BIOCHEMICAL PHARMACOLOGY 75 (2008) 923–930

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Cyclo(dehydrohistidyl-L-tryptophyl) inhibits nitric oxide production by preventing the dimerization of inducible nitric oxide synthase

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ARTICLE INFO

Article history:
Received 16 July 2007
Accepted 18 October 2007

Keywords:
Cyclo(dehydrohistidyl-L-tryptophyl)
iNOS
Dimerization
Nitric oxide
BV-2 microglia
LPS

ABSTRACT

Dimerization of inducible NOS has been known to be a potential therapeutic target for iNOS-mediated pathologies. Cyclic dipeptides are among the simplest peptides commonly found as by-products of food processing or metabolites of microorganisms. In this study, we found that cyclo(dehydrohistidyl-L-tryptophyl) (CDHT), a cyclic dipeptide from an unidentified fungal strain Fb956, prevents iNOS dimerization in activated microglial BV-2 cells. CDHT inhibited NO production with an IC_{50} of 6.5 μM in LPS-treated BV-2 cells. Western blot analysis and iNOS activity measurement of fractions from size-exclusion chromatography of cell lysates indicated that CDHT inhibits dimerization of iNOS, while it has no effect on iNOS expression or enzyme activity. The CDHT inhibition of iNOS dimerization was confirmed by partially denaturing SDS-PAGE analysis. In contrast, CDHT did not affect cGMP production in endothelial HUVEC cells, which indicates no inhibition of endothelial NOS activity. These results reveal that CDHT, one of the simplest and cyclic dipeptides, selectively inhibits NO production by inhibiting iNOS dimerization, and could be a useful therapeutic agent for inflammation-mediated diseases.

1. Introduction

Nitric oxide (NO) plays an important role in the physiology and pathophysiology of the central nervous, cardiovascular, and immune systems [1–5]. NO is produced by the oxidation of L-arginine to L-citrulline by each of three isoenzymes of nitric oxide synthase (NOS): neuronal (nNOS), endothelial (eNOS), and inducible (iNOS). nNOS and eNOS are constitutively expressed in neuronal and endothelial tissues, respectively, under noninflammatory conditions, and their activities are tightly regulated. In contrast, iNOS is a key mediator of inflammation and host defense systems. Expression of iNOS is induced by inflammatory stimuli such as bacterial lipopolysaccharide (LPS), interferon, interleukin-1, and tumor necrosis factor-α at a transcriptional level in macrophages or microglia [6–8]. Continuous expression of iNOS leads to a higher level of NO, which is implicated in the pathogenesis of various inflammatory diseases, including septic shock, rheumatoid

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Abbreviations: CDHT, cyclo(dehydrohistidyl-L-tryptophyl); NO, nitric oxide; iNOS, inducible nitric oxide synthase; nNOS, neuronal nitric oxide synthase; eNOS, endothelial nitric oxide synthase; LPS, lipopolysaccharide; HUVEC, human umbilical vein endothelial cells; L-NAME, N^G-nitro-L-arginine methyl ester; cGMP, guanosine-3′,5′-cyclic monophosphate; GTP, guanosine triphosphate.

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doi:10.1016/j.bcp.2007.10.021

arthritis, inflammatory bowel disease, and neurodegenerative diseases [9,10]. Thus, the intervention of iNOS-driven NO is believed to be effective for the prevention of these diseases. Inhibition of eNOS, however, is undesirable because of its role in the maintenance of vascular homeostasis [11,12]. In this regard, a specific inhibitor of iNOS has therapeutic potential for treating these diseases [13].

Dimerization of NOS proteins is essential to their activity [14–17]. NOS isoforms are homodimers that catalyze NADPH-dependent oxidation of L-arginine to NO and citrulline. NOS monomers consist of an oxidoreductase domain and an oxygenase domain. The reductase domain is homologous to cytochrome P450 reductase, and contains binding sites for NADPH, FAD, and FMN. The oxygenase domain has binding sites for L-arginine, the heme prosthetic group, and tetrahydrobiopterin (BH_4). Dimerization of NOS is required for fully coupled enzyme activity because the flow of electrons during catalysis occurs *trans* to the reductase domain of one monomer subunit to the oxygenase domain of the other monomer [18]. Since antifungal imidazoles such as clotrimazole were known as iNOS dimer inhibitors [19], some chemicals such as BBS-1 and 2 with a pyrimidineimidazole skeleton [20,21], PPA250 [22], FR26030 [23], and 2-imidazol-1-ylpyrimidine [24] have been reported to inhibit iNOS dimerization and exhibit *in vivo* efficacy. The structures of these chemically synthesized inhibitors, however, were very complex and safety of those inhibitors remained unknown.

Cyclic dipeptides are among the simplest peptide derivatives commonly found in nature [25]. Most cyclic dipeptides appear to have emerged as by-products of food processing or metabolites of microorganisms. In addition, cyclo(His–Pro) is present as an endogenous neuropeptide in humans [26]. Recently, cyclo(His–Pro) is reported to be related to glucose metabolism and to have antidiabetic activity [27,28].

In the course of screening for inhibitors of NO production in activated microglia from microbial extracts, we found that cyclo(dehydrohistidyl-L-tryptophyl) (CDHT), a cyclic dipeptide isolated from a culture of an unidentified fungal strain Fb956, prevented iNOS dimerization. Here, we report that CDHT inhibits NO production by preventing dimerization of iNOS in activated microglia, without having an effect on eNOS activity.

2. Materials and methods

2.1. Materials

LPS (*E. coli* 011:B4) was purchased from Calbiochem (La Jolla, CA, USA). Minimal essential medium (MEM), penicillin, streptomycin, glutamine, sodium pyruvate, nonessential amino acid, and horse serum were obtained from Life Technologies (Gaithersburg, MD, USA). Monoclonal antibody against iNOS was purchased from Transduction Laboratories (Lexington, KY, USA). The enhanced chemiluminescence (ECL) kit for immunodetection and cGMP enzymeimmunoassay system and goat anti-rabbit horseradish peroxidase conjugate was purchased from Amersham Biosciences (Piscataway, NJ, USA). Mouse microglia BV-2 cells were a kind gift from Dr. Jau-Shyong Hong (National Institute of Environmental Health Sciences, NC, USA). Human umbilical vein endothelial cells (HUVEC) and EGM-2 medium were obtained from Clonetics (Walkersville, MD, USA). Clotrimazole, genistein, and N^G-nitro-L-arginine methyl ester (L-NAME) were purchased from Sigma. Ionomycin and 3-isobutyl-1-methylxanthine were purchased from Calbiochem (La Jolla, CA, USA).

2.2. Purification of CDHT

CDHT (Fig. 1) was isolated from the fermented whole medium of an unidentified fungal strain Fb956. The fermented whole medium was extracted with 80% acetone, and the extract was concentrated *in vacuo* to an aqueous solution, which was then extracted three times with an equal volume of EtOAc. EtOAc extract was concentrated *in vacuo* to dryness. The crude extract was subjected to SiO_2 (Merck Art No. 7734.9025) column chromatography, followed by stepwise elution with $CHCl_3$–MeOH (50:1, 10:1, 5:1). The active fractions eluted with $CHCl_3$–MeOH (50:1) were pooled and concentrated *in vacuo* to give an oily residue. The active fraction dissolved in MeOH was further purified by reverse-phase HPLC column (20 mm × 150 mm, YMC C_{18}) chromatography with a photodiode array detector. The column was eluted with acetonitrile-H_2O (35:65) at a flow rate of 8 ml/min to yield CDHT as a white powder with >99% purity at retention times of 9.4 min. Its chemical structure was determined to be cyclo(dehydrohistidyl-L-tryptophyl) by mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectral data as follows; $[\alpha]_D$ + 155 (*c* 0.5, MeOH); ESI-MS *m/z* 322.1 $(M + H)^+$; ^1H NMR (600 MHz, pyridine-d_5) 11.9 (1H, brs, 1-NH), 11.4 (1H, brs, 15-NH), 9.99 (1H, brs, 12-NH), 8.00 (1H, d, *J* = 7.4 Hz, H-4), 7.86 (1H, s, H-20), 7.48 (1H, s, H-22), 7.42 (1H, s, H-2), 7.40 (1H, d, *J* = 7.8 Hz, H-7), 7.17 (1H, dd, *J* = 7.4, 7.8 Hz, H-6), 7.16 (1H, dd, *J* = 7.4, 7.8 Hz, H-5), 6.33 (1H, s, H-17), 4.82 (1H, dd, *J* = 4.0, 5.9 Hz, H-11), 3.75 (1H, dd, *J* = 5.9, 14.5 Hz, H_b-10), 3.60 (1H, dd, *J* = 4.0, 14.5 Hz, H_a-10); ^{13}C NMR (150 MHz, pyridine-d_5) 167.3 (C-16), 162.7 (C-13), 137.6 (C-9), 136.8 (C-20), 133.6 (C-22), 128.6 (C-8), 127.4 (C-18), 125.8 (C-2), 123.2 (C-14), 121.7 (C-6), 119.4 (C-4 and C-5), 111.9 (C-7), 109.1 (C-3), 108.2 (C-17), 57.4 (C-11), 31.9 (C-10). CDHT was previously reported as a metabolite of *Penicillium* sp. [29].

2.3. Cell culture

Mouse microglia BV-2 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 50 U/ml penicillin, and 50 μg/ml streptomycin. The cells were grown at 37 °C in 5% CO_2 in a humidified atmosphere. HUVEC cells were grown onto a gelatin-coated 75-cm^2 flask in EGM-2 at 37 °C in 5% CO_2 and

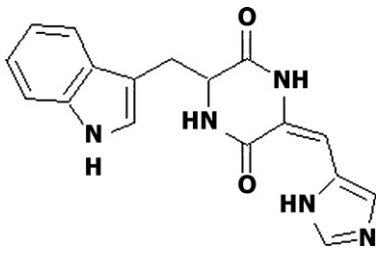

Fig. 1 – Chemical structure of cyclo(dehydrohistidyl-L-tryptophyl).

95% air. Test compounds were prepared in dimethylsulfoxide (DMSO), the final concentration of which did not exceed 0.05%. Cells were either treated with 0.05% DMSO as vehicle control.

2.4. Determination of NO concentration

BV-2 cells were cultured at an initial cell density of 5×10^4 cells/well in 24-well plates. After 48 h, the medium was replaced with DMEM supplemented with 10% FBS containing 1 μg of LPS/ml and various concentrations of CDHT, clotrimazole, genistein, or N^G-nitro-L-arginine methyl ester (L-NAME). Culture supernatants were collected 24 h after stimulation. NO production was assessed by measuring the concentration of nitrite, a stable degradation product of NO, with the Griess reagent.

2.5. Western blot analysis

BV-2 cells were seeded at 5×10^4 cells in a 10-cm dish and cultured for 2 days. Medium was then replaced with DMEM supplemented with 10% FBS containing stimulants and drugs described above. Cells were cultured for another 12 h and removed from the surface of the dish with a plastic scraper, and the cells were then washed twice with ice-cold phosphate-buffered saline (PBS), pH 7.4. Lysis buffer (200 μl) containing 50 mM Tris–HCl, pH 7.4, 1% NP-40, 1 mM phenylmethylsulfonyl fluoride (PMSF), 5 μg/ml aprotinin, 1 μg/ml leupeptin, and 150 mM NaCl was added and centrifuged at 12,000 × *g* for 10 min at 4 °C. The supernatant was stored at −70 °C and used as a cell lysate. Protein was determined using the Lowry protein assay. Cell lysates (5 μg of protein) were suspended in 10 μl of Laemmli reducing sample buffer consisting of 58 mM Tris–HCl, 6% glycerol, 1.67% SDS, 0.002% bromophenol blue, and 1% 2-mercaptoethanol, pH 6.8, and was boiled for 3 min, followed by SDS-polyacrylamide gel electrophoresis (PAGE) performed at room temperature using a 7.5% gel. For partially denaturing SDS-PAGE, cell lysates were mixed with sample buffer in an ice bath, non-boiled, and electrophoresed at 4 °C [9].

Proteins in the gel were electroblotted onto a polyvinylidene difluoride membrane for 120 min at 200 mA. The membrane was blocked with 5% nonfat dry milk and probed for 1 h with anti-iNOS monoclonal antibody (1:2000). Goat anti-rabbit horseradish peroxidase conjugate (1:3000) was used as the secondary antibody, and detection was carried out with the ECL detection system.

2.6. Gel filtration chromatography

The cell lysates prepared from 200 ml of BV-2 cells treated with LPS in the presence or absence of drugs were analyzed by size-exclusion chromatography, followed by western blotting of iNOS and the measurement of NOS activities. Cell lysates were chromatographed on a 10 mm × 50 cm Superdex 200 column (flow rate 1 ml/min) in 100 mM Tris–HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, and 1 mM PMSF. Fractions (1 ml) were collected, and 10 and 50 μl of each fractions were used for western blot analysis and NOS activity measurement, respectively.

2.7. NOS assay

The NOS assay was performed as described previously [19]. NO synthesis activity of cell lysates or column fractions was determined in 200 μl incubations containing aliquots of cell lysates or column fractions and 40 mM Tris buffer, pH 7.8, 1 mM NADPH, 2 mM L-Arg, 20 μM FAD, 20 μM FMN, and 0.5 mM BH_4. Incubations were run for 60 min at 37 °C, and nitrite was analyzed.

2.8. Determination of cGMP

The cGMP assay was performed as described previously [30]. HUVEC were cultured at an initial cell density of 1.25×10^3 cells/well in 96-well plates. After 48 h, the medium was replaced with EGM-2 containing 0.5 mM isobutylmethylxanthine and various concentrations of drugs. After 1 h, the cells were stimulated with 2 μM ionomycin for 4 h. Media were removed and the cGMP content was measured by an enzyme immunoassay system used according to the instructions of the manufacturer.

2.9. Statistical analysis

Data are presented as means ± S.E. for the indicated number of independently performed experiments. Statistical analysis was carried out using one-way analysis of variance (ANOVA), followed by the Bonferroni *t*-test for multi-group comparison tests. A p-value <0.05 was considered statistically significant. Analysis of the regression line test was used to calculate IC_{50} values.

3. Results

3.1. Inhibition of NO accumulation in culture by CDHT

The mouse microglia BV-2 cells stimulated with LPS were used as an *in vitro* model of activated microglia, and the effect of CDHT on NO release was determined. After stimulation with LPS (1 μg/ml), nitrite, a stable degradation product of NO, accumulated in the culture medium in a time-dependent manner, reaching a plateau with a concentration of 20–25 μM in 24 h (data not shown). CDHT inhibited the nitrite accumulation in a dose-dependent manner, as shown in Fig. 2. The IC_{50} was 6.5 μM. In addition, CDHT at 100 μM showed no cytotoxic effect based on both a microscopic observation and a MTT assay, which indicates that the inhibitory activity of CDHT was not due to cytotoxicity (data not shown). In addition, clotrimazole (inhibitor of iNOS dimerization) and genistein (inhibitor of iNOS expression), both well-known inhibitors of NO production, showed similar activity to CDHT in our system, while L-NAME (inhibitor of NOS enzyme) exhibited weaker activity (Fig. 2).

3.2. Effect on iNOS expression or activity

Since most known inhibitors of NO production in activated microglia or macrophages inhibit the signaling pathway to result in a decrease in the expression of iNOS protein [26], the

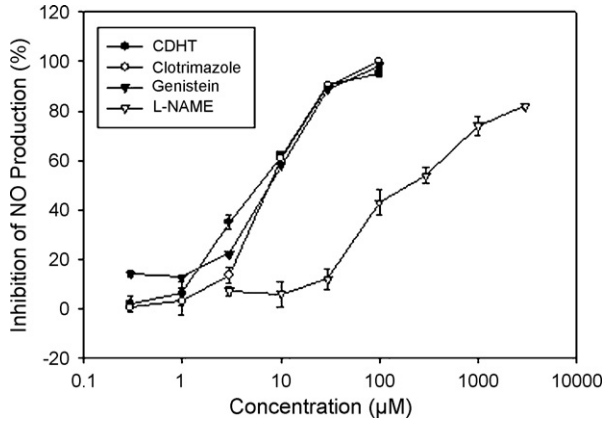

Fig. 2 – Inhibition of NO production in activated BV-2 cells by CDHT. BV-2 cells were cultured at an initial cell density of 5×10^4 cells/0.1 ml in DMEM supplemented with 10% FBS. After 48 h, the medium was replaced with DMEM supplemented with 10% FBS containing 1 μg of LPS/ml and the indicated doses of drugs. Culturing was continued for another 24 h. NO production was assessed by measuring the concentration of nitrite in culture supernatants with the Griess reagent. For reference, nitrite concentration in the LPS-stimulated cells was 22.1 ± 2.1 μM. The values were represented as the mean ± S.D. obtained from two independent experiments performed in triplicate.

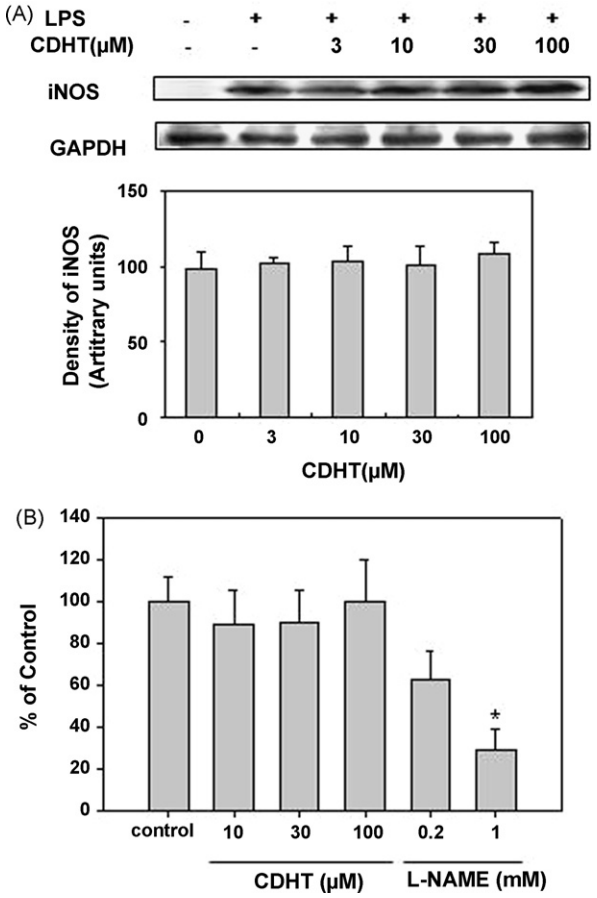

Fig. 3 – Effects of CDHT on iNOS protein expression (A) and enzyme activity (B). (A) BV-2 cells (5×10^4 cells/10 cm dish) were cultured under the conditions described in the legend to Fig. 2. Cells were harvested at 12 h after LPS stimulation in the presence or absence of CDHT, and cell lysates were prepared. Heat-denatured cell lysates (5 μg of protein) were subjected to SDS-PAGE and western blot analysis. Histograms show the densitometric analysis of iNOS protein expression normalized to GAPDH. (B) Effect of CDHT on iNOS activity in cell lysates was determined. Cell lysates were prepared from BV-2 cells stimulated with LPS for 12 h. As a control, nitrite concentration produced by the cell lysates was 4.34 ± 0.38 μM. The values were represented as the mean ± S.D. obtained from two independent experiments performed in triplicate. * $p < 0.01$ compared with the control.

effect of CDHT on iNOS expression was examined. As shown in Fig. 3A, CDHT had no effect on the expression of iNOS protein in BV-2 cells stimulated with LPS for 12 h, even at 100 μM. To investigate whether CDHT inhibits iNOS catalytic activity, the effect of CDHT on iNOS activity was examined. CDHT did not inhibit iNOS activity in cell lysates even at 100 μM, which were prepared from cells that had been stimulated with LPS, while L-NAME as a positive control inhibited iNOS activity in a dose-dependent manner (Fig. 3B).

3.3. *Inhibition of iNOS dimerization*

One potential inhibitory mechanism of CDHT was an effect on iNOS dimerization, which is essential for enzyme activity. To determine whether CDHT affects the intracellular ratio of iNOS monomers to dimers, cell lysates from BV-2 cells activated with LPS in the presence or absence of CDHT were fractionated by gel filtration chromatography in which the molecular weight of each fraction was estimated based on protein standards, and the fractions were analyzed by western blotting using anti-iNOS antibody. As shown in Fig. 4A, size-exclusion chromatography of cell lysates showed that more dimeric iNOS in fraction 11–19 was present than monomeric iNOS in fraction 21–29 when cells were grown with LPS in the absence of the inhibitor. In contrast, dimeric iNOS dramatically decreased in cells treated with LPS and CDHT at 30 μM, while monomeric iNOS increased. As a positive control, cells treated with LPS and clotrimazole at 20 μM showed a similar result to the LPS and CDHT-treated cells. To confirm whether the fractions containing dimeric iNOS have iNOS activity, NO synthesis activities of each fraction from gel filtration chromatography were measured because only dimeric form of iNOS has enzyme activity [15]. As expected, iNOS activity was detected in the fractions corresponding to dimeric iNOS, not monomeric iNOS (Fig. 4B). In LPS and CDHT-treated cells, much fewer fractions showed iNOS activity compared to the LPS-treated cells. These results clearly indicate that CDHT inhibited formation of iNOS dimer in the cells.

For a double check of the CDHT inhibition of iNOS dimerization, the intracellular ratio of iNOS monomers to dimers was determined by partially denaturing SDS-PAGE analysis [9,20,22]. As shown in Fig. 5A, partially denaturing

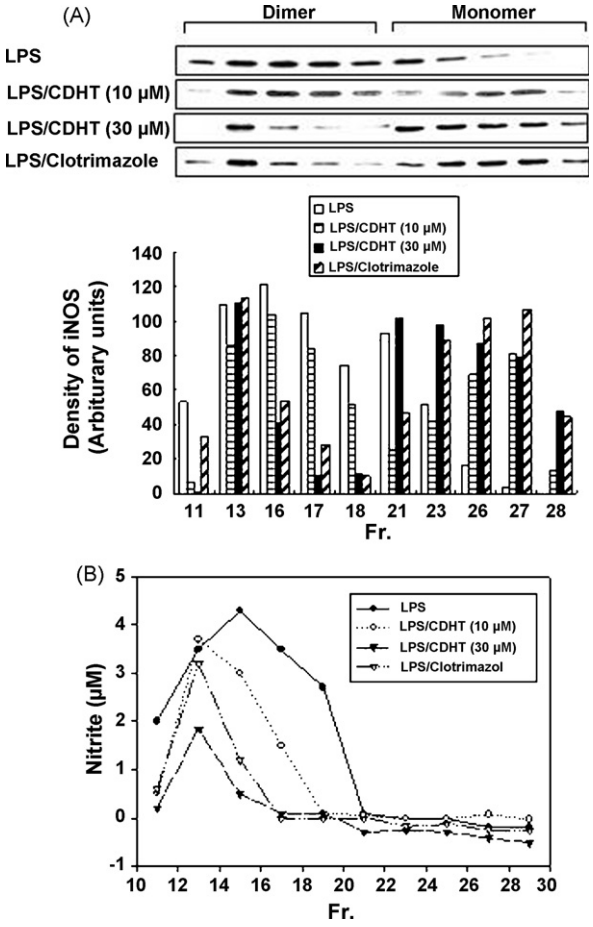

Fig. 4 – Effects of CDHT on cytoplasmic iNOS dimer–monomer ratio and NO synthesis activity. Cell lysates were prepared from 200 ml of BV-2 cells treated with LPS, LPS/CDHT (10 μM), LPS/CDHT (30 μM), or LPS/clotrimazole (20 μM) for 12 h as described in the legend to Fig. 3. The cell lysates were then chromatographed on a Superdex 200 column. (A) 10 μl of each fraction were analyzed by SDS-PAGE and western blotting to detect iNOS. Fractions were determined to contain iNOS dimer or monomer based on the estimated molecular weight of each fraction by comparison to protein standards. Histograms show the densitometric analysis of iNOS protein expression. (B) 50 μl of each fraction were used for the measurement of NO synthesis activity. Experiments shown are representative of two or three experiments.

SDS-PAGE experiments at 4 °C separated monomer and dimer of iNOS so that lots of dimer were detected in the LPS-treated cell lysates. No dimer of iNOS, however, was detected in the cells treated with LPS and CDHT at 15 μM, indicating that CDHT prevented formation of iNOS dimer. As a positive control, clotrimazole also prevented formation of iNOS dimer at 20 μM. To see whether CDHT have affected the expression level of iNOS, heat-denatured cell lysates were analyzed. The amount of iNOS in the LPS and CDHT-treated cell lysates is almost the same as that in the LPS-treated cell lysates (Fig. 5B). These results also indicate that the inhibition of NO production by CDHT in activated BV-2 cells occurs due to the prevention of iNOS dimerization.

3.4. *Effect on cGMP production in HUVEC*

It is well known that the small amount of NO produced by eNOS activates soluble guanylate cyclase by interacting with the catalytic heme groups of the enzyme and producing cGMP from GTP [4,5]. Therefore, cGMP production was measured in order to determine the effect of CDHT on endothelial cell NO production via eNOS. To determine whether CDHT inhibits NO production in endothelial cells, HUVEC were treated with different concentrations of CDHT, and NO production was determined by measuring cGMP production (Fig. 6). CDHT did not inhibit the production of cGMP by HUVEC, even at 100 μM, while as a positive control, L-NAME (1 mM), the nonselective inhibitor of NOS enzymes, inhibited cGMP production. This result indicates that CDHT does not inhibit eNOS activity.

4. Discussion

There is a great number of reports regarding the overproduction of NO and overexpression of iNOS in various inflammatory and autoimmune diseases [9,10]. A selective inhibitor of iNOS has therapeutic potential for these diseases, since the inhibition of eNOS could lead to side effects because of its role in the maintenance of vascular homeostasis [11–13]. iNOS dimerization has been recognized as a potential target for selective inhibition of iNOS because of the differences in dimer interactions and interfaces between iNOS and constitutive eNOS [31–33].

In this study, we found that CDHT [cyclo(dehydrohistidyl-L-tryptophyl)] strongly inhibits NO production by reducing the dimerization of monomeric iNOS. CDHT is a cyclic dipeptide that has been isolated from an unidentified fungal strain Fb956. CDHT has inhibitory activity on NO production in LPS-stimulated BV-2 cells, with an IC_{50} (μM) of 6.5, which showed similar activity to that of clotrimazole, the known inhibitor of iNOS dimerization. While CDHT did not show any level of inhibition on iNOS translation, CDHT inhibits iNOS dimerization as demonstrated by both gel filtration chromatography and partially denaturing SDS-PAGE analysis. However, CDHT does not inhibit the catalytic activity of iNOS, which indicates that CDHT does not dissociate dimeric iNOS but inhibit formation of iNOS dimer. This is consistent with the observations about other iNOS dimerization inhibitors including clotrimazole [19], FR260330 [23], and BBS-1 with a pyrimidineimidazole core [20]. Importantly, CDHT does not inhibit eNOS activity since it did not affect the production of cGMP by eNOS in endothelial HUVEC cells. This result suggests that CDHT could not have side effects on vascular homeostasis. It has been reported that the known inhibitors, FR260330 and pyrimidineimidazole, did not affect eNOS activity as demonstrated using arterial blood pressure assay in rats and cell-base assay, respectively [23,24]. Altogether, these results show that CDHT has selective inhibition for the iNOS isoform.

The selectivity of CDHT for iNOS isoform may be explained by considering the differences in dimer interfaces between

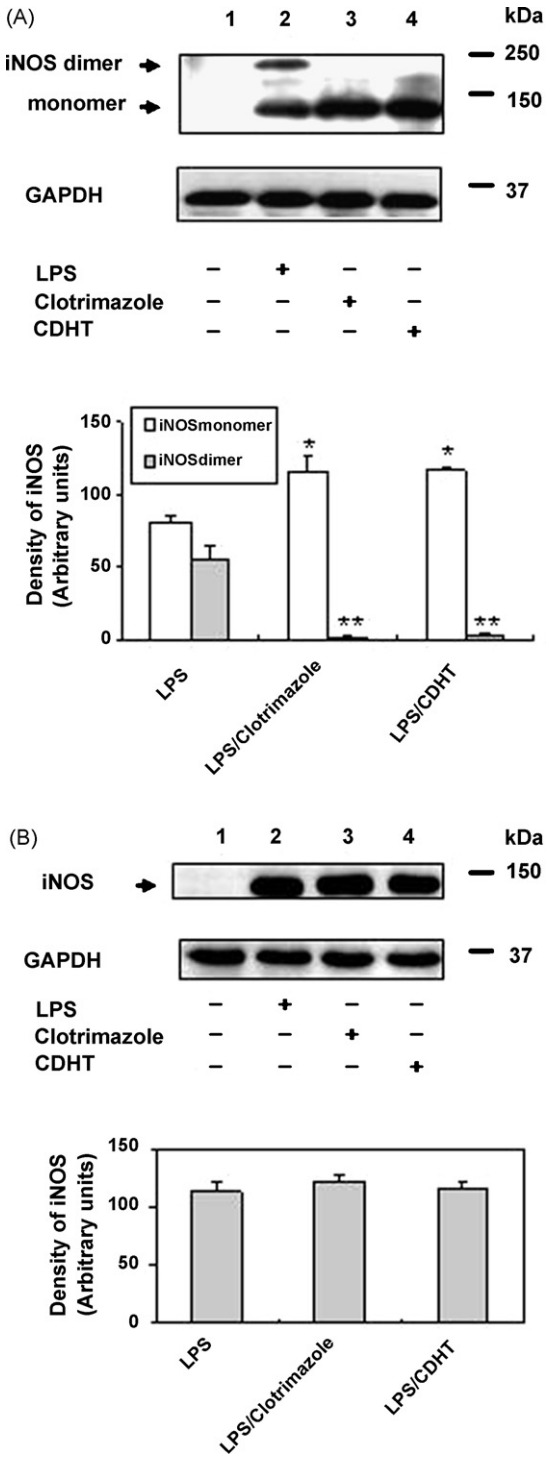

Fig. 5 – Western blot analysis of iNOS in BV-2 cells. BV-2 cells (5×10^4 cells/10 cm dish) were cultured under the conditions described in the legend to Fig. 2. Cells were harvested at 12 h after stimulation. Cell lysates were prepared from untreated cells (lane 1), cells treated with LPS alone (lane 2), cells treated with LPS and clotrimazole (20 μM) (lane 3), or cells treated with LPS and CDHT (15 μM) (lane 4). (A) Nondenatured cell lysates (5 μg of protein) were subjected to SDS-PAGE at 4 °C and western blot analysis. The positions of iNOS monomer and dimer are indicated based on protein size markers. Histograms show the densitometric analysis of iNOS protein expression normalized to GAPDH. (B) Heat-denatured cell lysates (5 μg of protein) were subjected to SDS-PAGE and western blot analysis. Histograms show the densitometric analysis of iNOS protein expression normalized to GAPDH. The values were represented as the mean ± S.D. obtained from three independent experiments. *$p < 0.001$ compared with iNOS monomer in LPS alone, **$p < 0.001$ compared with iNOS dimer in LPS alone.

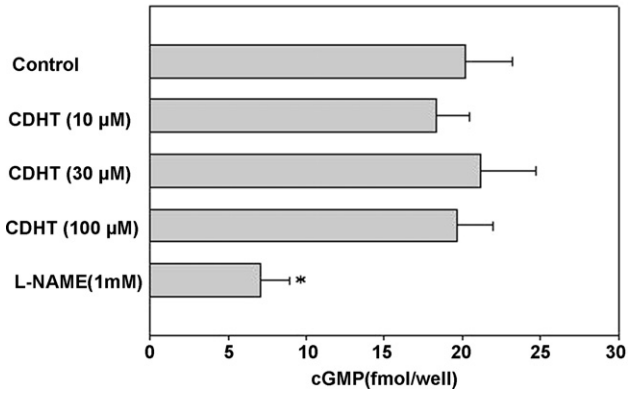

Fig. 6 – Effects of CDHT on cGMP production in HUVEC cells. HUVEC were cultured at an initial cell density of 1.25×10^3 cells/0.1 ml in EGM-2. After 48 h, the medium was replaced with EGM-2 containing 0.5 mM isobutylmethylxanthine and various concentrations of drugs. After 1 h, the cells were stimulated with 2 μM ionomycin for 4 h. Media were removed and the cGMP content was measured by an enzyme immunoassay system. The values were represented as the mean ± S.D. obtained from two independent experiments performed in triplicate. *$p < 0.01$ compared with the control.

iNOS and eNOS. Stuehr and co-workers in 2002 reported marked differences in the dimer interfaces among three NOS isoforms, in that the surface area buried in the eNOS dimer interface is larger than that in the iNOS dimer interface, and the dimer interfaces have great sequence variations among the three NOS isozymes. The crystal structure of the known inhibitor pyrimidineimidazole with the monomeric oxygenase domain of murine iNOS has shown that the imidazole moiety of pyrimidineimidazole coordinates directly to the heme and occupy the active site in the iNOS monomer in such a way to perturb key structural elements in the iNOS monomer that are critical for stable dimer formation [20]. Structurally, CDHT has one common feature with known iNOS dimerization inhibitors such as clotrimazole, BBS-1 and 2, PPA250, FR260330, and 2-imidazol-1-ylpyrimidine [19–24]. All of them are imidazole-containing structures except for FR260330.

All of the known iNOS dimerization inhibitors have been reported to be chemically synthesized. In addition, safety of those inhibitors remained unknown. CDHT, however, is one of the simple and cyclic dipeptides of natural origin. Cyclic dipeptides are among the simplest peptide derivatives commonly found in nature [31]. Most cyclic dipeptides appear to have emerged as by-products of food processing or metabolites of microorganisms. However, among them, a

few cyclic dipeptides have been known to show biological activities, including antibacterial and phytotoxic activities [34–36]. Synthetic histidine-containing diketopiperazines cyclo(-His–Ala) and cyclo(His–Gly) show anticancer and antithrombotic effects [37]. In humans, cyclo(His–Pro) is present as an endogenous neuropeptide [26]. Recently, cyclo(His–Pro) is reported to be related to glucose metabolism and to have antidiabetic activity [27,28].

In conclusion, CDHT, one of the simplest cyclic dipeptides, inhibits the production of NO in activated microglial BV-2 cells. Inhibition occurs due to the prevention of iNOS dimerization, an essential step for enzyme activity. Importantly, CDHT does not inhibit NO production by eNOS, suggesting that it is expected not to have side effects on vascular homeostasis. Thus, CDHT with a selective inhibition for the iNOS isoform could be a useful therapeutic agent for the treatment of inflammation-mediated diseases.

Acknowledgements

This work was supported by the 21C Frontier Microbial Genomics and Application Center Program, Ministry of Science & Technology (Grant MG05-0308-3-0), Republic of Korea.

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