Sesquiterpene Lactones Specifically Inhibit Activation of NF-κB by Preventing the Degradation of IkB-α and IkB-β*

(Received for publication, June 23, 1997, and in revised form, September 2, 1997)

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Extracts from certain Mexican Indian medicinal plants used in traditional indigenous medicine for the treatment of inflammations contain sesquiterpene lactones (SLs), which specifically inhibit the transcription factor NF-κB (Bork, P. M., Schmitz, M. L., Kuhnt, M., Escher, C., and Heinrich, M. (1997) FEBS Lett. 402, 85–90). Here we show that SLs prevented the activation of NF-κB by different stimuli such as phorbol esters, tumor necrosis factor-α, ligation of the T-cell receptor, and hydrogen peroxide in various cell types. Treatment of cells with SLs prevented the induced degradation of IkB-α and IkB-β by all these stimuli, suggesting that they interfere with a rather common step in the activation of NF-κB. SLs did neither interfere with DNA binding activity of activated NF-κB nor with the activity of the protein tyrosine kinases p59fyn and p60src. Micromolar amounts of SLs prevented the induced expression of the NF-κB target gene intracellular adhesion molecule 1. Inhibition of NF-κB by SLs resulted in an enhanced cell killing of murine fibroblast cells by tumor necrosis factor-α. SLs lacking an exomethylene group in conjugation with the lactone function displayed no inhibitory activity on NF-κB. The analysis of the cellular redox state by fluorescence-activated cell sorter showed that the SLs had no direct or indirect antioxidative properties.

The transcription factor NF-κB is one of the key regulators of genes involved in the immune and inflammatory response (for review, see Ref. 2). In mammalian cells, NF-κB is composed of a homo- or heterodimer of various DNA-binding subunits. Five different DNA-binding subunits share a N-terminal homology domain, which confers DNA binding, dimerization, nuclear translocation, and interaction with the inhibitory IκB proteins (for review, see Refs. 3 and 4). In most cell types these proteins sequester NF-κB, which is frequently a heterodimer of the p50 and p65 (RelA) subunits, in the cytoplasm by masking their nuclear localization sequence. Constitutive NF-κB activity in the cell nucleus can only be detected in certain neurons, some cells of the monocyte/macrophage lineage and B cells (for review, see Refs. 5 and 6). Stimulation of cells with a variety of pathogenic agents including inflammatory cytokines, phorbol esters, UV irradiation, and oxidants finally leads to the intracellular generation of reactive oxygen intermediates (ROIs) as a key event and results in the activation of NF-κB (for review, see Refs. 7 and 8). The two major forms of IκB proteins, termed IκBα and IκBβ, can be inducibly phosphorylated and ubiquitylated (9–11). These post-translational modifications tag the molecule for the subsequent proteolytical degradation by the ubiquitin-26 S proteasome pathway (12–14). This induced degradation of IκB proteins unmasksthe nuclear localization sequences of the DNA-binding subunits of the NF-κB dimer and allows NF-κB to enter the nucleus, to bind to its DNA sequence, and to induce transcription. The target genes whose transcription is mainly regulated by NF-κB include many cytokines, cell adhesion molecules, such as intercellular adhesion molecule 1 (ICAM-1), and vascular cell adhesion molecule 1, as well as acute-phase proteins and immunoreceptors (for review, see Ref. 15).

Among its many different biological activities NF-κB seems to play an important role in cell killing. NF-κB has been shown recently to counteract the induction of apoptosis by the cytokine TNF-α, ionizing radiation, and the cancer chemotherapeutic agent daunorubicin (for review, see Ref. 16). However, there is also evidence for apoptosis-promoting properties of NF-κB. Glutamate was found to induce NF-κB in neuronal cells and acetylsalicylic acid (aspirin) protected these cells from NF-κB-induced cell death (17, 18). Along this line, the overexpression of the cellular and viral anti-apoptotic proteins Bcl-2 and E1B 19 K both negatively interfered with the activation of NF-κB under these conditions (19–21).

The role of NF-κB in the immune response is also evident from gene disruption experiments. The targeted deletion of the p50, p65, RelB, and c-Rel subunits resulted in an impaired immune response and/or in a reduced viability of the mice (22–26). These findings and the immunological relevance of most of the NF-κB target genes make this transcription factor an interesting therapeutical target for the identification of inhibitors. One group of NF-κB inhibitors exerts its inhibitory effects by scavenging ROIs. These inhibitors include N-acetyl-L-cysteine (27, 28), pyrrolidine dithiocarbamate (29), acetylsalicylic acid (30, 31), or curcumin (32). All these compounds are structurally unrelated, but share the property of being anti-oxidative. Another group of inhibitors

*This work was supported by a European Union Biomed-2 grant (to M. L. S.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1The abbreviations used are: ROIs, reactive oxygen intermediates; CHUK, conserved helix-loop-helix ubiquitous kinase; DFCH, dichlorofluorescein diacetate; EMSA, electrophoretic mobility shift assay; ICAM-1, intercellular adhesion molecule-1; IKK, IκB kinase; JNK, c-Jun N-terminal kinase; MEKK-1, mitogen-activated protein kinase/ERK kinase kinase-1; MKK4, mitogen-activated protein kinase kinase 4; PMA, phorbol 12-myristate 13-acetate; PAGE, polyacrylamide gel electrophoresis; FACS, fluorescence-activated cell sorter; SLs, sesquiterpene lactones; TNF, tumor necrosis factor.
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We recently identified SLs isolated from extracts of Mexican Indian medicinal plants as specific inhibitors of NF-κB (1). SL-containing plant extracts are frequently used in the traditional Mexican Indian medicine for the treatment of infections of the skin and other organs (for review, see Ref. 38). The SL parthenolide is also contained in drugs such as Feverfew® (Tanacetum parthenium) used against migraine, an illness that has been implicated with neurogenic inflammatory processes (39). The anti-inflammatory activity of the SL-containing plant extracts was confirmed in the hen egg tests where they showed a delay in cell culture experiments (40) and the onset of capillary reactions of the allantois membrane (1).

This study shows that SLs prevent a common step in NF-κB activation. They did not interfere with the generation of oxygen radicals, but prevented the induced degradation of IκB-α and IκB-β as well as the induced expression of the NF-κB target gene ICAM-1. Structural studies identified the exomethylene group in conjugation with the lactone group as the decisive structural feature for the inhibitory activity.

**EXPERIMENTAL PROCEDURES**

**Cell Culture—**Jurkat T leukemia cells (subclone JR “Würzburg”) were maintained in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum, and 1% (w/v) penicillin/streptomycin (all purchased from Life Technologies, Inc., Grand Island, NY). HeLa cells and L929 fibroblasts were grown in Dulbecco's modified Eagle's medium containing 10% fetal calf serum and 1% (v/v) penicillin/streptomycin. All cells were grown in an incubator at 37 °C and 5% CO₂. TNF-α and poly(dI-dC) were obtained from Boehringer Mannheim (Mannheim, Germany). Parthenolide, isohelenin, isophoronoxide, limonenoxide, caryophyllenoxide, sclareolide, and santonin were from Sigma. Antibodies directed against IκB-α and NF-κB were from Santa Cruz Inc. (Santa Cruz, CA), α-CD28 antibodies were isolated from a hybridoma (Pharmingen Inc., San Diego, CA), and α-CD3 antibodies were obtained from Pharmingen Inc. (San Diego, CA). All other chemicals were either from Sigma, Aldrich (Steinheim, Germany), or Roth (Karlsruhe, Germany).

**Electrophoretic Mobility Shift Assay (EMSA)—**HeLa or L929 cells (5 x 10⁶) were grown overnight on 10-cm dishes, Jurkat cells (approximately 1 x 10⁵/ml) in cell culture flasks. One hour prior to stimulation by TNF-α, phorbol-12-myristate 13-acetate (PMA), or hydrogen peroxide, cells were preincubated with the indicated amounts of the tested substances for 60 min at 37 °C. The tested substances were dissolved in dimethyl sulfoxide as a solvent. In the following cells were stimulated for 20 min with PMA at a final concentration of 50 ng/ml, TNF-α (2000 units/ml), α-CD3/α-CD28 (1 μg/ml) antibodies or for the indicated periods with the specified amounts of hydrogen peroxide. Cells were harvested by centrifugation and washed twice with cold TBS buffer (25 μM Tris/HC1, pH 7.4, 137 mM NaCl, 5 mM KCl, 0.7 mM CaCl₂, 0.1 mM MgCl₂). The pellet was resuspended in TOTEX buffer (20 mM Heps/ KOH, pH 7.9, 0.35 M NaCl, 20% (v/v) glycerol, 1% (v/v) Nonidet P-40, 1 mM MgCl₂, 0.5 mM EDTA, 0.1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride) and incubated on ice for 30 min. The samples were carefully vortexed every 10 min. The cell debris was pelleted upon centrifugation with 14,000 rpm at 4 °C for 10 min. Equal amounts of supernatant were tested for DNA binding activity as described in detail elsewhere (1). Briefly, the extracts were incubated with 2 μg of poly(dI-dC), 2 μg of bovine serum albumin, and 10,000 cpm of a ³²P-labeled oligonucleotide the position of a constitutively DNA-binding protein and the open arrowhead points to the unbound oligonucleotide.

**FIG. 1.** SLs inhibit NF-κB activation by different stimuli. A, inhibition of NF-κB activated by H₂O₂. Jurkat JR cells were preincubated with the indicated amounts of parthenolide for 1 h and stimulated with various concentrations of H₂O₂ for 90 min. Subsequently total cell extracts were prepared and tested for DNA binding of activated NF-κB by EMSA. B, inhibition of NF-κB activated by CD3/CD28 ligation. Jurkat J16 cells were preincubated with the indicated amounts of parthenolide for 1 h and activated by immobilized α-CD3 antibodies and cross-linked α-CD28 antibodies. Ten minutes after stimulation cells were harvested and assayed for NF-κB activity by EMSA. The SLs were kept with the cells during both stimulations. The filled arrowhead indicates the location of the DNA-NF-κB complex, the circle indicates
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**Fig. 2.** SLs do not interfere with DNA binding of activated NF-\( \text{kB} \). HeLa cells were stimulated for 20 min with PMA and total cell extracts of the stimulated cells were pooled. These extracts were incubated for 1 h with various concentrations of parthenolide as indicated. Subsequently these extracts were tested together with a protein extract from unstimulated HeLa cells as a control for DNA binding activity of NF-\( \text{kB} \) by EMSA. Bound and free oligonucleotides were separated by electrophoresis on a native gel, dried, and exposed. The symbols used are as explained in Fig. 1.

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**Fig. 3.** Effects of SLs on DNA binding of Oct-1 and the fragmentation of IeB-\( \alpha \). Mouse L929 fibroblasts were preincubated with parthenolide for 1 h prior to stimulation with murine TNF-\( \alpha \). Subsequently cell extracts from these cells were prepared, together with extracts from unstimulated and TNF-\( \alpha \)-stimulated cells as indicated. Equal amounts of the protein extracts were simultaneously tested for DNA binding activity of NF-\( \text{kB} \) (A), Oct-1 (C), and for the presence of IeB-\( \alpha \) in Western blots (B). A, DNA binding activity of NF-\( \text{kB} \). Cells were treated as indicated and total cell extracts were tested for NF-\( \text{kB} \) binding by EMSA. The symbols used are as described for Fig. 1. B, IeB-\( \alpha \) Western blot. Total cell extracts were separated by SDS-PAGE and transferred to a polyvinylidene difluoride membrane. The arrow points to the IeB-\( \alpha \) protein which was detected with an anti-IeB-\( \alpha \) antibody. The positions of prestained protein markers are indicated. C, DNA binding activity of Oct-1. The indicated cell extracts were tested for the activity of the constitutively DNA-binding protein Oct-1 by EMSA. The open arrowhead points to the position of the unbound oligonucleotide, the filled arrowhead indicates the position of the DNA-protein complex.
RESULTS

SLs Inhibit a Common Step in NF-κB Activation—From a previous study it was evident that SLs prevent the PMA-induced activation of NF-κB (1). Therefore we studied whether SLs can inhibit NF-κB activation in response to stimuli different from PMA. As an example for a receptor-mediated pathway, mouse L929 cells were stimulated with TNF-α with or without preincubation with various amounts of isohelenin or parthenolide. After 1 h of preincubation cells were stimulated for 20 min with murine TNF-α and total extracts were tested for DNA binding activity by EMSA. The TNF-induced binding of NF-κB was completely prevented by preincubation of cells with 5 μM parthenolide and 20 μM isohelenin, respectively (data not shown). Since it is known that most inducers of NF-κB lead to the formation of ROIs, we tested whether SLs would interfere with H₂O₂-induced NF-κB binding activity. Therefore Jurkat JR cells were preincubated for 1 h with various amounts of parthenolide and isohelenin and treated with various concentrations of H₂O₂ for 90 min. The extracts prepared from these stimulated cells were subsequently analyzed by EMSA (Fig. 1A). Only H₂O₂ concentrations between 100 and 250 μM activated NF-κB, whereas the addition of 1 mM H₂O₂ failed to efficiently induce NF-κB, probably due to an oxidative destruction of the protein. The H₂O₂-induced DNA binding activity of NF-κB was completely prevented by preincubation with the two SLs in low micromolar concentrations. Another stimulus of NF-κB with special relevance in T-cells is the activation of the CD3/CD28 pathway, which also leads to an increased concentration of ROIs (44). T-cell specific activation of NF-κB in Jurkat T-cells by cross-linking the CD3 and CD28 receptors was prevented by preincubation with low micromolar concentrations of the two SLs (Fig. 1B). All these experiments indicate that SLs interfere with one or more common steps during NF-κB activation in different cell types rather than with one single event specific for an individual stimulus. They are therefore likely to display their inhibitory properties after the

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**Fig. 4.** SLs inhibit the degradation of IκB-α and IκB-β induced by various stimuli. Cells were preincubated with 10 μM parthenolide 1 h prior to stimulation with 2000 units of TNF-α (A), 50 ng/ml PMA (B), ligation of the CD3/CD28 receptors (C), and 100 μM H₂O₂ (D). Cells were harvested at the indicated time points and total cell extracts were prepared. Equal amounts of protein were tested by EMSAs for DNA binding of NF-κB and by Western blot experiments for the occurrence of IκB proteins. The shifted DNA-protein complexes are shown in the upper parts of the respective panels with the filled arrowhead pointing to the position of the NF-κB-DNA complex. The same extracts were simultaneously tested for the occurrence of IκB-α and IκB-β in Western blots with the arrow pointing to the respective IκB proteins. Representative experiments are shown, for further details see text.
point of integration of the different signals.

**SLs Do Not Affect Activity of Src Family Protein Tyrosine Kinases**—Tyrosine kinases of the Src family have been implicated in NF-κB activation in response to various stimuli including UV radiation, T-cell receptor ligation, and stimulation with pro-oxidants. We therefore investigated the effect of parthenolide and isohelenin on kinase activity of recombinant p60<sup>src</sup> and p59<sup>src</sup>. After pretreatment with various concentrations of SLs, kinase activities were determined by incubation of the protein kinases with the substrate rabbit muscle enolase and [γ<sup>32</sup>P]ATP. Tyrosine-phosphorylated proteins were then separated by SDS-PAGE and visualized by autoradiography. Neither increasing amounts up to 20 μM isohelenin nor parthenolide affected the ability of p59<sup>src</sup> to phosphorylate itself or the substrate enolase. Also the recombinant p60<sup>src</sup> kinase was completely unchanged in its phosphorylating activity in the presence of both SLs (data not shown). These results suggest that SLs do not prevent NF-κB activation by inhibiting Src tyrosine kinases. Furthermore, these data indicate that SLs do not unspecifically alter the activity of cytoplasmatic enzymes by reacting with the sulfhydryl group of cysteine residues, because both Src family tyrosine kinases possess exposed and redox-reactive cysteines which were shown to be important for their activity (45).

**SLs Do Not Prevent DNA Binding of NF-κB**—To investigate the mechanism of action of SLs we first tested the potential effects of the SLs parthenolide and isohelenin on the DNA binding activity of NF-κB in band shift experiments. Therefore HeLa cells were stimulated with PMA and cell extracts were prepared 20 min after stimulation. These extracts, which contain the activated nuclear form of NF-κB, were pooled and incubated either with various concentrations of the two SLs or with the solvent dimethyl sulfoxide as a control. The preincubated extracts were analyzed for DNA binding activity of NF-κB in an EMSA. Increasing concentrations of parthenolide completely abrogating NF-κB activation in the intact cell (see Fig. 1) did not influence DNA binding of activated NF-κB in vitro (Fig. 2). The same amounts of isohelenin also failed to reduce the DNA binding activity of NF-κB (data not shown). This experiment excludes that SLs directly inhibit the DNA binding activity of activated NF-κB, e.g. by modifying reactive amino acids such as cysteine residues in the DNA-binding or dimerization domains. The induced DNA-binding complex was confirmed to be a p50/p65 NF-κB dimer by competition assays with unlabeled oligonucleotides and antibody reactivity (data not shown).

**SLs Prevent the Induced Degradation of IκB-α and IκB-β**—The part of the NF-κB activation cascade that is influenced by SLs was further analyzed by following the fate of IκB-α in Western blots. Mouse L929 cells were preincubated for 1 h with 5 μM of either isohelenin or parthenolide. Subsequently murine TNF-α was added without changing the medium and kept for 20 min on the cells. Total cell extracts were tested for the presence of IκB-α in Western blots and simultaneously for DNA binding of NF-κB and Oct-1 by EMSAs (Fig. 3). The TNF-α-induced DNA binding of activated NF-κB in the EMSA experiment coincided with the degradation of IκB-α as detected by Western blotting (Fig. 3, A and B). Preincubation of cells with 5 μM parthenolide completely prevented the induction of the DNA-binding form of NF-κB and protected IκB-α from proteolysis by the 26 S proteasome. Identical results were obtained for HeLa cells preincubated with 5 μM isohelenin (data not shown). In a control experiment the DNA binding

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**Fig. 5.** Reversibility of SL treatment. HeLa cells were preincubated with 5 μM parthenolide for 1 h. Subsequently the medium was replaced by parthenolide-free medium and the cells were grown for the indicated time periods prior to stimulation with PMA for 15 min. NF-κB activity was then assessed by EMSA and the amount of DNA-bound NF-κB was quantitated in a PhosphorImager (Molecular Dynamics). A, schematic outline of the experimental strategy. The incubation time with parthenolide is symbolized by the black bars, the stimulation period is highlighted by hatching. B, time dependence of NF-κB inhibition. The diagram shows the percental inhibition of NF-κB activity in dependence of the time period between parthenolide preincubation and PMA stimulation. The complete inhibition of NF-κB in the experiment without incubation in parthenolide-free medium was set as 100%. A typical experiment is shown.

**Fig. 6.** Effect of parthenolide on the cell killing by TNF-α. L929 cells were incubated with 5 μM parthenolide (*Φ*), 2000 units/ml TNF-α (*▲*), or a combination of both (*■*). At the indicated time points, cell viability was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay. Viable cells remaining after treatment with mouse TNF-α are shown as a percentage of viable untreated cells. A representative experiment is shown.
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In a more detailed analysis we tested the behavior of IκB-α and IκB-β proteins in the presence or absence of SLs with different NF-κB-inducing conditions at various time points (Fig. 4, A-D). The rapid TNF-α-induced DNA binding of NF-κB in L929 cells remained unchanged up to 120 min (Fig. 4A). Preincubation with parthenolide completely prevented the induced degradation of IκB-α and IκB-β as monitored by Western blotting. Sixty minutes after the addition of TNF-α only the IκB-α protein was re-synthesized. The PMA-induced DNA binding of NF-κB in HeLa cells took place with a significantly slower kinetic when compared with TNF-α. DNA binding was complete 30 min after stimulation with PMA and resulted in a full degradation of IκB-α, which was not apparent in the presence of parthenolide (Fig. 4B). The IκB-β protein was not inducibly degraded by PMA, suggesting the importance of alternative PMA signaling pathways for IκB-β. The effects of CD3/CD28 receptor ligation on NF-κB and IκB proteins were monitored in peripheral blood lymphocytes. Various time points after receptor ligation, total cell extracts were prepared and tested for DNA binding activity and IκB degradation. The DNA binding of NF-κB was significantly enhanced already 5 min after receptor triggering. The observed constitutive DNA binding activity is most likely due to the constitutively active form of NF-κB from mature B-cells, which are contained in the peripheral blood lymphocytes. The stimulation of NF-κB by CD3/CD28 ligation is reflected by a degradation of the IκB-α and IκB-β proteins, which again can be prevented by preincubation with parthenolide (Fig. 4C). The activation of NF-κB by 100 μM H2O2 in Jurkat JR cells appeared only after an incubation period of 90 min. As already seen for the other tested stimuli, the (in the case of H2O2 incomplete) degradation of IκB-α and IκB-β proteins was abrogated by preincubation with parthenolide (Fig. 4D). Identical results were obtained for all the stimuli described here with the SL isohelenin (data not shown). These experiments indicate that SLs prevent the induced degradation of IκB-α and IκB-β by diverse stimuli and therefore interfere with a common step in the signaling cascade leading to the activation of NF-κB.

SLs Irreversibly Inhibit NF-κB Activation—We next tested whether SLs act as competitive or irreversible inhibitors. HeLa cells were incubated with 5 μM parthenolide for 1 h. Subsequently the cells were washed with medium void of SLs and further grown for various periods as schematically displayed in Fig. 5A. After stimulation with PMA for 20 min, cells were lysed and the extracts were assayed for NF-κB activity by EMSA. The amount of DNA-bound NF-κB dimers was quantitated with a PhosphorImager and the results are displayed graphically (Fig. 5B). The total inhibition of NF-κB binding activity seen after immediate stimulation of cells following the preincubation with parthenolide was set as 100%. The inhibition was still almost complete after 2 h of incubation and decreased to approximately 50% after 18 h of incubation in parthenolide-free medium (Fig. 5B). This kinetic behavior suggests that the SLs act by covalently and thus irreversibly modifying their target molecule(s), presumably by their reactive Michael system in the lactone ring. The partial restoration of inducible NF-κB activation, which is occurring 18 h after SL treatment, is most probably due to the de-synthesis of the inactivated protein(s).

SLs Promote Killing of Mouse L929 Cells by TNF-α—The role of NF-κB during the TNF-α-induced cell death is still not clear and seems to depend on the tested cell line (for review, see Refs. 16 and 46). We therefore wanted to address the question whether cell death of L929 cells in response to TNF-α is influenced by parthenolide. Mouse L929 cells were incubated either with 5 μM parthenolide or 2000 units/ml TNF-α alone or by a combination of both. After various incubation times the cell viability was measured (Fig. 6). Treatment with parthenolide alone did not influence the cell viability and TNF-α-induced cell death was enhanced in the presence of parthenolide. This experiment shows that the NF-κB inhibitor parthenolide is also enhancing the TNF-α-induced cell killing of mouse L929 cells.

SLs Prevent the Induced Expression of the NF-κB Target

Fig. 7. SLs prevent the expression of ICAM-1. A, effect of TNF-α on the expression of ICAM-1. Jurkat JR cells were stimulated for 10 h with 2000 units/ml TNF-α, stained with an α-ICAM-1 antibody coupled to phycoerythrine, and analyzed by flow cytometry. The profiles of untreated (white) and TNF-α-stimulated (black) cells are shown. B, effect of parthenolide on ICAM-1 expression. Jurkat cells were treated as in A in the presence of 10 μM parthenolide. Profiles of untreated (white) and stimulated (black) cells are displayed.
Gene ICAM-1—The inducible transcription of the ICAM-1 gene in response to TNF-α, interleukin-1β, and PMA is controlled by a NF-κB-binding site in its promoter (for review, see Ref. 47). This feature makes ICAM-1 surface expression a good read-out to test the effect of SLs on the expression of endogenous NF-κB target genes. The induction of ICAM-1 gene expression and the appearance of the protein on the cell surface was analyzed by FACS. Treatment of Jurkat T-cells with TNF-α resulted in a strong induction of ICAM-1 expression, as displayed in Fig. 7A. Preincubation with either 5 mM parthenolide almost completely prevented the induction of ICAM-1 expression, as displayed in Fig. 7A. In a control experiment, parthenolide or isohelenin did not influence the amount of the T-cell receptor CD3 protein on the surface of Jurkat cells in the presence or absence of TNF-α (data not shown). These results indicate that the induced transcription of NF-κB target genes is specifically inhibited by SLs.

Structural Determinants for the Inhibitory Activity of SLs—We next investigated the structural features of the SLs which confer inhibitory activity on NF-κB activation pathways. Two structural hallmarks of SLs are an isoprenoide ring system and a lactone ring. In many cases this lactone ring contains a conjugated exomethylene group. Both groups together form a reactive Michael system which is a target for nucleophilic substrates, e.g., for cysteine residues in proteins. Various isoprenoide substances lacking either the lactone ring or the exomethylene group were tested for their effects on NF-κB activation. The structure of these tested compounds is given in Table I. None of these substances showed cytotoxic effects on HeLa cells at concentrations of 5 and 10 μM after 10 h of incubation time. One hour after preincubation with 5 and 10 μM of the respective drugs, HeLa cells were stimulated for 20 min with PMA. Subsequently total extracts of these cells were tested by EMSA on NF-κB activation. All tested isoprenoide substances lacking either the lactone ring or the exomethylene group in the α-position to the lactone function displayed no inhibitory effect on the pathway leading to the activation of NF-κB (see Table I). Another interesting structural element especially of parthenolide is its epoxide ring which is also a likely site for the addition
of nucleophilic reagents. To investigate the importance of this epoxide ring we also tested substances with this structural feature, even in combination with exomethylene groups to provide a bireactive substrate for (the) target molecule(s). Again none of the tested substances displayed inhibitory properties on NF-κB as assessed by EMSA (Table I). This failure of inhibition occurred irrespective of a synthetic or natural origin of the tested substances.

SLs Inhibit NF-κB Activation without Having Anti-oxidative Properties—The chemical structure of the SLs suggests that they do not have anti-oxidative properties such as many other inhibitors of NF-κB. To exclude that SLs display any direct or
The role of NF-κB in the induction of cell death is still not yielding a homogenous picture. The inhibition of NF-κB promotes TNF-α-mediated cell death in HeLa cells, macrophages, fibroblasts, fibrosarcoma cells, and Jurkat cells (60–63), while it is ineffective in other cell lines such as human breast carcinoma cells (64). Part of the discrepancies may be explained by differences of the cell type studied and the nature of the apoptosis-inducing stimulus. This study shows that the incubation with parthenolide facilitates cell killing by TNF-α in mouse L929 cells, although only additional experiments using further inhibitors of NF-κB can really prove the protecting role of this transcription factor for L929 cells. The mechanism of this cell-protecting effect of NF-κB is still not clear. It might rely on the induced expression of anti-apoptotic genes such as the zinc finger protein A20 or manganese superoxide dismutase (for review, see Ref. 16).

The SLs tested in this study display a high degree of specificity for their inhibitory activity, since they did not influence the activity of other transcription factors such as AP-1, RBP-Jκ, and Oct-1 (Ref. 1 and this study). Furthermore, the SLs did not impair the activity of the T-cell kinases p59Fyn and p60c-src.

These results show that the SLs do not interfere in a nonspecific manner with transcription factors or signaling molecules. A potential target specificity of SLs may well be explained by considering the fact that the combination of the reactive Michael acceptor system together with the oxygen-substituted isoprenoide rings forms a pattern of potential noncovalent binding sites (e.g. hydrogen bonds). These binding sites would allow the SLs to interact with complementary sites on the surface of the target molecule(s). The relative positions of SLs and other inhibitors of NF-κB in the signaling cascade are schematically displayed in Fig. 9. This allows the usage of this drug for the...
analysis and dissection of the diverse signal transduction pathways finally resulting in the activation of NF-kB. Since the various classes of inhibitors interfere with NF-kB activation at different levels in signal transduction it is tempting to speculate that a mixture of these different inhibitors might be highly effective in NF-kB inhibition. Furthermore, the individual doses required for an optimal blocking may presumably be reduced in such a blend, thereby reducing the respective side effects.

The inhibition of NF-kB may be of therapeutic use for the treatment of chronic diseases such as rheumatoid arthritis or for the acute situations such as septic shock. There is emerging evidence for the role of NF-kB during rheumatoid arthritis (for review, see Ref. 2) and the importance of NF-kB during Crohn's disease. Here, the local administration of antisenesce phosphorothioate oligonucleotides to the p65 subunit of NF-kB abrogated established experimental colitis in mice (65). Also other chronic inflammatory diseases such as Alzheimer's disease involve the activation of NF-kB. The amyloid β peptide, which is a major component of the plaque, induces an increase in ROS and activates the nuclear translocation of the p50 and p65 subunits of NF-kB in the neurons directly surrounding the plaque (5). Moreover the septic shock syndrome is associated with a massive activation of NF-kB. Septic shock occurs when microbial products such as LPS stimulate the expression of inflammatory cytokines. This massive production of cytokines leads to failure of circulation and general organ function. Therefore it is of therapeutic interest to develop drugs that are able to interfere with the activity of NF-kB.

The complete inhibition of NF-kB is lost only to 50% after 18 h post-treatment, suggesting an irreversible mechanism such as a covalent modification of proteins. Also other drugs such as aspirin and omeprazole (Antra®), lead to an irreversible inactivation of their target molecules (for review, see Refs. 66 and 67). The covalent modification of a target protein by a drug reduces the required frequency of drug application. The determination of the structural requirements of SLs for NF-kB inhibition presented in this study provides the basis for a rational development of a new generation of anti-inflammatory substances interfering with NF-kB.

Acknowledgments—We thank Dr. Alexey Ushmorov for help with the FACS analysis and Andreas Dumont for carefully reading the manuscript.

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