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

Clarisse Cuaz-Pérolin, Ludivine Billiet, Eric Baugé, Corinne Copin, Daniel Scott-Algara, Felicitas Genze, Berhold Büchele, Tatiana Syrovets, Thomas Simmet, Mustapha Rouis

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 approximately 100%, and treatment with AKβBA significantly reduced it by approximately 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. Although markers of chronic inflammation, such as C-reactive protein, are clearly predictive of clinical atherosclerosis, 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. 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. 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) and activates a number of intracellular signal pathways leading to the release of proinflammatory cytokines and reactive oxygen metabolites. 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.

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.10,11 Thus, LPS-treated apoE−/− mice seem to be a model of special interest to study inflammation and anti-inflammatory compounds in atherosclerosis.

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.12 NF-κB is therefore believed to be a good target for antiinflammatory 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.13 Some preparations from the oleogum resin of Boswellia species, commonly known as frankincense, have been used in traditional medicine as antiinflammatory remedies and clinical pilot trials with extracts from Boswellia oleogum resins in patients with rheumatoid arthritis or inflammatory bowel diseases have yielded promising results.14 Only recently we have shown that chemically pure acetyl-boswellic acids such as acetyl-11-keto-β-boswellic acid (AKβBA) inhibited the cytokine production in human monocytes in vitro.15 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.16 Interestingly, apoptosis induction was not observed in normal nontumor cells such as MRC-5 fibroblasts suggesting specificity for tumor cells.16 Consistent with this observation, the traditional remedies from frankincense oleogum resins were never observed to induce any nonspecific cytotoxicity.14

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.17 The compound was further characterized by mass spectrometry and 1- and 2-dimensional nuclear magnetic resonance spectroscopy.17 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.16

**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 hematoxin 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).18 Mouse peripheral blood mononuclear cells (PBMCs) were isolated from blood obtained by cardiac puncture and anticoagulated with EDTA 5 mmol/L by density gradient centrifugation using Nycodenz 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.19 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 G 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 [γ-3P]-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 pre-treated 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.
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 quantitated by video microscopy. Data are mean ± SD. *P < 0.05.

**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 al., 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 \((\sim 50\%)\), 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 monocytes and cancer cells, 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, IκB\(\alpha\), leading to its degradation and subsequent nuclear translocation of NF-κB proteins such as p65. We therefore analyzed the phosphorylation of IKK and IκB\(\alpha\) as parameters of NF-κB activity. Indeed, our results showed that phospho-IKK and -IκB\(\alpha\) 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. 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 IκB\(\alpha\) (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 IA). 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
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 in vivo.

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 IIB).

**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, 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 chemotactant protein-1 (MCP-1), MCP-3, IL-1α, macrophage inflammatory protein-2 (MIP-2), lymphotactin (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 1).

**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 Pharmacopoeiae 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. 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.

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. Antiatherosclerotic efficacy has recently also been reported for parthenolide, another NF-κB inhibitor of natural origin. 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.20 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, Figure 2C) or vascular smooth muscle and endothelial cells, indicating that MCP-3, like MCP-1, is possibly involved in atherogenesis. This is in agreement with what has been 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.29 Our results show that the treatment of LPS-challenged apoE−/− mice with AKβBA also significantly reduces IL-1α, TNF-α, and VEGF levels, which are potent inflammatory cytokines, 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 atherosclerosis30 and plaque instability, respectively.31 All these genes were reported to be under the control of NF-κB.32

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.33 However, in our current as well as in our previous study,20 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 IIB).

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 medical treatments for chronic inflammatory diseases such as atherosclerosis.

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Disclosures
None.

References
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Data Supplement

Anti-inflammatory and anti-atherogenic effects of the NF-κB inhibitor acetyl-11-keto-β-boswellic acid in LPS-challenged apoE<sup>−/−</sup> mice.

Clarisse Cuaz-Pérolin, Ludivine Billiet, Eric Baugé, Corinne Copin, Daniel Scott-Algara, Felicitas Genze, Berhold Büchele, Tatiana Syrovets, Thomas Simmet and Mustapha Rouis

Methods

Experimental animals
At 8 weeks of age, female apoE<sup>−/−</sup> mice were randomly divided into several groups (n=8 per group). The first group was challenged intraperitoneally with LPS (Escherichia coli, serotype 055:B5, Sigma-Aldrich, St. Louis, MO, USA) (50 μg) once every week and with daily γ-cyclodextrin (control complex) during 5 weeks, the second group was injected with LPS (50 μg) once every week and daily with 100 μmol/kg AKβBA γ-cyclodextrin (AKβBA) during 5 weeks. These dosages of control complex and AKβBA have previously been shown to induce no overt toxicity in mice<sup>1</sup>. All mice were fed a chow diet. For blood collection, mice were food-deprived for 4 hours. After isofluorane anesthesia, blood samples were collected from the retro-orbital sinus on EDTA tubes and kept at 4°C. At the end of the study, animals were killed by exsanguination, perfused transcardially with PBS and the heart, liver and spleen were excised. All procedures involving animal handling and their care were in accordance with the Institut Pasteur Guidelines for Husbandry of Laboratory Mice.
**Evaluation of aortic lesions**

Hearts and proximal aortas were removed and fixed. Hearts were cut directly under and parallel to the leaflet, and the upper portions were embedded in OCT medium and kept at 4°C. Ten μm thick sections were cut through the aortic sinus. Twenty-one sections per animal were stained for lipids with Oil-red O and counter-stained with Hematoxylin Harris. The first section analyzed for each animal corresponded to the origin of the aortic sinus. The intermediate sections were used for immunohistochemical analyses.

**Plasma lipid quantification**

Plasmas were separated by centrifugation at 630xg for 20 min at 4°C. Lipids were determined enzymatically using a commercial kit for triglycerides (triglycerides PAP 1000, Biomerieux), cholesterol (cholesterol RTU, Biomerieux).

**Cytokine and other biological marker determinations**

Blood was collected in EDTA tubes from the retro-orbital sinus of control- and AKβBA-treated LPS-challenged apoE⁻/⁻ mice. Plasmas were separated by centrifugation at 630xg for 20 min at 4°C and frozen in 0.5 ml aliquots at -80°C until tested. For biological parameters quantification, samples were thawed and 74 parameters were evaluated using the Test multi-analyte murine MAP1.4 (Rules-Based Medicine, Inc. Austin, TX) according to the manufacturer instructions.

*Lymphocytes*: Animals were killed as above and peripheral blood and spleen cells were isolated from each animal by density centrifugation and were washed three times in PBS. Hepatic lymphocytes were isolated by a method described elsewhere. The liver was removed and cut in two pieces; one half was passed through 200-gauge steel mesh, and then suspended in RPMI medium with 5% of heat-inactivated calf serum. Once the cells were
washed, the lymphocytes were isolated by a gradient of Percoll (35% Percoll containing 100 U/ml heparin) (Sigma). Purified lymphocytes were washed three times before culture. Human umbilical vein endothelial and smooth muscle cells were isolated as described ³.

Flow cytometric cytokine detection assays

Purified cells were incubated overnight at 37°C with 5% CO₂ in brefeldin A (2 μg/ml) (Sigma). Cells were recovered and incubated for 30 min at 25°C with FITC-conjugated monoclonal antibodies directed against CD4, CD8 and NK1.1 (Pharmingen, Boston, MA, USA). At the end of incubation, the cells were washed twice in PBS and fixed with 200 μl of 2% formaldehyde solution for 15 min at 25°C. The cells were washed two more times and 150 μl of permeabilization solution (0.5% of saponin) (Sigma), in PBS solution with 5% of fetal calf serum, for 10 min. After incubation, cells were centrifuged for 5 min and the supernatants discarded. 2.5 μl of anti-cytokines monoclonal antibodies (anti-IFN-γ, tumor necrosis factor (TNF)-α, and IL-4, Pharmingen, Boston, MA, USA) diluted in permeabilization buffer were added for 30 min at 20°C. Cells were washed twice in PBS and 500 μl of PBS was added before analysis. At least 25,000 lymphocytes were analyzed in order to determine the proportions of populations.
References


Figure I. AKβBA inhibits NF-κB activity in atherosclerotic plaques of LPS-challenged apoE⁻/⁻ mice. ApoE⁻/⁻ mice were challenged for 5 weeks once a week with 50 µg LPS per mouse and were treated with daily i.p. injections of either control complex or AKβBA (100 µmol/kg). Mice were sacrificed, the aortic roots were removed, fixed, and cut into 5 µm sections, and analyzed immunohistochemically. A, immunohistochemistry of the phosphorylated form of IκBα. B, immunohistochemical analysis of nuclear p65. The sections were counterstained with hematoxilin. Magnification x50, small insert x400.
Figure II. AKβBA inhibits NF-κB signaling in vitro. A, inhibition of IKK by AKβBA was analyzed by in vitro kinase assays using recombinant IkBα as substrate. Mouse macrophages were pretreated with AKβBA or left untreated (control) followed by stimulation with LPS (100 ng/mL) to activate IKK; IKK was immunoprecipitated and substrate and ³²P-ATP were added. The samples were resolved by PAGE and phosphorylated substrate was visualized by phosphorimaging. IKK - loading control. IP, immunoprecipitation; KA, kinase assay; WB, Western blot. B, AKβBA inhibits phosphorylation of IkBα in human endothelial (EC) and vascular smooth muscle cells (SMC) activated with LPS (100 ng/mL and 10 µg/mL, respectively). Cells were treated with AKβBA or solvent for 30 min before addition of LPS. Samples were analyzed by Western blotting. Actin - loading control. All results show 1 of 3 independent experiments.
Figure III. AKβBA has no effect on lymphocytes-derived cytokines. The lymphocytes were isolated from blood of control mice or of AKβBA-treated LPS-injected mice by a gradient of Percoll and purified cells were washed and incubated overnight at 37°C in the presence of brefeldin A (2 μg/ml). Cells were recovered and incubated for 30 min at 25°C with FITC-conjugated monoclonal antibodies directed against CD4, CD8 and NK1.1. At the end of incubation, the cells were washed, fixed with 2% formaldehyde solution and permeabilized with saponin solution. After incubation, cells were centrifuged for 5 min and the supernatants were discarded. 2.5 μl of diluted anti-cytokine monoclonal antibodies were added. At least, 25 000 live lymphocytes were analyzed in order to determine the proportions of populations. Three independent experiments were performed. Values are the means ± SD. NS: Not significant.
Table I. AKβBA has no effect on plasma levels of triglycerides and cholesterol in LPS-challenged apoE⁻/⁻ mice. ApoE⁻/⁻ mice were challenged intraperitoneally with LPS (50 μg) once every week and daily either with control complex (n=8, control) or 100 μmol/kg AKβBA (n=8) during 5 weeks. The plasma concentrations of cholesterol and triglycerides were determined in each mouse. Data are mean ± SD. # :NS. However, p<0.05 when compared to the cholesterol level in mice not treated with LPS. §: NS. However, p<0.01 when compared to the TG level in mice not treated with LPS.

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<th>Cholesterol (mg/dL)</th>
<th>Triglycerides (mg/dL)</th>
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<tr>
<td>Without LPS</td>
<td>401.10 ± 65.8</td>
<td>56.40 ± 10.2</td>
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<tr>
<td>Control</td>
<td>362.40 ± 57.7</td>
<td>125.48 ± 48.6</td>
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<tr>
<td>AKβBA</td>
<td>359.73 ± 11.8</td>
<td>114.99 ± 64.5</td>
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