherosclerotic efficacy has recently also been reported for parthenolide, another NF-κB inhibitor of natural origin.23 In this study, we therefore investigated the effect of the natural NF-κB inhibitor AKβBA on atherosclerotic lesion development enforced by
weekly LPS injection in C57Bl/6 apoE−/− mice. In pilot studies we observed that injection of mice with LPS alone increases atherosclerotic lesion size, which strongly suggests that the activation of the innate immune system promotes atherogenesis. This is in agreement with what has been published previously. Similar results were obtained when mice were treated after LPS injection with control complex. By contrast, mice injected with LPS and treated with AKβBA have a significantly reduced lesion size (Figure 1). Plasma triglycerides and total cholesterol levels were not affected by the AKβBA treatment (supplemental Table I) and, therefore, cannot account for the observed beneficial effect. However, the activity of the nuclear transcription factor NF-κB, which is well known as the key actor in the development and progression of chronic inflammatory diseases such as atherosclerosis, was reduced in these mice as judged by the decreased phosphorylation of both IKK and IκBα and the decreased nuclear staining of the p65 subunit (Figure 2B, supplemental Figure 1A and IIB, respectively). Additional biochemical experiments demonstrated the inhibitory effect of AKβBA on the NF-κB activity in inflammatory cells of human and murine origin, strengthening our immunohistochemical data (Figure 3A, 3B, 3C, and 3D). As a consequence, a downregulation of the expression of a wide range of genes, which are under the control of the NF-κB system, and which play a role in this chronic inflammatory disease, was observed. Among such genes, MCP-1 and MCP-3 were significantly reduced (Table 1). MCP-1, a chemokine which triggers migration of monocytes into the arterial intima, has been proposed to play an important role in the establishment and progression of atherosclerosis. MCP-3 is chemotactic for monocytes, lymphocytes, eosinophils, basophils, natural killer cells, and dendritic cells. MCP-3 can interact with CCR2 and CCR3 receptors. CCR2 is known to be shared with MCP-1, and its absence is associated with markedly decreased atherosclerotic lesion areas in apoE−/− mice suggesting that MCP-3, like MCP-1, is possibly involved in atherogenesis. In addition, lymphotactin, a lymphocyte and particularly T cell–specific chemotactic protein, was also found to be significantly reduced in AKβBA-treated mice (Table 1). As a consequence of the decrease of these chemoattractant proteins one might expect a decrease of monocyte and T lymphocyte abundance in the vascular wall of the treated animals.

Previous studies analyzed the effects of the consumption of green and black tea, red wine, and a mixture of catechin, caffeic acid, and resveratrol on atherosclerosis in various animal models in which a reduction of atherosclerotic lesions was observed. In the study of Norata et al this reduction was associated with a decrease in expression markers of macrophages and lymphocytes (Th1 and Th2) as well as a reduction of several inflammatory molecules such as MCP-1, MIP-1α, and MIP-1β. Our results show that the treatment of LPS-challenged apoE−/− mice with AKβBA also significantly reduces IL-1α, TF, and VEGF levels, which are a potent inflammatory cytokine, an important coagulation factor and a vascular growth factor, respectively. Both vascular endothelial growth factor and tissue factor are reported to be associated with the progression of atherosclerosis and plaque instability, respectively. All these genes were reported to be under the control of NF-κB.

One could question whether apoE−/− mice subjected to 5 weeks of LPS treatment might develop LPS tolerance. However, this phenomenon usually concerns a second activation within minutes or hours of the first one, ie, it is a receptor desensitization. This is not applicable here. Our data show that macrophages respond after 1-week treatment. In addition, downregulation of TNF-α appears to be a consequence of LPS tolerance. However, in our current as well as in our previous study, we did not observe any downregulation of TNF-α in a CD3+CD8− subset of lymphocytes from weekly LPS-treated apoE−/− mice as stated in the methods section.

Our results clearly show that AKβBA acts by reducing the NF-κB activity after LPS stimulation of murine macrophages or peripheral blood mononuclear cells (Figure 3A, 3B, and 3C) or vascular smooth muscle and endothelial cells, indicating that the AKβBA effect is not restricted to macrophages (supplemental Figure 1IB).

Taken together, these results indicate that cells isolated from apoE−/− mice stimulated with LPS exhibit increased NF-κB activity and that AKβBA is able to reduce such activity. All these data clearly indicate that AKβBA reduces chronic inflammation mimicked by LPS injection in apoE−/− mice through the inhibition of the NF-κB system. Therefore,
therapeutic approaches targeting this transcription factor to treat chronic inflammation in atherosclerosis could be developed. It’s important to note that statins and peroxisome proliferator-activated receptor-α (PPAR-α) agonists have been shown to reduce cardiovascular morbidity and mortality in various studies. Although the salutary effects of these agents may be explained by their beneficial actions on the lipid profile, increasing evidence suggests that statins and PPAR-α agonists such as fibrates may also exhibit effects unrelated to lipid reduction such as the inhibition of inflammation through the attenuation of the activity of the NF-κB system. Finally, herbal therapies and plant resins from species of the Boswellia family might represent an alternative for classical medicine treatments for chronic inflammatory diseases such as atherosclerosis.

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Disclosures

None.

References