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Incensole Acetate, a Novel Anti-Inflammatory Compound Isolated from *Boswellia* Resin, Inhibits Nuclear Factor-κB Activation[S]

Arieh Moussaieff, Esther Shohami, Yoel Kashman, Ester Fride, M. Lienhard Schmitz, Florian Renner, Bernd L. Fiebich, Eduardo Munoz, Yinon Ben-Neriah, and Raphael Mechoulam

Department of Medicinal Chemistry and Natural Products, Medical Faculty (A.M, R.M.) and Department of Pharmacology and Experimental Therapeutics, School of Pharmacy (A.M., E.S.), Hebrew University, Jerusalem, Israel; School of Chemistry, Raymond and Beverly Sackler Faculty of Exact Sciences, Tel-Aviv University, Israel (Y.K.); Departments of Behavioral Sciences and Molecular Biology, the College of Judea and Samaria, Ariel, Israel (E.F.); Institute of Biochemistry, Medical Faculty, Justus-Liebig-University, Giessen, Germany (M.L.S., F.R.); University of Freiburg Medical School, Department of Psychiatry, Germany (B.L.F.); Departamento Biologia Celular, Fisiologia e Inmunologia, Facultad de Medicina, Universidad de Cordoba, Spain (E.M.); and the Lautenberg Center for Immunology, Hebrew University-Hadassah Medical School, Jerusalem, Israel (Y.B.)

ABSTRACT

Boswellia resin is a major anti-inflammatory agent in herbal medical tradition, as well as a common food supplement. Its anti-inflammatory activity has been attributed to boswellic acid and its derivatives. Here, we re-examined the anti-inflammatory effect of the resin, using inhibitor of nuclear factor-κBα (IκBα) degradation in tumor necrosis factor (TNF)α-stimulated HeLa cells for a bioassay-guided fractionation. We thus isolated two novel nuclear factor-κB (NF-κB) inhibitors from the resin, their structures elucidated as incensole acetate (IA) and its nonacetylated form, incensole (IN). IA inhibited TAK/TAB-mediated IκB kinase (IKK) activation loop phosphorylation, resulting in the inhibition of cytokine and lipopolysaccharide-mediated NF-κB activation. It had no effect on IKK activity in vitro, and it did not suppress IκBα phosphorylation in costimulated T-cells, indicating that the kinase inhibition is neither direct nor does it affect all NF-κB activation pathways. The inhibitory effect seems specific; IA did not interfere with TNFα-induced activation of c-Jun N-terminal kinase (JNK) and p38 mitogen-activated protein kinase. IA treatment had a robust anti-inflammatory effect in a mouse inflamed paw model. Cembrenoid diterpenoids, specifically IA and its derivatives, may thus constitute a potential novel group of NF-κB inhibitors, originating from an ancient anti-inflammatory herbal remedy.

Boswellia species are natives of Eastern Africa, where their resin, commonly known as "frankincense" or "olibanum," is used and exported as incense. It has been extensively used for many centuries for various medical purposes, especially for the treatment of inflammatory diseases, in European, Middle East-ern, and African medical traditions. In India, *Boswellia* resin is widely used in the treatment of inflammatory conditions, including Crohn's disease, arthritic diseases, and asthma; hence, a considerable amount of work has been done on the anti-inflammatory properties of *Boswellia* (for example, see Gupta et al., 1998; Gerhardt et al., 2001; Altmann et al., 2004). Numerous previous reports attribute the anti-inflammatory and cytotoxic properties of *Boswellia* resin solely to boswellic acid and its derivatives (e.g., Gerhardt et al., 2001; Altmann et al., 2004; Xia et al., 2005; Khanna et al., 2007).

NF-κB is an inducible transcription factor that plays a central role in the mammalian innate immune response and

ABBREVIATIONS: NF-κB, nuclear factor κB; IκB, inhibitor of nuclear factor-κB; IKK, IκB kinase; TAK, transforming growth factor β-activated kinase; TAB, transforming growth factor β-activated kinase-binding protein; IA, incensole acetate; IN, incensole; PE, petroleum ether; HPLC, high-performance liquid chromatography; GC-MS, gas chromatograph-mass spectrometry; LTR, long-terminal repeat; TNFα, tumor necrosis factor-α; PBS, phosphate-buffered saline; PMA, phorbol 12-myristate 13-acetate; NP-40, Nonidet P-40; WB, Western blotting; GST, glutathione transferase; JNK, C-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; WB, Western blotting.

chronic inflammation (Karin, 2005; Perkins, 2007). Ubiquitously expressed and involved in the activation of a multitude of genes in response to various stress stimuli, NF-κB plays a pivotal role in immune and inflammatory responses (Karin and Ben-Neriah, 2000). This effect is exerted through the regulation of target genes that encode proinflammatory cytokines, adhesion molecules, chemokines, growth factors, and inducible enzymes (Lawrence et al., 2001). Inappropriate regulation of NF-κB is thus directly involved in a wide range of human disorders, including arthritis, asthma, inflammatory bowel disease, a variety of cancers, ataxia telangiectasia, and neurodegenerative diseases (Karin and Ben-Neriah, 2000; Ben-Neriah and Schmitz, 2004). Hence, identification of drugs allowing modulation of the NF-κB transduction pathway is of considerable interest (Bremner and Heinrich, 2002; Calzado et al., 2007). In nonstimulated cells, NF-κB is normally sequestered in the cytoplasm and must be translocated into the nucleus for the exertion of its function. This subcellular localization is controlled by IκB proteins, a family of inhibitory proteins that bind NF-κB, inhibit its DNA binding, and prevent its nuclear accumulation. Specific extracellular stimuli lead to the rapid phosphorylation, ubiquitination, and ultimately proteolytic degradation of IκB, which frees NF-κB to translocate to the nucleus, where it regulates gene transcription (Karin and Ben-Neriah, 2000; Perkins, 2007). Cytokines act through distinct signaling pathways that converge on the activation of IKK. IκB degradation, after its phosphorylation by the IKK complex at Ser-32 and Ser-36, is considered to be the major step in NF-κB regulation (Karin and Ben-Neriah, 2000). Thus, activation of IKK is a key event in canonical NF-κB activation (Häcker and Karin, 2006). The core IKK complex consists of the kinases IKKα and IKKβ and the regulatory IKKγ/NEMO protein. The activation of both IKKs depends on phosphorylation of serines at their activation loop. This process probably involves transautophosphorylation of IKKs and phosphorylation by upstream kinases such as transforming growth factor β-activated kinase (TAK) 1. TAK1 is recruited to the IKK complex via the ubiquitin-binding adaptor proteins TAK-binding protein (TAB) 2 and TAB3 (Häcker and Karin, 2006).

We revisited the anti-inflammatory properties of *Boswellia* resin and examined the mechanism by which the active ingredients of the resin inhibit NF-κB activation. A bioassay-guided fractionation, testing the inhibition of IκBα phosphorylation/degradation, led to the identification and isolation of incensole acetate (IA) and its nonacetylated form, incensole (IN) as inhibitors of NF-κB activation. Although IA and IN have previously been identified in *Boswellia* species (Corsano and Nicoletti, 1967) and are considered to be biomarkers of these species (Hamm et al., 2005), their biological activities have not yet been studied.

Materials and Methods

Extraction and Isolation of IA. *Boswellia carterii* resin (20 g; Pamir, Tel-Aviv, Israel) was extracted with petroleum ether (PE) (three times with 150 ml). PE extract was washed with NaOH 5% solution (three times with 200 ml). The non–acid-containing PE fraction was acidified with HCl (1 M) and then washed with a saturated NaCl solution and dried over MgSO₄. After evaporation, the residue was chomatographed on a silica column. Fractions were assayed for their activity on IκBα degradation as described under *IκBα Phosphorylation and Degradation*. A fraction eluted with 3%

diethyl-ether in PE, which contained IA, showed activity. Pure IA was obtained by HPLC separation, using an HPLC system with a UV absorbance detector (Spectra-Physics 783; Applied Biosystems, Foster City, CA) and a C18 column (10 × 250 mm; Vydac, Hesperia, CA). Acetonitrile and water were used as mobile phase for HPLC, and the gradient consisted of 90 to 99% acetonitrile for 30 min.

Structure Elucidation. To analyze the purification process, the HPLC system consisted of a pump (660; Waters, Milford, MA) and a Photo-Diode Array detector (996; Waters) with an analytical C18 symmetry column (4 × 250 mm).

Electrospray ionization and high resolution mass spectral analyses (Bruker APE×3 ICRMS) as well as several NMR methods (¹H-NMR, ¹³C-NMR, distortionless enhancement by polarization transfer, correlation spectroscopy, heteronuclear single quantum correlation, heteronuclear multiple-bond correlation spectroscopy, total correlation spectroscopy, and nuclear Overhauser effect spectroscopy) were used for the structure elucidation of the isolated active compounds. NMR spectra were recorded both in CDCl₃ and in C₆D₆ solutions using an Avance spectrometer (Bruker, Newark, DE) at 400 MHz and repeated using a Unity Inova spectrometer (Varian, Inc., Palo Alto, CA) at 500 MHz.

GC-MS analysis was performed using a gas chromatograph detector system (G1800A; Hewlett Packard, Palo Alto, CA) with a gas chomatograph with an electron ionization detector (HP5971; Hewlett Packard). An SPB-5 (30 m × 0.25 mm × 0.25 μm film thickness) column was used. The following method was used for analysis: The column was held at 70°C for 4 min, after which a temperature gradient was applied from 70 to 280°C, at a rate of 50°/min (inlet temperature, 280°C; detector temperature, 280°C; splitless injection; gas, helium, 1 ml/min).

Cell Lines. HeLa cells and 293T cells were grown in Dulbecco′s modified Eagle's medium supplemented with 10% fetal calf serum and 1% (v/v) penicillin/streptomycin (all from Biological Industries, Kibbutz Beit Haemek, Israel) in a humidified incubator at 37°C.

The RAW 264.7 macrophage cell line derived from BALB/c mice was obtained from American Type Culture Collection (Manassas, VA). Cells were cultured in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum (Hyclone, Logan, UT), 1% (v/v) penicillin/streptomycin (Biological Industries, Beit Haemek, Israel), nonessential amino acid (Sigma, St. Louis, MO), 1% glutamine (Beit Haemek, Israel), and 1% pyruvate (Beit Haemek, Israel). Cells were grown in a humidified incubator at 37°C.

Jurkat T leukemia cells were grown at 37°C in RPMI 1640 medium containing 10% (v/v) heat-inactivated fetal calf serum, 10 mM HEPES, 1% (v/v) penicillin/streptomycin (all from Invitrogen, Eggenstein, Germany) and 2 mM glutamine.

The 5.1 Jurkat and HeLa-Tat-Luc cell lines have been described previously (Sancho et al., 2004). 5.1 cells is a Jurkat derived clone stably transfected with a plasmid containing the luciferase gene driven by the HIV-1 LTR promoter, responsive to the NF-κB activator cytokine TNFα. The HeLa-Tat-Luc contains the luciferase gene driven by the HIV-1 LTR promoter and the Tat gene regulated by the CMV promoter. Therefore, the HIV-1 LTR is highly activated in this cell line because of high levels of intracellular Tat protein, and the luciferase activity is on the order of 10⁷ relative light units/10⁵ cells (considered 100% activation).

A549 cells (10⁵/ml) were transiently cotransfected with the KBF-Luc reporter (0.2 μg/ml) together with empty vectors or overexpressing vectors for IKKα/IKKβ (0.5 μg/ml each), TRAF-2 (1 μg/ml), and TAK1/TAB2 (0.5 μg/ml each). The transfections were performed using Lipofectamine Plus reagent (Invitrogen) for 24 h, according to the manufacturer's recommendations.

Isolation of Human Monocytes. Human peripheral monocyte*s* from healthy human donors were prepared following a standardized protocol (Ficoll gradient preparation; GE Healthcare, Freiburg, Germany) using a completely endotoxin-free cultivation as described previously (Noble et al., 1968; English and Andersen, 1974). By using 50-ml tubes, 25 ml of Ficoll was loaded with 25 ml of the buffy coats

from the blood of healthy donors. The gradient was established by centrifugation at 1800 rpm, 20°C for 40 min by using slow acceleration and brakes. Peripheral blood mononuclear cells in the interphase were carefully removed and resuspended in 50 ml of prewarmed phosphate-buffered saline (PBS; Invitrogen, Karlsruhe, Germany) followed by centrifugation for 10 min at 1600 rpm and 20°C. The supernatant was discarded, and the pellet was washed in 50 ml of PBS and centrifuged as described above. The pellet was then re-suspended in 50 ml of RPMI-1640 low endotoxin-medium (Invitrogen, Karlsruhe, Germany) supplemented with 10% human serum (PAA, Coelbe, Germany).

Animals. Female Sabra mice (Harlan, Jerusalem, Israel; 15–20 weeks old) were used for in vivo anti-inflammatory assessments. Ten mice were housed in each cage. The animal care and protocols met the guidelines of the U.S. National Institutes of Health, detailed in the *Guide for the Care and Use of Laboratory Animals*, and were applied in conformity with the Institutional Ethics Committees. Temperature in the animal room was maintained between 20 and 22°C, and there was a 12-h light/dark cycle (light from 8:00 AM–8:00 PM).

IκBα Phosphorylation and Degradation. HeLa cells were preincubated with tested compounds (dissolved in ethanol) for 2 h, and then stimulated for 20 mins with 20 ng/ml TNFα (Cetus, Emeryville, CA) or costimulated with 20 ng/ml phorbol 12-myristate 13-acetate (PMA) and 100 ng/ml ionomycin for 15 min. After removing the slides from plates for immunostaining (see *p65 Subunit Immunostaining*), proteins were extracted in NP-40 lysis buffer [50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 10 mM NaF, 0.5 mM sodium vanadate, 10 μg/ml leupeptin, 1% (v/v) NP-40, and 10% (v/v) glycerol] from remaining cells in the plates. Total protein concentration was determined using the Bradford method (Bradford, 1976). Lysates were then analyzed either by Western blotting (WB) or by in vitro kinase assays (see below). Boswellic acid mixture (α and β) was obtained from the laboratory of Dr. Gerald Culioli, Université de Toulon et du Var, La Valette-du-Var, France.

Kinase Assays. The IKK complex was isolated from precleared NP-40 HeLa cell extracts (see *IκBα Phosphorylation and Degradation*) by immunoprecipitation with 2 μg of αIKKγ antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) and 25 μl of protein A/G Sepharose. The precipitate was washed three times in the above NP-40 lysis buffer and two times in kinase buffer (20 mM HEPES/KOH, pH 7.4, 25 mM β-glycerophosphate, 2 mM dithiothreitol, and 20 mM MgCl$_2$). The kinase assay was performed using glutathione transferase (GST) fusion proteins as substrates, in a final volume of 20 μl of kinase buffer containing 2 μg of bacterially expressed GST-IκB-α (1–54), 20 μM ATP, and 5 μCi of [γ-^{32}P]ATP. After incubation for 20 min at 30°C, the reaction was stopped by the addition of 5× SDS sample buffer. After separation by SDS-polyacrylamide gel electrophoresis, the gel was fixed, dried, and autoradiographed. The JNK assays were performed with a similar protocol, except that the immunoprecipitation was done with αJNK1 and αJNK2 antibodies (Santa Cruz Biotechnology) and that GST-c-Jun (5–89) was used as a substrate protein.

IKK Phosphorylation Assay. After binding of an appropriate secondary antibody coupled to horseradish peroxidase, proteins were visualized by enhanced chemiluminescence according to the instructions of the manufacturer (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK).

p65 Subunit Immunostaining. HeLa Cells were preincubated with IA and then stimulated with TNFα, as described under *IκBα Phosphorylation and Degradation* above. Cells were then fixed with formaldehyde 1%, permeabilized with 0.25% Triton X-100, stained with rabbit anti-p65 (Santa Cruz Biotechnology) and visualized with anti-rabbit Rhodamine Red-labeled secondary antibody (Jackson ImmunoResearch Laboratories Inc., West Grove, PA). The cells were also stained with 4,6-diamidino-2-phenylindole for nuclei location (data not shown). The cells were examined under an Axioscope Zeiss microscope with a plan-Neofluor 60× lens.

Electrophoretic Mobility Shift Assay. Cells were preincubated for 1 h with IA and stimulated for 15 min as shown. The oligonucleotides were synthesized at MWG Biotech, Germany, and the single-strand oligonucleotides were annealed according to standard procedure by heating and subsequent cooling down to 50°C in 10 mM Tris/HCl, pH 7.5, and 100 mM NaCl. Equal amounts of protein contained in TOTEX buffer [20 mM HEPES/KOH, pH 7.9, 0.35 M NaCl, 20% (v/v) glycerol, 1% (v/v) Nonidet P-40, 1 mM MgCl$_2$, 0.5 mM EDTA, 0.1 mM EGTA, and 1 mM phenylmethylsulfonyl fluoride] were incubated with a ^{32}P-labeled double-stranded oligonucleotide containing an NF-κB recognition site for 15 min. Bound and free oligonucleotides were separated by electrophoresis on a native 0.5× TBE 4% polyacrylamide gel. The dried gel was then exposed to X-ray film.

Luciferase Assays. The various cell lines were preincubated with the compounds and stimulated as specified in the figure legends. Cells were harvested, washed with PBS and then lysed in a luciferase lysis buffer (25 mM Tris-phosphate, pH 7.8, 8 mM MgCl$_2$, 1 mM dithiothreitol, 1% Triton X-100, and 7% glycerol). Luciferase activity was measured using an Autolumat LB 953 luminometer (Berthold Technologies, Bad Wildbad, Germany) following the instructions of the luciferase assay kit (Promega, Madison, WI).

Inflamed Paw Model. Vehicle (isopropanol/Emulphor/saline = 1:1:18) or vehicle containing IA (50 mg/kg) was administered by i.p. injection 30 min before applying the inflammatory stimulus. Emulphor, a polyethoxylated vegetable oil, is a commercial emulsifier. Hind paws were injected with 50 μl of saline (left or right alternating) or λ-carrageenin (4%, right or left alternating), using 26G needles. The resulting inflammatory swelling was measured by increase in foot volume in a plethysmometer (Ugo-Basile, Italy) as described previously (Calhoun et al., 1987). Paw volume as well as redness (as a measure of erythema) and licking (as a measure of pain) were assayed before carrageenin application and every 60 min until 4 h.

Data Analysis. Dose response data were plotted and analyzed using GraphPad Prism 4.01 software (San Diego, CA). Differencees were considered statistically significant if the p value was <0.05.

Results

Isolation of the Active Components of *Boswellia* Resin that Inhibit NF-κB Activation. The PE extract of *B. carterii* inhibited the TNFα-induced degradation of IκBα in HeLa cells; solvent partition of the extract into acid and nonacid fractions resulted in the localization of the active components in the nonacid fraction. Further fractionation, guided by IκBα degradation assay, led to the isolation of the active compounds.

Structure Elucidation of the Active Compounds. The major active component was found to exhibit a molecular ion at 349.2745 *m/z* on high-resolution mass spectrometry, indicating an elemental composition of C$_{22}$H$_{36}$O$_3$. IA (Fig. 1), which has the same elemental composition, is a known constituent of *Boswellia* species. We therefore compared the ^{13}C NMR spectrum of the active compound isolated by us with a published ^{13}C NMR spectrum of IA (Gacs-Baitz et al., 1978). The spectra were identical (Table 1 in Data Supplement). The ^{13}C NMR spectrum of the isolated IA, compared with the known spectrum of IA, together with proton NMR and several 2D NMR experiments (see *Materials and Methods*) and the high-resolution mass spectrometry analysis, fully elucidated the structure of the active compound. A full NMR assignment of IA is given in Table 2 in the Data Supplement. A second active compound was isolated from *B. carterii* resin and its structure was elucidated as IN, the nonacetylated form of IA. The ^{13}C NMR spectrum of the isolated IN was

compared with the known spectrum of IN (Gacs-Baitz et al., 1978; see Table 1 in Data Supplement). Together with proton NMR and several two-dimensional NMR experiments (see *Materials and Methods*), these spectra confirmed the structure of the compound. For further validation of structure, we hydrolyzed IA (using LiAlH$_4$ as a reducing agent) and compared the GC-MS and ^{13}C-NMR spectra of the resultant IN to that of isolated IN. They were found to be identical (see Fig. 1 in Data Supplement for a GC-MS comparison).

IA and IN, but Not Boswellic Acid, Inhibited IκBα Degradation. We compared the effect of the main component of *B. carterii* resin that inhibited IκBα degradation with that of a mixture of α and β boswellic acids. Whereas the inhibition of IκBα degradation by IA was statistically significant, boswellic acid did not inhibit the degradation of IκBα (Fig. 2). We assayed IA and IN at different concentrations for their effect on IκBα degradation in TNFα-stimulated HeLa cells. Both compounds inhibited IκBα degradation in a similar dose-dependent manner (Fig. 3).

IA Inhibited IκBα and p65 Phosphorylation by Impairment of IKK Activation. Because IKK is also essential for the phosphorylation of p65 at serine 536 (Sizemore et al., 2002; Yang et al., 2003), we further analyzed whether IA affects the TNFα-induced phosphorylation of p65 in addition to its activity on the degradation of IκBα. IA inhibited the p65 subunit phosphorylation as well as the phosphorylation of IκBα and the phosphorylation by IKKs in TNF-stimulated HeLa cells (Fig. 4A, top). The inhibitory effect of IA on IKK activity is apparently specific, because JNKs and p38 MAPK, which are also activated after treatment with TNFα (Rizzo and Carlo-Stella, 1996; Kang et al., 2004), were unaffected by IA (Fig. 4A, bottom). In contrast to IκBα phosphorylation and subsequent degradation in TNF-stimulated HeLa cells, IA did not inhibit IκBα phosphorylation in human Jurkat T leukemia cells, costimulated by PMA in combination with ionomycin (Fig. 4B, top). We examined the effect of IA on TNF-stimulated Jurkat cells and found a robust inhibition of IκBα phosphorylation and degradation (Fig. 4B, bottom). The lack of IA-mediated IKK inhibition in costimulated T cells raises the possibility that this compound does not directly target the IKKs (see Mattioli et al., 2004). Accordingly, in

vitro phosphorylation experiments showed full functionality of IKKs in the presence of IA (Fig. 4C), suggesting that IA targets an upstream event. Together, these data suggest that IA inhibits the NF-κB pathway upstream of IKK. To address this possibility with an experiment, we examined the effect of IA on TNFα-induced phosphorylation of IKK. These experiments showed inhibition of IKKα/IKKβ activation loop phosphorylation by IA (Fig. 4D), attributing its effect to an upstream event.

IA Interfered with TAK/TAB Mediated Phosphorylation of IKKα/β Activation Loop. To further examine the mechanism of the effect of IA on IKK activation, we assayed A549 cells, transfected with KBF-luc alone or in combination with IKKα/IKKβ, TRAF2, or TAK1/TAB2 expression vectors. Treatment with IA did not interfere with NF-κB activation triggered by TRAF2, whereas TAK1/TAB2-stimulated NF-κB activation was significantly and dose-dependently inhibited in the presence of IA (Fig. 5A). In contrast, the effect of IA on NF-κB activation in IKKα/β-overexpressing cells was very mild and can presumably be attributed to the interaction of IKKα/β with the endogenous TAK/TAB module. To determine whether IA can interfere with TAK/TAB-induced phosphorylation and thus activation of IKKα/β, HA-TAK1 and Myc-TAB1 were overexpressed in 293T cells and IKK α/β phosphorylation was examined by immunoblotting. IA exerted a dose-dependent reduction of IKKα/β phosphorylation in TAK/TAB-overexpressing cells, as displayed in Fig. 5B. These experiments demonstrate that IA interfered with a critical step relaying the TAK/TAB module with IKKα/β activation loop phosphorylation.

IA Inhibited NF-κB Accumulation in Cell Nuclei and DNA Binding. Immunostaining of the p65 subunit of NF-κB in TNF-stimulated HeLa cells illustrated the inhibition of the nuclear accumulation of NF-κB by IA (Fig. 6A). IA also inhibited NF-κB DNA-binding in LPS-stimulated human pe-
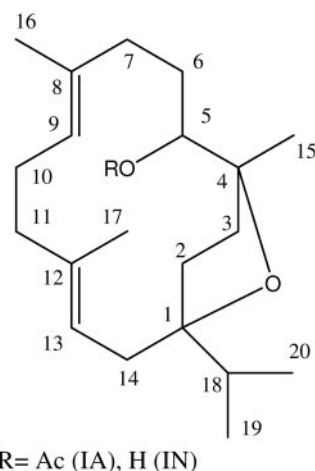

Fig. 1. The structures of IA (R = Ac.) and IN (R = H). Structures elucidation was done according to NMR (see *Materials and Methods*; Tables 1 and 2 in Data Supplement) and MS data (see *Materials and Methods*; Fig. 1 in Data Supplement).
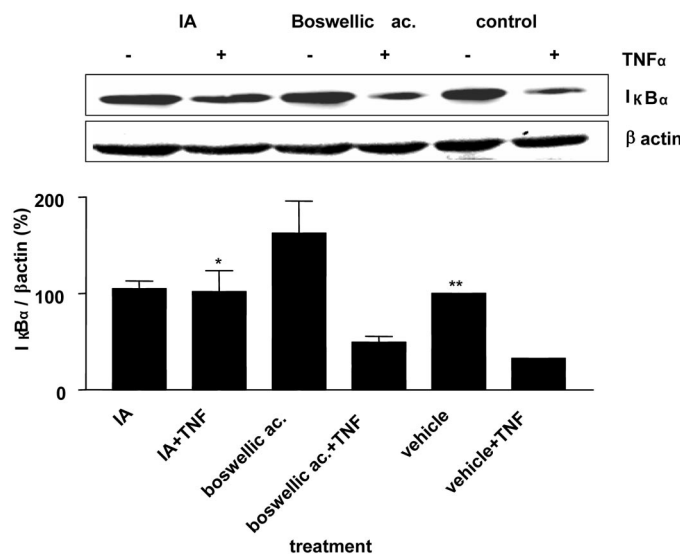
R= Ac (IA), H (IN)

Fig. 2. Comparison of isolated IA with boswellic acid for their effect on IκBα degradation in TNFα-stimulated HeLa cells. Cells were treated with nontoxic concentrations of IA (220 μM) and boswellic acid (280 μM) and stimulated with TNFα (20 ng/ml for 20 min). Equal amounts of protein were separated by SDS-polyacrylamide gel electrophoresis and further analyzed by immunoblotting. IκBα/β-actin ratio of untreated cells was considered as 100%. ∗, $p < 0.05$; ∗∗, $p < 0.001$ by t test (versus vehicle + TNFα stimulated cells). The error bars represent S.E.M.

ripheral monocytes, as examined by electrophoretic mobility shift assay and depicted in Fig. 6B.

IA Inhibited Gene Expression by NF-κB. The HIV-1 promoter contains two high affinity binding sites for NF-κB and is highly responsive to both the TNFα-induced NF-κB pathway and the Tat/TAR-dependent pathway. Using stably transfected cell lines with a plasmid, in which the luciferase gene is driven by the HIV-1 LTR promoter, we found that IA inhibits TNFα-induced (Fig. 7A) but not Tat-mediated HIV-1 LTR *trans*-activation (Fig. 7B) in a dose-dependent manner.
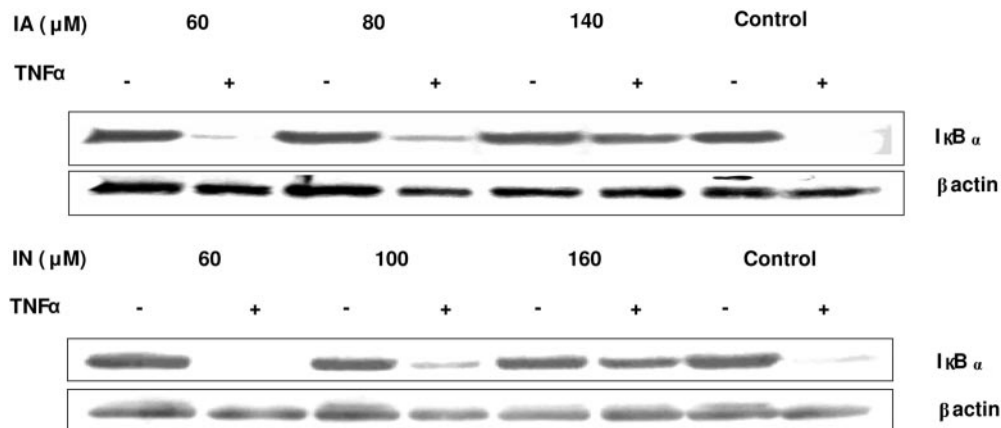
Fig. 3. IA and IN inhibited IκBα degradation in a dose-dependent manner. HeLa cells were preincubated with IA (top) or IN (bottom) at the indicated concentrations for 2 h before 20-min exposure to TNFα (20 ng/ml). A representative experiment is shown.
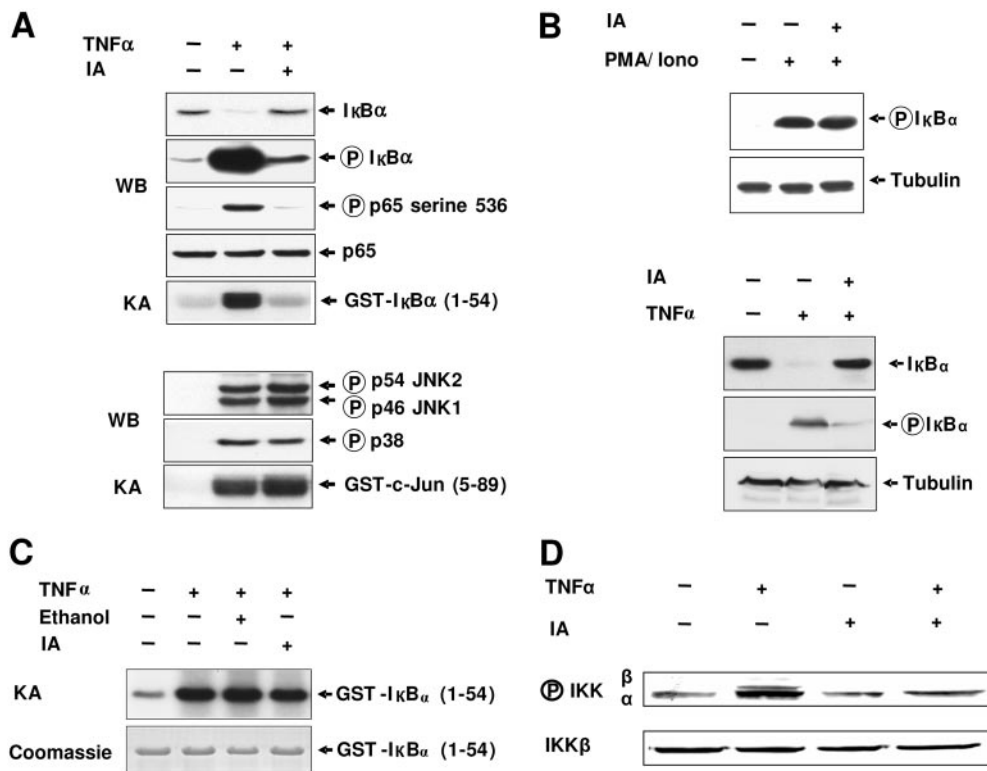

Fig. 4. IA inhibited the phosphorylation and subsequent degradation of IκBα by impairment of IKK phosphorylation and activation. A, HeLa cells were stimulated with TNFα (20 ng/ml for 20 min) in the absence or presence of IA (140 μM) as shown. Subsequently, whole-cell extracts were prepared and aliquots thereof analyzed either for the stability and phosphorylation of the indicated proteins by WB or for IKK activity by kinase assays. IKKγ/NEMO was immunoprecipitated from cell lysates and IKK activity was determined by immune complex kinase assays using recombinant GST-IκB-α (1–54) as substrate. An autoradiogram from a reducing SDS gel is shown. Bottom, the effect of IA on the phosphorylation of p38 and JNK1/2 and JNK in vitro kinase activity. The experiment shown is representative of three independent experiment sets. B, IA does not impair IκBα phosphorylation in costimulated T cells. Human Jurkat T leukemia cells were left untreated or incubated with IA (560 μM). T cells were costimulated by treatment with 20 ng/ml PMA in combination with ionomycin (100 ng/ml) as shown. After 15 min, cell extracts were prepared and analyzed by immunoblotting for the phosphorylation of IκBα. TNF-stimulated Jurkat cells (bottom) were left untreated or incubated with IA in the same manner as costimulated cells. C, IA does not inhibit IKK activity in vitro. HeLa cells were stimulated with TNFα as shown, and the IKK complex was isolated by immunoprecipitation with αIKKγ/NEMO antibodies. Immune complex kinase assays using the GST-IκBα substrate protein were performed in the presence of IA (150 μM) or ethanol as a solvent control. An autoradiogram from a reducing SDS gel (top) and a Coomassie staining of the GST-IκBα fusion proteins (bottom) are shown. D, IA inhibits the phosphorylation of IKK activation loop. HeLa cells were stimulated with TNFα (20 ng/ml for 20 min) in the presence of IA (140 μM) or ethanol as a solvent control. Whole-cell extracts were prepared and analyzed for the phosphorylation of IKKα/β by WB.

The lack of interference with Tat-induced transcription rules out potential effects of IA on the basal transcriptional machinery or other nonspecific effects. In contrast to IA, the CDK9 inhibitor 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole riboside effectively inhibited luciferase activity in HeLa-Tat-Luc cells.

IA Suppressed Inflammation in the Mouse Paw Model. Having established that IA inhibited the NF-κB pathway in vitro, we studied the anti-inflammatory properties of IA in vivo and found that IA significantly reduced inflammation in the inflamed paw model in mice (n = 5 per group) during a 4-h period. The decreased inflamed paw volume in the treated mice reflected a decrease in edema, which is a component of the inflammatory response. There were highly significant effects of treatment (F = 11.7, df = 3,64, p < 0.001), time (F = 10.6, df = 4,64, p < 0.0001), and interaction (F = 3.9, df = 12,64, p < 0.001) as seen in Fig. 8. IA also significantly reduced other inflammatory parameters: redness (scored by visualization) and pain (as measured by the paw-licking frequency) (data not shown).

Together, these data show that IA inhibited NF-κB activation and exerted anti-inflammatory properties in an in vivo model of inflamed paw.

Discussion

In the current study, we demonstrate that the major NF-κB inhibitory components in *Boswellia* resin are IA and its derivative IN. IA inhibits IKK phosphorylation and activation in vivo, but not in vitro, implying that it exerts its action upstream of IKK. IA blocks NF-κB activation in response to TNFα and LPS but does not inhibit IKK activation in costimulated Jurkat T cells. TAK1 plays a critical role in TNFα-induced NF-κB activation (Blonska et al., 2005). TAB2 and TAB3 are adaptors that link the kinase TAK1 to upstream regulators in the proinflammatory TNF signaling pathway (Hong et al., 2007). IA attenuates TAK/TAB-induced phosphorylation of the IKKα/β activation loop by interfering with a step that couples TAK to IKK phosphorylation and activation. However, this inhibition appears to be specific, because IA does not impair TNFα-induced activation of JNK and p38 MAPK. This specificity might suggest that IA can serve as a pharmacological tool in the intensive research conducted on the activation of IKK by upstream events.

The inhibition of IκBα phosphorylation and of subsequent degradation, as well as the inhibition of p65 phosphorylation at serine 536 can be attributed to the inhibitory effect of IA on IKK, because IKK plays a major role both in IκBα and p65
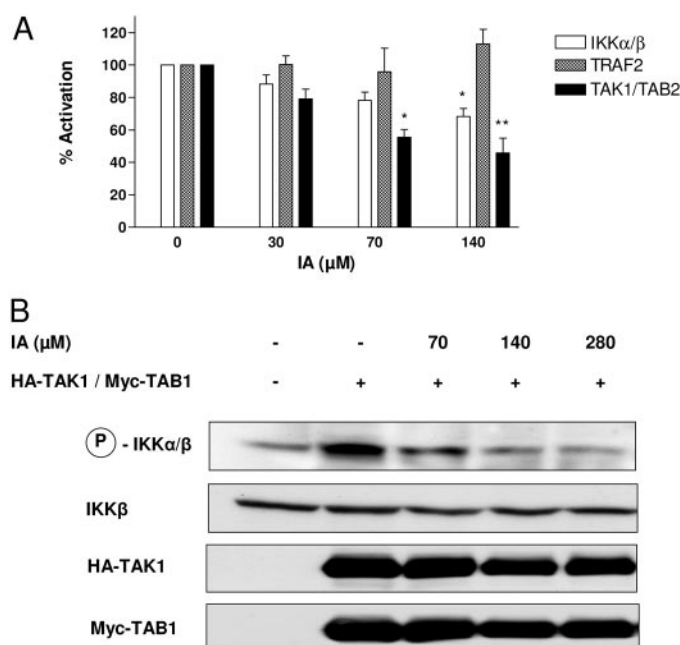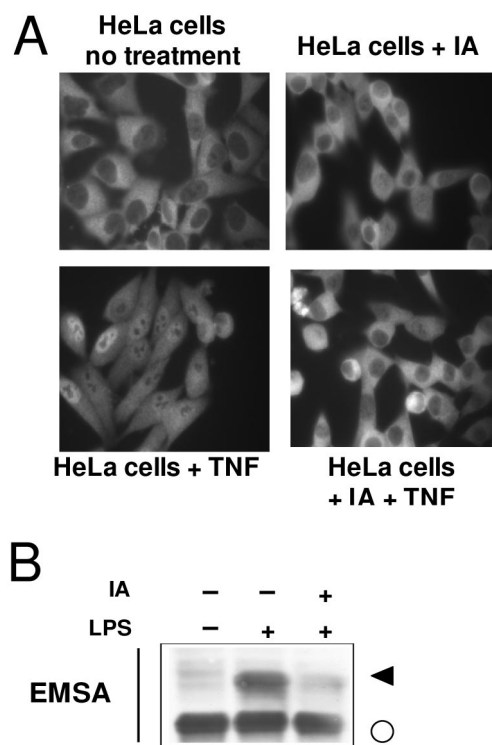

Fig. 5. IA inhibited TAK/TAB-triggered IKK activation. A, A549 cells were transiently transfected with KBF-luc alone or in combination with IKKα/IKKβ, TRAF2, or TAK1/TAB2 expression vectors. After 24 h of transfection, cells were treated with IA for 6 h and luciferase activity was assayed. To allow comparability, activation by IKKα/IKKβ, TRAF2, or TAK1/TAB2 in untreated cells were given a value of 100%, and the IA inhibitory effect is represented as percentage of activation. Average values from three independent experiments are shown; error bars show S.E.M. The data represent the results of five independent experiments. *, p < 0.05; **, p < 0.001 compared with nontreated cells (two-way ANOVA, followed by a Bonferroni multiple comparison test). B, IA inhibits the phosphorylation of IKKα/β induced by TAK/TAB overexpression. 293T cells were transfected with expression vectors for HA-TAK1 and Myc-TAB1 or with a control vector. Thirty-six hours after transfection, cells were treated for 6 h with increasing concentrations of IA as shown and lysed. Equal amounts of protein were examined for phosphorylation of IKKα/β activation loop by immunoblotting. Similar results were obtained when transfecting cells with TAK1/TAB2 (data not shown).

Fig. 6. IA inhibited the accumulation of p65 in cell nuclei of TNFα-stimulated HeLa cells and the NF-κB DNA-binding of LPS-stimulated human peripheral monocytes. A, HeLa cells were stimulated with TNFα (20 ng/ml for 20 min) in the presence of IA (140 μM) or ethanol as a solvent control. Cells were fixed and then stained with rabbit anti-p65 followed by anti-rabbit Rhodamine Red-labeled secondary antibody and with 4,6-diamidino-2-phenylindole for nuclear location (data not shown). The cells were examined under an Axioscope Zeiss microscope with a plan-Neofluor 60× lens. Results of one of three independent experiments are shown. B, human peripheral monocytes extracts in the presence or in the absence of IA (100 μM) were tested by electrophoretic mobility shift assay for NF-κB DNA binding. The filled arrowhead indicates the location of the DNA-NF-κB complex; the circle indicates the position of a constitutively DNA-binding protein.

phosphorylation (Sizemore et al., 2002). Downstream of IκB, IA inhibits the accumulation of NF-κB in TNF-stimulated HeLa cell nuclei, NF-κB DNA binding in LPS-stimulated human peripheral monocytes, and the induction of NF-κB dependent gene expression.

The inhibition of IKK activation by IA resembles that of

Fig. 7. IA inhibited NF-κB activation in TNFα-stimulated 5.1 Jurkat cells. A, 5.1 Cells were preincubated with IA for 30 min at the indicated concentrations and stimulated with TNFα (2 ng/ml) for 6 h. The luciferase activity was then measured. There was no effect of solvent on cell viability or on the enzymatic activity of luciferase at the highest concentration (data not shown). B, HeLa-Tat-Luc cells were incubated with either 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole riboside (50 μM) or IA at the indicated concentrations for 18 h. Tat-induced LTR-luciferase activity was measured. Luciferase activity is given as -fold induction or relative light units (RLU); standard deviations are given. **, $p \leq 0.01$ to -fold values in the absence of IA by t test.
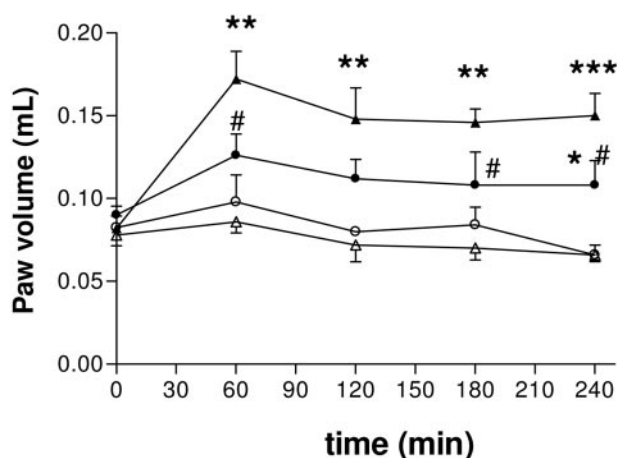
Fig. 8. IA inhibited inflammation in the inflamed paw model after injection of carrageenin. IA (50 mg/kg) or vehicle was injected i.p. to Sabra female mice (five per group) 30 min before induction of the inflammatory stimulus. Hind paws were then injected with 50 μl of saline or λ-carrageenin (4%). Ensuing inflammatory swelling was measured by increase in foot volume in a plethysmometer. IA also reduced paw redness (as a measure of erythema) and licking (as a measure of pain) (data not shown). There were highly significant effects of treatment ($F = 11.7$, $df = 3,64$, $p < 0.001$). △, vehicle + saline; ▲, vehicle + carrageenin; ○, IA + saline; ●, IA + carrageenin; ∗, different from IA + saline, $p < 0.05$; ∗∗, different from vehicle + saline at $p < 0.01$, respectively;∗∗∗, different from vehicle + saline at $p < 0.001$, respectively; #, different from vehicle + carrageenin, $p < 0.05$.

the antiapoptotic protein embelin, recently demonstrated to be an NF-κB inhibitor. Like Embelin (Ahn et al., 2007), IA mediates its effects on IKK activation through impairment of a step connecting TAK to the IKK activation loop phosphorylation. TRAF2 apparently also recruits the IKK complex directly via the complex of TRADD, TRAF2, TRAF5, and RIP1 (Häcker and Karin, 2006). Thus, the lack of inhibitory effect by IA on TRAF2-overexpressing cells supports a specific IA intervention at the TAK1-IKK activation step. The effect of IA on IKK also resembles that of the tetracyclic kaurene diterpenes as shown by Castrillo et al. (2001), as both are signaling rather than direct inhibitors. However, it appears that IA's activity is more specific to the NF-κB pathway, because kaurenes also inhibit the phosphorylation of p38, ERK1, and ERK2 MAPK.

Diterpenoids are natural compounds with a backbone of 20 carbon atoms biosynthesized from geranylgeranyl pyrophosphate (Hanson, 2005). It is an important and chemically diverse group of natural products, which share some common biosynthetic steps and are of considerable biological importance (Hanson, 2005). It is noteworthy that although members of this group of natural products share no common chemical moiety, a large arsenal of biologically active compounds has been identified among them (see Ojo-Amaize et al., 2002; Tempeam et al., 2005; Zhang et al., 2005 for some examples), and several diterpenoids are known as inhibitors of NF-κB activation (Castrillo et al., 2001; Leung et al., 2005; Yinjun et al., 2005). The mechanism by which terpenes impair IKK activation has so far been poorly characterized (Castrillo et al., 2001). The mechanism of action of IA, however, is completely different from several other anti-inflammatory diterpenoids that inhibit the NF-κB pathway (for example, oridonin, ponicidin, xindongnin A, and xindongnin B). These diterpenoids, isolated from *Isodon rubescens*, directly interfere with the DNA-binding activity of NF-κB to its response DNA sequence (Leung et al., 2005), whereas IA inhibits IKK activation. Even with kaurene diterpenoids that inhibit IKK activation (Castrillo et al., 2001), there are differences, in the mechanism of action, such as the specificity of action and the effect on p65 phosphorylation. The observation that some members of a large group of natural products are a source of NF-κB modulators by a multiplicity of pathways implies that they can serve as a valuable tool for the examination of the NF-κB pathway, especially upstream of IKK, where this pathway is still to be unfolded.

Based on our findings, we attribute the main NF-κB inhibitory effect of *Boswellia* resin to IA and its derivatives. The resin of *Boswellia* species, containing IA derivatives, has been used to treat inflammatory conditions for many centuries in traditional medicine in Europe, Asia, and Africa and is still in such use, besides its common religious use as incense. One interesting example of its use as an ingredient of an important anti-inflammatory remedy that has been common in Europe and Asia for hundreds of years is the Jerusalem Balsam (Moussaieff et al., 2005). *Boswellia* extracts are also marketed as food supplements for the treatment of arthritis in the US and Europe; in view of our current data, we propose that these products should be standardized for IA and its derivatives as well as boswellic acids. Moreover, the possible synergistic effects of these compounds need to be investigated. We propose the GC-MS fingerprinting depicted in
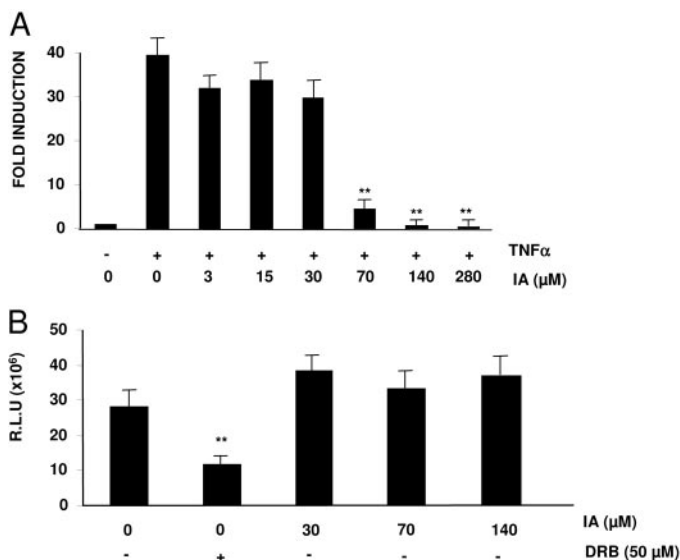
Data Supplement Fig. 1 as a simple method for the standardization of *Boswellia* resin for IA and its derivatives.

IA demonstrates a robust anti-inflammatory effect in a mouse inflamed paw model. This effect is within the range of drugs such as salicylates injected i.p. (Siqueira-Junior et al., 2003). However, IA and IN are practically insoluble in water and poorly soluble in other solvents used for injection to animals or in cell systems. Hence, the actual active concentrations of these compounds are probably considerably lower. The identification of the NF-κB inhibitory effect of cembrenoid diterpenes, an important group of common natural products, present in tobacco among other herbs and marine creatures, may further open these fields to the discovery of novel drugs for the treatment of diseases that pose unanswered challenges and affect a large segment of the population.

Acknowledgments

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Address correspondence to: Arieh Moussaieff, Department of Medicinal Chemistry and Natural Products, Medical Faculty, Hebrew University, Jerusalem 91120, Israel. E-mail: ariehm@ekmd.huji.ac.il