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SHORT COMMUNICATION

Inhibition by Parthenolide of Phorbol Ester-Induced Transcriptional Activation of Inducible Nitric Oxide Synthase Gene in a Human Monocyte Cell Line THP-1

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ABSTRACT. Excessive nitric oxide production by inducible nitric oxide synthase (iNOS) in stimulated inflammatory cells is thought to be a causative factor of cellular injury in inflammatory disease states. Compounds inhibiting iNOS transcriptional activity in inflammatory cells are potentially anti-inflammatory. An assay method for estimating iNOS transcriptional activity in the human monocyte cell line THP-1 was established using a luciferase reporter gene system. In this study, we demonstrate that parthenolide, the predominant sesquiterpene lactone in European feverfew (*Tanacetum parthenium*), exerts potent inhibitory effects on the promoter activity of the *iNOS* gene in THP-1 cells. Parthenolide effectively suppressed iNOS promoter activity in a dose-dependent manner at concentrations higher than 2.5 μM, with an IC_{50} of about 10 μM. A tumor-promoting phorbol ester, 12-*O*-tetradecanoylphorbol-13-acetate (TPA), significantly increased the iNOS promoter-dependent reporter gene activity, and the TPA-induced increase in iNOS promoter activity was effectively suppressed by parthenolide, with an IC_{50} of approximately 2 μM. The present findings may further explain the anti-inflammatory property of parthenolide. BIOCHEM PHARMACOL **60**;4:595–600, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. parthenolide; nitric oxide synthase; transcriptional activity; luciferase; THP-1 cells; 12-*O*-tetradecanoylphorbol-13-acetate

Tanacetum parthenium (L) Sch. Bip., commonly known as "feverfew," is a popular herbal remedy advocated for fever, arthritis, and migraine [1]. Feverfew products have been shown to exhibit potent anti-inflammatory properties. For example, feverfew inhibits the generation of oxyradicals, prostaglandins, and proinflammatory cytokines from activated macrophages and leukocytes [2, 3]. These anti-inflammatory effects are mainly attributable to sesquiterpene lactones present in the plant [3, 4]. Parthenolide, the predominant sesquiterpene lactone in feverfew, inhibits the expression of inducible cyclooxygenase and proinflammatory cytokines (tumor necrosis factor-α and interleukin-1) in the LPS‖-stimulated murine macrophage cell line RAW264.7 [3].

Macrophages and monocytes produce oxyradicals and NO radicals under inflammatory conditions, and these are thought to cause oxidative damage of cellular components in inflammatory sites [5]. Therefore, suppression of the generation of oxyradicals and NO radicals is thought to be beneficial. Brown *et al.* reported that feverfew extract and parthenolide inhibit the phorbol ester-induced oxidative burst in polymorphonuclear leukocytes [2]. However, the effects of parthenolide on NO production in inflammatory cells have not yet been reported. In this study, a reporter gene assay was established to evaluate the promoter activity of the iNOS gene in a human monocyte cell line THP-1. We found that parthenolide is a potential inhibitor of *iNOS* gene transcription induced by TPA.

MATERIALS AND METHODS

Cells and Culture Conditions

THP-1, a human monocyte cell line obtained from the RIKEN Cell Bank, was cultured in phenol red free RPMI-1640 medium containing 5% heat-inactivated fetal bovine serum and antibiotics. TPA, a tumor-promoting phorbol ester, and parthenolide were obtained from Sigma-Aldrich, Japan K.K. TPA and parthenolide were dissolved in di-

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‖ *Abbreviations:* LPS, lipopolysaccharide; NO, nitric oxide; iNOS, inducible nitric oxide synthase; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; BSD, blasticidin S deaminase; PKC, protein kinase C; MAPK, mitogen-activated protein kinase; and NF-κB, nuclear factor-κB.

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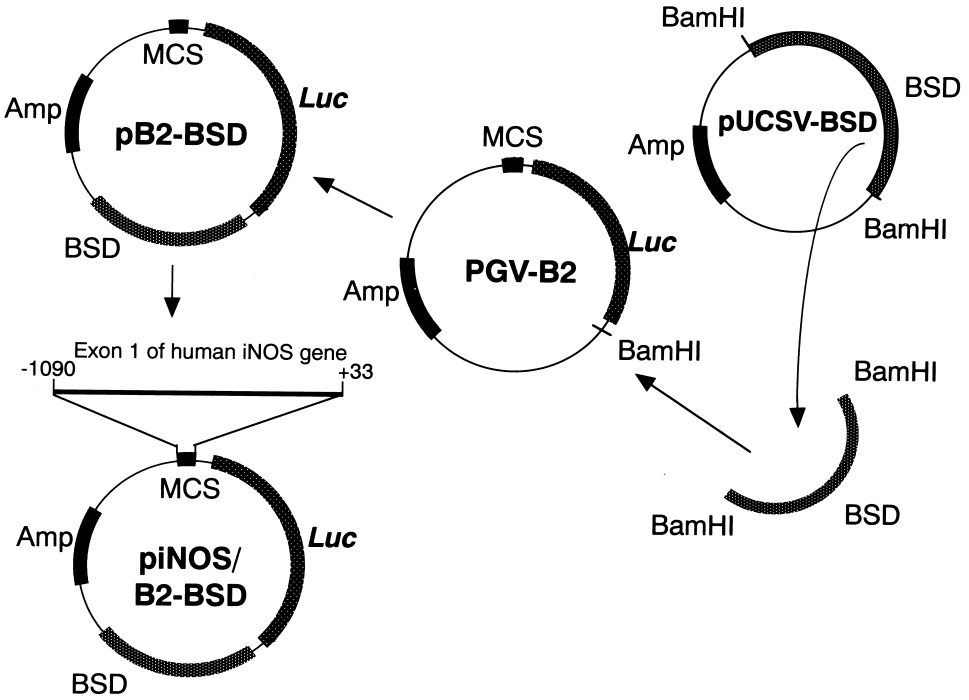

FIG. 1. Construction of plasmid DNA for luciferase reporter gene assay with stable transfection. *Luc*: firefly luciferase gene; BSD: blasticidin S deaminase; Amp: ampicillin resistance gene; MCS: multicloning sites.

methylsulfoxide at concentrations of 10 mg/mL and 100 mM, respectively. Further dilutions were made in culture medium.

Construction and Transfection of Reporter Gene Plasmids

Luciferase-blasticidin S deaminase fusion plasmid, designated as pB2-BSD, was constructed as shown in Fig. 1. In brief, the 1.7-kb *Bam*HI fragment containing the *BSD* gene, which was isolated from the pUCSV-BSD vector (Kaken Pharmaceutical), was inserted into the *Bam*HI site of the PicaGene luciferase basic vector PGV-B2 (TOYO INK MFG. Co., Ltd.). Human genomic DNA was isolated from peripheral lymphocytes obtained from a healthy volunteer. A 1123-nucleotide human iNOS gene promoter fragment stretching from −1090 to +33 (relative to the transcription start site) was amplified by polymerase chain reaction (PCR). PCR primers corresponding to sequences at the 5′ end (forward primer; 5′-CATATGTATGGGAATACTGTATTTCAGGCATTA-3′) and the 3′ end (reverse primer; 5′-GGAGCCTCAGTTTTCGACTCGCTACAAAGTTAT-3′) of the 5′-flanking region, from −1090 to +33 sites, of the human gene [6] were used. The resultant PCR product was inserted upstream of the luciferase reporter gene at the multicloning sites of the pB2-BSD basic vector. The resultant fusion plasmid was designated as piNOS/B2-BSD.

For transfection, 2×10^6 THP-1 cells were transfected either with 15 μg of pB2-BSD or piNOS/B2-BSD plasmid DNA using Tfx™-20 (Promega Corporation). Stable transfectants, in which reporter genes are integrated into the chromosomal DNA, were selected in a medium containing 20 μg/mL of blasticidine S hydrochloride (Kaken Pharmaceutical). Stable transfectant cells containing pB2-BSD in the genome DNA of THP-1 cells were designated as THP-1/B2-BSD, and cells containing piNOS/B2-BSD were designated as THP-1/iNOS-B2-BSD.

Quantification of Cell Viability and Luciferase Reporter Gene Activity

Cells were seeded at a density of 4×10^5 cells per well in 24-well plates and treated with test reagents. Changes in cell viability after each treatment were evaluated by a colorimetric method using a WST-1 assay (Dojindo Laboratories). WST-1 is a tetrazolium compound that produces water-soluble formazan dye on reduction. Spectrophotometric measurement of formazan products reflects the reducing effect of cells originating from various dehydrogenase activities. Cells were then collected by centrifugation and lysed in 200 μL detergent lysis solution. The luciferase activity of cell lysates was quantified by chemiluminescent detection using a luminometer after mixing with the PicaGene luciferase substrate solution (TOYO INK MFG. Co., Ltd.). All assays were carried out in quadruplicate and each experiment repeated at least three times.

RESULTS AND DISCUSSION

The 5′-flanking region of the *iNOS* gene contains several consensus sequences for the binding of transcriptional

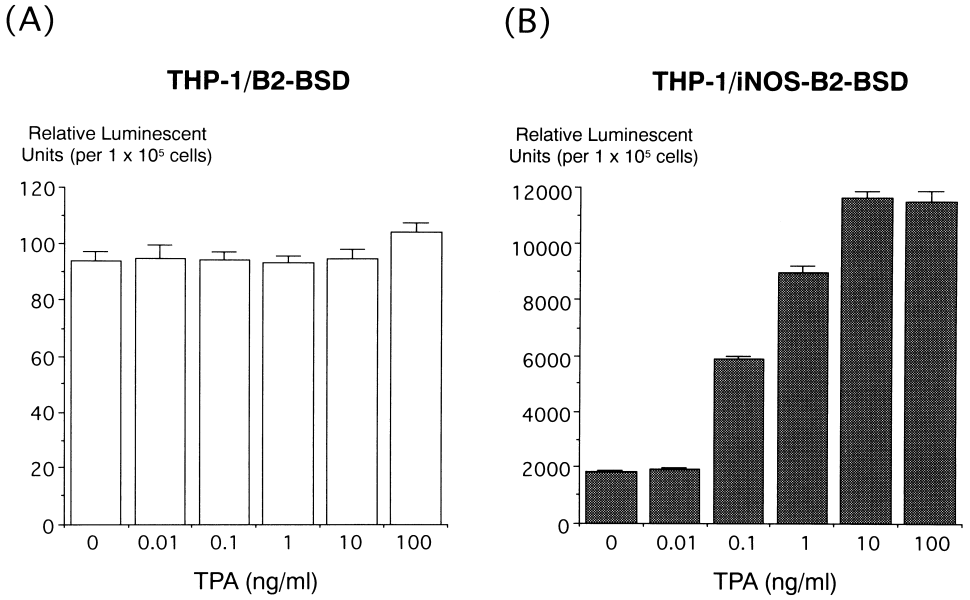

FIG. 2. Basal levels and inducibility by TPA of reporter luciferase activity in THP-1/B2-BSD (A) and THP-1/iNOS-B2-BSD (B) cells. Cells were seeded in 24-well multiwell plates at a density of 4 × 10^5/mL and cultured either in the absence or presence of TPA at the concentrations indicated. After 6-hr treatment, iNOS promoter-dependent reporter gene activity was assessed by measuring the luciferase activity of cell lysates obtained from each well. The values represent the means of quadruplicate wells and are expressed as relative chemiluminescent units per 1 × 10^5 cells. Bars indicate the standard errors. The data are representative of an experiment repeated three times with similar results.

factors involved in the inducibility of the gene by cytokines and inflammatory stimuli [6]. The luciferase activity in THP-1/iNOS-B2-BSD cells reflects the activity of the transcriptional factors that bind to the regulatory sites of the *iNOS* gene. THP-1/B2-BSD cells, which contain the basic reporter plasmid DNA, expressed little luciferase activity (Fig. 2A). On the other hand, THP-1/iNOS-B2-BSD cells showed a greater than 40-fold increase in luciferase activity compared to THP-1/B2-BSD cells (Fig. 2B).

Phorbol ester TPA is an intermediate of phosphatidyl inositol metabolism and an activator of PKC [7]. PKC is physiologically activated by receptor-mediated hydrolysis of inositol phospholipids induced by various external signals such as hormones, neurotransmitters, and some growth factors, and plays an important role in the control of cell function and proliferation [8]. A key role in stimulation by TPA is also played by various MAPKs [9]. Through PKC and MAPKs, TPA regulates the activity of many transcriptional factors, such as NF-κB, to alter the expression of cellular genes including *iNOS*. The effects of TPA on reporter gene activity were investigated in THP-1/B2-BSD and THP-1/iNOS-B2-BSD cells. As shown in Fig. 2, TPA increased the luciferase activity in THP-1/iNOS-B2-BSD cells in a dose- and time-dependent fashion, but the induction by TPA was not observed in THP-1/B2-BSD cells. A significant increase in luciferase activity in THP-1/iNOS-B2-BSD cells was observed at concentrations of TPA as low as 0.1 ng/mL, and the induction reached a plateau at 20 to 50 ng/mL. An appreciable increase in luciferase activity was first detected at 2 hr and activity increased with time, reaching a maximum at 12 hr after addition of TPA (data not shown).

The present experimental design is a whole cell system relevant to the inflammatory processes. The luciferase reporter gene assay system using the iNOS promoter region thus appears to be a sensitive method for evaluating anti-inflammatory activities. Using this assay system, effects of parthenolide were investigated. Parthenolide suppressed both the steady-state levels and the TPA-induced increase in iNOS promoter-dependent reporter gene activity, the suppression being more evident in the latter case (Fig. 3). When THP-1/iNOS-B2-BSD cells were cultured in the presence of parthenolide for 6 hr, a dose-dependent decrease in iNOS promoter-dependent luciferase reporter gene activity was observed at concentrations higher than 2.5 μM, with an IC_{50} value (the concentration that reduced luciferase activity by 50%) of 10 μM (Fig. 3, open column). At a concentration of 20 μM, reporter gene activity in THP-1/iNOS-B2-BSD cells was decreased to less than 20% of the non-treated cells after 6-hr incubation. When THP-1/iNOS-B2-BSD cells were cultured in the presence of 10 ng/mL of TPA, luciferase activity increased more than 5-fold compared to the non-treated cells after 6 hr. Parthenolide exhibited a more potent inhibitory effect on TPA-induced iNOS promoter activity, the dose–response curve showing an IC_{50} of about 2 μM (Fig. 3, filled column). Luciferase activity was markedly suppressed to non-treated levels on exposure to 5 μM parthenolide for 6 hr. No obvious decrease in cell viability, as assessed by WST-1 assay, was observed at concentrations up to 10 μM when the analysis was carried out after 6-hr incubation (data not

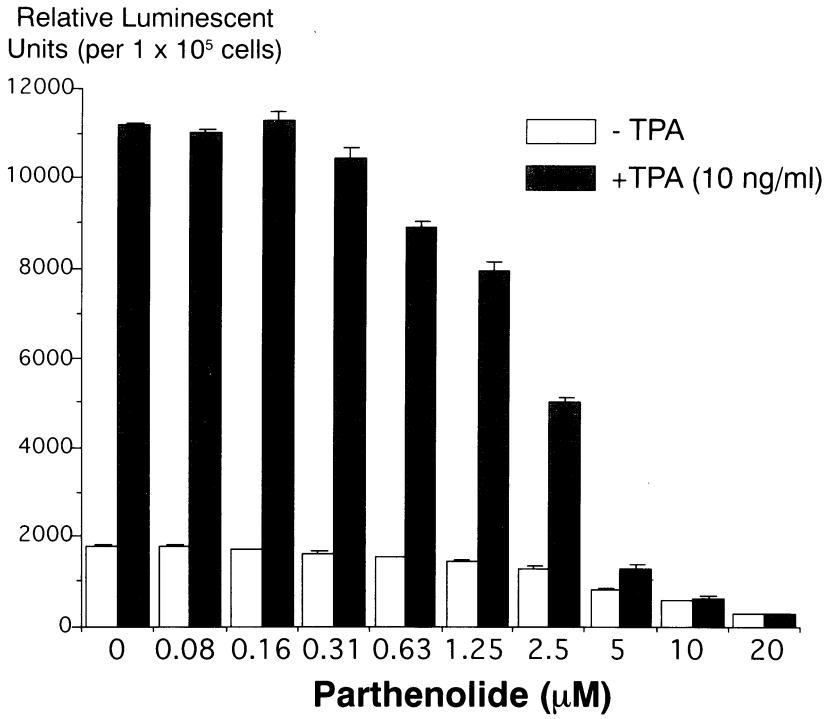

FIG. 3. Decrease in reporter gene activity by parthenolide treatment in non-stimulated and TPA-stimulated THP-1/iNOS-B2-BSD cells. THP-1/iNOS-B2-BSD cells were seeded in 24-well multiwell plates at a density of 4×10^5/mL and cultured in the absence (open column) or presence (filled column) of 10 ng/mL of TPA in medium containing parthenolide at the concentrations indicated. After 6-hr treatment, iNOS promoter-dependent reporter gene activity was assessed by measuring the luciferase activity of cell lysates obtained from each well. The values represent the means of quadruplicate wells and are expressed as relative chemiluminescent units per 1×10^5 cells. Bars indicate the standard errors. The data are representative of an experiment repeated three times with similar results.

shown). However, a decrease in cell viability was observed when cultured cells were exposed to parthenolide at concentrations of 20 μM or higher for more than 12 hr. Decreased cell viability was evident after 12-hr exposure to 20 μM parthenolide, and a 50% decrease in cell viability was observed after 24-hr exposure (data not shown).

The mechanism by which parthenolide inhibits iNOS promoter activity still needs to be elucidated. Parthenolide contains α-methylene-gamma-lactone (MGL) and an epoxide in its structure [10]. These moieties can interact with biological nucleophiles such as the sulfhydryl group of target proteins [3]. The present study also showed that sulfhydryl groups such as L-cysteine, dithiothreitol, and 2-mercaptoethanol effectively abrogated the inhibitory effects of parthenolide on iNOS promoter-dependent reporter gene activity (Fig. 4). In the present experimental system, the tyrosine protein kinase inhibitor genistein did not significantly inhibit iNOS promoter-dependent reporter gene activity, but a PKC inhibitor, 3,4,5-trimethoxybenzoic acid 8-(diethylamino)octyl ester (TMB-8), effectively inhibited reporter activity.* It may thus be possible that parthenolide inhibits TPA-induced iNOS promoter activity via targeting the protein(s) in PKC signaling pathways.

Parthenolide was recently found to be a potent inhibitor of transcription factor NF-κB activation [11, 12]. NF-κB-dependent transcriptional regulation plays a key role in the TPA-induced PKC signaling pathways [12]. The NF-κB binding consensus sequence is present in the 5′-flanking promoter region of the *iNOS* gene [6]. NF-κB, which resides in an inactive cytoplasmic complex in unstimulated cells, is activated by phosphorylation and degradation of its inhibitory subunit, IκB [12]. Hehner *et al.* recently reported that parthenolide inhibits NF-κB activation by targeting the IκB kinase complex and blocking the degradation of IκB [12, 13]. Thiol-depleting agents are also known to inhibit the activation of NF-κB by interfering with the degradation of IκB [14]. It is thus possible that the inhibitory effect of parthenolide may be due to a general effect of thiol depletion and not specific for the compound tested. It would be interesting to investigate the effects of other thiol-depleting agents on iNOS promoter activity using the present reporter gene assay to find more effective inhibitors.

Production of NO at inflammatory sites is involved in tissue damage and contributes to worsening of inflammatory disease states [15]. NO and superoxide anions, both formed in inflamed tissue, rapidly react to produce the peroxynitrite anion ($ONOO^-$), which possesses stronger oxidant activity than its precursors and causes DNA and tissue damage [16]. It is therefore suggested that inhibition of excessive NO production from inflammatory cells could be beneficial in the treatment of inflammatory diseases. Cytostatic and cytocidal effects of parthenolide against

* Fukuda F, unpublished observation.

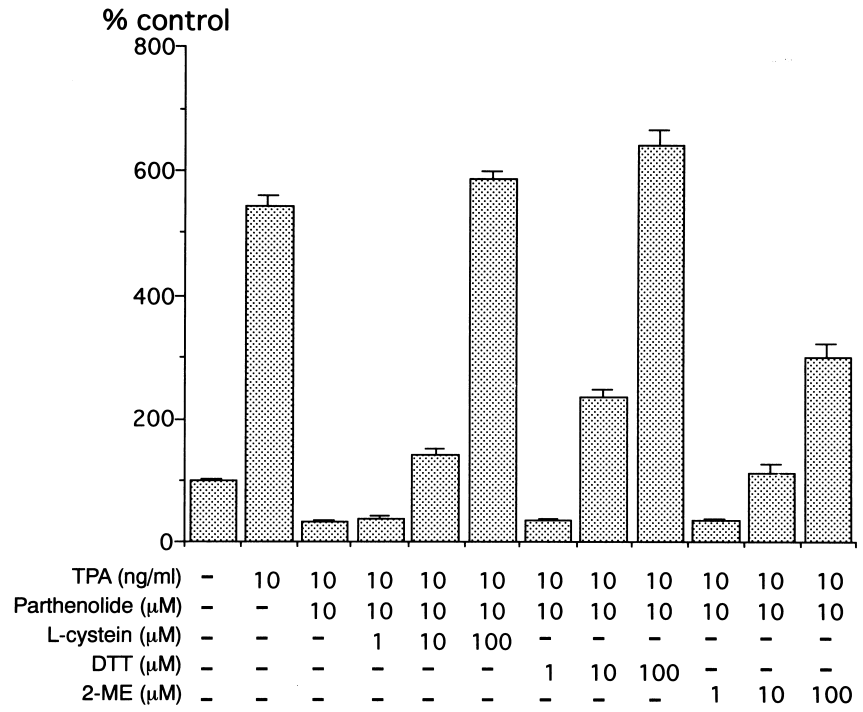

FIG. 4. Effects of sulfhydryl groups on the inhibitory effects of parthenolide against iNOS promoter-dependent reporter gene activity. THP-1/iNOS-B2-BSD cells were treated with TPA (10 ng/mL) and parthenolide (10 μM) in the presence of dithiothreitol (DTT), 2-mercaptoethanol (2-ME), or L-cysteine (Cys) at the concentrations indicated. Cells were incubated for 6 hr and the luciferase activity analyzed. The data are presented as a percent (%) of the control value obtained from non-treated THP-1/iNOS-B2-BSD cells. Data shown are means ± SE of quadruplicate wells. The data are representative of an experiment repeated three times with similar results.

tumor cells have been reported [17]. Ross *et al.* reported that parthenolide inhibited cell growth irreversibly at concentrations above 5.0 μM and after 24-hr exposure. However, this effect was reversible at lower concentrations [18]. Thiol depletion and/or inhibition of NF-κB may be responsible for the reduction in cell viability observed after long incubation with parthenolide. In the present study, parthenolide's inhibitory effects on iNOS-dependent promoter activity were demonstrated at doses that did not significantly decrease cell viability. Cell viability was moderately reduced only after 12 hr of continuous exposure to 20 μM parthenolide, which was a substantially longer exposure compared to that needed for inhibition of iNOS promoter activation. This suggests that parthenolide's inhibitory properties are not simply due to cytotoxicity. Wong and Menendez recently reported that parthenolide inhibits *iNOS* gene expression in cultured rat aortic smooth muscle cells treated with LPS and interferon-γ [11]. Thus, the inhibitory activity of parthenolide is applied to other iNOS inducers. Hehner *et al.* reported that parthenolide does not influence the activity of other transcription factors such as activating protein 1 [12], indicating that parthenolide does not interfere in a non-specific manner with transcription factors or signaling molecules. Taken together, the present findings extend our understanding of the molecular mechanisms underlying the anti-inflammatory activity of parthenolide and feverfew.

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