Inhibition of Lipopolysaccharide-induced Inducible Nitric Oxide (iNOS) mRNA Expression and Nitric Oxide Production by Higenamine in Murine Peritoneal Macrophages

Hoi Young Lee[1,3], Jang-Soon Lee[1], Eun Ju Kim[2], Jeung Whan Han[2], Hyang Woo Lee[2], Young Jin Kang[3] and Ki Churl Chang[3]

[1]Department of Pharmacology, College of Medicine, Konyang University, Nonsan 320-711, Korea, [2]College of Pharmacy, Sungkyunkwan University, Suwon, 440-745 and [3]Department of Pharmacology, College of Medicine, Gyeongsang University, Chinju 660-280, Korea

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Nitric oxide synthesized by inducible nitric oxide synthase (iNOS) has been implicated as a mediator of inflammation in rheumatic and autoimmune diseases. The effects of higenamine, a tetrahydroisoquinoline compound, on induction of NOS by bacterial lipopolysaccharide (LPS) were examined in murine peritoneal macrophages. LPS-induced nitrite/nitrate production was markedly inhibited by higenamine which at 0.01 mM, decreased nitrite/nitrate levels by 48.7±4.4%. This was comparable to the inhibition of LPS-induced nitrite/nitrate production by tetrandrin (49.51±2.02%) at the same concentration. Northern and Western blot analysis of iNOS expression demonstrated that iNOS expression was significantly attenuated following co-incubation of peritoneal macrophages with LPS (10 μg/ml; 18hrs) and higenamine (0.001, 0.01 mM; 18hrs). These results suggest that higenamine can inhibit LPS-induced expression of iNOS mRNA in murine peritoneal macrophages. The clinical implications of these findings remain to be established.

Key words : Higenamine, Inflammation, Nitric oxide, iNOS mRNA, Murine macrophages

INTRODUCTION

Nitric Oxide (NO) is a small membrane-permeable gas that serves as a mediator of many physiological events. It was first identified as an endothelium-drived relaxation factor (Palmer *et al.*, 1987). However, it is now recognized to be an intra- and extracellular mediator of cell functions (Nathan and Xie, 1994; Schmidt, 1994). Three distinct types of NOS have been identified. Neuronal NOS (nNOS), which is found in the brain and peripheral nervous system and non-neuronal tissue as well, is involved in a non-adrenergic, non-cholinergic neurotransmitter regulation, long term potentiation, and long-term depression. Endothelial NOS (eNOS), which is found predominantly in membrane, plays a key role in the regulation of vascular tone and platelet aggregation. Cytokine-induced NOS (iNOS) is a Ca^{2+} insensitive protein containing calmodulin as a tightly bound subunit and is an important mediator including antimicrobial and antitumor activities.

Correspondence to: Hoi Young Lee, Dept. of Pharmacology, College of Medicine, Konyang University, Nonsan 320-711, Korea

It has been known that NO formation is increased during inflammation, and several classic inflammatory symptoms are reversed by NOS inhibitors (Marletta, 1994). Furthermore, there is increasing evidence to suggest that L-arginine-derived NO and its derivatives, such as peroxynitrite and nitrogen dioxide, play a role in inflammation and also possibly in the multistage process of carcinogenesis (Ohshima and Bartsch, 1994; Halliwell, 1994). Benzylisoquinoline and tetrahydroisoquinoline alkaloids were isolated from a traditional Chinese herbal medicine and have been used in China for several decades for the treatment of silicosis and arthritis, two disease states associated with considerable inflammatory mediator release (Rachmilewitz *et al.*, 1994; Routledge *et al.*, 1993). Activation of macrophages, which may result in the production of large amounts of NO, is thought to provoke critical or lethal toxicity. Higenamine is a tetrahydroisoquinoline derivative and important cardiotonic constituent of *Aconiti radix* (Chang *et al.*, 1986; 1992; 1994). While the cardiotonic action of higenamine is well established through extensive investigations for several years, it is not known yet why it can be used for anti-inflammtory purpose. There was a report showing that higenamine possesses marked

antiinflammtory action on swelling induced by histamine in the rat (Zhang *et al.*, 1985). The mechanism of action of higenamine was postulated that it can scavenge free radicals and inhibit peroxidation of lipids so to protect synovial fluids. Recently, it has been reported that higenamine inhibits LPS induced iNOS mRNA expression in rat aorta (Kang *et al.*, 1997). Furthermore, tetrandrine, a benzylisoquinoline alkaloid with very similar structure as higenamine, exhibits antifibrotic and anti-inflammatory activities in rat alveolar macrophage (Chen *et al.*, 1997). Therefore, it is highly possible that higenamine could be used as an antiinflammtory drug through its effect on NO generation. In this report we investigated the effects of higenamine on the induction of iNOS in peritoneal macrophages activated with LPS and compared those effects with the results from tetrandrine treatment.

METHODS

Cell culture and treatment with higenamine

Murine macrophage was activated by injecting thioglycollate (40 mg/ml) to specific pathogene-free BALB/c at 5 to 8 wk of age. Peritoneal macrophages (2×10^6 cells/ml) were cultured in RPMI 1640 containing 10% fetal bovine serum, 100 U/ml penicillin, and 100 g/ml streptomycin at 37°C in a humidified incubator. Cells were dispensed on 60 mm culture dish and incubated for 2 hrs at 37°C. Cells were treated with LPS (10 μg/ml) for 18 hrs. with or without different concentrations of drugs.

Determination of NO generation

Nitrite concentrations in the medium were measured as an indicator of NO production by the Griess reaction. One hundred microliters of supernatant was mixed with 200 μl of Griess reagent (mixture of 1 part of 1% sufanilamide in 5% phosphoric acid and 1 part of 0.1% naphthylethylenediamine dihydrochloride in water) in triplicate. The plates were read using an ELISA plate reader at 550 nm.

Northern blot analysis

Macrophages were activated with LPS (10 μg/ml) in the presence or absence of drugs. Total RNA was isolated from macrophages using TriZol (Life Technology, Inc., MA, USA) following the manufacturer's suggested protocols. Ten micrograms of RNA were fractionated on 1% formaldehyde agarose gel and transferred to an immobilization membrane (Maximum strength Nytran Plus, Schleicher & Schuell, NH, USA) by capillary method using 20 X SSC (3 M NaCl, 0.3 M Sod. citrate, pH 7.0). RNA was immobilized on the membrane by using the UV crosslinking technique. Membrane was pre- and hybridized at 68°C in QuickHyb solution (Stratagene, Inc., WI, USA) following the manufacturer's suggested protocols in the presence of salmon sperm DNA (Research Genetics, Inc, AL, USA). After 1 h hybridization, the membrane was washed with 2 X SSC, 0.1% SDS for 15 min twice at room temperature and once with 0.2 X SSC, 0.1% SDS at 65°C. Membrane was exposed to X-ray film with intensifying screen at -70°C. Radioactive probe was prepared with Radpriming kit (Life Technology, Inc., MA, USA) following the manufacturer's protocols in the presence of 25 ng of DNA fragments. Two oligomers, Ir-1S (CACAAGGCCACATCGGATTTC) and IR-1A (TGCATACCACTTCAACCCGAG) were used to amplify iNOS probe from murine iNOS cDNA. As a control, DNA fragment of glyceraldehyde 3-phosphate-dehydrogenase (GAPDH) was amplified using a sense oligomer, TCCCTCAAGATTGTCAGCAA (GPDS), and an antisense oligomer, AATGTATCCGTTGTGGATCT (GPDA). DNA fragments were labeled with [α-^{32}P]dCTP.

Western blot analysis

Whole cell proteins (5 μg) were subjected to electrophoresis in a 7.5% SDS-polyacrylamide gel. Resolved proteins were transferred to a nitrocellulose membrane and incubated with a specific anti-iNOS monoclonal antibody, as specified by Transduction Laboratories (KY, USA). After treating with alkaline phosphatase conjugated goat anti-rabbit antibody, the membrane was developed with alkaline phosphatase substrate (Bio-Rad, Inc., USA) following the manufacturer's suggested proocols.

Measurement of cell viability

Cell viability was measured by tetrazolium reduction by using MTT assay (Denizot *et al.*, 1986). After each experiment, drug-treated samples showing higher than 85% of nontreated control were used.

Statistical analysis

All values are expressed as the means±standard deviation (SD). To compare the multiple means of histologic scores, Student-t test and ANOVA analysis were used. Probability value of less than 0.05 was considered to be statistically significant.

RESULTS

Effects of higenamine on NO generation from murine macrophages

Incubation of murine peritoneal macrophage with LPS (10 μg/ml) stimulated nitric oxide production, elevating nitrite/nitrate levels from a basal value of 2.53±1.35 to 106.61±3.20 μM. The increase in nitrite/

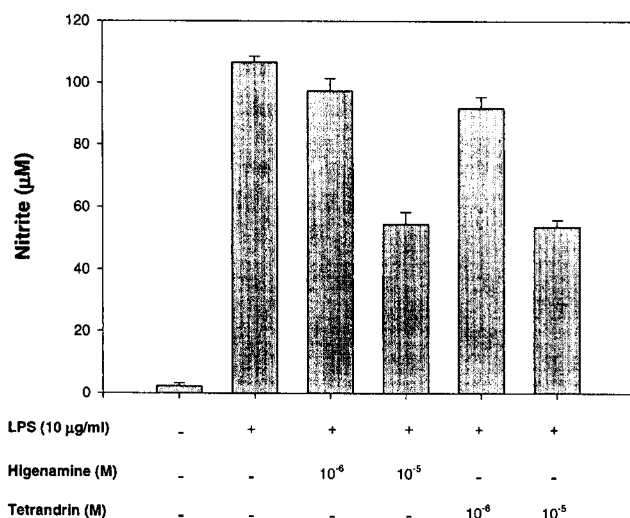

Fig. 1. Inhibition of NO synthesis from macrophages by higenamine and tetrandrin. Thioglycollate-elicited murine peritoneal macrophages were activated with LPS (10 μg/ml) in the presence of two different concentrations of higenamine and tetrandrin (0.001, 0.01 mM). After 18 h incubation, the concentrations of nitrite were measured in culture supernatants by Griess reaction as described. Values are the mean±SD of 3 independent experiments. Asterisk denotes significant difference comparing with the production of nitrite/nitrate by LPS ($p<0.05$).

nitrate production was markedly inhibited by higenamine which at 10 μM, decreased nitrite/nitrate levels by 48.7±4.4% (n=3). The inhibition of nitrite/nitrate production by tetrandrine was 49.51±2.02% at the same concentration as higenamine (Fig. 1).

Effects of higenamine on NO synthase mRNA expression

To determine whether the reduction of NO production by higenamine was caused by reduced expression of iNOS mRNA, we isolated total RNA of each samples and performed Northern hybridization. The level of mRNA for iNOS was markedly reduced in higenamine treated cells. In the presence of 10 μM of higenamine, the iNOS mRNA expression was reduced by 67% when the relative intensity (iNOS/GAPDH) of the higenamine treatment was compared to that of LPS. Tetrandrine reduces the iNOS mRNA expression by 44% (Fig. 2).

Effects of higenamine on NO synthase expression

To determine whether the inhibitory effect of higenamine was due to the inhibition of iNOS expression, Western blot analysis was carried out on whole cell lysates using a monoclonal antibody for murine iNOS. In lysates from LPS-activated but not from untreated cells the iNOS antibody recognized a protein band which migrated at a molecular weight of ~130 KDa

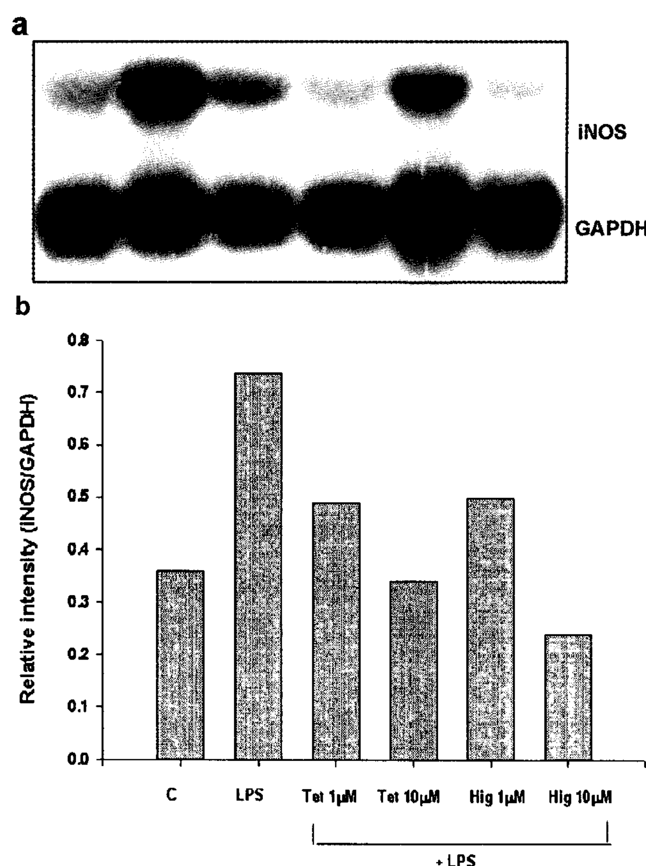

Fig. 2. Northern-blot analysis of mRNA expression of iNOS. (a) Peritoneal macrophages were activated with LPS (10 μg/ml) in the presence of two different concentrations of higenamine and tetrandrin (0.001, 0.01 mM). After 18 h incubation, total RNA was prepared as described. Ten micrograms RNA were subjected to a formaldehyde agarose electrophoresis (1%), transferred to a nylon membrane, hybridized with 32P-labeled DNA fragment, and analyzed by autoradiography. (b) Relative intensity of mRNA (iNOS/GAPDH) bands quantified by scanning densitometry.

(Fig. 3). While the level of iNOS protein, determined by scanning densitometry, was reduced by 68% in lysates from the murine peritoneal macrophage cells activated with LPS in the presence of higenamine (10 μM), tetrandrine inhibited iNOS expression by 41% at the same concentration.

DISCUSSION

This study has examined the effects of higenamine on the inducible nitric oxide production in the murine peritoneal macrophages. We observed that higenamine inhibited nitrite/nitrate production after LPS treatment and our data are consistent with the report by Kang *et al.* (1997) showing that higenamine at 0.1 mM concentration inhibited nitrite/nitrate production in rat aorta. We reduced the concentration of higenamine to 10-100 fold (0.001 to 0.01 mM), still we could see significant reduction of LPS-induced

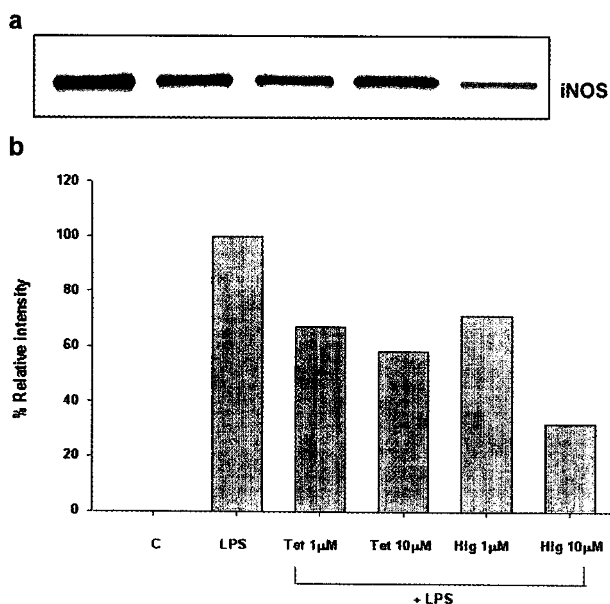

Fig. 3. Western-blot analysis of iNOS in murine peritoneal macrophage. (a) Whole cell extract (5 μg) from untreated cells (C) and from cells incubated with LPS (10 μg/ml) alone and in combination with either tetrandrin (1, 10 μM; Tet) or higenamine (1, 10 μM; Hig) were separated by SDS-PAGE, transferred to a nitrocellulose and blotted with a mouse monoclonal anti-iNOS antibody. (b) Relative intensity of iNOS protein bands quantified by scanning densitometry. Data are expressed as a percentage of the maximum value obtained for cells treated with LPS alone.

nitrite/nitrate production. While Kang *et al.* focused on the vascular smooth muscle, present study shows the action of higenamine on murine peritoneal macrophages which are supposed to directly involved in many inflammatory diseases including arthritis. Furthermore, we directly compared the effects of higenamine to those of tetrandrine, a well known inhibitor of LPS-induced nitrite/nitrate production.

Tetrandrine, a benzylisoquinoline alkaloids, has similar structure as higenamine. Tetrandrine was reported to suppress hepatic injury (Kondo *et al.*, 1993) and exhibited antifibrotic and anti-inflammatory activities through the inhibition of iNOS mRNA expression in rat alveolar macrophage (Chen *et al.*, 1997). Therefore, it is desirable to study whether structurally related compounds as benzylisoquinoline alkaloids can produce the similar effect. In present study, we compared higenamine and tetrandrine for their effects on the production of nitrite/nitrate and iNOS expression after LPS-induced activation. Higenamine inhibited iNOS mRNA expression and nitrite/nitrate production almost similarly to those caused by tetrandrine.

Tetrahydroisoquinoline alkaloids including higenamine have been used widely for the treatment of rheumatoid arthritis in traditional medicine. It has been reported that higenamine possesses marked anti-inflammtory action on swelling induced by histamine in the rat through scavenging free radicals and inhibiting peroxidation of lipids to protect synovial fluids (Zhang *et al.*, 1985). Cardiotonic effect of higenamine through the activation of beta adrenoceptor is well established (Park *et al.*, 1984, Chang *et al.*, 1992). Higenamine could reduce the mortality of mouse model from endotoxin shock (Yun and Kim, 1994). Recently, Chong *et al.* (1998) showed that higenamine increased heart rate and contractile force. Therefore, higenamine has been suggested to be used for septic shock through its positive inotropic action and inhibition of LPS-induced nitrite/nitrate production. Our data showed that higenamine significantly suppressed nitrite/nitrate production (>45%) at micromolar concentrations. Therefore, our results provide the basic information for future study of tetrahydroisoquinoline compounds for their use against septic shock and inflammatory diseases.

Although our data suggest that higenamine may be acting at the molecular level to inhibit iNOS induction, the precise mechanism by which this effect is mediated remains to be established. Tetrandrine inhibits the activation of nuclear factor kappa B (NF-kB), a transcription factor activated by pro-inflammatory mediator. Whether higenamine exert its effects by inhibiting activation of NF-kB remains a critical experiment to be carried out.

In conclusion our data have identified higenamine as an inhibitor of the iNOS in murine peritoneal macrophages, exerting its effects presumably by inhibiting the expression of iNOS mRNA. This finding provides an additional mechanism by which the anti-inflammatory property of this compound could be mediated. However, further studies regarding the effect of higenamine to NF-kB are required to understand the action mechanisms of tetrahydroisoquinoline compounds.

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