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Parthenolide Inhibits Activation of Signal Transducers and Activators of Transcription (STATs) Induced by Cytokines of the IL-6 Family[1]

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Progression of inflammatory processes correlates with the release of cell-derived mediators from the local site of inflammation. These mediators, including cytokines of the IL-1 and IL-6 families, act on host cells and exert their action by activating their signal transduction pathways leading to specific target gene activation. Parthenolide, a sesquiterpene lactone found in many medical plants, is an inhibitor of IL-1-type cytokine signaling that blocks the activation of NF-κB. Here we show that parthenolide is also an effective inhibitor of IL-6-type cytokines. It inhibits IL-6-type cytokine-induced gene expression by blocking STAT3 phosphorylation on Tyr705. This prevents STAT3 dimerization necessary for its nuclear translocation and consequently STAT3-dependent gene expression. This is a new molecular mechanism of parthenolide action that additionally explains its anti-inflammatory activities.

Key Words: **parthenolide; inflammation; IL-6; STAT3; tyrosine phosphorylation.**

Infection, injury or tumor growth induces a series of host defense reactions resulting in a state known as inflammation. During the inflammatory processes, which finally lead to return to normal functions, a variety of cell-derived mediators, including cytokines, are released from a local site of injury and act on other host cells inducing a systemic reaction [1]. Proinflammatory cytokines play a very important role in the development and progression of inflammatory processes. Among them the family of IL-1-type cytokines (IL-1α, IL-1β, TNFα, TNFβ and IL-18) is crucial in early stages of inflammation [2, 3]. These cytokines bind to their surface receptors on target cells and activate two major transcription factors: nuclear factor κB (NF-κB) and activating protein-1 (AP-1). Signal transduction pathways used by IL-1-type cytokines involve adapter proteins (TRAFs, RIP, MyD88) and kinases with homology to kinases from MAPK pathway (IRAK, NIK, IKK). Cytokine-activated AP-1 and NF-κB induce the expression of many target genes indispensable for development of an inflammatory state. These include genes coding for IL-6-type cytokines (IL-6, LIF, OSM, IL-11, CT-1 and CNTF) [4], in turn involved in further progression of inflammation and induction of acute phase proteins in liver [5]. These cytokines also bind to their surface receptors on target cells and activate JAK-STAT and MAPK pathways with STATs and C/EBPs as major transcription factors regulating expression of IL-6-type responsive genes [6].

Although inflammation represents a physiological response to injury and helps to restore tissue homeostasis, acute or prolonged inflammatory processes may lead to increased tissue destruction and uncontrolled amplification of inflammatory response. Therefore, a number of anti-inflammatory mediators, such as glucocorticoids or anti-inflammatory cytokines, have evolved. These mediators down-regulate and eventually terminate inflammation. For the same reason an extensive search is focused on the development and characterization of pharmacological compounds that exhibit anti-inflammatory activities. Recently, such activities have been described for sesquterpene lactones obtained from several medical plants [7]. Parthenolide,

Abbreviations used: ACT, α_1-antichymotrypsin; AP-1, activating protein-1; APP, acute phase protein; βME, β-mercaptoethanol; CAT, chloramphenicol acetyltransferase; IKK, IkB kinase; IL, interleukin; IRAK, IL-1 receptor associated kinase; JAK, Janus kinase; LIF, leukemia inhibitory factor; MAPK, mitogen activated protein kinase; NIK, NF-κB inducing kinase; NF-κB, nuclear factor κB; OSM, oncostatin M; SIE, *cis*-inducible element of c-fos gene; STAT, signal transducer and activator of transcription; TNFα, tumor necrosis factor α; TRAF, TNF-receptor associated kinase.

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the major sesquterpene lactone in European feverfew (*Tanacetum parthenium*), has already been shown to inhibit IL-1- and TNFα-induced NF-κB activation [7, 8]. Here, we examined the effect of parthenolide on the activity of IL-6-type cytokines and found that it inhibits its activation of STAT proteins by blocking their phosphorylation on tyrosine residue, a step indispensable for dimerization and target gene activation.

MATERIALS AND METHODS

Cells and stimulation. Human hepatoma HepG2 cells were obtained from ATCC (Rockville, MD). Cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and antibiotics. Cells were stimulated with: 50 ng/ml human IL-6 (kindly provided by Dr. P. C. Heinrich, RWTH, Aachen, Germany), 10 u/ml human LIF (a gift of Dr. H. Baumann (Roswell Park Cancer Institute, Buffalo, NY) or 25 ng/ml human OSM (R&D Systems Minneapolis, MN). Dexamethasone (10^{-7} M) was included in the media to enhance cytokine action. Parthenolide (Sigma, St. Louis, MO) was added, at 5 μM (50 μM concentration was not toxic), 1 h before cytokine treatment.

Northern blot analysis. Total RNA was prepared using the phenol extraction method [9]. 5 μg samples of RNA were subjected to formaldehyde gel electrophoresis using standard procedure [10] and transferred to Hybond-N membranes (Amersham), according to the manufacturer's instructions. The filters were prehybridized at 68°C for 3 h in 10% dextran sulfate, 1 M sodium chloride and 1% SDS, and hybridized in the same solution with a 1.4 kb *Eco*RI–*Eco*RI fragment of ACT cDNA (a gift of Dr. H. Rubin, University of Pennsylvania) labeled by random priming [11]. After the hybridization, nonspecifically bound radioactivity was removed by washing in 2× SSC at room temperature, followed by two washes in 2× SSC/1%SDS at 68°C for 20 min.

Transient transfections. Plasmids pACT-352CAT and p6x(ACT-A)tkCAT containing STAT binding elements of the human α_1-antichymotrypsin gene (ACT) have been described [12]. Cells were transfected in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum in 12 well cluster dishes using FuGene6 reagent (Roche Molecular Biochemicals, Indianapolis, IN), with 1 μg of plasmid DNA and 0.4 μg of internal control plasmid pCH110 (Pharmacia, Piscataway, NY). One day after transfection, cells were stimulated, then cultured another 24 h and harvested. Protein extracts were prepared by freeze thawing [13], and protein concentration was determined by the BCA method (Sigma Chemical Co., St. Louis, MO). Chloramphenicol acetyltransferase (CAT) and β-galactosidase assays were performed as described [14, 15]. CAT activities were normalized to the internal control β-galactosidase activity and are means ± SEM (3 determinations).

Extract preparation and gel retardation assays. Whole cell extracts were prepared as described [16]. Double-stranded high affinity STAT binding SIE(m67) probe [16] was labeled by filling in 5′ protruding ends with Klenow enzyme using [α^{32}P]dCTP (3000 Ci/mmol). Gel retardation assays were carried out according to published procedures [17]. 5 μg of extracts and approx. 10 fmol (10,000 cpm) of probe were used.

Western blotting. HepG2 cells growing in 6 cm dishes were lysed in 800 μl of boiling 1% SDS, 10 mM Tris pH 7.4 v 1 mM sodium vanadate. Samples (30 μl) were subjected to SDS–PAGE and electroblotted onto nitrocellulose (Schleicher & Schuell, Keene, NH). STAT3, Tyrosine- and Serine-phosphorylated STAT3 were detected using monoclonal anti-STAT3 antibodies (Transduction Laboratories, Lexington, KY) and polyclonal Phospho-STAT3 (Tyr705 or Ser727) antibodies (New England Biolabs, Inc., Beverly, MA) respectively. Antigen-antibody complexes were visualized by enhanced chemiluminescence (Amersham, Arlington Heights, IL).

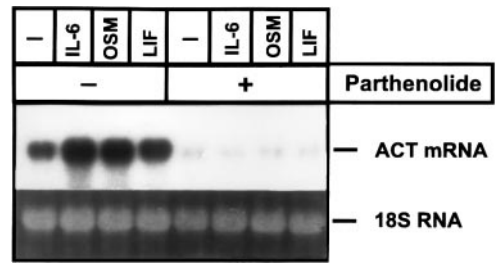

FIG. 1. Parthenolide inhibits the ACT gene expression. HepG2 cells were incubated with 5 μM parthenolide for 1 h and then stimulated with 50 ng/ml IL-6, 25 ng/ml OSM, or 10 U/ml LIF. Total RNA was isolated after 18 h and subjected to Northern blot analysis using ACT cDNA probe. Lower panel shows 28S ribosomal RNA stained with ethidium bromide on the membrane.

RESULTS

Human hepatocarcinoma HepG2 cells, broadly accepted model cells used to study hepatic gene regulation, synthesize a broad range of liver-derived acute phase proteins (APPs) [18]. In liver the expression of APPs drastically change during inflammatory response [1]. These changes are strictly controlled by cytokines of the IL-1 and IL-6 families together with glucocorticoids. The ACT gene encoding α_1-antichymotrypsin, one of the major positive APPs, is regulated by cytokines of the IL-6 family with minor role of the IL-1 type cytokines [19]. To investigate the effect of parthenolide on the cytokine-mediated gene activation we stimulated HepG2 cells with cytokines of the IL-6 family (IL-6, OSM and LIF) in the presence of 5 μM parthenolide and monitored activation of the ACT gene by Northern blotting. Figure 1 shows 4–5-fold increase in the amount of ACT mRNA in response to the IL-6-type cytokines as previously reported [19]. Treatment of the cells with parthenolide resulted in suppression of basal ACT mRNA expression and totally blocked activation by all tested cytokines. This effect was not restricted to HepG2 cells since similar results were obtained using human astrocytoma U373 cells (data not shown).

Cytokines of the IL-6 family have been shown to enhance the expression of the ACT gene on the level of transcription [12]. This transcriptional regulation is mediated by two regulatory elements (ACT-A and ACT-B) within the promoter region of the ACT gene. Both elements have been shown to bind STAT3 and STAT1 complexes in response to IL-6 type cytokines and contribute to full transcriptional activity of the ACT gene [12]. Since parthenolide inhibited ACT mRNA expression we tested its effect on the transcriptional activity of the ACT gene promoter. HepG2 cells were transiently transfected with construct containing

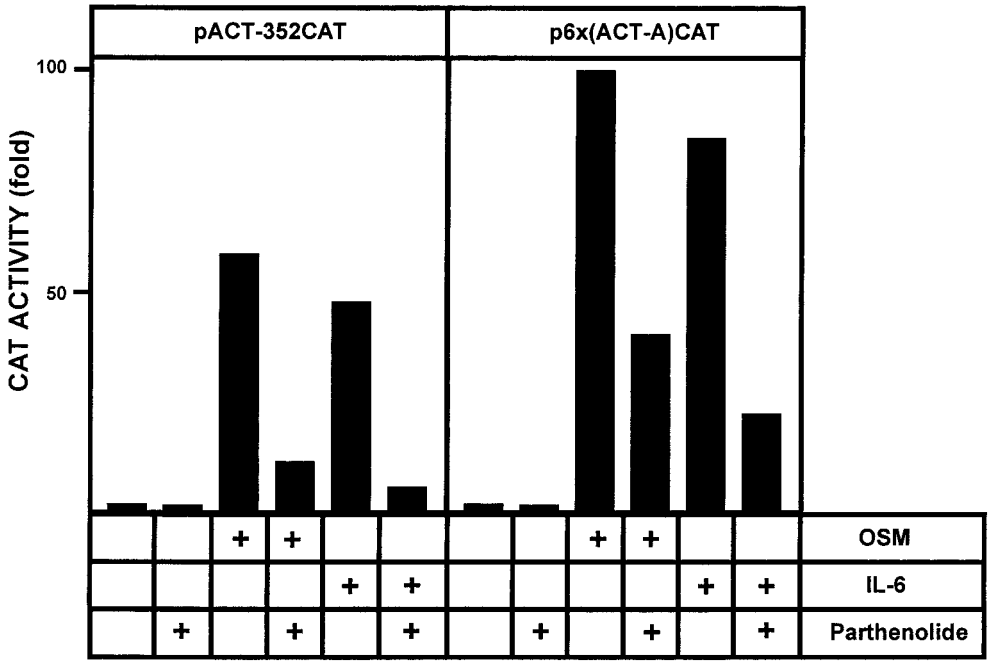

FIG. 2. Inhibition of STAT-mediated promoter activity by parthenolide. HepG2 cells were transfected with plasmids pACT-352CAT or p6x(ACT-A)CAT and a control expression vector pCH110. One day after transfection cells were pretreated with 5 μM parthenolide for 1 h and then stimulated with 50 ng/ml IL-6 or 25 ng/ml OSM. After 24 h cells were harvested. CAT activity was normalized to β-galactosidase activity (cpm/units) and shown means $\pm$ SEM. (three experiments).

the 352 bp long ACT gene promoter or construct containing six copies of high affinity ACT-A element linked to the minimal thymidine kinase (tk) promoter both driving the transcription of bacterial CAT gene. Transcriptional activity of these constructs has previously been shown to increase by 20-fold in response to cytokines of the IL-6 family [12]. Figure 2 shows that parthenolide inhibited IL-6- or OSM-enhanced transcription of the ACT promoter. Furthermore, parthenolide blocked cytokine-enhanced transcription of the construct containing multiple copies of the ACT-A element known to bind STAT3 and STAT1. The observed inhibition of cytokine-induced transcription was less drastic then observed on the mRNA level. We concluded that this difference results from higher cytokine sensitivity observed in transient transfection experiments in comparison to induction of particular mRNA as described before [12]. Alternatively, parthenolide can inhibit other, not identified, transcription factors binding outside the promoter fragments we used. However, inhibition of STAT-element-dependent response was clearly seen. These results suggested that STAT proteins might be possible targets of parthenolide action.

STATs become phosphorylated, dimerize, translocate to nucleus and bind to the specific regulatory elements within target genes in response to many cytokines and growth factors, including IL-6 type cytokines [20]. We investigated the effect of parthenolide on binding of STATs to the high affinity *cis*-inducible element of the *fos* gene (SIE). Cells were treated with OSM with or without parthenolide and binding to the SIE probe was analyzed. OSM induced strong binding of STAT3 and STAT1 homo- and heterodimers to the probe (Fig. 3). However, this binding was totally inhibited by pretreatment with parthenolide. Since β-mercaptoethanol (βME) has previously been shown to suppress inhibition of IL-1-induced activation of NF-κB by parthenolide [8] we tested parthenolide together with βME. As expected βME blocked inhibition of OSM-induced binding by parthenolide. The same results were also obtained when astrocytoma U373 cells were analyzed (data not shown). These experi-

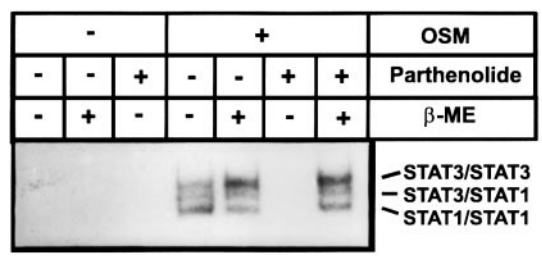

FIG. 3. Parthenolide inhibits binding of STAT3. HepG2 cells were incubated with 5 μM parthenolide and/or 50 μM βME for 1 h and then stimulated with 25 ng/ml OSM. After 15 min whole cell extracts were prepared. 5 μg of extract was incubated with ^{32}P-labeled SIE probe. The formed DNA–protein complexes were separated on a native 4% polyacrylamide gel. After drying, the gel was exposed to an X-ray film. The positions of induced complexes are indicated.

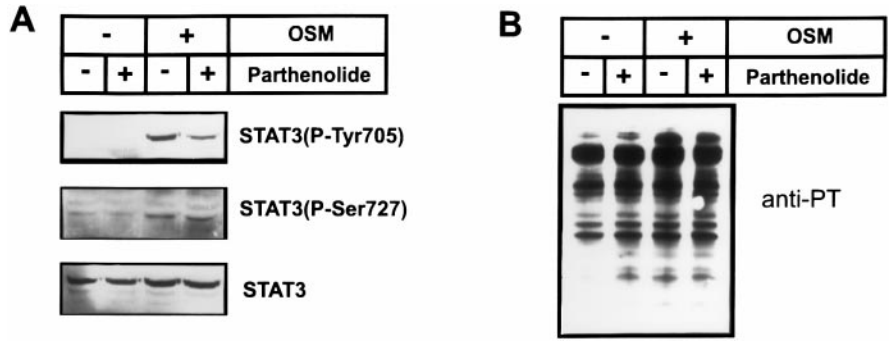

FIG. 4. Immunoblot analysis of HepG2 cells. HepG2 cells were incubated with 5 μM parthenolide for 1 h and then stimulated with 25 ng/ml OSM for 10 min. Cells were lysed under denaturing conditions. Lysates were separated by SDS–PAGE and electroblotted to nitrocellulose membrane. Blots were probed with anti-phosphoSTAT3(Tyr705), anti-phosphoSTAT3(Ser727), anti-STAT3, or anti-PY antibodies.

ments show that parthenolide blocks activation of STATs by IL-6 type cytokines.

STATs are activated by specific phosphorylation on crucial tyrosine and serine residues. Phosphorylation of STATs on tyrosine results in dimerization by reciprocal SH2-PY interactions between two phosphorylated STATs. In contrast phosphorylation on serine residues is not required for dimerization or binding to regulatory elements; however, is necessary for full transactivation by STATs [20]. We examined phosphorylation of STAT3 in response to OSM in the presence or absence of parthenolide by Western blotting. The results are shown in Fig. 4. OSM treatment of HepG2 cells induced both tyrosine and serine phosphorylation of STAT3. In the presence of parthenolide phosphorylation on tyrosine was inhibited with some but little decrease in serine phosphorylation. The inhibition of STAT3 tyrosine phosphorylation was specific since the overall pattern of tyrosine phosphorylation for other proteins was not drastically changed as detected by Western blotting using anti-phosphotyrosine antibodies (Fig. 4B).

DISCUSSION

Parthenolide, know so far as an inhibitor of NF-κB activation and IL-1/TNFα-induced signaling, can effectively block expression of proinflammatory cytokines including IL-6 [7]. This is an important step limiting the progression of inflammation. Now, we describe an additional anti-inflammatory activity of parthenolide: inhibition of IL-6-type cytokine signaling by blocking signal transduction through STATs.

Cytokines of the IL-6 family have pleiotropic activities including control of proliferation of various cells as well as induction of expression of the APP genes in liver cells [6]. The control of proliferative response of cells is dependent on the activation of MAP kinase pathway while the regulation of the APP gene expression requires activation of STAT proteins (with a predominant role of STAT3) [21]. Here we have shown that parthenolide can effectively block expression of ACT mRNA. This is achieved by inhibition of the ACT gene transcription. By applying the promoter-CAT constructs containing multiple copies of STAT binding elements linked to the tk promoter we demonstrated that parthenolide inhibits STAT-mediated response. Since expression of the ACT gene is STAT3-dependent we concluded that STAT3 is a target of parthenolide action. The observed inhibition by parthenolide was in fact achieved by blocking of STAT3 (and STAT1) binding to the regulatory elements as shown by EMSA. Furthermore, parthenolide inhibited binding of STAT3 due to the inhibition of OSM-induced phosphorylation on tyrosine 705. This prevents STAT3 dimerization, a step required for its translocation to nucleus and subsequently gene activation. The precise mechanism responsible for this inhibition is not known; however, the most likely explanation would be the inhibition of JAKs through conjugation with their SH groups (or other target proteins that affect their activity). This hypothesis is supported by the results obtained in the presence of βME since this reducing agent restores responsiveness to cytokines most likely by preventing interaction of parthenolide with SH groups of target proteins.

These new data complete previous findings demonstrating inhibition of the IL-1-type cytokine signaling. Inhibition of both IL-1- and IL-6-type cytokine action also correlates with a common folk knowledge. In many parts of the world, including Europe, leaves of certain plants, i.e., European feverfew, have been used to heal wounds for ages. This anti-inflammatory activities can be now explained on the molecular level and attributed to at least two events; inhibition of IL-1-type signaling by blocking NF-κB activation and a new step, inhibition of IL-6-type signaling by blocking phosphorylation of STAT3 on tyrosine 705.

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