

Hypoestoxide, a Novel Anti-inflammatory Natural Diterpene, Inhibits the Activity of I κ B Kinase

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Received February 6, 2001; accepted April 17, 2001

Most inflammatory agents activate nuclear factor- κ B (NF- κ B), resulting in induction of genes coding for cytokines, chemokines, and enzymes involved in amplification and perpetuation of inflammation. Hypoestoxide (a bicyclo [9,3,1] pentadecane) is a diterpene from *Hypoestes rosea*, a tropical shrub in the family Acanthaceae, several members of which are used in folk medicine in Nigeria. Here, we demonstrate that hypoestoxide (HE) abrogates the production of pro-inflammatory cytokines (IL-1 β , IL-6, and TNF- α) in lipopolysaccharide (LPS)-activated normal human peripheral blood mononuclear cells. Moreover, HE inhibits the production of nitric oxide (NO) by IL-1 β - or IL-17-stimulated normal human chondrocytes. *In vivo*, oral administration of HE to mice significantly ameliorated hind paw edema induced by antibodies to type II collagen plus LPS. Furthermore, topical administration of HE to mice also significantly inhibited phorbol ester-induced ear inflammation. The anti-inflammatory activity of HE may be due in part to its ability to inhibit NF- κ B activation through direct inhibition of I κ B kinase (IKK) activity. Thus, HE could be useful in treating various inflammatory diseases and may represent a prototype of a novel class of IKK inhibitors.

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Key Words: hypoestoxide; inflammation; nuclear factor- κ B; I κ B kinase; arthritis.

INTRODUCTION

Plants and their products have been used for medicinal purposes for more than 3500 years (1). Drugs of natural origin have often been discovered on the basis

of ethnobotanical information provided by “native doctors” practicing folk medicine and living in regions of the world rich in bioresources. Hypoestoxide (HE) is a natural diterpene isolated from the shrub *Hypoestes rosea* (Acanthaceae), a plant indigenous to the rain forest regions of Nigeria (2–4). The natives have long used the *H. rosea* leaf extracts in folk medicine to treat skin rashes and fungal infections (5). The use of plants from this same family has been reported in local herbal medicine (5). HE was identified from a group of compounds isolated from *H. rosea* as possessing potential anti-inflammatory activities, namely, inhibition of the synthesis of pro-inflammatory cytokines and mixed leukocyte reaction (MLR). Because interference with the biosynthesis or action of pro-inflammatory cytokines, such as tumor necrosis factor alpha (TNF- α), interleukin 1 beta (IL-1 β), and interleukin 6 (IL-6), has become an important strategy for pharmacological intervention in a variety of inflammatory and fibrotic diseases (6, 7), we examined the anti-inflammatory effects of HE in more detail.

Inflammation is a complex series of vascular, leukocyte, and plasma-interactive events that occur in response to injury. Nitric oxide (NO) and nuclear factor κ B (NF- κ B) are the subjects of intense investigation because they are involved in the inducible expression of a variety of cellular genes that regulate inflammatory response (8). Constitutive and inducible isoforms of nitric oxide synthase (NOS) exist in endothelial cells and activated macrophages and other inflammatory/immunocompetent cells (8). NF- κ B is a group of transcription factors that belong to the Rel family, which share a DNA binding and dimerization domain that interacts with a class of specific inhibitory proteins termed I κ Bs (9). The major form of regulation for NF- κ B is inducible degradation of the I κ Bs, which otherwise retain NF- κ B in the cytoplasm (9). Both TNF- α and IL-1 β as well as byproducts of bacterial and viral infections, for instance LPS, activate signaling path-

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ways that stimulate transcription factors AP-1 and NF- κ B and thereby induce expression of genes coding for cytokines, chemokines, adhesion molecules, and enzymes that catalyze the synthesis of secondary inflammatory mediators (10, 11). Because HE inhibits synthesis of pro-inflammatory cytokines, it was of interest to study how it may affect the NF- κ B signaling pathway and NO production. We demonstrate that HE significantly inhibits IL-1 β - or IL-17-induced NO production by normal human chondrocytes and also inhibits TNF-induced NF- κ B activation in HeLa cells through direct inhibition of IKK activity. Furthermore, type II collagen-induced arthritis (CIA) and phorbol ester-induced topical inflammation are significantly ameliorated by oral and topical administration, respectively, of HE in mice. These results suggest a mechanism whereby this novel class of natural agents acts and demonstrate the potential usefulness of HE as an anti-inflammatory agent.

METHODS

Hypoestoxide Extraction from H. rosea Plant Material

HE was prepared by the general procedure of Okogun *et al.* (2). Briefly, dried plant material was subjected to solid/liquid extraction with boiling hexanes in a large soxhlet apparatus. The crude extract was subjected to flash silica gel column chromatography using a step gradient solvent system beginning with petroleum ether (30–60 bp) and stepping to 5, 10, and then 20% ethyl acetate. At 30% ethyl acetate, HE was eluted from the column. Appropriate fractions were combined and concentrated to dryness and petroleum ether or hexanes added to obtain crystalline HE. See Fig. 1 for the structure of HE.

Inhibition of macromolecular synthesis. CEM cells were incubated with 3 and 10 times the IC₅₀ of either 4-hydroperoxycyclophosphamide (4-HPOCY) or HE. After 30 min of incubation at 37 C, the cells were incubated with [³H]thymidine, [³H]uridine, or [³H]leucine. One, 2, or 4 h after addition of radiolabel, a sample of each incubation was collected and the radioactivity in the acid-insoluble material was determined. For [³H]uridine, the radioactivity in the alkali-labile/acid-insoluble fraction (RNA) was also determined.

Pro-inflammatory Cytokine Induction in Vitro

PBMC were isolated from normal human peripheral blood by Ficoll-Hypaque and cultured with 3 μ g/ml of the B-cell mitogen *Escherichia coli* LPS (GIBCO) at 2×10^6 /ml in 1.0-ml volumes in 24-well plates. Cultured cells were incubated in the presence of varying concentrations (100, 10, 1, 0.1 μ M) of HE or medium only for 24 or 48 h. Culture supernatants were col-

lected at the end of incubation period and assayed by ELISA for the cytokines IL-1 β , IL-6, and TNF- α . The amounts of the respective cytokines present in the supernates are expressed in pg/ml.

Effect of Hypoestoxide on PBMC Cultures

PBMC were cultured for varying periods of time (24–72 h) in the presence of varying concentrations of hypoestoxide (100–1.25 μ M) or 4-HPOCY (100 μ M). Cytotoxicity was determined by a colorimetric MTT assay. Briefly, 1×10^3 cells per well (100 μ l) in a 96-well flat-bottom microtiter plate were cultured either with the culture medium alone or with various concentrations of drug at 37°C in a 5% CO₂ 95% air humidified incubator for 24–72 h. At the end of the culture, 10 μ l of 5 mg/ml sterile solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) in PBS was added per well and the incubation was resumed for an additional 4 h. Acid-isopropanol (100 μ l of 0.04 N HCL in isopropanol) was added to all wells and kept at room temperature for 30 min. Mixing with a multichannel pipetter dissolved the dark blue crystals and the absorbance was measured at 545–650 nm using an ELISA plate reader. Cytotoxicity was also determined by the trypan blue dye exclusion method.

NO Production

Production of NO was stimulated in normal human articular chondrocytes with either IL-1 β or IL-17 in the absence or the presence of HE or dexamethasone (DEX). Following 48 h of culture, media were collected for measurement of NO levels by the Griess reaction (12).

NF- κ B Activity

Cell culture, transfection, and treatments. Cells were cultured in Dulbecco's modified minimum essential medium (DMEM) (HeLa or HEK 293 cells), supplemented with 10% fetal calf serum and antibiotics. Cells were stimulated with either 20 ng/ml recombinant human TNF- α or 10 ng/ml IL-1 β (Sigma). Transfections were performed as described, using Lipofectamine Plus (Gibco) for HeLa cells. Xpress-tagged NIK, HA-tagged IKK α , and HA-tagged IKK β and IKK β (EE) were described (13, 14). Cells were lysed in buffer B (50 mM Tris-HCl, pH 7.5, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton, 0.5% NP-40, 10% glycerol, supplemented with 20 mM β -glycerophosphate, 19 mM PNPP, 500 mM Na₃VO₄, 1 mM PMSF, 20 μ g/ml aprotinin, 2.5 μ g/ml leupeptin, 8.3 μ g/ml bestatin, and 1.7 μ g/ml pepstatin and 2 mM DTT) and extracts were used for the assays described below.

Electrophoretic mobility-shift assay (EMSA). Aliquots of total extracts (15 μ g of protein) in buffer B were incubated with ³²P-labeled κ B DNA, followed by

analysis of DNA binding activities by EMSA on 4% polyacrylamide gels (15). The specificity of protein–DNA complexes was verified by supershift with polyclonal antibodies specific for p65 (Rel A).

IKK Assays

Kinase assay, immunoprecipitation, and immunoblotting. Lysates were prepared as described and incubated with anti-IKK α (PharMingen), anti-HA (12CA5), anti-JNK1 (333.8) (PharMingen), and anti-p38 (New England Biolabs) for 2 h and 15 μ l of protein A–Sepharose (Sigma) was added for 1 h. After extensive washing, kinase assays were performed as described (11). Endogenous IKK, HA-tagged IKK α , HA-tagged IKK β , HA-tagged IKK β (EE), endogenous JNK1, and p38 activities were determined with GST-I κ B α (1–54), GST-c-Jun(1–79), and GST-ATF2 as substrates.

Phorbol Ester-Induced Inflammation in Mice

Topical inflammation was induced in mice according to the method of Chang *et al.* (16). Briefly, phorbol 12-myristate 13-acetate (PMA, 5 μ g in 20 μ l of absolute alcohol) was applied topically to the anterior and posterior surfaces of the right ear of groups of five ICR derived female mice weighing about 22 g. Equipotent (a dose of HE required to induce an equivalent level of inhibition induced by a specific dose of a known anti-inflammatory drug, DEX) doses of HE (3 mg/ear), DEX (1 mg/ear), and vehicle (ethanol/acetone, 20 μ l/ear) were applied topically on the anterior and posterior surfaces of the right ear of test animals 30 min before and 15 min after PMA was applied. Ear swelling was measured by a Dyer model micrometer gauge, 6 h after PMA application as an index of inflammation. The percentage of inhibition was calculated according to the formula $I_c - I_t/I_c \times 100$, where I_c and I_t refer to the increase of ear thickness (mm) in vehicle control and treated mice, respectively. Inhibition of 30% or more ($\geq 30\%$) relative to the vehicle-treated group was considered significant.

Type II CIA in Mice

Arthritic inflammation was induced in mice essentially as previously described by Terato *et al.* (17). Briefly, groups of five Balb/cByJ strain mice, 6–8 weeks of age, were used for the induction of inflammation (arthritis) by monoclonal antibodies (mAbs) responding to type II collagen, plus LPS. A combination of four different mAbs (D8, F10, DI-2G, and A2) was administered intravenously at a total of 4 mg/mouse at day 0. This was followed by the intravenous administration of 25 μ g of LPS 72 h later (day 3). From day 3, 1 h after LPS administration, equipotent doses of 30 mg/kg HE, 3 mg/kg indomethacin, and/or vehicle (2%

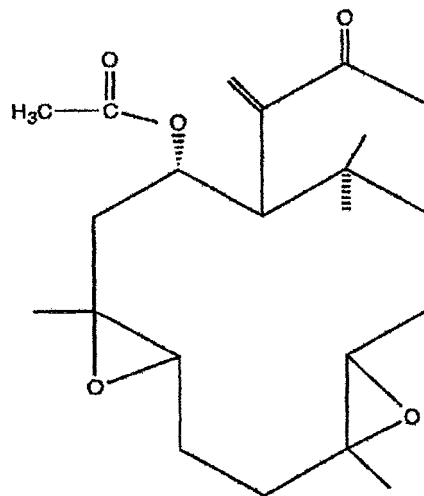


FIG. 1. Bicyclic structure of HE which features two epoxide moieties, an $\alpha\beta$ unsaturated ketone and an acetyl ester function.

Tween 80/distilled water) were administered orally once daily for 3 consecutive days. The maximum tolerated dose for HE in mice was determined in prior experiments to be greater than 750 mg/kg (MTD > 750 mg/kg) ip or po. At day 5, one or two paws (particularly the hind paws) began to appear red and swollen, and by day 7, arthritis symptoms of the hind paws were present; they were severely red and swollen. A plethysmometer (Ugo Basile, Cat. No. 7150) with a water cell (12 mm in diameter) was used for the measurement of volume in microliters (μ l) of the two hind paws on day 0, 5, and 7.

The percentage of inhibition of increase in volume relative to vehicle control was calculated by the formula Inhibition (%): $[1 - (T_n - T_0)/(C_n - C_0)] \times 100$, where C_0 (C_n) is the volume on day 0 (day n) in vehicle control group and T_0 (T_n) is the volume on day 0 (day n) in the test compound-treated group. Inhibition of edema in both hind paws by more than 30% was considered significant anti-inflammatory (anti-arthritis) activity.

RESULTS

Structural Features and Activity

HE contains two functional groups normally associated with alkylation properties, i.e., $\alpha\beta$ unsaturated ketones and epoxides (Fig. 1) (2, 3). Preliminary results indicate that HE does not exhibit activities expected of a typical alkylating agent. Thus, we compared HE to 4-HPOCY for the effects *in vitro* on macromolecular synthesis, i.e., DNA, RNA, and protein synthesis, using tritiated thymidine, uridine, and leucine, respectively. While 4-hydroperoxycyclophosphamide was a potent inhibitor of all three parameters. HE had little or no effect on any of them, even at concentrations up to 20 μ M (data not shown).

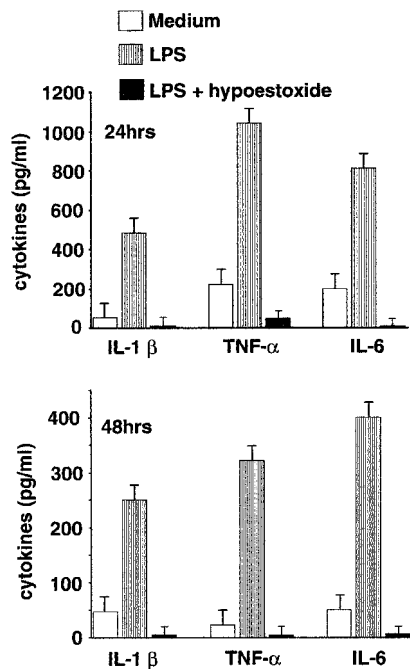


FIG. 2. Inhibitory effect of HE on pro-inflammatory cytokine synthesis *in vitro*. PBMC were cultured for 24 and 48 h, respectively, either in medium only or in the presence of LPS only or HE (100, 10, 1, 0.1 μ M) and LPS (3 μ g/ml). The results of 10 μ M cultures are shown. Total inhibition was achieved with 10 and 100 μ M, respectively, while 1 μ M had a moderate effect, and 0.1 μ M had no effect. Culture supernatants were collected and assayed by EIA for the presence of the designated cytokines.

Anti-inflammatory Activity of Hypoestoxide in Cultured Cells

Three separate experiments demonstrate the inhibitory effect of HE on pro-inflammatory cytokine synthesis in PBMC stimulated with LPS (Fig. 2). Significant inhibition of the synthesis of IL-1 β , TNF- α , and IL-6 was achieved at several doses of HE (100, 10, 1, 0.1 μ M, respectively). The results obtained at 10 μ M of HE are shown. Total inhibition was achieved only at concentrations of 10 and 100 μ M, respectively. HE, unlike 4-HPOCY, was not toxic to normal human PBMC in a 72-h culture at any of the tested concentrations (100–1.6 μ M) as determined by colorimetric MTT assay (Table 1). In contrast, 4-HPOCY reduced viability of PBMC by about 40% (Table 1). These results support earlier reports, which demonstrated that CY could reduce the viability of normal human lymphocytes in culture (18). Cytotoxic effects of HE on normal PBMC was further examined by DNA staining and apoptosis assays. Results indicated that HE did not induce apoptosis in normal PBMC even at 100 μ M (data not shown). A trypan blue dye exclusion assay also ruled out toxicity on normal PBMC cultured for 72 h at varying concentrations of HE (Table 1). Thus, the potent inhibitory effect of HE on pro-inflammatory

cytokine synthesis by LPS-activated PBMC could not be due to cytotoxic effects. Besides the inhibitory effects of HE on LPS-stimulated cultures, similar, but less dramatic, inhibitory effects have also been observed with other stimuli of cytokine production, i.e., PWM, PHA, CON-A, and MLR, but only at high HE concentrations (≥ 100 μ M) (data not shown).

HE inhibited NO production by normal human articular chondrocytes stimulated with IL-1 β (Fig. 3a). Because IL-17 was shown to be a potential player in the cytokine networks involved in arthritis (19), it was of interest to ascertain whether HE would inhibit IL-17-induced NO production. HE also inhibited IL-17-stimulated NO production in a dose-dependent fashion (Fig. 3b). The fivefold difference observed between the inhibitory effect of HE on cytokine and NO production may be due to differences in the ability of different cell types to respond to HE. This may also explain why higher concentrations of HE are required to inhibit T-cell mitogenic/antigenic responses as noted above.

Inhibition of Type II Collagen-Induced Arthritis in Mice

To test *in vivo* efficacy, HE was administered orally to type II collagen-induced arthritic mice. No treatment-related deaths or untoward effects occurred during this study. HE ameliorated collagen-induced hind paw edema by 58 and 46% on days 5 and 7, respectively (Fig. 4). By comparison, the classical nonsteroidal anti-inflammatory drug indomethacin showed 60 and 52% inhibition on corresponding days after induction of arthritis. Although serum levels of HE in the HE-treated mice were not measured, results of earlier pilot pharmacokinetics studies indicate that after oral dosing,

TABLE 1
Lack of Toxicity of Hypoestoxide against Cultured Human Peripheral Blood Mononuclear Cells

HE concentration (μ M)	Trypan blue dye ^a (% viability \pm SE)	MTT ^b (OD 545–650 nm) \pm SE
Medium control	98.0 \pm 2.0	0.14 \pm 0.02
1.25	98.1 \pm 1.5	0.14 \pm 0.01
2.5	96.4 \pm 3.2	0.13 \pm 0.02
5.0	97.0 \pm 2.6	0.14 \pm 0.01
10.0	96.8 \pm 1.3	0.14 \pm 0.01
20.0	96.3 \pm 1.2	0.13 \pm 0.02
100.0	94.2 \pm 4.8	0.11 \pm 0.01
Positive control (4-HPOCY)	60.0 \pm 2.6	0.08 \pm 0.01

^a Cytotoxicity of HE was determined against normal human PBMC in a 72-h culture by the trypan blue dye exclusion method. The number of viable cells per total number of cells counted is expressed as the percentage of total cells \pm standard error (SE).

^b Cytotoxicity of HE against normal human PBMC was also determined in a 72-h culture by a colorimetric MTT assay. Results are expressed in optical density (OD) units \pm standard error (SE).

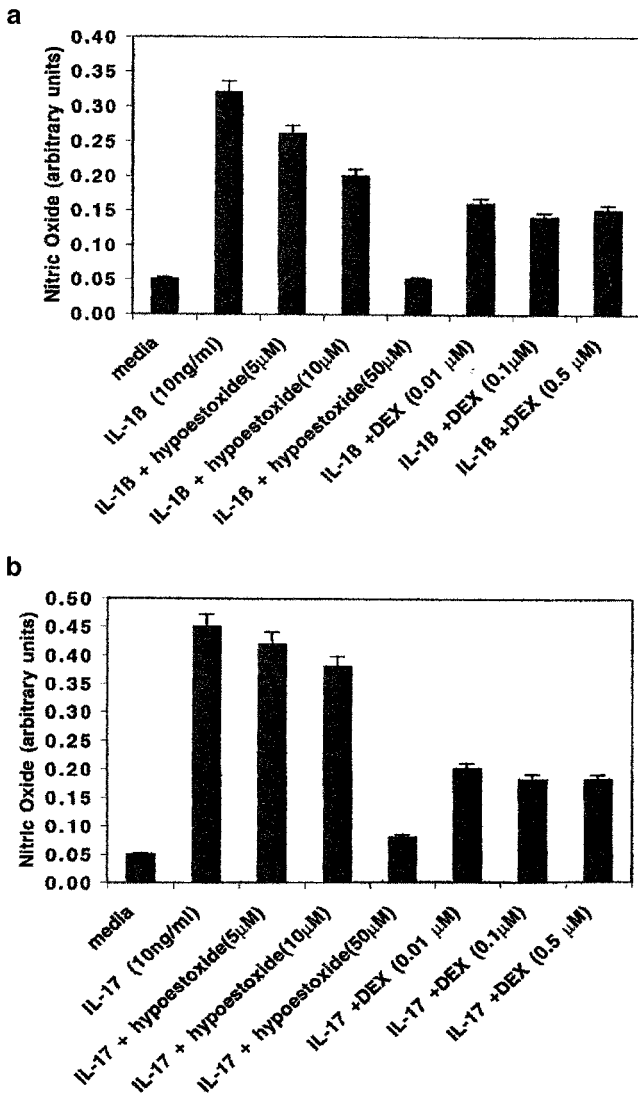


FIG. 3. (a) HE inhibits IL-1 β -induced NO production. IL-1 β stimulated normal human articular chondrocytes treated with either HE (5, 10, and 50 μ M) or DEX (.01, 0.1, and 0.5 μ M) for 48 h. Following incubation, media were collected for measurement of NO levels by the Griess reaction. (b) HE inhibits IL-1 β -induced NO production. IL-17-stimulated normal human articular chondrocytes treated with either HE (5, 10, and 50 μ M) or DEX (.01, 0.1, and 0.5 μ M) for 48 h. Following incubation, media were collected for measurement of NO levels by the Griess reaction.

the bioavailability of HE at 24 h was only 6.4% or greater in plasma (data not shown). However, HE had a large volume of distribution and a high clearance, which suggested that it might have an extensive tissue distribution and metabolism, which accounts for its oral bioactivity.

Inhibition of Phorbol Ester-Induced Topical Inflammation by Hypoestoxide in Mice

To test the topical activity of HE, the compound was evaluated for its ability to inhibit phorbol ester-in-

duced inflammation in mice. As shown in Table 2, HE exhibited significant inhibition (57%) of ear swelling relative to vehicle control. Concurrently tested dexamethasone, serving as a positive control, showed a 63% inhibition of ear swelling.

Inhibition of NF- κ B and IKK Activation by Hypoestoxide

To determine whether NF- κ B activation was prevented by HE in an established system, HeLa cells were pretreated with HE and stimulated with TNF- α (Fig. 5). HE inhibited NF- κ B activation at concentrations similar to those that inhibit cytokine production. In addition, HE did not inhibit the DNA binding activity of the transcription factors Oct-1 and SP-1 (Fig. 5). The half-maximal inhibitory (IC_{50}) concentration of NF- κ B DNA binding activity for HE was 11 μ M (Fig. 5).

Upon TNF or IL-1 β stimulation, I κ B α is phosphorylated by the IKK complex, which contains two catalytic subunits (IKK α and IKK β) and the IKK γ or NEMO regulatory subunit (20, 21), at sites that trigger ubiquitination and degradation via the proteasome (11, 13). To determine whether IKK could be the target for HE in an established system, HeLa cells were pretreated with HE and stimulated with either TNF- α or IL-1 β (Fig. 6a). IKK activity was rapidly stimulated by both TNF- α and IL-1 β , reaching a maximum after 10–15 min. HE inhibited IKK activity with an IC_{50} of 24 μ M (Fig. 6b). Similarly, HE inhibited IKK activity and prevented I κ B α degradation in HEK 293 cells treated with TNF- α (data not shown), suggesting that the inhibitory effect of HE is independent of cell type and

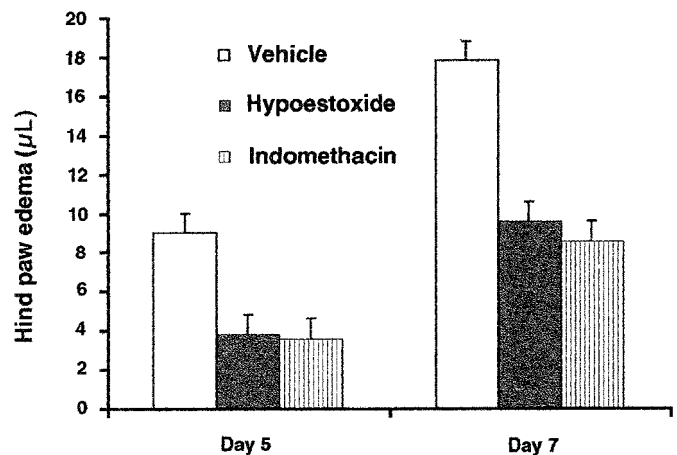


FIG. 4. HE ameliorates CIA in mice. Group of five Balb/c mice were used for the induction of CIA. Following induction of CIA, mice were treated orally with vehicle (2% Tween 80/distilled water), 30 mg/kg HE, or 3 mg/kg indomethacin. The volume of both hind paws was measured by plethysmometer on days 5 and 7. The increase in volume at each time point was determined by comparison with the respective day 0 paw volume. Reduction of edema obtained for each time point by more than 30% was considered significant.

TABLE 2
Hypoestoxide Inhibits Phorbol Ester-Induced Topical Inflammation in Mice

Compound	Route	Dose	Mouse No.	Thickness ($\times 0.01$ mm)			% Inhibition
				Right ear	Left ear	Net	
Vehicle (ethanol/acetone)	TOP	20 μ l/ear $\times 2$	1	41	20	21	
	TOP	20 μ l/ear $\times 2$	2	42	21	21	
	TOP	20 μ l/ear $\times 2$	3	43	22	21	
	TOP	20 μ l/ear $\times 2$	4	44	23	21	
	TOP	20 μ l/ear $\times 2$	5	45	24	21	
				Mean	43	22	21
			SEM	1.6	1.6	0	
Hypoestoxide	TOP	3 mg/ear $\times 2$	1	25	17	8	
	TOP	3 mg/ear $\times 2$	2	28	18	10	
	TOP	3 mg/ear $\times 2$	3	27	19	8	
	TOP	3 mg/ear $\times 2$	4	28	18	10	
	TOP	3 mg/ear $\times 2$	5	27	18	9	
				Mean	27.0	18.0	9.0
			SEM	1.2	0.7	1.0	
Dexamethasone-21-acetate	TOP	1 mg/ear $\times 2$	1	25	19	6	
	TOP	1 mg/ear $\times 2$	2	24	18	6	
	TOP	1 mg/ear $\times 2$	3	33	20	13	
	TOP	1 mg/ear $\times 2$	4	28	19	9	
	TOP	1 mg/ear $\times 2$	5	25	20	5	
				Mean	27.0	19.2	7.8
			SEM	3.7	0.8	3.3	

Note. Hypoestoxide and vehicle (ethanol/acetone) were applied topically 30 min before and 15 min after phorbol ester (PMA) application for inducing inflammation and then ear swelling was measured 6 h after PMA application.

stimulus. Though IKK activity was inhibited, HE did not inhibit Jun N-terminal kinase (JNK) and p38 MAP kinase, suggesting that HE targets a selected subset of kinase pathways that are involved in inflammation. To show that the inhibition of IKK was direct, we transfected HeLa cells with a constitutively active form of IKK β , IKK β (EE), where the two serine residues in the activation loop have been replaced by glutamic acid and then treated the cells with HE for 60 min. HE inhibited the constitutively active form of IKK β (Fig. 6c). HE did not affect IKK β expression as determined by Western blot analysis, whereas it strongly inhibited IKK β activity. These results suggest that hypoestoxide may be a direct inhibitor of IKK rather than targeting upstream components of the TNF or IL-1 β signaling pathways. To determine whether HE inhibits NIK-induced IKK activation (22), HeLa cells were transiently cotransfected with Xpress-tagged NIK and IKK- α or IKK- β (Fig. 6d) for 24 h and treated with HE for 60 min. Under the conditions used, HA-IKK α and HA-IKK β are incorporated into functional cytokine-responsive 900-kDa complexes (20). NIK-induced IKK activity was inhibited by HE under all conditions (Fig. 6d). We next investigated whether recombinant IKK β expressed in sf9 cells using a baculovirus vector was also sensitive to HE. We found that a dose of 100 μ M HE was sufficient to completely inhibit IKK β (Fig. 6e). Furthermore, we tested whether HE was able to inhibit IKK activity *in vitro*. HeLa cells were stimulated

with TNF- α for 10 min. Endogenous IKK was immunoprecipitated with anti-IKK α antibody and kinase activity was determined in the presence of different concentrations of HE. IKK activity was inhibited in a dose-dependent fashion following HE treatment for 30 min (Fig. 6f). No inhibition of endogenous activities of JNK or p38 activity was observed in the same experiment after HE treatment *in vitro*. Taken together, these results strongly suggest that HE inhibits IKK directly.

DISCUSSION

Substantial *in vitro* data suggest that activation of NF- κ B is a critical step in the inflammatory response (9). Additionally, several studies have demonstrated a link between *in vivo* NF- κ B activation with cytokine production and the generation of inflammation in animal models of inflammatory diseases (23, 24). It is also well established that NF- κ B is involved in induction of iNOS (25). Administration of NO inhibitors decreases the adherence and emigration of leukocytes in postcapillary venules, an early event in inflammation (26, 27). In the present study, we investigated the effects of a novel natural diterpene, HE (Fig. 1), on the synthesis of the pro-inflammatory cytokines IL-1 β IL-6, and TNF- α and NO production *in vitro*. The results clearly demonstrate that HE completely inhibited the synthesis of the cytokines (Fig. 2) without exerting cytotoxic

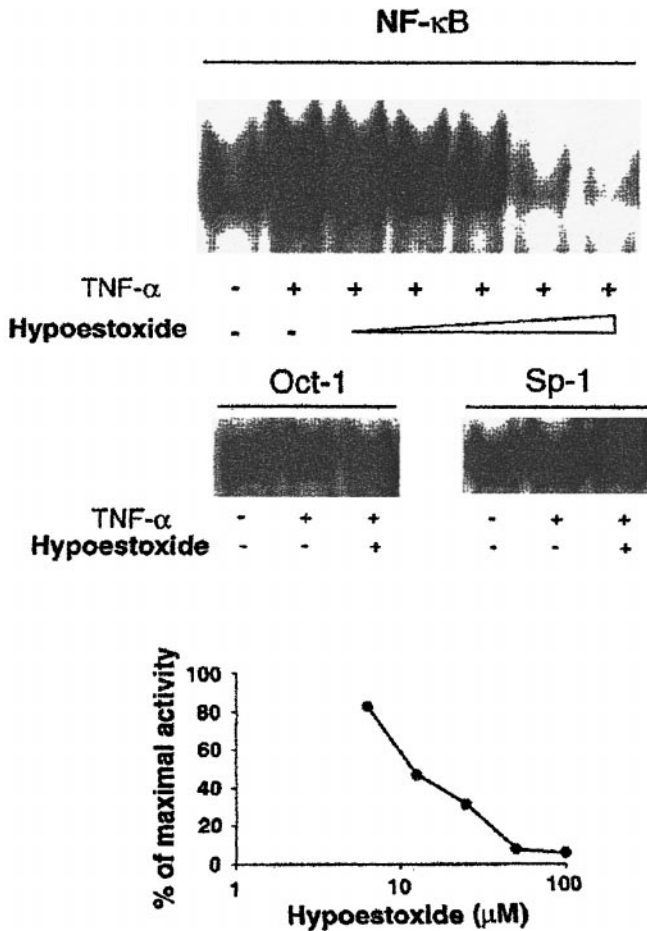


FIG. 5. HE inhibits TNF- α -induced NF- κ B activity. HeLa cells treated with HE (0, 6.25, 12.5, 25, 50, 100 μ M) for 1 h and then stimulated with TNF- α for 30 min. After lysis, extracts were assayed for NF- κ B activation by EMSA. No effects of HE (50 and 100 μ M) on constitutive Oct1 and Sp1 DNA binding activities were observed. The dose response curves for NF- κ B binding activity were obtained by calculating NF- κ B binding activity, which was quantified by phosphoimaging and expressed as a percentage of the maximal activity achieved in the absence of the inhibitor.

effects on PBMC (Table 1). Production of NO following stimulation of normal human articular chondrocytes with either IL-1 β or IL-17 was also inhibited by HE in a dose-dependent fashion (Fig. 3). However, DEX was more active than HE in inhibiting NO production. Nevertheless, the inhibitory effect of HE on NF- κ B translocation and IKK activation in HeLa cells closely parallels its inhibitory effect on NO production in chondrocytes. We examined whether NF- κ B activation with TNF- α was inhibited by HE and found that HE inhibited NF- κ B activation at concentrations that inhibit cytokine production with an IC₅₀ of about 10 μ M (Figs. 2 and 5). These results are consistent with results obtained on nonsteroidal anti-inflammatory drugs (NSAID), such as aspirin, sodium salicylate, and indomethacin, which also inhibited NF- κ B activation (28). Because of the potent inhibitory effect of HE on NF- κ B

activation, it was of interest to determine whether IKK could be the target for HE. We found that HE inhibited IKK activity at an IC₅₀ of 24 μ M in HeLa cells (Fig. 6b). Again, these results are consistent with those obtained on aspirin, which showed that aspirin inhibited IKK (28).

Topical application of phorbol ester to the back of mice induces strong inflammation resulting in erythema, leukocyte infiltration, edema, and epidermal hyperplasia (29). IL-1 β plays a pivotal role in phorbol ester-induced inflammatory cell infiltration (29). We found that the inhibitory effect of HE on phorbol ester-induced topical inflammation was comparable to that of dexamethasone (57% versus 63% inhibition) (Table 2), an established NF- κ B inhibitor. The inhibitory effect of HE on NF- κ B activation (Fig. 5) and the various reports which demonstrate the involvement of NF- κ B activation in rheumatoid arthritis (30) and CIA (31) prompted us to examine the effect of HE on CIA. Our results clearly demonstrate that HE significantly inhibited CIA in mice at doses comparable to the cyclooxygenase (COX) inhibitor indomethacin (Fig. 4). Interestingly, HE does not inhibit either COX 1 or COX 2 activity even when tested at concentrations as high as 100 μ M (data not shown). Recent studies have shown that indomethacin and dexamethasone do not inhibit endogenous IKK kinase activity (28), suggesting that HE is a novel NSAID that specifically inhibits IKK and that dexamethasone and indomethacin most likely operate via different mechanisms; thus, combinations of these could possibly be more effective for treating various inflammatory diseases. The mechanism by which hypoestoxide inhibits CIA or topical inflammation is presently unknown. Although it is tempting to speculate on the possibility that specific inhibition of IKK may be responsible for HE's activity *in vivo*, the present results are preliminary and cannot be extrapolated to responsibility for its *in vivo* anti-inflammatory activity. Pilot pharmacokinetic results from earlier studies (data not shown) indicate that oral bioavailability of HE at 24 h was greater than 6.4% in plasma but had a large volume of distribution and high clearance, which indicated that HE might have an extensive tissue distribution and metabolism which accounts for its effective biological activity. Furthermore, preliminary results indicate that HE is a potent inhibitor of the cellular motility machinery or of a signal transduction cascade required for effective cell migration (data not shown). This, too, may be another mechanism by which HE induces anti-inflammatory activity *in vivo*.

The exact mechanism by which HE inhibits IKK is unknown. However, it is possible that direct covalent modification of the IKK β subunit by the reactive functional groups (i.e., α/β unsaturated ketone and/or epoxides) found on HE could occur through a Michael addition-type mechanism. This would likely involve

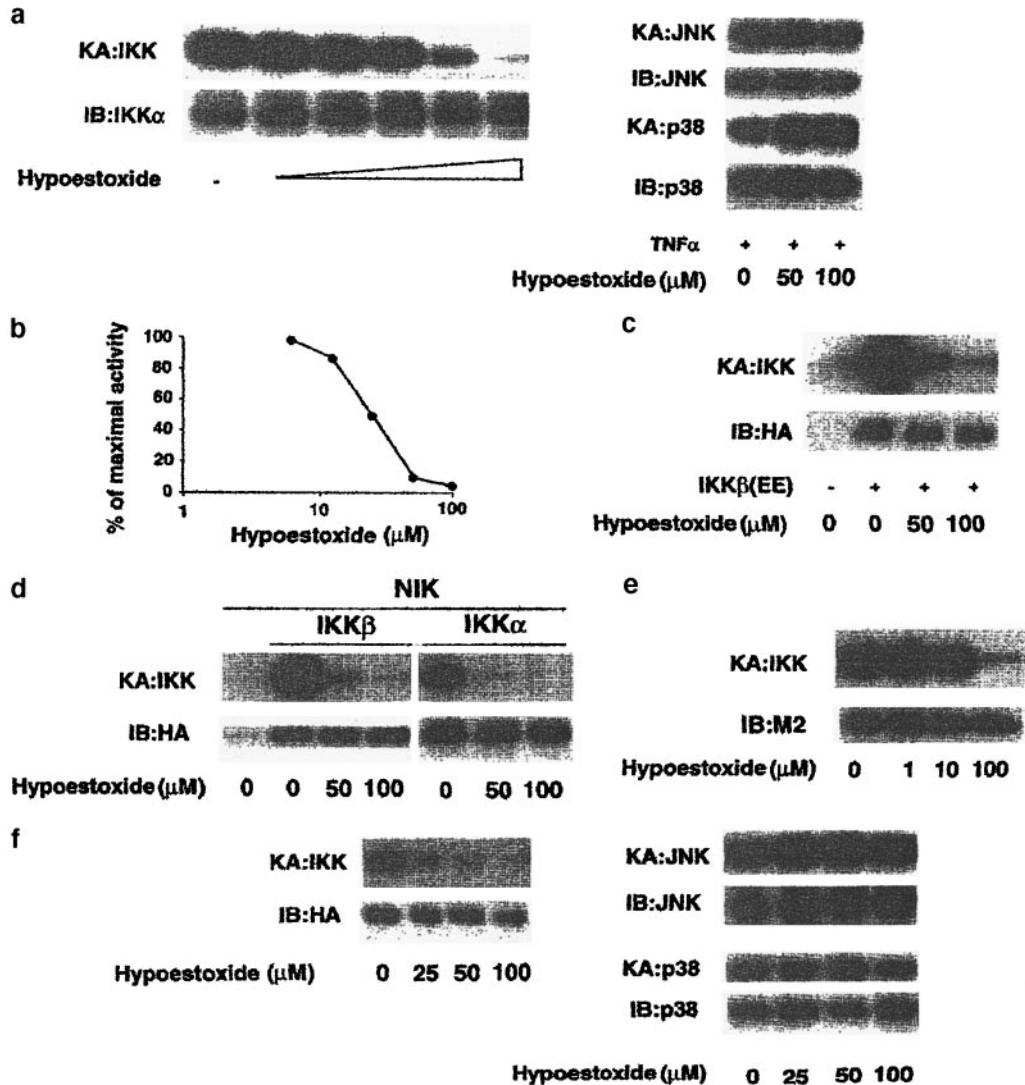


FIG. 6. HE inhibits IKK activity directly. (a) HeLa cells pretreated with various concentrations of HE for 1 h and then stimulated with TNF- α for 15 min. Endogenous IKK (KA:IKK) and recoveries (IB:IKK- α) were determined. Extracts from HeLa cells that were treated with HE (0, 50, 100 μ M) and TNF- α for 15 min were analyzed for JNK1 (KA:JNK) and p38 (KA:p38) activities. Immunoblots of immunoprecipitated JNK (IB:JNK) and p38 (IB:p38) are shown. (b) The dose response curves for IKK activity were obtained by quantifying IKK activity by phosphoimaging and expressed as a percentage of the maximal activity achieved in the absence of the inhibitor. (c) HE inhibits constitutively active IKK- β . Cells were transfected with constitutively active HA-tagged IKK β (EE) and treated with HE for 60 min. Cells were lysed and assayed for HA-tagged IKK β -associated kinase activity and recovery 24 h after transfection. (d) HE inhibits kinase activity of IKK- α or IKK- β activated by NIK. HeLa cells cotransfected with Xpress-tagged NIK expression vector and IKK- α or IKK- β were treated with HE for 60 min. Cells were lysed and assayed for endogenous IKK activity and recovery 24 h after transfection. (e) Sf9 cells infected with IKK β baculovirus were incubated with various concentrations of hypoestoxide for 1 h at 28°C. Kinase activity (KA) was measured after immunoprecipitating with M2 antibody. Kinase expression was determined by immunoblotting (IB). (f) HeLa cells were stimulated with TNF- α for 10 min. Cells were lysed and IKK, JNK, or p38 was immunoprecipitated and incubated *in vitro* with the indicated concentrations of HE. This was followed by kinase assay and immunoblot analysis.

the thiol groups of cysteine residues in the activation loop of IKK, as was demonstrated recently for the activity of the anti-inflammatory cyclopentenone prostaglandin J2 (32), which has been shown to be associated with resolution in late phases of inflammation (33) and also contains an α/β unsaturated ketone moiety. To test this possibility, the cysteine at position 179 in the activation loop of IKK β was replaced with an alanine residue by site-directed mutagenesis (32).

Wild-type IKK β and the mutant form of IKK β , IKK β (C179A), were coexpressed with NIK in HeLa cells, and their sensitivity to HE was examined. While both constructs were responsive to NIK, resulting in similar levels of kinase activity, both were also sensitive to HE, indicating that the cysteine at 179 in IKK β is not critical for HE activity (data not shown). Thus, HE does not appear to act by direct modification of the same site on IKK β as that of prostaglandin J2 in spite

of their similarities in reactive functional groups, therefore establishing HE as a novel NSAID that not only inhibits IKK specifically, but does not affect prostaglandin synthesis as well. Although we have shown that HE appears to have a direct inhibitory effect on IKK, some questions regarding its exact mode of action still remain to be resolved. For instance, it would be worthwhile to know if the inhibition is reversible or competitive with ATP. These and other possible mechanisms are presently under investigation. In conclusion, this report demonstrates the anti-inflammatory activity (*in vitro* and *in vivo*) of a novel, naturally occurring diterpene and provides, for the first time, some underlying scientific basis for the use of the decoction of the plant *H. rosea* in folk medicine.

ACKNOWLEDGMENTS

We thank A. Kumar and F. Hanna for their technical assistance on the drug screening and cytokine assays and Taamrat Amaize for processing the manuscript. M.K. is the Frank and Else Schilling American Cancer Society Research Professor.

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