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Withaferin A Strongly Elicits IκB Kinase β Hyperphosphorylation Concomitant with Potent Inhibition of Its Kinase Activity*[S]

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The transcription factor NFκB plays a critical role in normal and pathophysiological immune responses. Therefore, NFκB and the signaling pathways that regulate its activation have become a major focus of drug development programs. *Withania somnifera* (WS) is a medicinal plant that is widely used in Palestine for the treatment of various inflammatory disorders. In this study we show that the leave extract of WS, as well as its major constituent withaferin A (WA), potently inhibits NFκB activation by preventing the tumor necrosis factor-induced activation of IκB kinase β via a thioalkylation-sensitive redox mechanism, whereas other WS-derived steroidal lactones, such as withanolide A and 12-deoxywithastramonolide, are far less effective. To our knowledge, this is the first communication of IκB kinase β inhibition by a plant-derived inhibitor, coinciding with MEK1/ERK-dependent Ser-181 hyperphosphorylation. This prevents IκB phosphorylation and degradation, which subsequently blocks NFκB translocation, NFκB/DNA binding, and gene transcription. Taken together, our results indicate that pure WA or WA-enriched WS extracts can be considered as a novel class of NFκB inhibitors, which hold promise as novel anti-inflammatory agents for treatment of various inflammatory disorders and/or cancer.

The search for anticancer drugs and anti-inflammatory agents from natural products represents an area of great interest worldwide (1). In the Palestinian traditional folk medicine, more than 700 plant species are known for their use as medicinal herbs or as botanical pesticides (2, 3). As revealed by a recent ethnobotanical survey carried out in the West Bank, at least 63 plant species are still in use (4). Plants like *Withania somnifera* (WS)[4] are applied in different forms (decoctions, infusions, ointments, powder, syrup, etc.) (5, 6) to treat burns, wounds, and/or dermatological disorders, to prevent infections, and to treat various diseases including gastrointestinal diseases, infertility, and cutaneous abscesses (7).

WS belongs to the Solanaceae family and is being used in many indigenous systems of medicine (8). It is a small, semi-woody shrub with ovate leaves and greenish orange fruit. It grows wild to about 1.5 m in height and can be found growing in Palestine as well as in some other Middle East countries, in Africa, and in India (where it is alternatively called ashwagandha in ayurvedic medicine). As a result of this wide growing range, there are considerable morphological and/or chemotypical variations in terms of local species, which may affect the composition of its metabolites and/or its activities (6, 9–11). The biologically active constituents of WS are alkaloids (isopelletierine and anaferine), saponins (sitoindosides), and the C_{28} steroidal lactones (withanolides) (12). Although some evidence on immunostimulatory and anti-inflammatory activities of WS has been reported before, the molecular mechanisms by which its constituents exert their immunomodulatory effects have not been studied and characterized sufficiently.

Because the NFκB transcription factor is a key regulator of cellular processes involved in the immune response, differentiation, cellular proliferation, and apoptosis (13, 14) and because the constitutive activation of NFκB contributes to multiple pathophysiological conditions such as rheumatoid arthritis, asthma, inflammatory bowel disease (15), AIDS (16), and cancer (17), we investigated the effect of the WS leaf extract and its major constituent withaferin A (WA) on the NFκB signaling pathway.

In mammalian cells, the NFκB/Rel family consists of five members: RelA (p65), RelB, c-Rel, p105/p50 (NFκB1), and

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Fig. 1.

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[4] The abbreviations used are: WS, *Withania somnifera*; WA, withaferin A; NFκB, nuclear factor κB; IκB, inhibitory subunit of NFκB; IKK, IκB kinase; TNF, tumor necrosis factor; IL, interleukin; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; DTT, dithiothreitol; EMSA, electrophoretic mobility shift assay; MEK, mitogen-activated protein kinase; RANTES, regulated on activation normal T cell expressed and secreted; HPLC, high performance liquid chromatography; JNK, c-Jun NH_2-terminal kinase; DEX, dexamethasone; GST, glutathione *S*-transferase; P-, phospho-; 12DW, 12-deoxywithastramonolide; WdA, withanolide A; PSI, proteasome inhibitor.

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p100/p52 (NFκB2). Each family member has a conserved Rel homology domain specifying DNA binding, protein dimerization, and nuclear localization. In most cells, NFκB is composed of a heterodimer of p65 and p50 where the p65 protein is responsible for the transactivation potential. In unstimulated cells, NFκB is sequestered predominantly in the cytoplasm in an inactive complex through interaction with IκB inhibitor proteins. In response to stimulation by a variety of potent activators, such as tumor necrosis factor (TNF)-α, interleukin (IL)-1, or lipopolysaccharide (18), IκBα is rapidly phosphorylated at two conserved NH_2-terminal serines (Ser-32 and Ser-36) and degraded through a ubiquitin-dependent proteolysis, resulting in the release of NFκB, translocation into the nucleus, and induction of gene transcription. NFκB regulates a wide variety of important target genes encoding cytokines (IL-6, TNF, and IL-1), chemokines (IL-8 and RANTES), adhesion molecules (E-selectin), and even its own inhibitors, IκBα and IκBβ (19, 20).

IκB kinase (IKK) is the protein kinase complex responsible for IκB phosphorylation in response to proinflammatory stimuli, resulting in ubiquitination and degradation of the latter. IKK is a multisubunit complex that contains two catalytic subunits, IKKα and IKKβ, and a regulatory subunit, IKKγ/NEMO (NFκB essential modulator) (20, 21). Knock-out studies have revealed that IKKβ is responsible for the proinflammatory cytokine-induced activation of NFκB (22). The mechanisms that activate the IKK complex are not completely understood, but it is known that activation requires phosphorylation of Ser-177 and Ser-181 in the activation loop of IKKβ (23). The physiological molecular mechanism for postinduction inactivation of IKK remains a mystery; regulated IKK inhibitors such as A20 (24) or other IKK inhibitors such as PP2Cβ, PP2A, CYLD (cylindromatosis), hTid-1, and Hsp70 might be involved (25–30). Alternatively, regulation of the conformational state of IKKβ via hyperautophosphorylation of its COOH terminus could lead to IKK inactivation (23). Importantly, the disruption of the COOH-terminal sites in IKKβ leads to persistently active IKK upon stimulation (23), suggesting inhibitory autophosphorylation as the major source of fast IKK down-regulation. Considerable work has been directed at identifying small molecules that inhibit IKKβ as possible targets for the development of anti-inflammatory and antineoplastic drugs (31).

In this study, we highlight the potential of pure WA compound or WA-containing fractions of the WS leaf extract from Palestine as potent and specific inhibitors of the NFκB pathway. We observed that WA inhibits NFκB activation through inhibition of IKKβ activity depending on thioalkylation-sensitive IKKβ regulation concomitant with IKKβ Ser-181 hyperphosphorylation. This results in stabilization of cytoplasmic IκBα and hence the reduction of nuclear translocation, diminished NFκB/DNA binding, and blockage of NFκB-driven gene expression.

MATERIALS AND METHODS

Plant Collection and Preparation—The leaves of WS were collected during the months of January to April from the surrounding area of Ramallah, Palestine. The shade-dried and ground leaves of WS were extracted with a mixture of dichloromethane and methanol (1:1, v/v) for 24 h at room temperature. After filtration, the residue was covered with 100% methanol for 20 min and then drained into the same flask. The solvent was dried by rotary evaporation under reduced pressure and at a maximal temperature of 45 °C. The resulting crude extracts were stored at −20 °C until assayed. Before running the bioassay the extract was dissolved in Me_2SO, diluted in Dulbecco's modified Eagle's medium, and added to the cell culture at the dose or concentration range indicated in figure legends.

Chromatography—The crude extract was fractionated via solid phase extraction using Bond Elut C_{18} cartridges (Varian, St.-Kathelijne Waver, Belgium). After activation with methanol and conditioning with water, the extract (dissolved in methanol and diluted with 9 volumes of water) was applied to the cartridge. Fractions were eluted, respectively, with solvent of increasing strengths: water, water/methanol (3:1, v/v), water/methanol (1:1), water/methanol (1:3), and finally methanol. Appropriate volumes were used depending on the scale of the fractionation. All extracts and fractions were analyzed using HPLC. Analyses were carried out using a Waters 2695 Alliance Separations Module equipped with a Waters 996 Photodiode Array detector. The column was a Varian Omnispher (C_{18}, 250 × 4.6 mm, 5 μm) and was maintained at 35 °C; the injection volume was 20 μl. Gradient elution over 60 min was applied from 15% solvent B (a methanol/acetonitrile mixture with 0.025% formic acid) in solvent A (water with 0.025% formic acid) to 95% solvent B in solvent A. Another gradient from 45% solvent C (methanol with 0.025% formic acid) in solvent A to 85% solvent C in solvent A was used for the quantitative analysis of WA in the WS extract and in the fractions. Chromatograms at 220 nm were extracted from the three-dimensional data, and peaks were characterized based on their UV spectra and retention times and compared with authentic standards. Peak integrations were carried out using standard parameters. Isolation of WA and withanolide A was carried out using a Gilson 322 pump equipped with a Gilson UV-visible 156 detector and a Gilson 206 fraction collector. Solvents and gradients were similar to those used for analytical purposes. The column was a Varian Omnispher (C_{18}, 350 × 21.4 mm, 10 μm), and fractions were collected either manually or based on peak intensity. The organic solvent remaining in the collected fractions was removed under reduced pressure using a rotavapor, and fractions were taken to dryness via lyophilization. Samples were analyzed with mass spectroscopy using flow injection on a Micromass Quattro Micro. The purified materials were also subjected to ^1H NMR and ^{13}C NMR using a Bruker 300 MHz spectrometer.

Cell Culture—Murine fibrosarcoma L929sA cells and human embryonic kidney 293T cells were maintained in Dulbecco's modified Eagle's medium supplemented with 5% newborn calf serum, 5% fetal calf serum, 100 units/ml penicillin, and 0.1 mg/ml streptomycin. Twenty-four hours before induction, cells were seeded in multiwell dishes such that they were confluent at the time of the experiment. IKKα- and IKKβ-deficient mouse embryonic fibroblasts (kindly provided by Dr. Emmanuel Dejardin) (32) and cervix cancer cells (HeLa) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. MDA-MB-231 human breast cancer cells

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were cultured in L-15 medium supplemented with 10% fetal calf serum.

Cytokines, Inducing Compounds, Antibodies, and Reagents—Recombinant murine TNF, produced in *Escherichia coli* and purified in our laboratory to at least 99% homogeneity, had a specific biological activity of 8.58×10^7 IU/ml of protein as determined in a standard TNF cytolysis assay. Reference TNF (code 88/532) was obtained from the National Institute of Biological Standards and Control (Potters Bar, UK). U0126 was supplied by Promega (Madison, WI); SB203580 was purchased from Alexis (Lausanne, Switzerland); SP600125 was purchased from Calbiochem; cycloheximide was purchased from Sigma. Work solutions of the reagents were routinely prepared in culture medium. Control experiments verified that the final concentration of organic solvents did not interfere with any of the assays.

Withaferin A, withanolide A, and 12-deoxywithastramonolide were purchased from ChromaDex (Santa Ana, CA) and were stored as 1 mg/ml solution in methanol at −20 °C. PSI was purchased from Affiniti Research Products Ltd. and was stored as a 10 mM solution in Me_2SO at −20 °C. MG132 was purchased from Calbiochem, and lipopolysaccharide was purchased from Sigma. Anti-IκBα (C-21), anti-IKKα, anti-IKKβ, anti-p65, anti-IKKγ, and the phosphospecific anti-IκBα and anti-IKKα(Ser-180)/IKKβ(Ser-181) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). The phosphospecific anti-p38 (Thr-180/Tyr-182), anti-p42/p44 (Thr-202/Tyr-204), and anti-stress-activated protein kinase/JNK (Thr-183/Tyr-185) MAPK polyclonal rabbit antibodies only detect the dual phosphorylated form of MAPK; they were purchased from New England Biolabs, Inc. (Beverly, MA).

Plasmids—The full-size IL-6 promoter reporter gene construct p1168hu.IL6P-luc+ and the recombinant plasmids pAP1luc and $p(IL\text{-}6\kappa B)_3$50hu.IL6P-luc+ were described previously (33, 34). p1481.IL8P-luc+, containing an IL-8 promoter fragment of 1481 bp, was a gift from Dr. N. Mukaida (Cancer Research Institute, Kanazawa, Japan). pELAMP-luc+, containing the E-selectin promoter, was a kind gift from D. Goeddel (Tularik, San Francisco, CA).

Biological IL-6 Assay—Secreted IL-6 was quantified according to its growth-stimulating effect on 7TD1 cells as described previously (33).

Transfection Procedure—Stable transfection of L929sA cells was performed by the calcium phosphate precipitation procedure according to standard protocols (33).

Reporter Gene Analysis—Luciferase and galactosidase reporter assays were carried out according to the manufacturer's instructions (Promega) and have been described previously (33). Normalization of luciferase activity was performed by measurement of β-galactosidase levels in a chemiluminescent reporter assay Galacto-Light kit (Tropix, Bedford, MA). Light emission was measured in a luminescence microplate counter (Packard Instrument Co.). Luciferase activity, expressed in arbitrary light units, was corrected for the protein concentration in the sample by normalization to the co-expressed β-galactosidase levels. β-Galactosidase protein levels were quantified with a chemiluminescent reporter assay Galacto-Light kit (Tropix).

Electrophoretic Mobility Shift Assay (EMSA)—L929sA, MDA-MB231, HeLa, and human embryonic kidney 293T cells were seeded in 6-well plates at 3×10^5 cells/well. After appropriate induction, cells were washed with ice-cold phosphate-buffered saline (PBS), harvested with a rubber policeman, and precipitated in 1 ml of PBS by centrifugation for 10 min at 2600 rpm (4 °C). Preparation of total, cytoplasmic, or nuclear cell extracts has been described previously (35, 36). For EMSA, equal amounts of protein were incubated for 25 min with an NFκB-specific ^{32}P-labeled oligonucleotide and binding mixture as described previously (36, 37). Labeling of the oligonucleotides was performed with [α-^{32}P]dCTP by using Klenow enzyme (Roche Applied Science). The NFκB oligonucleotide comprises the sequence 5′-AGCTATGT**GGGTTTTCCC**AT-GAGC-3′ in which the single IL-6 promoter-derived NFκB motif is bold and underlined. Samples were loaded on a 6% polyacrylamide gel run in 0.5× Tris borate-EDTA buffer (pH 8). The gel was dried after electrophoresis, and complexes formed were analyzed using PhosphorImager technology.

Western Blot Analysis—L929sA cells were seeded in 6-well plates at 3×10^5 cells/well. Cells were pretreated with the extract/compound for 1 h followed by incubation with TNF for the indicated time periods. Cells were washed with ice-cold PBS and lysed in SDS sample buffer (62.5 mM Tris-HCl, 2% (w/v) SDS, 10% glycerol, 50 mM dithiothreitol (DTT), and 0.01% (w/v) bromphenol blue). To shear DNA and reduce sample viscosity, lysates were sonicated for 1 min in a water bath sonicator and then heated to 95 °C for 5 min after which they were immediately cooled in ice and microcentrifuged for 5 min. The lysates were separated by 10% SDS-PAGE and electrotransferred onto a nitrocellulose membrane. Blots were probed using the appropriate antibodies, and the immunoreactive protein was detected using enhanced chemiluminescence reagents (Eastman Kodak Co.).

Zymosan-induced Inflamed Paw Model—Eight-week-old C57BL/6J mice were purchased from Iffa Credo. The experimental setup contained three groups with 10 animals per group. Group 1 received an intraperitoneal injection of 60 μl of Me_2SO (sterile) followed 30 min later by a subcutaneous injection of zymosan solution (15 mg/ml in phosphate-buffered saline, sterilized) in the right footpad; the left footpad was left uninjected. Group 2 was treated intraperitoneally with 60 μl of 0.5 mg/mouse withaferin A (dissolved in Me_2SO) followed 30 min later with zymosan injection. Group 3 was treated with 60 μl of (150 μg/mouse) dexamethasone (DEX) followed 30 min later with zymosan injection. 6, 12, 24, and 48 h after the zymosan treatment, the thickness of both footpads was measured by using a caliper, and the difference between zymosan- and non-injected footpads was compared for all three experimental groups.

Determination of Systemic IL-6 Levels in Blood—Blood samples were taken by sinus retro-orbital punction under isoflurane anesthesia from mice 6 h after zymosan injection, which followed pretreatment with Me_2SO (60 μl/mouse), withaferin A (0.5 mg/mouse), or DEX (150 μg/mouse) injected intraperitoneally (six mice per group) for 30 min. IL-6 protein levels were tested by the biological 7TD1 assay as described elsewhere (33).

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Reverse Transcription-PCR—Reverse transcription-PCRs were carried out according to the instructions of the manufacturer (Promega). Briefly, an aliquot of 5 μg of total RNA, 2 μl of oligo(dT), and *x* ml of DEPC-bidi (diethyl pyrocarbonate-treated bidistilled water) (to adjust the final volume to 10 μl) was placed in a microcentrifuge tube and incubated at 70 °C for 10 min. The sample was briefly centrifuged and placed on ice for 10 min. A 20-μl reaction mixture (containing 6 μl of buffer 5×, 2.5 μl of 100 mM DTT, 2.5 μl of 2.5 mM dNTP, 0.5 μl of RNasin®, 1 μl if avian myeloblastosis virus reverse transcriptase, and 7.5 μl of DEPC-bidi) was added to the annealed RNA sample. The reverse transcription was conducted using the following conditions: 5 min at 70 °C followed by a decrease of 0.1 °C/s to 42 °C, 60 min at 42 °C, 5 min at 90 °C per cycle. The obtained cDNA fragments were then further analyzed with quantitative PCR. cDNA-specific primer sets are available upon request. The PCR products were analyzed by agarose gel electrophoresis. After staining the gel with ethidium bromide, the products were detected under UV light.

Northern Blot Analysis—After appropriate inductions, RNA isolations were obtained by using TRIzol™ reagent (Invitrogen). Briefly 10×10^6 L929sA cells were grown to subconfluency in 14-cm Petri dishes. Cells were pretreated with WS for 1 h before TNF induction for 6 h. Total RNA was isolated with TRIzol; denaturation was achieved with a combination of Me_2SO and deionized glyoxal (Sigma). A total of 23 μg of RNA was separated on a 1% agarose gel in 20 mM phosphate buffer (pH 7). RNA was transferred to Hybond-N+ membranes (Amersham Biosciences) by standard capillary blotting and cross-linked by UV irradiation. Hybridization was done by sequentially probing with murine IL-6, human IκBα, RANTES, and murine glyceraldehyde-3-phosphate dehydrogenase cDNA fragments. The membrane was stripped (with boiling 0.5% SDS, left on the membrane until it is cold) before each new hybridization step. All fragments were labeled with [α-^{32}P]dCTP by using a Random Primed labeling kit (Roche Applied Science).

In Vitro Kinase Assay—IKK complex from whole-cell extracts was precipitated with antibody against IKKγ for 2 h followed by treatment with protein A-Sepharose beads (Pierce). After 2 h of incubation, the beads were washed with IP buffer (50 mM Tris-HCl (pH 8), 250 mM NaCl, 3 mM EDTA, 3 mM EGTA, 0.1% Igepal, 1 mM DTT, 10 mM NaF, 1 mM Na_3VO_4, 25 mM β-glycerophosphate, and 10 mM *p*-nitrophenyl phosphate) and assayed in 40 μl of kinase assay mixture containing kinase buffer (50 mM Tris-HCl (pH 8), 100 mM NaCl, 2 mM $MgCl_2$, 1 mM DTT, 10 mM NaF, 1 mM Na_3VO_4, 25 mM β-glycerophosphate, 10 mM *p*-nitrophenyl phosphate, and 0.5 mM phenylmethylsulfonyl fluoride, 1 mM unlabeled ATP, and 2 μg of substrate glutathione *S*-transferase (GST)-IκBα. After incubation at 30 °C for 30 min, the reaction was terminated by boiling with SDS sample buffer for 5 min. Finally, the protein was resolved by 10% SDS-PAGE, electrotransferred onto a nitrocellulose membrane, and blotted with an anti-P-IκBα antibody.

RESULTS

WS Leaf Extract Inhibits Inflammatory Gene Expression—IL-6 is a pleiotropic cytokine involved in acute phase and immune reactions and inflammatory responses. Previously, we have demonstrated potent IL-6 gene activation in response to TNF in the mouse fibrosarcoma cell line L929sA that can be reversed by a plethora of anti-inflammatory compounds, such as chemical kinase inhibitors, natural products, hormones, etc. (33–40).

Taking into account the traditional use of WS in inflammatory affections, we explored the effect of WS leaf extract on TNF-induced IL-6 gene expression in L929sA mouse fibroblasts. As shown in Fig. 1*A*, the elevated levels of IL-6 protein detected after TNF treatment were strongly repressed in the presence of the extract in a concentration-dependent manner.

WS Inhibits NFκB-driven Gene Expression—To verify whether the decrease in IL-6 protein levels by WS was due to transcriptional repression at the promoter level, L929sA cells stably transfected with various TNF-inducible reporter gene constructs, driven by the natural full-size IL-6 promoter (p1168hu.IL6P-luc+), the IL-8 promoter (p1481hu.IL8P-luc+), and the E-selectin promoter (pE-selectin-luc+), respectively, were tested for WS effects in comparison with a constitutively expressed reporter gene construct (pPGKbGeobpA) controlled by the phosphoglycerokinase promoter (34). Enhanced luciferase expression levels were measured in response to TNF for the various reporter gene constructs, whereas pretreatment with WS was found to strongly inhibit reporter gene expression, leaving the constitutive housekeeping promoter phosphoglycerokinase expression unaffected under all conditions tested (Fig. 1*B*). Because we previously demonstrated an essential role for NFκB in triggering IL-6 gene transcription in response to TNF, we also measured WS effects on a recombinant promoter with multiple NFκB-responsive elements, *i.e.* p(IL-6κB)$_3$50hu.IL6P-luc+ (Fig. 1*C*). The expression levels observed with the recombinant promoter or the various NFκB-driven reporter gene constructs (IL-6, IL-8, E-selectin, and NFκB) are in agreement with the drastic repression observed with the endogenous IL-6 protein levels and reconfirm the central role of NFκB in TNF-induced IL-6 gene expression. In contrast, upon testing WS effects on another recombinant promoter construct, pAP1luc, with multiple AP1-responsive elements, it appeared that WS did not repress but slightly elevated AP1-driven gene expression, further illustrating the NFκB-selective activities of WS (Fig. 1*C*). Furthermore and along the same line, mRNA analysis of various endogenous NFκB target genes was evaluated in the presence or absence of the WS extract by Northern blot analysis and reverse transcription-PCR. As expected and shown in Fig. 1*D*, pretreatment of WS extract potently suppressed mRNA levels of basal and TNF-induced IL-6, RANTES, and IκBα, whereas the glyceraldehyde-3-phosphate dehydrogenase housekeeping transcription remained unaffected.

WS Inhibits NFκB/DNA Binding—We further investigated whether the inhibition at the promoter level is due to the inhibition of NFκB/DNA binding. As shown in Fig. 1*E*, TNF-induced NFκB/DNA binding could be seen as early as 15 min and was maintained until 120 min. Pretreatment with WS caused a complete inhibition of NFκB/DNA binding at all time points, whereas there was no effect on the levels of recombination signal sequence-binding protein Jκ, binding onto the same DNA

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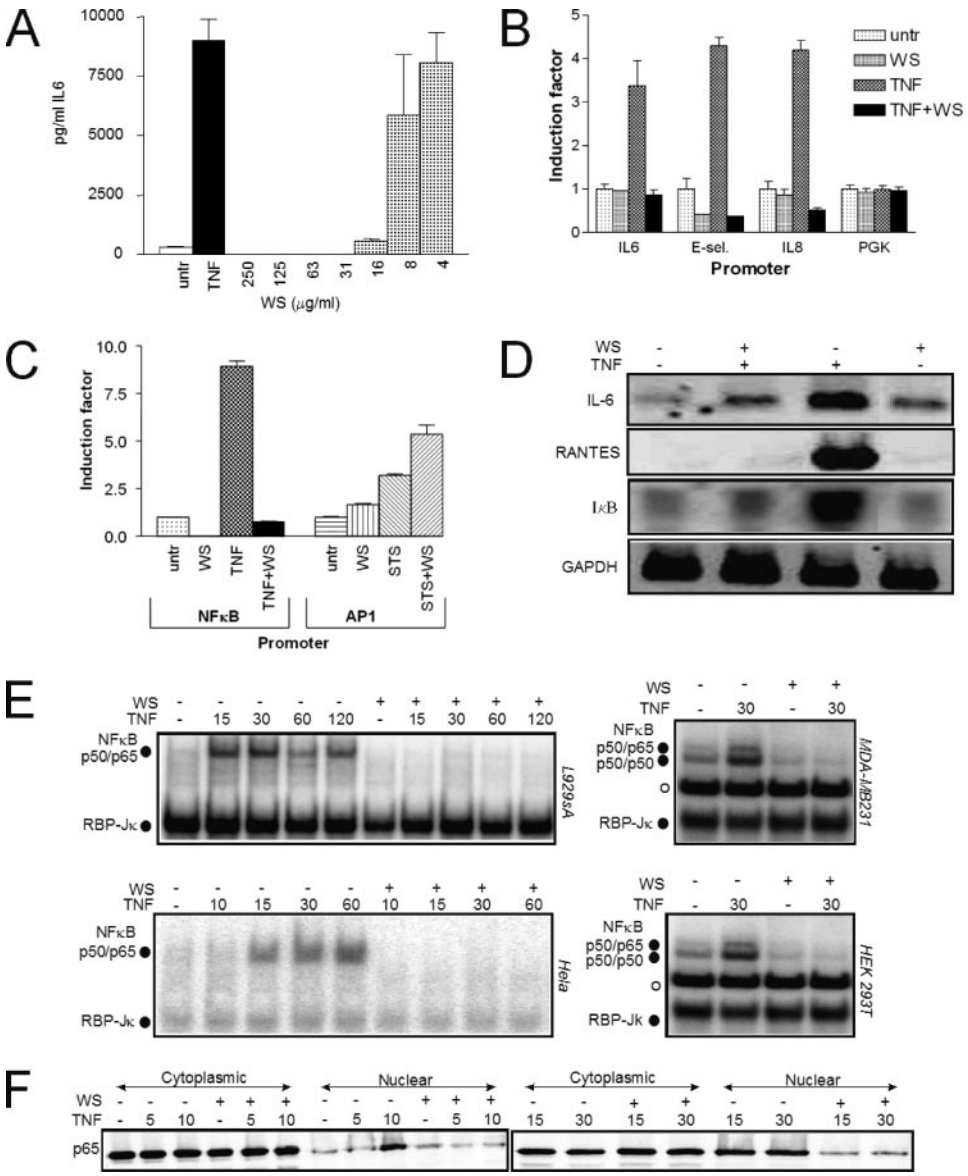

FIGURE 1. *A*, WS inhibits expression of the inflammatory cytokine IL-6. L929sA cells were pretreated for 1 h with the indicated concentrations of WS followed by 6-h treatment with 2000 IU/ml TNF. Corresponding levels of secreted IL-6 protein were quantified by the biological 7TD1 assay. *B*, WS inhibits NFκB-driven reporter gene expression. L929sA cells were stably transfected with reporter gene plasmids containing the natural IL-6, IL-8, E-selectin (*E-sel.*), and phosphoglycerokinase (*PGK*) promoter luciferase constructs. L929sA transfectants were left untreated (*untr*) or were treated with 2000 IU/ml TNF for 6 h either alone or following a 1-h pretreatment with WS extract (63 μg/ml). Lysates were prepared for quantification of reporter gene expression levels and normalization for protein concentration. The induction factor is defined as the amount of luciferase produced in TNF-treated cells after normalization for β-galactosidase expression compared with the non-induced state. *C*, WS inhibits NFκB- but not AP1-driven reporter gene expression. Stable L929sA transfectants of recombinant reporter gene constructs, driven by either NFκB- or AP1-responsive elements in front of a minimal promoter, were left untreated or were treated respectively with 2000 IU/ml TNF or 60 nM staurosporine (*STS*) (34) for 6 h either alone or following a 1-h pretreatment with WS extract (63 μg/ml). Lysates were prepared for reporter gene analysis as described in *B*. *D*, WS represses endogenous NFκB-regulated gene expression. L929sA cells with or without WS pretreatment (63 μg/ml for 1 h) were stimulated with 2000 IU/ml TNF for 6 h. Total cytoplasmic RNA was isolated, and reverse transcription-PCR was performed with mRNA-specific primer sets for IL-6, IκBα, RANTES, and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), respectively. *E*, effect of WS on TNF-induced NFκB/DNA binding. L929sA cells were left untreated or treated with 2000 IU/ml TNF alone or following a 1-h pretreatment with 63 μg/ml WS for the indicated time points. Total cell lysates were incubated with a ^{32}P-labeled IL-6 κB site-containing probe. Binding complexes formed were analyzed by EMSA. Loading of equal amounts of protein was verified by comparison with the binding activity of the repressor molecule recombination signal sequence-binding protein Jκ (*RBP-Jκ*) (36). Similar experiments were performed with human embryonic kidney (*HEK*) 293T, MDA-MB231, and HeLa cells. *F*, WS inhibits TNF-induced p65 nuclear translocation. L929 cells were left untreated or treated with 63 μg/ml WS for 1 h and then stimulated with (2000 IU/ml) for the indicated time periods. Nuclear and cytoplasmic extracts were prepared, and the p65 protein levels were measured by Western blot analysis.

motif (36). Similar results were obtained in the human cell lines MDA-MB-231, HeLa, and human embryonic kidney 293T pointing to a cell-independent action mechanism.

WS Inhibits NFκB Translocation—Because NFκB activation requires nuclear translocation of the NFκB p65 subunit prior to NFκB/DNA binding, we next measured the level of p65 in the cytoplasm and nucleus under the various experimental conditions tested. As illustrated in Fig. 1*F*, and as expected, TNF induction resulted in an increase of p65 levels in the nucleus. Pretreatment of cells with WS extract abolished this effect, demonstrating that WS inhibits the release of p65 from its inhibitor molecule, IκBα, in line with the above mentioned observations that WS interferes with TNF-induced NFκB/DNA binding.

WS Inhibits TNF-induced Phosphorylation and Degradation of IκBα—Because IκBα degradation is required for translocation of NFκB to the nucleus, we next determined whether inhibition of NFκB/DNA binding by WS was due to the inhibition of IκBα degradation or of new IκBα synthesis. We found that 15 min after TNF induction, IκBα was completely degraded, whereas it fully reappeared after 90 min. However, IκBα degradation was completely blocked upon incubation with WS extract (Fig. 2*A*). The observation that inhibition of NFκB/DNA binding by WS still persists in the presence of the protein synthesis inhibitor cycloheximide (Fig. 2*B*) further proves that WS interferes with the processing of IκB rather than with IκB protein synthesis.

As IκBα needs to be phosphorylated by the IKK complex prior to its polyubiquitination and proteasomal degradation (14), we next investigated whether WS blocks either the TNF-induced IκBα phosphorylation event or the degradation subsequent to its phosphorylation. To evaluate the level of IκBα phosphorylation in Western blot analysis, we used the proteasomal inhibitors MG132 and PSI to block the degra-

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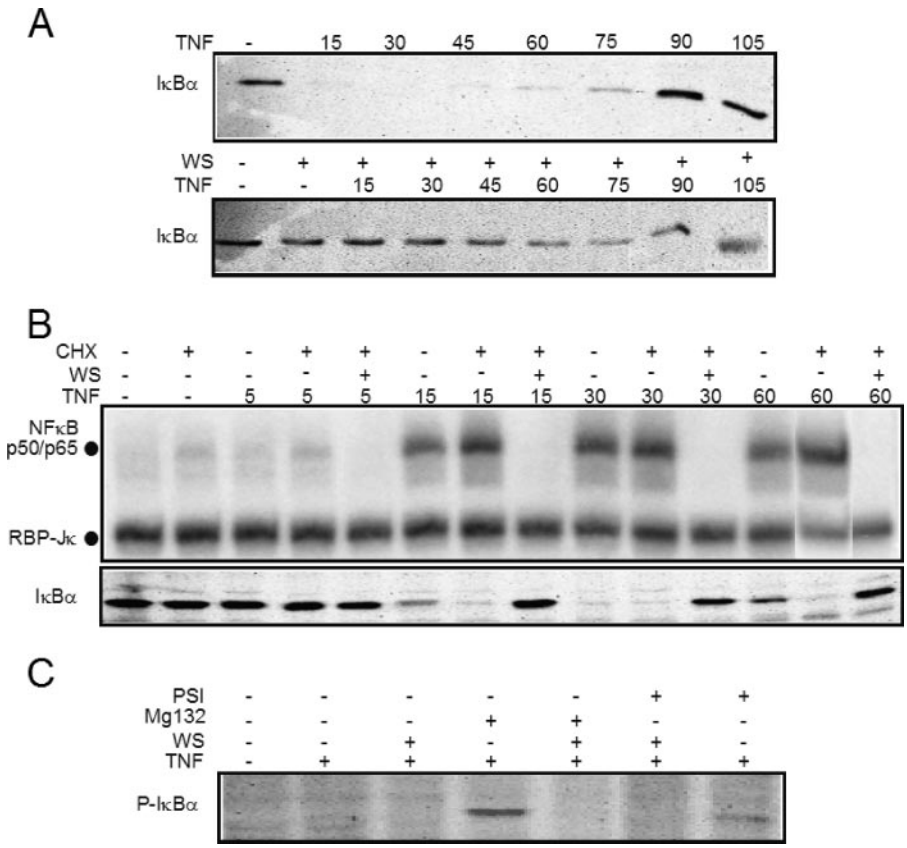

FIGURE 2. **Effect of WS on IκBα phosphorylation and degradation.** *A*, WS inhibits TNF-induced degradation of IκBα. L929sA cells were left untreated or were treated with 2000 IU/ml TNF either alone or following 1-h pretreatment with 63 μg/ml WS at the indicated time intervals. Whole-cell lysate was prepared, and equal amounts of protein were loaded onto an SDS-polyacrylamide gel for Western blot analysis. The levels of IκBα were detected using an anti-IκBα antibody. *B*, the experiment of Fig. 1*E* was repeated, but L929sA cells were incubated with 100 μg/ml cycloheximide (*CHX*) for 1 h before incubating with 63 μg/ml WS for another 1 h and then stimulated with 2000 IU/ml TNF for the indicated time. Whole-cell lysates were analyzed for NFκB/DNA binding by EMSA and for IκBα levels by SDS-polyacrylamide gel electrophoresis and Western blotting, respectively. *C*, WS blocks TNF-stimulated phosphorylation of IκBα. L929sA cells were untreated or treated with a 10 μM concentration of the protease inhibitors MG132 or PSI for 1 h, with or without a subsequent 1-h treatment with 63 μg/ml WS, and then stimulated with 2000 IU/ml TNF for 30 min. Whole-cell extracts were prepared and analyzed by Western blot analysis using an anti-P-IκBα antibody. *RBP-Jκ*, recombination signal sequence-binding protein Jκ.

dation of phosphorylated IκBα. In the presence of MG132 or PSI there was distinct phosphorylation upon TNF induction, whereas IκBα phosphorylation was abolished in the presence of WS (Fig. 2*C*). Thus, WS inhibits the degradation of IκBα most probably through the inhibition of its phosphorylation.

WS Inhibits TNF-induced IKKβ Activity Coinciding with Its Hyperphosphorylation—In response to a multitude of factors, such as inflammatory cytokines, bacterial products, viruses, and irradiation, the IKK complex is activated, leading to IKKβ-dependent phosphorylation of IκBα (41–43). Because IκBα phosphorylation and degradation are inhibited by WS, we assessed whether WS extract affects the TNF-induced IKK kinase activity. As shown in Fig. 3*A*, the IKK activity in the TNF-stimulated cells was totally inhibited when cells were pretreated with WS, providing conclusive evidence that the main inhibitory effect of WS is through the inhibition of the IKK complex.

To unravel whether WS directly blocks IKKβ kinase activity or rather abrogates upstream signaling cascades toward IKKβ Ser-181 phosphorylation in the activation loop, we performed phosphospecific Western blot analysis. Surprisingly (see Fig. 3*B*), pretreatment with WS induced hyperphosphorylation of IKKβ at Ser-181. Because the antibody recognizes the phosphorylation of both IKKα Ser-180 and IKKβ Ser-181, specificity of the Ser(P)-181 IKKβ signal was confirmed in knock-out cells using IKKα- and IKKβ-null mouse embryonic fibroblast cells. As shown in Fig. 3*C*, cells that no longer express IKKα exhibited normal IKKβ activation in response to TNF, and pretreatment with WS equally induced the sustained phosphorylation of IKKβ as apparent in wild type cells. However, and as expected, the signal was completely lost in cells that no longer express IKKβ.

WS Induces the Phosphorylation of IKKβ through the MEK/ERK Pathway—Because MAP3K, Tpl2/ERK, MEK/ERK/p90RSK, and TAK1 all have been described to act upstream of IKK (44–48), we further investigated which pathway may be involved in WS-dependent IKKβ hyperphosphorylation.

To address this question, L929sA cells were starved for 48 h in serum-free medium and treated with TNF alone or following a pretreatment with WS for 1 h before addition of TNF for different time periods. Pretreatment with WS was found to increase the basal and TNF-induced phosphorylation of p38, ERK, and JNK kinases (Fig. 4*A*). To further investigate potential cross-talk of MAPK pathways with sustained phosphorylation of IKKβ in the presence of WS, L929sA cells were treated with TNF, WS, or their combination in the presence of SB20538, U0126, or SP600125, which are p38, MEK/ERK, and JNK inhibitors, respectively. In the presence of the MEK1 inhibitor the sustained phosphorylation of IKKβ, induced by WS, was strongly reversed (Fig. 4*B*). Similarly treatment with the MEK1 inhibitor also reversed WS-induced phosphorylation of ERK activity (Fig. 4*B*). In contrast, no significant inhibition of WS-induced IKKβ hyperphosphorylation could be observed in the presence of the p38 and JNK inhibitors used (Fig. 4*C*). From these results we can conclude that WS-dependent IKKβ hyperphosphorylation strongly depends on MEK1 signaling. Whether MEK1-dependent IKKβ hyperphosphorylation by WS is responsible for inhibition of IKKβ kinase activity was further evaluated by IKKβ kinase assays. As shown in Fig. 4*D*, treatment with U0126 did not reverse IKKβ kinase inhibition by WS, although IKKβ hyperphosphorylation was abrogated as

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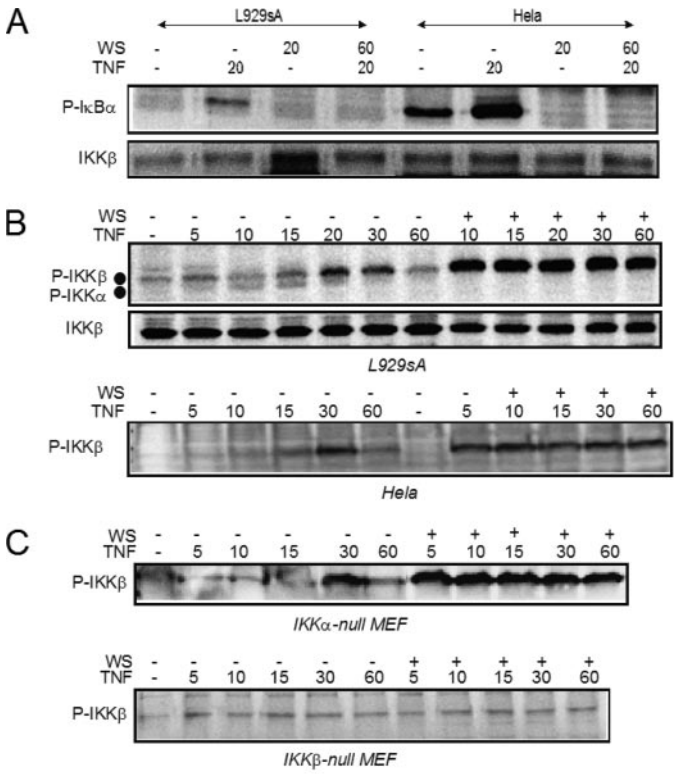

FIGURE 3. *A*, WS inhibits the TNF-induced IKKβ kinase activity. L929sA and HeLa cells were left untreated or were pretreated for 1 h with 63 μg/ml WS and then stimulated with 2000 IU/ml TNF for 20 min after which IKK protein was immunoprecipitated with anti-IKKγ antibody. Subsequently an IKK kinase assay was performed with the immunoprecipitated IKK complex, using GST-IκBα as a substrate and an excess of ATP, and the reaction complex was assayed by Western blot analysis with a P-IκBα antibody. The same nitrocellulose membrane was reprobed with anti-IKKβ antibody to detect the level of immunoprecipitated IKK protein. *B*, WS induces the phosphorylation of IKKβ. L929sA and HeLa cells were left untreated or were treated with 63 μg/ml WS for 1 h and then stimulated with 2000 IU/ml TNF at the indicated time intervals. Total cell extracts were assayed by Western blot analysis using anti-P-IKKα/β (Ser-180/Ser-181) antibody, and the blot was reprobed with anti-IKKβ antibody to detect the levels of IKKβ protein. *C*, an experiment similar to that described in *B* was performed on IKKα- and IKKβ-null mouse embryonic fibroblast cells.

shown above. Altogether this suggests that WS-induced IKKβ hyperphosphorylation and kinase inhibition can be functionally uncoupled.

Withaferin A Is the Major Active Component of W. somnifera, Which Is Responsible for NFκB Inhibition—Next we subfractionated the WS leaf extract by solid phase extraction using C_{18} cartridges (supplemental Fig. 1A). Different fractions were evaluated for their potency of inhibiting NFκB in a reporter gene assay (supplemental Fig. 1B). In the most active fraction, two peaks were identified. Based on retention times, UV spectra, and spiking with authentic standards (Chromadex), these peaks were characterized as the steroidal lactones WA (Fig. 5*A*) and withanolide A. The identities of these peaks were confirmed after isolation by semipreparative HPLC by mass spectrometry, ^{1}H NMR, and ^{13}C NMR. The purity as estimated by HPLC was ≥98%. Interestingly 12-deoxywithastramonolide (12DW), which has been demonstrated in WS, seems to be absent in the Palestinian chemotype of WS (9–11).

Next dose-response curves for both the isolated WS compounds and 12DW in the NFκB reporter gene assays were elaborated. Because WA belongs to the class of steroidal lactones, we also included the glucocorticoid DEX and the sesquiterpene lactone helenalin (49) as reference compounds for NFκB inhibition. As shown in Fig. 5*B*, it is apparent that WA with an IC_{50} of 250 nM is the major and most active NFκB-inhibiting compound in WS, whereas both WdA (50 μM) and 12DW (90 μM) required 200–400-fold higher concentrations to elicit similar NFκB repression. Furthermore NFκB reporter gene assays confirmed that essentially all NFκB-repressing activity is concentrated in the WA peak fraction as no repression could be observed in pre- or postpeak fractions as prepared by semipreparative HPLC (supplemental Fig. 1B). Interestingly in our assay system withaferin A revealed a stronger potency than the sesquiterpene lactone helenalin (IC_{50}, 2.5 μM) and a stronger efficacy than glucocorticoids. Although glucocorticoids revealed a lower IC_{50} (80 nM) than WA, their maximal efficacy reached only 50% NFκB inhibition as compared with 95% with WA. The strong differences in NFκB inhibition between different WS constituents are remarkable and illustrate high specificity of the effects (Fig. 5*B*). With respect to structure function analysis, important roles have been attributed to the double bond (C-2=C-3) configuration and the C-26 lactone moiety for its biological activities (12, 50), but all tested WS-derived reference compounds share these features. Thus, the presence of a C-5–C-6 epoxide and the absence of a C-20 hydroxyl seem critical for optimal activity.

Upon quantifying the amount of withaferin A present in the Palestinian WS leaf extract and NFκB repression levels obtained with pure withaferin A, it was observed that almost 100% of the NFκB-inhibiting activity in the WS extract originated from withaferin A (Fig. 5*C*). Finally in analogy with effects of WS extract on IKKβ regulation, we measured the effects of single WS constituents on IKKβ phosphorylation and IκBα degradation. Interestingly strong IKKβ Ser-181 hyperphosphorylation by WA again coincided with lack of IκBα degradation, whereas similar concentrations of WdA and 12DW showed much weaker IKKβ hyperphosphorylation and failed to significantly inhibit IκB degradation (Fig. 5*D*). This demonstrates that IKKβ hyperphosphorylation and inhibition of IKKβ-dependent IκBα degradation are similarly mediated by the single constituent WA in the WS extract.

Reducing Agents Reverse WA-mediated IKKβ Hyperphosphorylation and Suppression of TNF-induced NFκB Activation—Of special note, it has been suggested that withaferin A may be involved in Michael addition thioalkylation reactions through either its epoxide or its lactone ring (51, 52). Interestingly lactones have been described to directly suppress IKKβ kinase activity upon attack of Cys-179 in the kinase domain activation loop or of Cys-662/716, which affects IKKα/β complex formation (31, 53–56) independently of IKKβ phosphorylation regulation. Furthermore considering the observed cross-talk of MEK/ERK signaling with IKKβ hyperphosphorylation, resorcylic acid lactones were described to target mitogen-activated protein kinase pathways at four levels: mitogen receptors, mitogen-activated protein kinase kinases MEK1/2, and ERK1/2, and certain substrates downstream of ERK (57). Also dual specificity tyrosine phosphatases were found to be susceptible to thioalkylation due to a conserved "*X*H**C***XXG***X**SRS" motif in the

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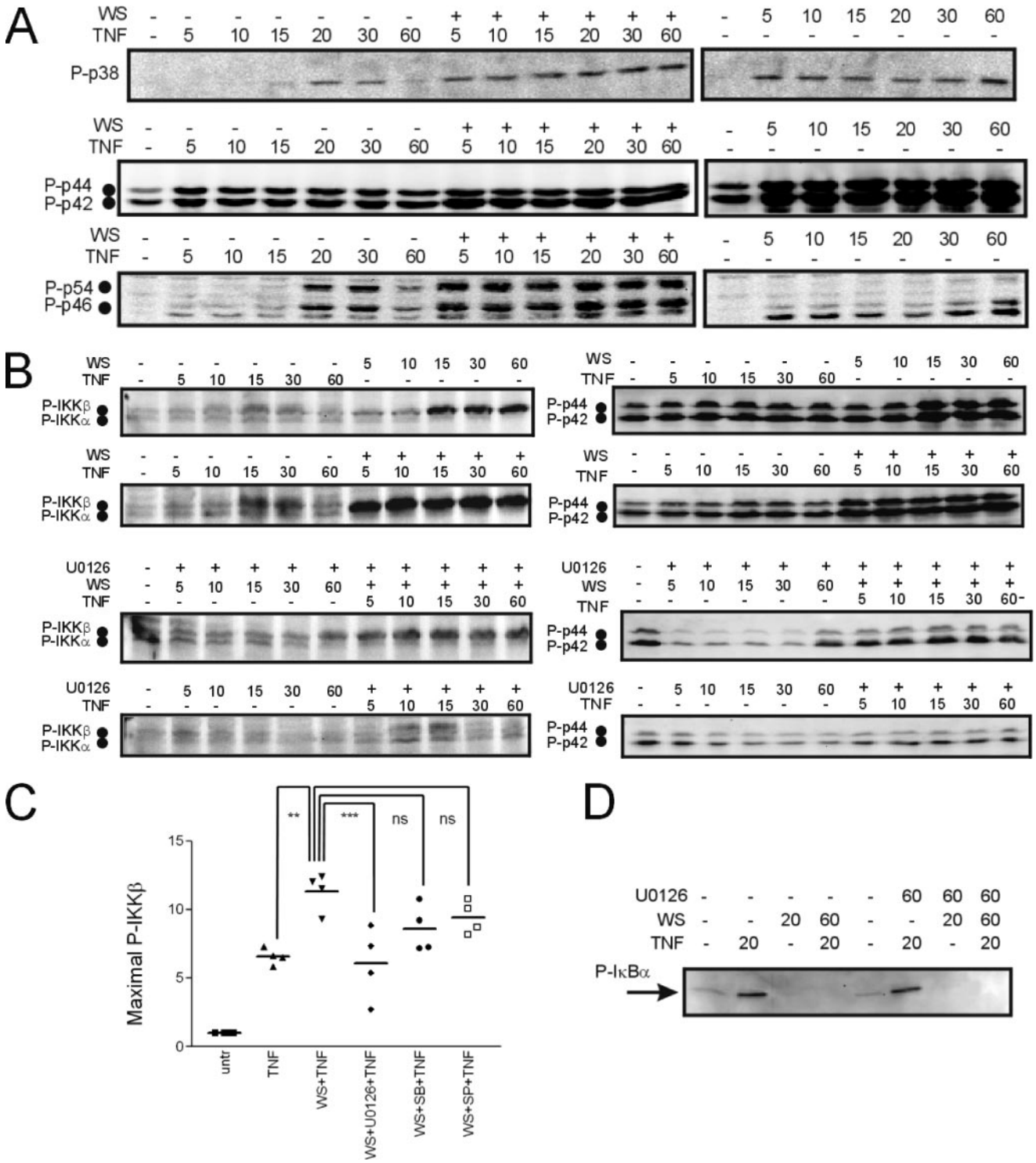

FIGURE 4. **Effect of WS on MAPK.** *A*, WS induces the phosphorylation of MAPKs. L929sA cells were starved for 48 h in serum-free medium and either left untreated or treated for 1 h with 63 μg/ml WS followed by stimulation with 2000 IU/ml TNF for the indicated time points. Total cell extracts were assayed by Western blot analysis using anti-P-p38, anti-P-JNK (p46/p54), and anti-P-ERK (p42/p44) antibodies. *B*, MEK1/ERK inhibitor (U0126) suppresses WS-induced hyperphosphorylation of ERK and IKKβ. L929sA cells were left untreated or were treated with 10 μM U0126 for 1 h followed by treatment with 63 μg/ml WS for 1 h and then stimulated with 2000 IU/ml TNF for the indicated times. Total cell lysates were assayed by Western blot analysis using anti-P-IKKα/β (Ser-180/Ser-181) or anti-P-ERK (p42/p44) antibodies. *C*, L929sA cells were left untreated (*untr*) or were pretreated with U0126, SB203580, or SP600125 inhibitors for 1 h followed by WS treatment (63 μg/ml) for 1 h with or without subsequent cell stimulation with TNF (2000 IU/ml) for 5, 10, 20, or 30 min. Total cell lysates were assayed by Western blot analysis using anti-P-IKKα/β (Ser-180/Ser-181), and specific signal intensities were subsequently quantified by free ImageJ software (National Institutes of Health). The P-IKK signal intensities obtained with the various treatments during 5–30-min TNF treatment are represented as *dots* in a column scatter plot. Statistical significant differences between the various treatments were calculated through the analysis of variance. Pairwise comparison of selected pairs of columns revealed the following significances: no significance (*ns*; $p > 0.05$), $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***). *D*, L929sA cells were left untreated or were treated with 10 μM U0126 for 1 h followed with 63 μg/ml WS for 1 h and then stimulated with 2000 IU/ml TNF for 20 min after which the IKK complex was immunoprecipitated with anti-IKKγ antibody. Subsequently IKKβ kinase activity present in the immunoprecipitated IKK complex was determined by *in vitro* kinase assay using GST-IκBα as a substrate and an excess of ATP. The final yield of phosphorylated IκBα in the reaction mixture was revealed by Western blot analysis with a P-IκBα antibody.

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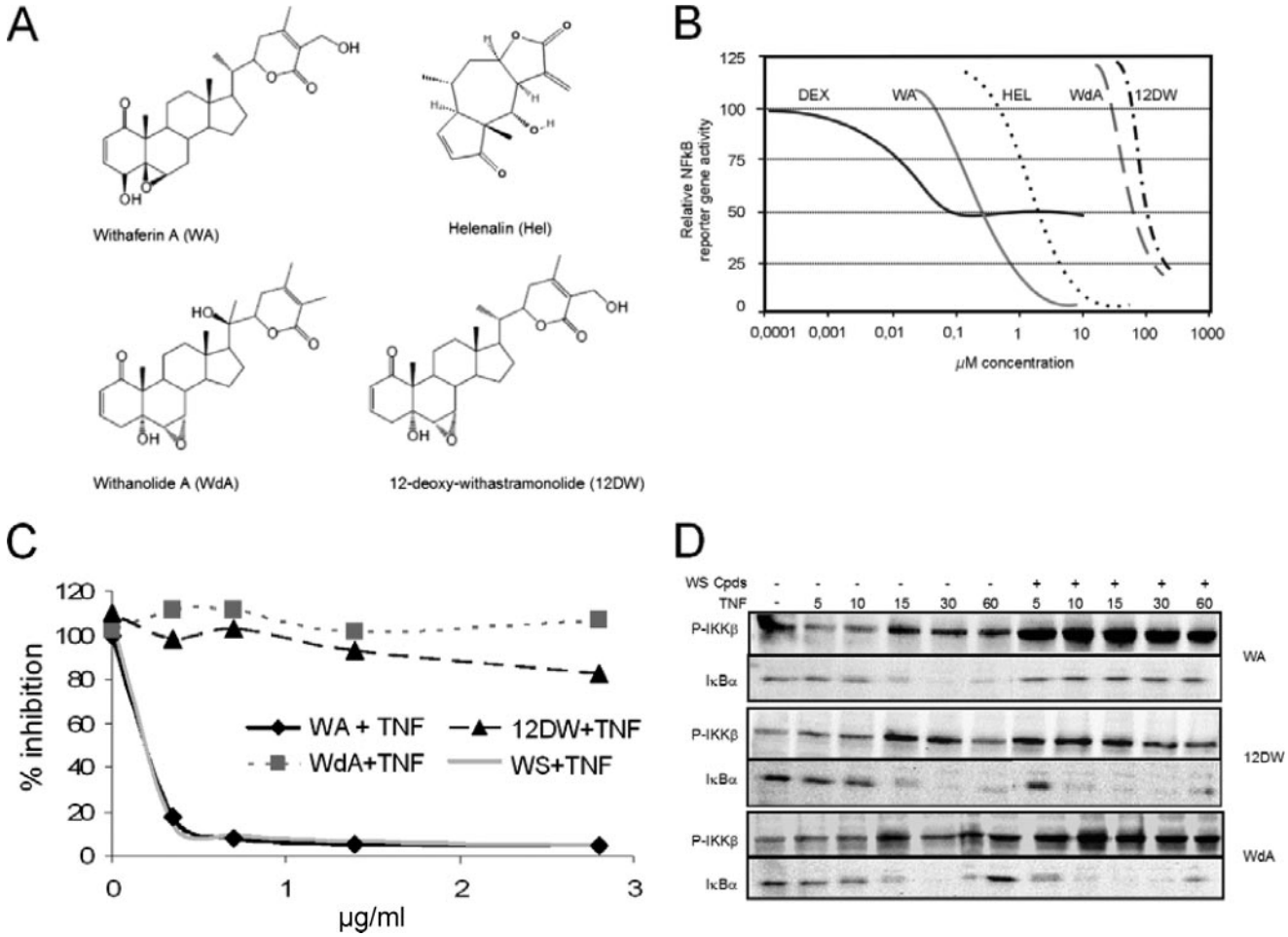

FIGURE 5. *A*, chemical formulas of WA, 12DW, WdA, and helenalin (*Hel*). *B*, cells, stably transfected with p(IL-6κB)350hu.IL6P-luc+, were left untreated or were pretreated with various concentrations of WA, WdA, 12DW, helenalin (*HEL*), or DEX for 1 h and then stimulated with 2000 IU/ml TNF for 6 h. Lysates were prepared for quantification of reporter gene expression levels and normalization for protein concentration. *C*, L929sA cells, stably transfected with p(IL-6κB)$_3$50hu.IL6P-luc+, were left untreated or were pretreated for 1 h either with crude extract of WS or with equimolar amounts of WS compounds (*Cpds*) as can be measured in the Palestinian WS leaf extract followed by stimulation with 2000 IU/ml TNF for 6 h. Lysates were prepared for quantification of reporter gene expression levels and normalization for protein concentration. *D*, WA, but not WdA or 12DW, elicits hyperphosphorylation of IKKβ. L929sA cells were left untreated or were pretreated for 1 h with a 2.8 μg/ml concentration of either WA, WdA, or 12DW and then stimulated with 2000 IU/ml TNF for the indicated time points. Whole-cell extracts were prepared, and Western blot analysis was performed with anti-P-IKKα/β and anti-IκBα.

catalytic domain, which may result in sustained MEK/ERK signaling (58–63). Finally NFκB p65 DNA binding itself was demonstrated to be sensitive to Cys-38 alkylation too (31, 49, 64). Therefore, we further investigated whether WA mediates part of its NFκB-inhibitory effects by alkylation of thiol-sensitive redox pathways. Therefore, L929sA cells were co-treated with WA and DTT for 1 h, and then TNF-induced NFκB activation was examined. We found that DTT had no effect on TNF-induced NFκB activation but that it significantly reversed the inhibitory effects of WA on NFκB activation (Fig. 6*A*). Furthermore DTT also prevented WA-dependent IKKβ hyperphosphorylation (Fig. 6*B*). This suggests that WA may target various cysteine residues of multiple kinases/phosphatases (25, 65–72) that affect the phosphorylation status of p38, MEK/ERK, JNK, and IKKβ, ultimately resulting in inhibition of IKKβ kinase activity upon attack of critical cysteine residues involved in kinase activation and/or complex formation.

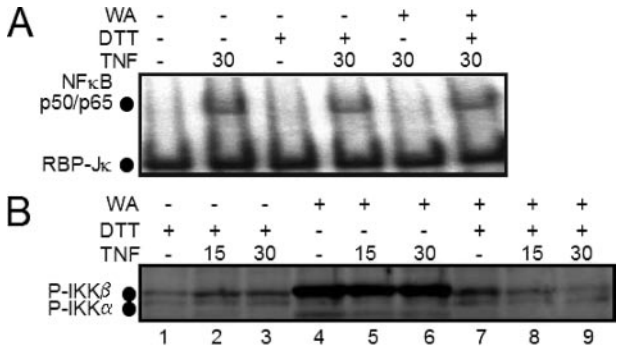

FIGURE 6. *A*, DTT reverses WA-mediated suppression of TNF-induced NFκB/DNA binding. L929sA cells were co-treated with 250 μM DTT and 2.8 μg/ml WA for 1 h and then treated with 2000 IU/ml TNF for 30 min. Total protein extract was prepared and analyzed for NFκB/DNA binding by EMSA. *B*, DTT reverses WA-induced phosphorylation of IKKβ. L929sA cells were co-treated (*lanes 7–9*) with 2.8 μg/ml WA and 5 mM DTT for 1 h and then treated with TNF (2000 IU/ml) for the indicated time periods.

WA Displays Anti-inflammatory Properties in Vivo—Because NFκB is a critical player in the inflammatory signaling pathway and because WA inhibits NFκB activation, we further investigated the possible anti-inflammatory effects of WA *in vivo* using the zymosan-induced inflamed paw model. Mice are injected subcutaneously with zymosan in the footpad, and

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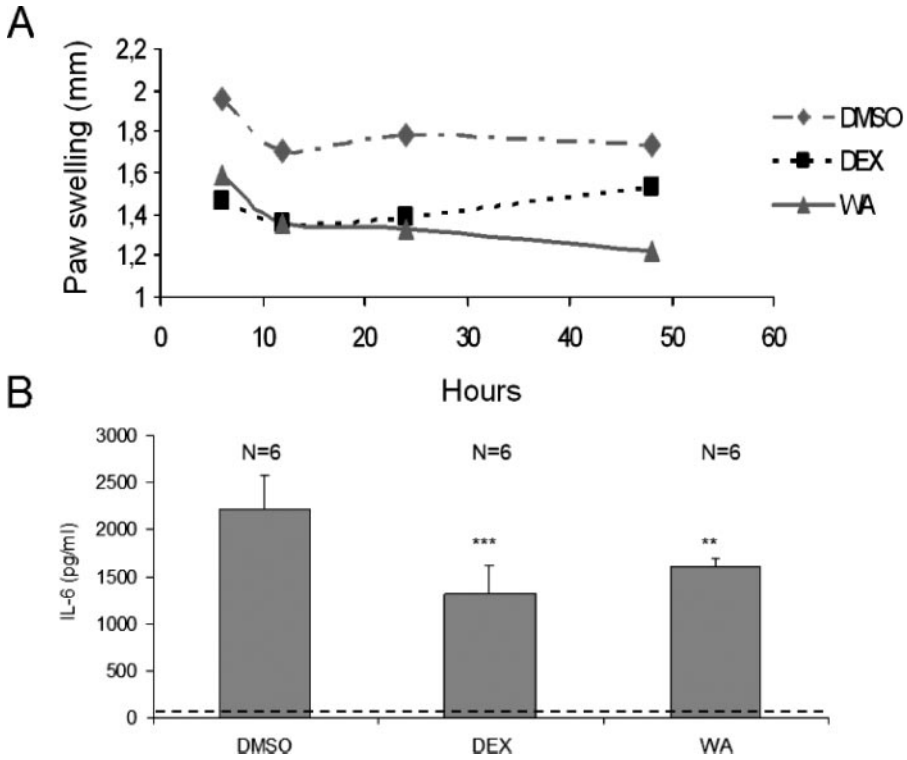

FIGURE 7. *A*, mice were injected subcutaneously with zymosan in the footpad, and swelling was determined at 6, 12, 24, and 48 h after the injection. Mice were preinjected intraperitoneally with WA, DEX, or Me_2SO (*DMSO*) 30 min before zymosan injection. *B*, effect of WA or DEX on blood IL-6 protein levels. Blood IL-6 protein concentration was determined at 6 h after zymosan injection from the same mice as used in Fig. 6*A*. The *dashed line* indicates background IL-6 levels of untreated mice. The statistical significance between the treated groups and the control group was calculated through the analysis of variance. *p* values less than 0.05 were considered as indicative of significance. ***, $p = 0.0004$; **, $p = 0.001$.

swelling is determined at several time points after the injection. Pretreatment with WA (intraperitoneal) was performed to evaluate anti-inflammatory effects, whereas DEX (intraperitoneal) was chosen as a positive control. Results in Fig. 7*A* show the mean of the differences between the zymosan-treated and untreated footpad for all the three groups. Both DEX and WA pretreatment showed clear anti-inflammatory activities. At the first time intervals DEX and WA were more equally active, whereas they become less equally active, and a significant difference can be observed at later time points (48–72 h). These results show that WA displays an anti-inflammatory activity that is more persistent than that of DEX. Moreover IL-6 protein levels were measured at an early stage, and as can be observed in Fig. 7*B*, pretreatment with DEX or with WA significantly reduced the levels of IL-6 compared with Me_2SO, which is in line with our results obtained *in vitro*.

DISCUSSION

The transcription factor NFκB regulates the expression of cytokines, chemokines, adhesion factors, and inducible proinflammatory receptors (73). The abnormal activation of NFκB has been established for a series of inflammatory diseases and cancer (74, 75). Thus, NFκB is an ideal target for anticancer and anti-inflammatory drug development. In fact, several anti-inflammatory agents such as aspirin, sulfasalazine, and steroids have been suggested to act at least partially by inhibiting NFκB activation (73, 76).

Use of *W. somnifera* as a traditional remedy for several illnesses is widespread in many countries. Today this plant is used as a constituent in more than 100 herbal preparations (77). However, the molecular mechanism of its immunomodulatory action is poorly understood. In this report we demonstrate that pure WA or WA-containing extract of WS completely suppressed NFκB activation induced by inflammatory agents irrespective of the cell type, but not AP1 activity. Indeed we showed that pure WA and WS extract block IKKβ kinase activity, which in turn abrogates IκBα phosphorylation and degradation, p65 translocation, NFκB/DNA binding, and subsequent NFκB-driven gene expression, which finally results in the down-regulation of various NFκB-regulated gene products induced by TNF.

Although Mohan *et al.* (78) previously reported that WA might inhibit NFκB activity by impaired IκB degradation and ubiquitination, we clearly show that WS and WA inhibit NFκB activation by directly suppressing IKKβ kinase activity, which blocks IκBα phosphorylation prior to its ubiquitination and degradation. Our results show that, unlike the previously described IKKβ inhibitors (79–81), WA and WA-containing preparations of WS inhibit the activity of IKKβ kinase via a thioalkylation-sensitive redox mechanism concomitant with inducing Ser-181 phosphorylation in a MEK1/ERK1-dependent way. This is surprising as IKKβ mutants (S176E/S181E) that mimic constitutive IKKβ phosphorylation are constitutively active (14, 43); however, in the case of WA, we cannot exclude cross-talk of Ser-181 with other less well defined COOH-terminal IKKβ phosphorylations, which have been described to decrease general IKK activity (23). Furthermore various critical cysteine residues have already been identified in IKKβ that interfere with kinase activity and complex conformation and may overrule phosphorylation-dependent control of the kinase activity (31). Surprisingly we were unable to completely block WA-dependent NFκB inhibition by either IKKβ C179A or C662A/C716A mutants (data not shown), which suggests that WA may target multiple IKKβ cysteine residues to mediate its full inhibitory activity. Furthermore independently of IKKβ regulation, WA/WS may elicit (hyper)phosphorylation of other kinases too that depend on cysteine-sensitive regulation of alternative phosphatase targets (66–72).

In summary, these experiments demonstrate that pure WA or WA-containing preparations of WS potently inhibit TNF-induced NFκB activation via inhibition of IKKβ kinase activity in the nanomolar range. We further demonstrate that WA

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clearly displays anti-inflammatory characteristics not only *in vitro* but also *in vivo*, *i.e.* in an acute inflammatory mouse model. Taken together, the results of this study extend our understanding of the molecular mechanisms underlying the anti-inflammatory and the antitumor activity of WS plant extracts that are used in traditional medicine and thus provide scientific support for the use of WA preparations of WS as a folk remedy for the treatment of inflammation. Finally WA is a promising lead compound for the design of potent IKKβ inhibitors for anti-inflammatory, antitumoral, and/or chemopreventive applications.

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