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Inhibition of Tumor Necrosis Factor-α mRNA Expression by a Limited Series of Tetrahydroisoquinolines in Mouse Peritoneal Macrophages

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Tumor necrosis factor-α (TNF-α) plays important roles in inflammatory responses. Some of tetrahydroisoquinoline (THI) compounds exhibited to inhibit iNOS expression in animal studies and RAW 264.7 cells, but the action of THI on inflammatory reaction was not fully investigated. In the present study, we examined a limited series of THIs (higenamine, YS-51 and THI-52) on the TNF-α mRNA expression in mouse peritoneal macrophages by Northern analysis. When thioglycollate-stimulated peritoneal macrophages were incubated with LPS (100 ng/ml), expression of TNF-α mRNA was evident and reached its maximum at 2.5 h, which was reduced concentration-dependently by treatment with THIs. When the TNF-α activity of macrophage-conditioned media was measured using a TNF-sensitive L929 fibroblast cell line, CCL 1, all THIs increased the cell viability in a concentration dependent manner. The concentrations of THIs used are not cytotoxic by itself when analysed by MTT. Furthermore, nitrite/nitrate level was significantly reduced by the presence of THIs in cells treated with LPS+ interferon-γ (IFN-γ). It is concluded, thus, that these results strongly indicated that THIs can suppress the TNF-α expression and reduce NO, which may be useful for the inflammatory disorders.

Key Words: Inducible nitric oxide synthase, Lipopolysaccharide, Tetrahydroisoquinolines, Tumor necrosis factor-α, Inflammation, Nitric oxide

INTRODUCTION

There is now a good evidence that an enhanced formation of nitric oxide (NO) by the inducible nitric oxide synthase (iNOS) contributes to the circulatory failure (hypotension and vascular hyporeactivity to vasopressor agents), multiple organ dysfunction and death caused by endotoxin in rodents (Thiemermann & Vane, 1990; Thiemermann et al, 1993). Inhibitors of NOS can reverse or prevent the hypotension induced in animals by lipopolysaccharide (LPS), hemorrhage and anaphylatic shock. It is suggested that iNOS plays a crucial role in LPS-induced death. The selective inhibitors of iNOS activity and/or iNOS protein expression may be beneficial for the treatment of systemic inflammatory disorders. Tetrandrine, isoquinoline analog, anti-inflammatory agent, was reported to inhibit NF-κB activation in rat alveolar macrophages by LPS (Chen et al, 1997). A series of isoquinoline alkaloids significantly suppressed NO production in murine peritoneal macrophages when stimulated with LPS, and they attenuated the LPS-induced hepatitis by suppression of tumor necrosis factor (TNF) production in mice (Kondo et al, 1993a, 1993b). We also recently reported that higenamine and YS-49, tetrahydroisoquinoline (THI) analogs, showed inhibitory action of iNOS expression in LPS-treated rat aorta and RAW 264.7 macrophage. (Kang et al, 1997; Kang et al, 1999a, 1999b). In order

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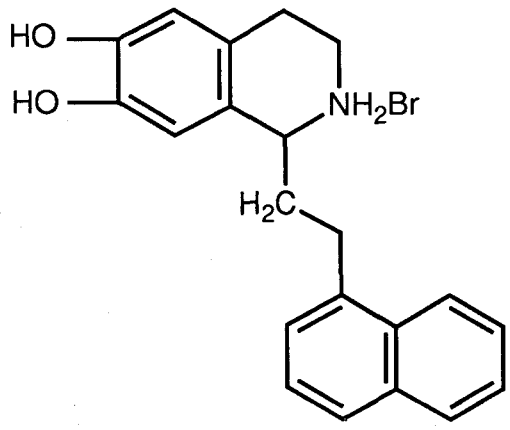

Fig. 1. The chemical structure of THI-52.

to find more potent and less toxic chemicals among THI series compound, recently we synthesized a new compound, THI-52 (Fig. 1) by slight modification of YS-49 structure. However, the effect of THIs, on TNF-α an important molecule in the induction of iNOS (Wu et al, 2000), expression was not investigated. Thus, the purpose of the present study was to know whether THIs can modulate TNF-α expression when mouse peritoneal macrophages were activated by LPS.

METHODS

Cell culture and stimulation

Mouse peritoneal macrophages were grown in Roswell Park Memorial Institute-1640 (RPMI-1640) medium supplemented with 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulphoneic acid (HEPES), 100 units/ml penicillin, 100 μg/ml streptomycin and 10% heat-inactivated fetal calf serum. The cells were stimulated with LPS (100 ng/ml) in the presence or absence of different concentrations of THIs (1, 5, 10, 30 and 100 μM). THIs were administered simultaneously or 1 h prior to stimulation with LPS. All THIs were dissolved in sterile distilled water.

Northern blot analysis

Total RNA was extracted from the cellular lysate. A sample 20 μg of total RNA per lane was subjected to electrophoresis on 1% agarose gels containing formaldehyde and transferred to nylon filters. The filter was then hybridized with a random-primed ^{32}P-labeled TNF-α cDNA probe in rapid hybridization solution (Quikhyb; Stratagene, CA, USA) at 68°C for 1 hr. The hybridized filter was subsequently washed twice for 15 min at room temperature with 2×SSC (sodium chloride/sodium citrate)/0.1% SDS (sodium dodecyl sulfate) and then twice for 15 min at 42°C with 0.2×SSC/0.1% SDS. The filter was then exposed to an X-ray film. The filter was subsequently stripped and rehybridized with a ^{32}P-labeled glycerladehyde-3-phosphate dehydrogenase (GAPDH) cDNA probe.

Assay for nitrite production

The cells were washed once with the RPMI, and 5×10^5 cells in the RPMI were then added to each well of 6 well plates. The cells were incubated overnight at 37°C in a humidified 5% CO_2 incubator. Various concentrations of THIs were added to the cells 1 hr before treatment of LPS (100 ng/ml). The cells were then incubated at 37°C in a humidified 5% CO_2 incubator for 24 hrs. NO was measured as its stable oxidative metabolites, nitrite. At the end of the incubation, 100 μl of the culture medium was mixed with an equal volume of Griess reagent (0.1% naphthylethylenediamine dihydrochloride and 1% sulfanilamide in 5% phosphoric acid). The absorbance at 550 nm was measured, and the nitrite concentration was determined using a curve calibrated on sodium nitrite standards.

Cell viability test

Mitochondrial respiration, an indicator of cell viability, was assessed by the mitochondrial-dependent reductioin of MTT to formazan (Mosman, 1983). Cells in 48-well plates were incubated (37°C) with MTT (0.2 mg/ml for 60 min). Culture medium was removed by aspiration and cells solubilized in dimethyl sulphoxide. Changes in absorbance at a wave length of 530 nm (OD_{530}) were measured by a microplate reader (Bio-Rad Model 550).

Cytotoxicity bioassay of TNF-α

The TNF-α activity of macrophage-conditioned media was measured by standard methods, using a TNF-sensitive L929 fibroblast cell line, CCL 1 (American Type Culture Collection, Rockville, MD).

Briefly, confluent monolayers of L929 cells were established by incubating fibroblast suspensions (4×10^5 cells/ml in M199 medium supplemented with 2% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin; medium pH 7.4) in sterile 96well plates for 24 h (37°C, 5% CO_2). The supernatants were discarded, and the fibroblast monolayers were covered with M199 containing 100 units/ml penicillin, 100 μg/ml streptomycin, and 5 mg/ml actinomycin D (pH 7.4). Aliquots of macrophage-conditioned media were added to the wells in serial dilution. Each assay included a blank of actinomycin D alone and internal standards of recombinant murine TNF-α. All samples were assayed in duplicate. The L929 cells were then incubated for a second 24-h period (37°C, 5% CO_2), at which time the cells were fixed and stained with 5% crystal violet. Dye uptake was determined at 590 nm using a microplate reader (Bio-Rad Model 550).

Materials

Roswell Park Memorial Institute-1640 (RPMI-1640), fetal calf serum, penicillin, streptomycin and glutamine were supplied by Gibco BRL (Gaithersburg, MD, USA). [3-(4,5-dimethyl-thiazol-2yl)-2,5-diphenyltetrazolium bromide] (MTT), 4-(2-hydroxyethyl)-1-piperazineethanesulphoneic acid (HE PES), lipopolysaccharide (*E.Coli*; serotype 0128: B12), sulphnilaminde, N-[1-naphthyl]ethylenediamine, sodium chloride, leupeptin, pepstatin A, phenylmethylsulfonyl fluoride, dithiothreitol and sodium citrate were from Sigma (St. Louis, MO. USA). THI-52 was synthesized by Dr. D.H.Lee, Sokang University, and the procedure of synthesis will be published in other journal.

Statistical evaluations

Data are expressed as mean±s.e.m. of number (n) of observations. Differences between data sets were assessed by one-way analysis of variance (ANOVA) followed by Student's unpaired *t*-tests. A level of $P<0.05$ was accepted as statistically significant.

RESULTS

Effects of THIs on TNF-α mRNA expression

When cells were treated with LPS, TNF-α mRNA expression was appeared from 30 min time point peaking at 2 h until 3 h and thereafter it decreased (data not shown). Fig. 2 shows the Northern data at 2.5 h time point, in which the increased TNF-α mRNA expression was decreased by incubation with THIs, concentration-dependently. For example, the TNF-α/GAPDH ratio for LPS was regarded as

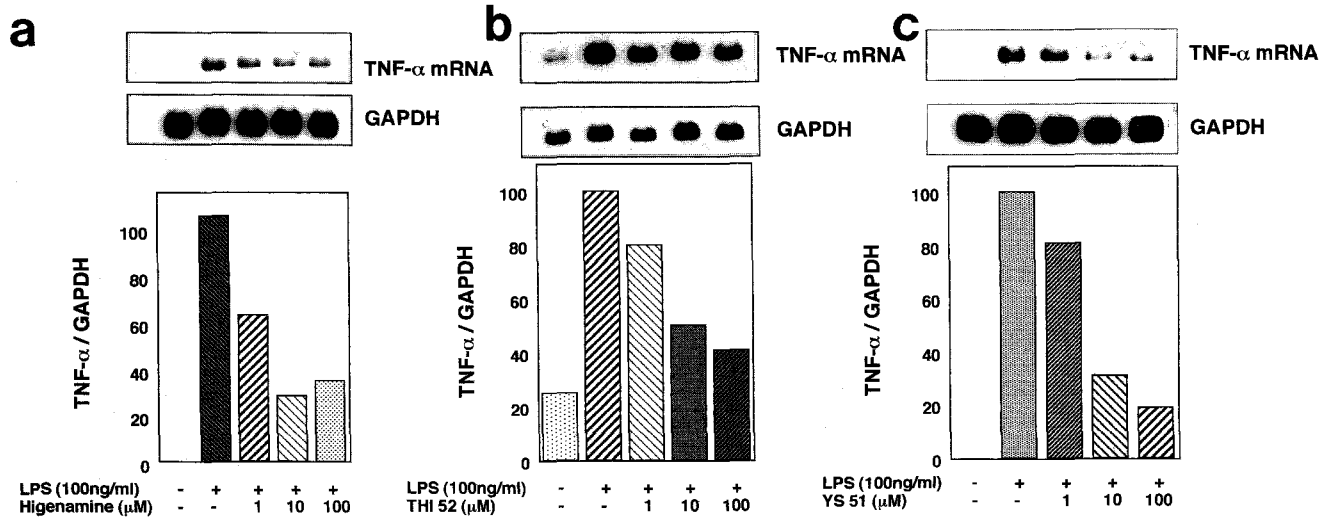

Fig. 2. Effects of THIs on the TNF-α mRNA expression in mouse peritoneal macrophages activated by LPS. Autoradiogram of TNF-α mRNA expression and GAPDH were shown at the top of the pannel. The TNF-α/GAPDH ratio was depicted as arbiturary unit (%), in which the ratio obtained by LPS-treatment was regarded as 100%.

100%, 10 μM higenamine was reduced to 29%, THI-52 to 50% and YS-51 to 30%. When the THI concentration was increased up to 100 μM, higenamine reduced to 38%, THI-52 to 40% and YS-51 reduced the TNF-α/GAPDH ratio to 20%.

Effects of THIs on LPS-treated L929 cells

The reduced TNF-α mRNA expression by THIs was related to reduced TNF-α toxicity, the TNF-α activity of macrophage-conditioned media was measured by standard methods, using a TNF-sensitive L929 fibroblast cell line, CCL 1. As shown in Fig. 3, the cell viability was increased by the presence of THIs, which was also concentration-dependent. Only 20% cells survived when incubated with media that had been treated with LPS without THIs. As incubated with the media that was treated with THIs, the survival rate of L929 cells was increased as the concentrations of THIs were increased.

Effects of THIs on nitrite/nitrate production

As shown in Table 1, nitrite/nitrate contents were elevated by LPS+IFN-γ, which was also concentration-dependently decreased by the THIs, indicating

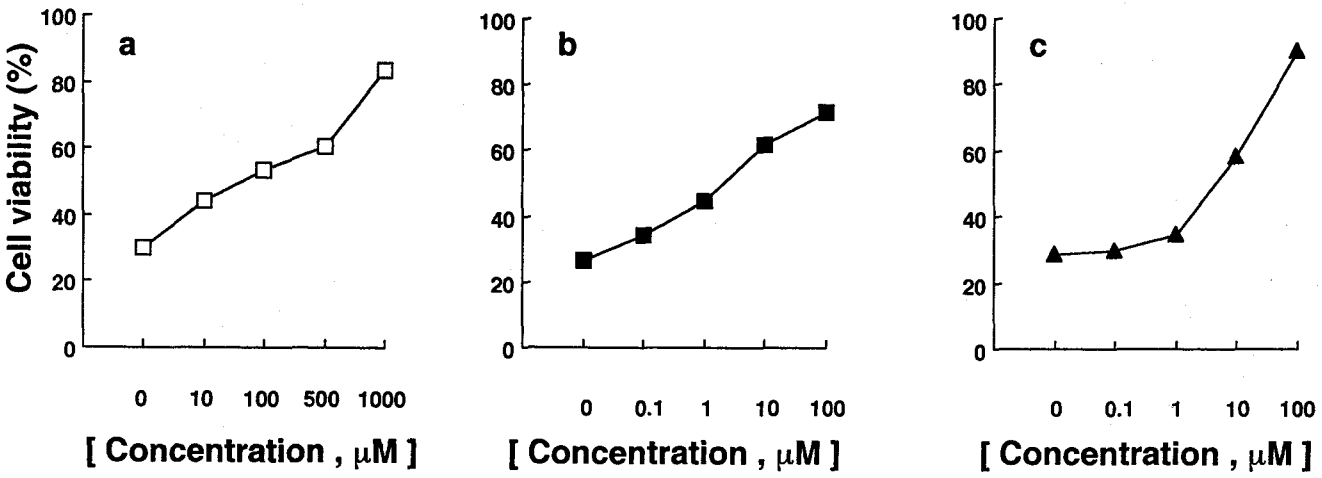

Fig. 3. Concentration-dependent increase of survival rate of L929 cells that incubated with media, which had been treated with LPS in the absence or presence of different concentrations of higenamine (a), THI-52 (b) and YS-51 (c). Represented data are one of the typical experiments performed by 3 separate experiments.

Table 1. Concentration-dependent decrease of nitrite/nitrate by THIs in the media of mouse peritoneal macrophages incubated with LPS+IFN-γ, which was measured after 18 h incubation

Drugs	Concentration						
	0	0	1	5	10	30	100 (μM)
	without CM	with CM					
Higenamine	8.2±3.0[a] (6)	79.7±8.2 (6)	50.4±6.1* (6)	N.D	37.8±3.2* (6)	N.D	10.5±2.1** (6)
THI-52	6.4±0.5 (4)	78.0±3.5 (4)	75±3.7 (4)	68.5±4.0* (4)	55.1±5.0* (4)	38.5±5.0* (4)	N.D
YS-51	5.9±1.2 (6)	81.3±7.5 (6)	54.7±3.3* (6)	N.D	33.5±4.0* (6)	N.D	9.8±3.5** (6)

CM: cytokine mixture (LPS+INF-γ), a: mean±sem (μM), N.D: not determined
*P<0.05 compared to CM, **P<0.01 compared to CM

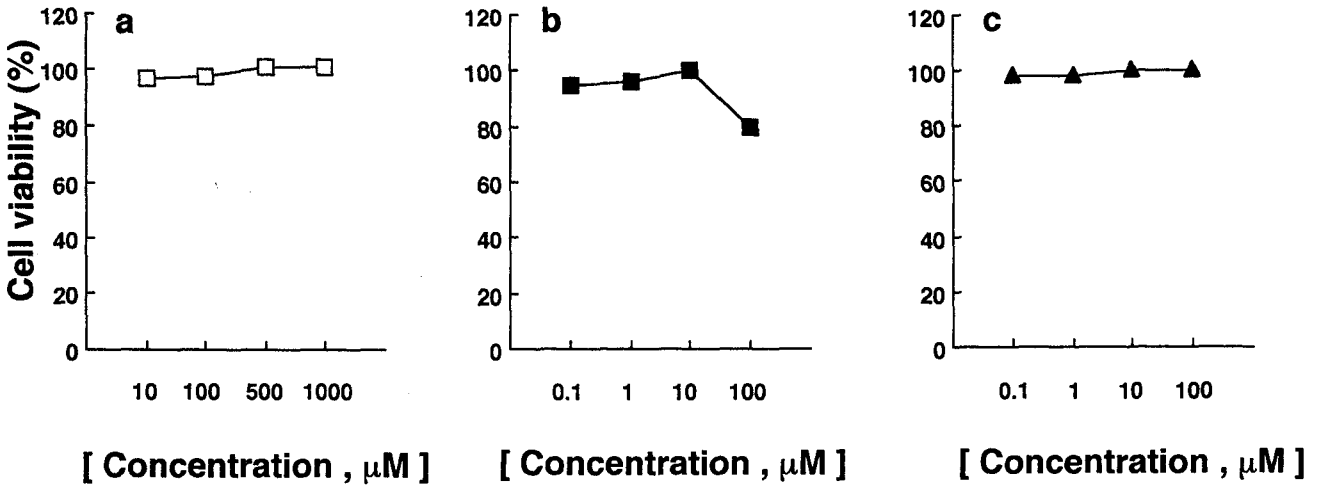

Fig. 4. Effects of higenamine (a), THI-52 (b) and YS-51 (c) on cell respiration. Represented data are one of the typical experiments. Similar results were obtained when tested for 3 separate experiments.

that THIs not only reduced TNF-α mRNA level but also decreased the production of NO in macrophages.

Effects of THIs on cell respiration

As to whether the reduced expression of TNF-α mRNA and NO production were due to cellular damage by THIs, MTT test has been performed. As shown in Fig. 4, up to 100 μM THIs did not significantly affect the cell viability. Furthermore, up to 1 mM higenamine still showed no harmful effect on the cells.

DISCUSSION

Bacterial lipopolysaccharide (LPS, endotoxin) and a number of cytokines including IFN-γ induce an isoform of NO synthase (iNOS) in macrophages, resulting in NO formation which destroys not only bacterial pathogens and tumor cells (Marletta et al, 1990) but modulates local cytotoxicity, edema formation and leukocyte traffic in the pathophysiology of inflammatory disorders (Middleton et al, 1993). Though some analogs of THI compounds are known to inhibit iNOS expression in rat vascular smooth muscles and RAW 264.7 cells (Kang et al, 1999a, 1999b), regulatory action of TNF-α by these chemicals has not been investigated so far. Therefore, the purpose of the present study was to know whether 1) newly synthesized THI analog, THI-52, shows inhibitory action on the production of NO, 2) do these THIs regulate TNF-α expression? A major finding of the present study is that THI-52, concentration-dependently, reduced not only TNF-α mRNA but also reduced NO production in which the IC_{50} was 53 μM. We confirmed that THI series of compound related to higenamine and YS-49 could inhibit production of NO. When the iNOS enzyme activity was tested, THI-52 also decreased the enzyme activity in (a) concentration-dependent manner (data not shown). Previously we along with others reported that higenamine, YS-49 and tetrandrine prevented iNOS expression by inhibiting the activation of NF-κB in RAW 264.7 cells, which is accounted for the mechanism of these chemicals (Kang et al, 1999a; 1999b; Chen et al, 1997). At the present experiment we did not test the effect on NF-κB activation, however, possible mechanism of action of these compounds also is involved in the inhibition of NF-κB activation by following reasons: 1) an autocrine stimulation induced by TNF-α, released after LPS treatment, represents a fundamental factor for iNOS expression in macrophage (Morris & Billar, 1994; Drapier et al, 1988; Saad et al, 1995), 2) the presence of anti-TNF-α antibodies in the culture media can partially inhibit the iNOS expression induced by LPS in macrophages (Drapier et al, 1988). 3) TNF-α is able to determine the activation of NF-κB (Hohmann et al, 1990), which is an important transcription factor involved in the control of iNOS gene expression (Xie et al, 1994). Therefore, there is a possibility of inhibition of TNF-α production by THIs via inhibiting the activation of NF-κB, which may play a

role to prevent iNOS protein expression in RAW 264.7 macrophage cells as shown by YS-49 and higenamine (Kang et al, 1999a, 1999b). Therefore, further studies are needed whether YS-51 and THI-52 can inhibit NF-κB activation, which can clarify the mechanism by which THIs prevent the expression of TNF-α in macrophage by LPS. Recent evidence suggests that TNF-α, a proinflammatory cytokine with potent negative inotropic properties, is elevated in septic shock (Tracey et al, 1986; Parrillo et al, 1990), acute myocarditis (Smith & Allen, 1992), reperfusion injury (Arbustini et al, 1991), and congestive heart failure (Levine et al, 1990). In septic shock, the release of cytokines that activate TNF-α, iNOS in cells such as macrophages and vascular smooth muscle is associated with extreme hypotension that has been reversed by iNOS inhibitors in animal shock model of shock (Kilbourne et al, 1990) and in humans (Petros et al, 1991). In this sense, higenamine and YS-51 may be more useful because they have strong positive inotropic action in concentrations used in the present study. From MTT test, among the three THI compounds, higenamine was the safest, while THI-52 was the most toxic and YS-51 was in between: when 100 μM was used, THI-52 showed some toxic effect, but YS-51 did not show any sign of toxic effect. Moreover, higenamine was still safe up to 1 mM, 10 times higer concentration. On the other hand, L929 cells were frequently used for the bioassay of TNF-α toxicity. In accordance with TNF-α mRNA expression experiments, THIs protected L929 cells concentration dependently, indicating that THI can reduce TNF-α excretion by reducing TNF-α mRNA at transcriptional level.

In summary and conclusion, we demonstrated THI compounds including newly sythesized THI-52 reduced NO production, and TNF-α mRNA expression in macrophage cells when activated with LPS. We concluded that THIs may be beneficial in systemic inflammatory disorders and other diseases, in which enhanced NO is a causative factor due to iNOS or TNF-α up-regulation.

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