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Chemistry & Biology 8 (2001) 759–766

Chemistry & Biology

www.elsevier.com/locate/chembiol

Research Paper

The anti-inflammatory natural product parthenolide from the medicinal herb Feverfew directly binds to and inhibits IκB kinase

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Received 8 March 2001; accepted 16 May 2001
First published online 22 June 2001

Abstract

Background: Biologically active natural products continue to be useful in the exploration and control of intracellular signaling processes. For example, the sesquiterpene lactone parthenolide from the anti-inflammatory medicinal herb Feverfew (*Tanacetum parthenium*) appears to inhibit the pro-inflammatory signaling pathway. Parthenolide's direct molecular target, however, remains unknown. We set out to identify the molecular mechanisms of parthenolide's anti-inflammatory activity.

Results: A parthenolide affinity reagent was synthesized and shown to bind directly to and inhibit IκB kinase β (IKKβ), the kinase subunit known to play a critical role in cytokine-mediated signaling. Mutation of cysteine 179 in the activation loop of IKKβ abolished sensitivity towards parthenolide. Moreover, we showed that parthenolide's in vitro and in vivo anti-inflammatory activity is mediated through the α-methylene γ-lactone moiety shared by other sesquiterpene lactones.

Conclusions: In recent years, the multi-subunit IKK complex has been shown to be responsible for cytokine-mediated stimulation of genes involved in inflammation and as such represents an attractive target for pharmaceutical intervention. Our finding that parthenolide targets this kinase complex provides a possible molecular basis for the anti-inflammatory properties of parthenolide. In addition, these results may be useful in the development of additional anti-inflammatory agents.

Keywords: Parthenolide; Feverfew; IκB kinase; Anti-inflammatory

1. Introduction

Nuclear factor-κB (NF-κB) is a dimeric transcription factor that activates the expression of many genes involved in the inflammatory process. In unstimulated cells, NF-κB is retained in the cytoplasm via interaction with its inhibitor IκB [1]. In response to various pro-inflammatory stimuli, IκB is phosphorylated by the IκB kinase complex (IKK). This leads to the ubiquitination and subsequent proteasome-mediated degradation of IκB, allowing NF-κB to enter the nucleus. The IKK complex is composed of two catalytic subunits, IKKα and IKKβ, as well as a third non-catalytic subunit, IKKγ or NEMO (NF-κB essential modulator) [2–6]. Recent murine gene 'knock-out' studies provide important insights into the understanding of the functions of the components of IKK complex. IKKα has been shown to be important in early embryonic development of the skin and skeletal system [7]. On the other hand, IKKβ and NEMO are indispensable for cytokine signaling. For example, in IKKβ knock-out cells, NF-κB is unresponsive to tumor necrosis factor α (TNFα) or interleukin-1 stimulation [8]. The recent identification of the additional putative upstream IKK kinases IKKε [9] and TBK1/NAK [10,11] suggests that the signaling cascade leading to IKK complex activation is not fully understood.

The popularity of medicinal herbs has grown significantly in recent years despite a dearth of information regarding their modes of action and continuing concerns

Abbreviations: IKK, IκB kinase; TNFα, tumor necrosis factor α; NF-κB, nuclear factor-κB

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1074-5521/01/$ – see front matter
PII: S1074-5521(01)00049-7

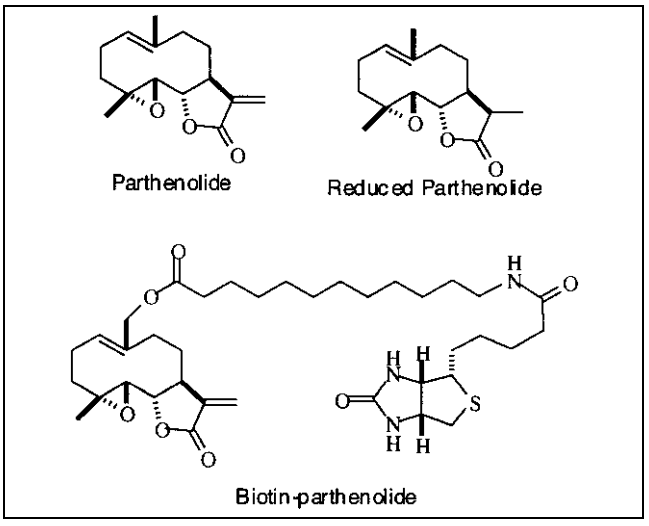

Fig. 1. Structure of parthenolide, reduced parthenolide (RP) and biotinylated parthenolide.

over their efficacy [12]. Recent efforts to elucidate the mechanisms of action of several anti-inflammatory herbs have focused on a class of compounds, sesquiterpene lactones, that are believed to be the active components of these herbal medicines [13–16]. Several plant-derived sesquiterpene lactones have been shown to inhibit the activation of NF-κB. Investigations into the molecular mechanisms of sesquiterpene lactone-mediated NF-κB inhibition have yielded interesting findings. For example, the sesquiterpene lactone helenalin from the medicinal herb *Arnica montana* has been suggested to alkylate selectively the p65 subunit of NF-κB, although no direct chemical proof has been offered in support of this hypothesis [17,18]. On the other hand, parthenolide (Fig. 1), an abundant sesquiterpene lactone found in the medicinal herb Feverfew (*Tanacetum parthenium*), has been reported to inhibit NF-κB activation via targeting an unknown signaling component upstream of IκB [19]. Given the lack of information regarding the exact molecular mode(s) of action of sesquiterpene lactones, we initiated our study to identify the direct target of parthenolide.

Here we show that parthenolide specifically binds to and inhibits IKKβ resulting in the loss of NF-κB activation. Moreover, parthenolide-mediated inhibition of a constitutively active IKKβ mutant demonstrates that parthenolide acts directly at the level of IKKβ to inhibit the NF-κB activation. This conclusion is supported by the finding that a single amino acid substitution in the activation loop (C179A) of IKKβ abolishes parthenolide's IKKβ inhibitory activity. Mass spectrometric analysis confirmed that the tryptic fragment of IKKβ containing C179 was modified by parthenolide. Furthermore, a parthenolide derivative that does not bind IKKβ lacks both in vitro and in vivo anti-inflammatory activity. Taken together, these data provide a molecular mechanism for the anti-inflammatory property of parthenolide.

2. Results

2.1. Parthenolide specifically binds to and inhibits IKKβ

The sesquiterpene lactone parthenolide has been reported to inhibit IκB phosphorylation, however, not through direct inhibition of IKKα, IKKβ or the upstream kinase MEKK1 [19]. To determine if parthenolide mediates its anti-inflammatory activity through IKK complex inhibition, human cervical carcinoma (HeLa) cells were treated with TNFα for different lengths of time alone or preceded by 1 h preincubation with parthenolide (Fig. 2). HeLa cells respond to TNFα treatment by rapidly induc-

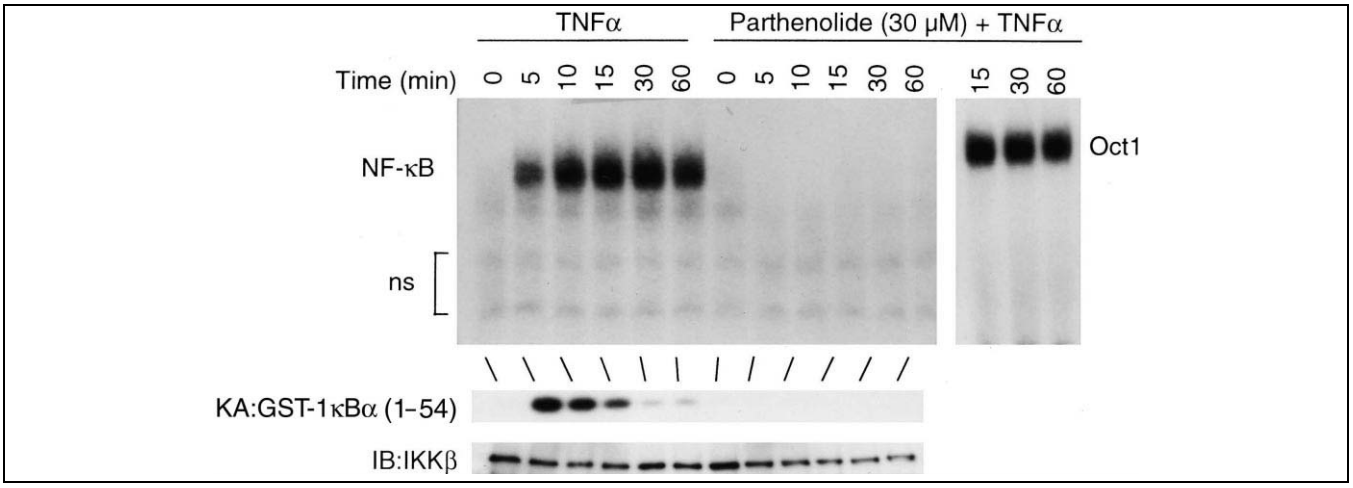

Fig. 2. Parthenolide inhibits TNFα-mediated NF-κB DNA binding activity and IKK activity. Cell lysates from HeLa cells treated with 30 μM parthenolide for 1 h prior to TNFα induction (20 ng/ml) were analyzed for NF-κB and Oct1 DNA binding activity by electrophoretic mobility shift assays (EMSA). Ns, non-specific bands. IKK complexes were immunoprecipitated (IP) from the same samples with anti-IKKα antibody and kinase activity (KA) was assayed using GST-IκBα (1–54) as substrate. The amount of IKK complex immunoprecipitated was verified by immunoblotting (IB) with anti-IKKβ antibody.

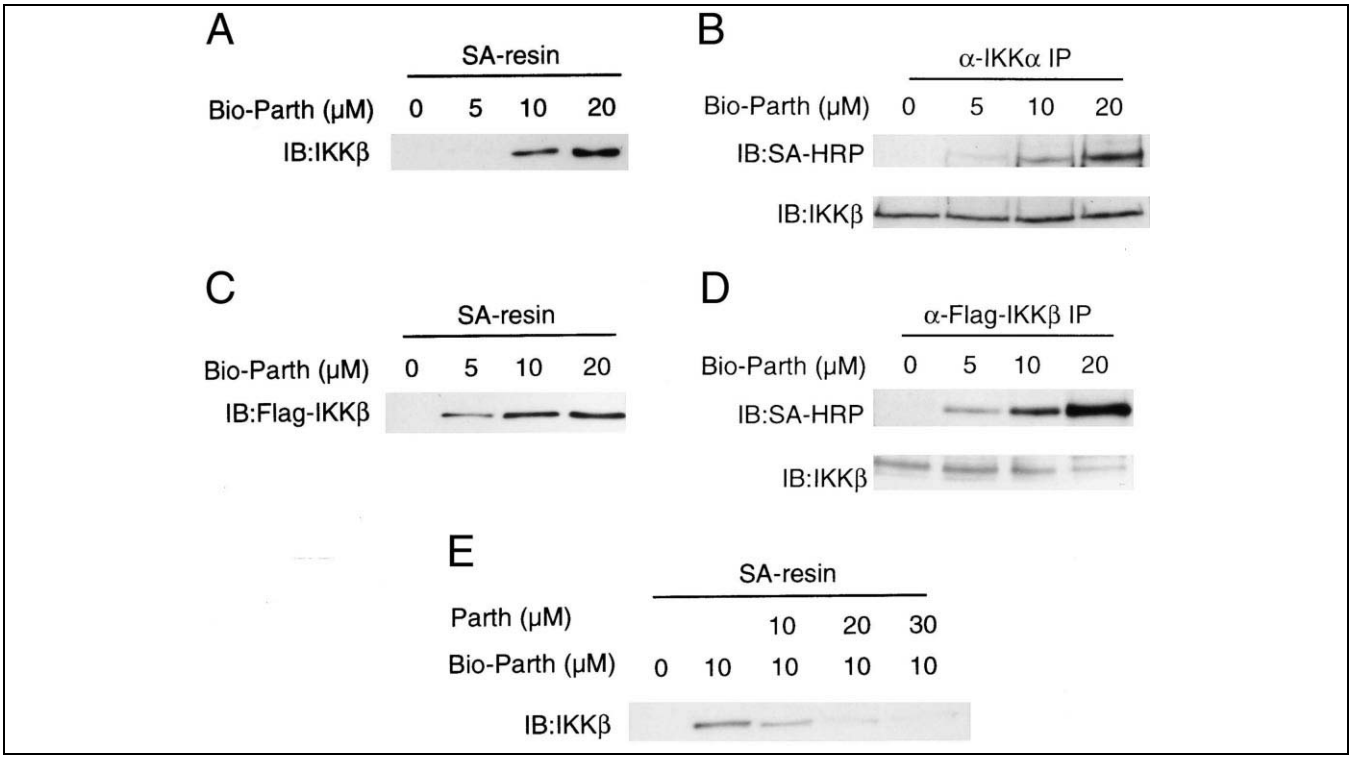

Fig. 3. Biotinylated parthenolide specifically labels endogenous and overexpressed IKKβ. HeLa cells alone (A,B,E) or transfected with Flag epitope-tagged IKKβ (C,D) were treated with various concentrations of biotinylated parthenolide for 1 h. The IKK complex was immunoprecipitated from cell lysates with (A,C,E) Soft-link™ avidin resin (SA) (Promega), (B) anti-IKKα, or (D) anti-Flag (M2) antibodies. (E) The specificity of the biotinylated parthenolide for IKKβ was shown using cells pretreated for 1 h with increasing concentrations of parthenolide before subsequent challenge with biotinylated parthenolide. Immunoprecipitated proteins were resolved by SDS–PAGE and analyzed by immunoblotting with anti-IKKβ (A,B,D,E), anti-Flag antibodies (C) or streptavidin-horseradish peroxidase conjugate (B,D).

ing NF-κB DNA binding activity. Using electrophoretic mobility shift assays (EMSAs), we found that TNFα-induced NF-κB DNA binding activity was first detected within 5 min and peaked at 30 min. In contrast, pretreatment with 30 μM parthenolide abolished the TNFα-stimulated NF-κB DNA activity but had no effect on the DNA binding activity of an unrelated transcription factor Oct1. The peak in NF-κB DNA binding activity was preceded by an increase in IKK activity as measured in IKK immunocomplex kinase assays using glutathione *S*-transferase (GST)-IκB (residues 1–54) fusion protein as substrate (Fig. 2). Parthenolide dramatically abolished this TNFα-stimulated kinase activity.

In order to identify the molecular target of this potent anti-inflammatory sesquiterpene lactone, we synthesized a biotinylated affinity derivative of parthenolide (Fig. 1). This analog, which also inhibits TNFα-stimulated NF-κB DNA binding activity (data not shown), was incubated with HeLa cells at increasing concentrations. Affinity purification of biotinylated proteins using streptavidin resin followed by anti-IKKβ immunoblot analysis demonstrated that parthenolide formed a covalent adduct with IKKβ, in a dose-dependent manner (Fig. 3A). Consistent with this finding, the converse experiment showed that biotinylated parthenolide was detected in immunoprecipitations of the

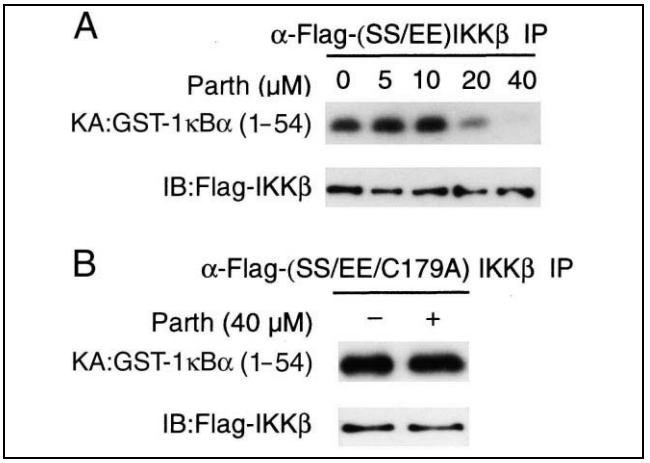

Fig. 4. Parthenolide inhibits constitutively active wild-type but not Cys179Ala (C179A) IKKβ. (A) HeLa cells transfected with Flag-tagged constitutively active (SS/EE) mutant IKKβ were treated with increasing concentrations of parthenolide for 1 h. IKK activity was measured in anti-Flag (M2) immunocomplex kinase assays using GST-IκBα (1–54). Immunoprecipitated IKKβ levels were determined by immunoblot analysis with anti-Flag antibodies. (B) Constitutively active SS/EE IKKβ with an additional mutation exchanging cysteine 179 for alanine (C179A) was overexpressed in HeLa cells. Transfected cells were incubated in the absence or presence of 40 μM parthenolide for 60 min and the resulting GST-IκB (1–54) kinase assay measured as in A.

IKK complex from biotinylated parthenolide-treated cells (Fig. 3B). Biotinylated IKKα was also detected in these experiments (data not shown) indicating an interaction between this second IKK catalytic subunit and parthenolide. The IKKβ:biotinylated parthenolide results were corroborated in transient IKKβ overexpression assays. Using epitope-tagged IKKβ (Flag-IKKβ), a direct covalent interaction between biotinylated parthenolide and IKKβ was observed in both streptavidin-resin pull-down (Fig. 3C) and anti-Flag immunoprecipitation experiments (Fig. 3D). Moreover, pretreatment with increasing concentrations of parthenolide blocked biotinylated parthenolide binding to endogenous IKKβ (Fig. 3E). This observation demonstrates that the covalent interaction between parthenolide and IKKβ is specific and saturable.

2.2. IKKβ inhibition by parthenolide is independent of upstream kinases

The IKK complex acts as a convergence point for a variety of upstream activating kinases, such as MEKK [20], NIK [21,22], and NAK [10,11]. To exclude the possibility that parthenolide prevents IKKβ activation via inhibition of an upstream kinase, constitutively active IKKβ was tested for sensitivity to parthenolide. An epitope-tagged IKKβ derivative (Flag-SS/EE IKKβ) was rendered constitutively active by substitution of serine residues 177 and 181, known to be phosphorylated in vivo, with negatively charged residues (i.e., glutamate) to mimic the activating phosphorylation at those sites [23,24]. Anti-Flag immunoprecipitates prepared from HeLa cells transfected

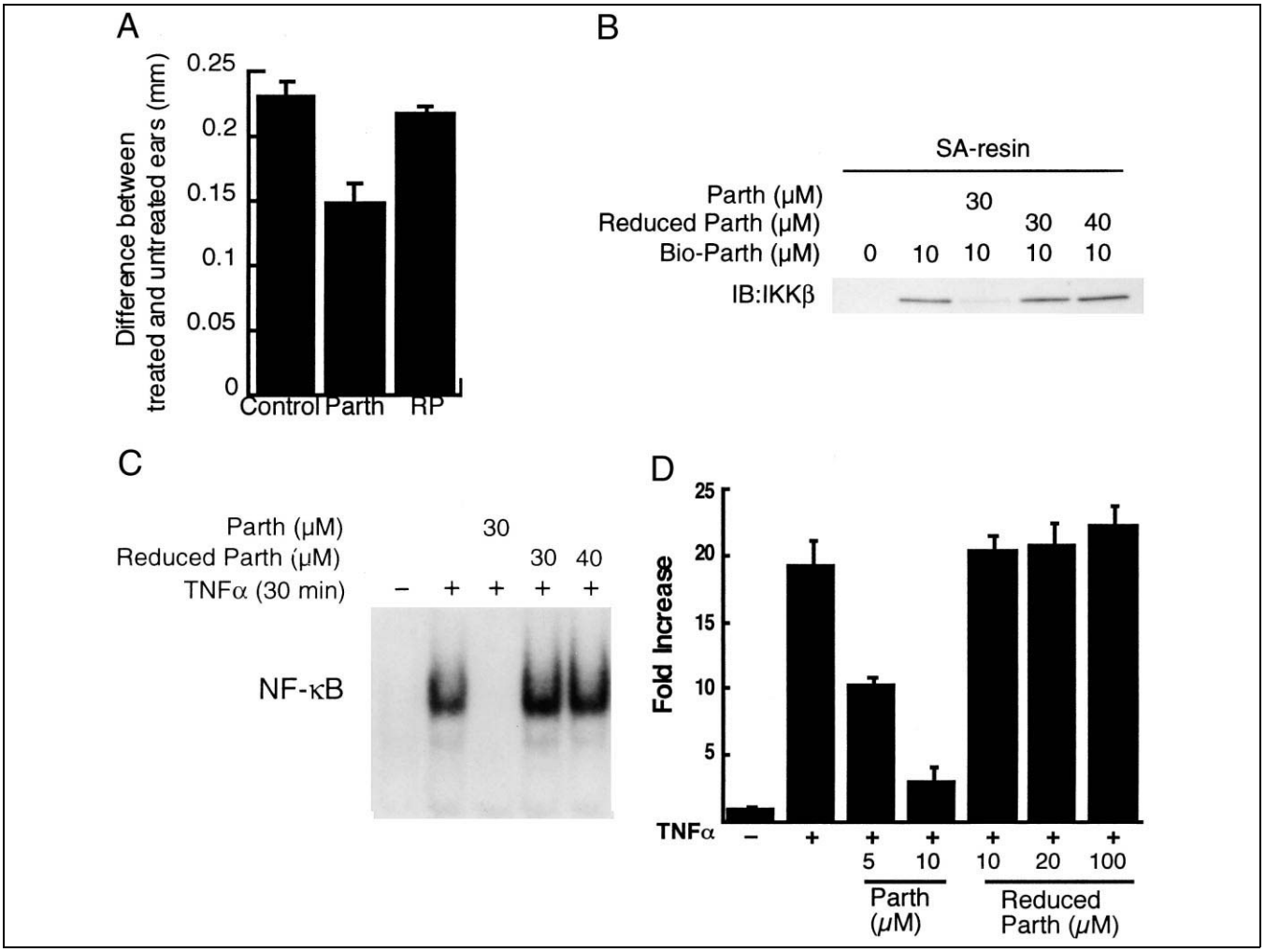

Fig. 5. Reduced parthenolide lacks the ability to inhibit in vivo and in vitro NF-κB signaling. (A) Murine ear edema assay. Parthenolide, reduced parthenolide (RP) or vehicle (DMSO:methanol, 1:3) was applied to the ears of mice for 15 min followed by PMA treatment. Ear thickness was measured 6 h after PMA application. (B) HeLa cells were pretreated with or without parthenolide or reduced parthenolide for 1 h prior to 1 h incubation with biotinylated parthenolide. Biotinylated proteins were purified with SA, resolved by SDS–PAGE and visualized by anti-IKKβ immunoblot analysis (IB). (C) The NF-κB DNA binding activity in HeLa cells treated with parthenolide, reduced parthenolide or vehicle (DMSO) for 1 h and induced with TNFα (20 ng/ml) for 30 min was tested by EMSA. (D) The effect of parthenolide and reduced parthenolide in inhibiting NF-κB-induced gene expression was tested using the Promega dual-luciferase reporter system. Transfected cells were then pretreated with parthenolide, reduced parthenolide, or vehicle (DMSO) for 1 h followed by a 5 h TNFα (10 ng/ml) stimulation.

with constitutively active IKKβ were found to induce GST-IκB (1–54) phosphorylation in immunocomplex kinase assays (Fig. 4A). More importantly, this constitutively active IKK activity was inhibited by parthenolide in a dose-dependent manner.

The fact that parthenolide inhibited this kinase activity, independently of any upstream activating kinases, indicates that parthenolide targets IKKβ directly rather than by inhibiting an activating kinase upstream of IKKβ. This is further supported by the use of an IKKβ derivative mutated at cysteine 179. This residue lies in the activation loop of the kinase between the two serine residues that are phosphorylated in response to pro-inflammatory cytokines. Moreover, cysteine 179 has recently been shown to be a site for modification by two IKKβ inhibitors, arsenite and cyclic prostaglandins [25,26]. Introduction of the C179A mutation into SS/EE IKKβ rendered a constitutively active IKKβ resistant to 40 μM parthenolide, a concentration that effectively inhibited the kinase activity of SS/EE IKKβ (Fig. 4B). We confirmed that the peptide containing C179 was modified by parthenolide by tandem mass spectrometric sequencing analysis of tryptic peptides from parthenolide-treated recombinant IKKβ (data not shown). These results demonstrate that IKKβ is the direct target mediating the anti-inflammatory activity of parthenolide.

2.3. Parthenolide's exocyclic methylene is required for in vivo and in vitro anti-inflammatory activity

Many anti-inflammatory sesquiterpene lactones possess an exocyclic methylene moiety as part of a γ-lactone group, which can serve as a site for covalent modification via a Michael addition reaction [27]. Parthenolide, however, also possesses an epoxide moiety, which is a second site for potential nucleophilic attack by an amino acid side chain. Given the presence of these two electrophilic sites on parthenolide, it was not possible a priori to determine the mechanism of covalent modification with IKKβ. To test whether the exocyclic methylene is responsible for parthenolide's anti-inflammatory activity, the natural product was modified to yield reduced parthenolide (Fig. 1). Employing the murine ear edema assay of inflammation, the tumor promoter phorbol myristate acetate (PMA) was applied with or without parthenolide to the ears of mice and the resulting swelling was measured 6 h later. As demonstrated previously [28], parthenolide significantly reduces the PMA-induced contact sensitivity in this in vivo assay. However, using reduced parthenolide, we show in parallel experiments that the in vivo anti-inflammatory activity of parthenolide is lost with the absence of the exocyclic methylene (Fig. 5A). We next tested reduced parthenolide for its ability to form the IKKβ:parthenolide protein adduct observed with the natural product. While pretreatment of cells with 30 μM parthenolide effectively blocked subsequent biotin–parthenolide interaction with IKKβ, 40 μM reduced parthenolide did not inhibit this interaction (Fig. 5B). In addition, reduced parthenolide also failed to inhibit both TNFα-induced NF-κB DNA binding activity (Fig. 5C) and NF-κB-mediated transcription (Fig. 5D) as measured by EMSA and luciferase reporter assays, respectively. Thus, protein:natural product adduct formation most likely occurs via nucleophilic attack by an amino acid side chain of IKKβ on the α,β unsaturated ester of parthenolide.

3. Discussion

The findings presented here provide the first evidence that parthenolide, the active component of the widely used anti-inflammatory herb Feverfew, directly targets IKKβ. Using biotinylated parthenolide, we demonstrate that parthenolide covalently binds IKKβ at similar concentrations required to inhibit NF-κB activation. Moreover, we show that this interaction is saturable and specific since excess parthenolide can compete for biotinylated parthenolide binding to IKKβ.

A number of kinases have been reported to be upstream of the IKK complex in the NF-κB signaling pathway. Overexpression of protein kinases MEKK or NIK leads to IKKβ activation [20,29,30] and, recently, additional candidate kinases proposed to be upstream of IKK have been identified [10,11,31]. Since IKKβ, IKKα, and the upstream kinase MEKK1 have been previously reported not to be the target of parthenolide [19], and we show here that parthenolide inhibits IκB phosphorylation in response to TNFα, it was important to determine if parthenolide directly inhibits IKKβ or the activity of an upstream IKK kinase. We show that parthenolide effectively inhibits the IKK activity of a constitutively active IKKβ (SS/EE mutant), thus demonstrating that parthenolide acts at the level of IKKβ. Moreover, a single amino acid substitution (C179A) in the kinase activation loop of this constitutively active IKKβ renders it insensitive to parthenolide. Given the lack of parthenolide sensitivity in the C179A mutant, this cysteine residue is a likely candidate for the site of covalent modification by parthenolide. This possibility is supported by tandem mass spectrometric tryptic peptide sequence analysis, which demonstrated that the tryptic peptide containing C179 was modified by parthenolide. Interestingly, two other IKKβ inhibitors have been recently shown to target this cysteine residue [25,26]. The α,β unsaturated carbonyl group of prostaglandin A_1 was shown to be essential for the covalent modification of IKKβ, putatively via interaction with cysteine 179 [26]. Similarly, modification of cysteine 179 of IKKβ has been proposed to mediate the pathological effects of arsenite [25]. Although additional IKKβ inhibitors have been described, it remains to be determined if cysteine 179 plays a role in mediating their inhibitory activities [23,32,33].

The molecular mechanism of IKKβ inhibition by par-

thenolide remains unknown. In general, phosphorylation of a kinase's activation loop induces a conformational change that allows substrate access to the active site [34]. Since the putative binding site, cysteine 179, lies between the two serines that are phosphorylated in response to TNFα, one model for parthenolide's IKKβ inhibitory activity is that the binding of parthenolide to IKKβ prevents phosphorylation of these serines by upstream activating kinases. However, the fact that parthenolide can inhibit a constitutively active IKKβ (SS/EE) mutant does not support this hypothesis as the sole mechanism. Alternatively, parthenolide could stabilize the kinase activation loop in the inactive conformation despite the presence of the neighboring negatively charged phosphorylated serines (or glutamates, in the case of the constitutively active SS/EE mutant). Structural studies of a parthenolide:IKKβ co-crystal will address this possibility. Parthenolide binding could also induce dissociation of the IKK complex, leading to a decrease in IKK activity. However, our preliminary data indicate that there is no dissociation of IKKα, IKKβ or NEMO from the core complex upon parthenolide binding (data not shown). Nevertheless, given the recent discovery of the additional complex component, CIKS/Act1 [35,36], this model remains a possibility.

Given the structural and biological similarities between parthenolide and other compounds found in anti-inflammatory herbal medicines, our findings shed light on the biological mechanism of this interesting class of natural products. Many biologically active sesquiterpene lactones possess an α-methylene γ-lactone moiety. We show that upon reduction of this functional group, parthenolide loses its ability to inhibit TNFα induction of NF-κB transcriptional activity. Furthermore, reduced parthenolide has no effect on the TNFα-induced DNA binding activity of NF-κB or on the PMA-induced ear edema in an in vivo model of inflammation. Reduced parthenolide's lack of biological activity also correlates with its inability to bind IKKβ. Thus, these findings demonstrate the importance of the α-methylene γ-lactone moiety and provide a molecular basis for the biological activity of other anti-inflammatory sesquiterpene lactones possessing an α-methylene γ-lactone moiety.

Given its importance as a critical player in the inflammatory signaling pathway, IKKβ is an attractive target for anti-inflammatory therapeutic development. Our finding that parthenolide directly targets IKKβ may serve as the basis for the generation of new anti-inflammatory agents.

4. Significance

Feverfew and several other anti-inflammatory medicinal herbs are rich in sesquiterpene lactones, a group of compounds thought to mediate the anti-inflammatory nature of these plants. Recently, parthenolide, a sesquiterpene lactone found in Feverfew, was suggested to target a component of the IKK complex. However, no direct target was identified. Earlier reports by Lyß et al. [17] and Rüngeler et al. [18] suggested that helenalin, another sesquiterpene lactone, directly alkylates p65, one subunit of the dimeric transcription factor NF-κB, yet no direct biochemical evidence was provided to prove this hypothesis. In this report, we show the direct interaction between parthenolide and IKKβ, one of the catalytic subunits of the IKK complex. Using a biotinylated derivative of parthenolide as an affinity reagent, we demonstrate covalent modification of IKKβ by biotinylated parthenolide. Mass spectrometric analysis revealed that cysteine 179 was modified by parthenolide. Moreover, by site-directed mutagenesis, we showed that an IKKβ mutant (C179A) abolished parthenolide's inhibitory effect on IKKβ. Since parthenolide could form a covalent adduct via nucleophilic attack on the epoxide or the exocyclic methylene, we further investigated the molecular interaction between parthenolide and its binding target by reducing the exocyclic methylene. Reduced parthenolide lacks both in vivo and in vitro anti-inflammatory properties. The results presented here provide a possible explanation for parthenolide's anti-inflammatory properties and may aid in the development of a new class of anti-inflammatory agents.

5. Materials and methods

5.1. Cell culture

All cell culture reagents were purchased from Gibco BRL (Gaithersburg, MD, USA). HeLa cells were cultured in Dulbecco's modified essential medium (DMEM) supplemented with 10% fetal bovine serum and PSF (an antibiotic-antimycotic (penicillin, streptomycin and Fungizone® from Gibco-BRL). Transfections were done using Fugene6 transfection reagent (Roche, Indianapolis, IN, USA) following the manufacturer's instructions. Cells were harvested 24 h after transfection. Parthenolide (Sigma) and biotinylated parthenolide were dissolved in dimethyl sulfoxide (DMSO) and used at the concentrations indicated. Cells were treated with parthenolide, biotinylated parthenolide or vehicle (DMSO) 1 h (or otherwise indicated) prior to harvesting. TNFα (Roche) was added at a final concentration of 20 ng/ml. Cells were harvested in NP-40 lysis buffer (50 mM Tris–HCl, 150 mM NaCl, 1% NP-40, 10% v/v glycerol) supplemented with pepstatin (10 μg/ml), leupeptin (10 μg/ml), aprotinin (5 μg/ml) and 1 mM phenylmethylsulfonyl fluoride at 4°C.

5.2. Syntheses of biotinylated parthenolide and reduced parthenolide

Biotinylated parthenolide was prepared first by oxidation of parthenolide (Aldrich) with selenium dioxide and *tert*-butylhydroperoxide to furnish the allylic alcohol, as previously described [37]. The allylic alcohol was then esterified with 12-(Fmoc amino) dodecanoic acid under Mitsunobu conditions. Removal of the Fmoc group with tetrabutylammonium fluoride followed by coupling with biotin using *N*-[dimethylamino)-1*H* -1,2,3-triazolo-

[4,5-b]pyridino-1-ylmethylene]-*N*-methylmethanaminimum hexafluorophosphate *N*-oxide/di-isopropylethylamine yielded biotinylated parthenolide. The biotinylated product was verified by ^{1}H nuclear magnetic resonance (NMR) and electrospray mass spectroscopy. ^{1}H NMR ($CDCl_3$, 500 MHz) δ 6.26 (d, 1H, J = 4.0 Hz), 5.87 (brs, 2H), 5.69 (t, 1H, J = 7.5, 9.0 Hz), 5.55 (d, 1H, J = 3.5 Hz), 5.19 (brs, 1H), 4.66 (d, 1H, J = 12.5 Hz), 4.54 (brs, 1H), 4.47 (d, 1H, J = 12.5 Hz), 4.32 (brs, 1H), 3.87 (t, 1H, J = 9.25 Hz), 3.24–3.14 (m, 3H), 2.95–2.91 (m, 2H), 2.87 (d, 1H, J = 9.5 Hz), 2.75 (m, 1H), 2.33–2.19 (m, 6H), 1.70–1.12 (m). ES-MS *m/z* (M+H) calcd. for $C_{37}H_{58}N_3O_7S$ 688.40, found 688.57.

Hydrogenation of parthenolide was carried out in ethyl acetate under 1 atm using a 5% molar ratio of palladium (10% wt on activated carbon) at room temperature to yield reduced parthenolide. The reduced product was verified by ^{1}H NMR spectroscopy.

^{1}H NMR ($CDCl_3$, 500 MHz) δ 5.20 (dd, 1H, J = 2.25, 11.9 Hz), 3.85 (t, 1H, J = 9.07 Hz), 2.70 (d, 1H, J = 8.96 Hz), 2.45–1.10 (m).

5.3. EMSA

Total HeLa cell lysate was prepared in NP-40 lysis buffer. Aliquots (10 μg protein) were mixed in binding buffer (10 mM Tris–HCl, pH 7.5, 1 mM $MgCl_2$, 0.5 mM ethylenediaminetetraacetic acid, 0.5 mM dithiothreitol (DTT), 4% glycerol, 0.05 mg/ml poly(dI-dC), 50 mM NaCl) at room temperature for 10 min followed by incubation with ^{32}P-radiolabeled NF-κB consensus oligos, 5′-AGTTGAGGGGACTTTCCCAGGC-3′ (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for an additional 20 min. DNA binding activity was analyzed by 4% non-denatured PAGE and autoradiography. The specificity of NF-κB binding was verified by supershift with (2 μg) p65 antibodies (Santa Cruz Biotechnology) (data not shown).

5.4. Immunoprecipitation, kinase assays and immunoblotting

Cell lysates prepared in NP-40 lysis buffer with phosphatase inhibitors (10 mM NaF, 0.5 mM sodium vanadate and 20 mM β-glycerophosphate) were incubated with antibodies (2–5 μg) for 1 h. Protein A- or G-conjugated agarose beads were then added and incubated overnight. Immunoprecipitated complex was then washed three times with lysis buffer and twice with 25 mM Tris–HCl pH 7.5. Kinase assays were performed by incubating the immunoprecipitate in kinase reaction buffer (25 mM Tris–HCl pH 7.5, 10 mM $MgCl_2$, 2 mM DTT, 50 μM ATP and phosphatase inhibitors) with 5 μCi [γ-^{32}P]ATP and 2 μg bacterially expressed GST-IκBα (1–54) in a reaction volume of 50 μl for 20 min at 30°C. The phosphorylated substrate was then affinity-purified with glutathione-Sepharose resin (Pharmacia) and analyzed by SDS–PAGE, autoradiography, and Western blotting. Anti-IKKβ and anti-Flag (M2) antibodies were purchased from Santa Cruz Biotechnology and Sigma, respectively.

5.5. Luciferase assay

HeLa cells were transfected with 100 ng each of pFLuc2xκB [38] and pRL-TK vector (Promega) DNA for 24 h. After incubation with parthenolide, reduced parthenolide, or vehicle (DMSO) for 1 h, transfected cells were stimulated for 5 h with TNFα (10 ng/ml). Luciferase assays were performed according to the manufacturer's instructions.

5.6. Mouse ear edema assay

Parthenolide (0.5 mg/ear), reduced parthenolide (0.5 mg/ear) or vehicle (DMSO:methanol 1:3) was applied to the right ears of three groups of five mice for 15 min before PMA treatment (5 μg in 20 μl ethanol/ear). Left ears of mice were treated with vehicle alone as a control. Six hours after PMA application, ear thickness was measured using a micrometer as described [38].

Acknowledgements

We thank S. Ghosh for Flag-IKKβ and NF-κB luciferase reporter plasmids, R. Gaynor for the IKKβ SS/EE and GST-IκB plasmids, William S. Lane (Harvard Microchemistry Laboratory) for mass spectrometric analyses, and members of our lab for many helpful discussions. M.I.N. is an UNCF/Pfizer Fellow. Supported by NIH Grant GM62120.

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