Essential amino acid tryptophan inhibits induction of inducible nitric oxide synthase gene expression in interleukin-1β stimulated hepatocytes

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ABSTRACT

Background: Tryptophan exerts protective effects against a variety of organ inflammation and injury, including the liver. However, there are few scientific reports about the mechanisms involved with the action. Pro-inflammatory cytokine interleukin (IL)-1β stimulates the induction of inducible nitric oxide synthase (iNOS) expression and NO production in cultured hepatocytes ("in vitro liver injury model"). Additionally, the prevention of iNOS expression and NO production is an indicator of liver protection. Thus, the study aimed to examine whether tryptophan influences the induction of iNOS gene expression and the mechanisms.

Methods: Tryptophan was added into primary cultures of rat hepatocytes stimulated by IL-1β. The iNOS induction, NO production, and its signaling pathway were analyzed.

Results: IL-1β induced iNOS gene expression, which was followed by iNOS expression and NO production. Tryptophan inhibited the expression of iNOS mRNA and protein, in addition to decreasing the production of NO. Transfection experiments with iNOS promoter-luciferase
The essential amino acid tryptophan plays an important role in immune system regulation, protein synthesis, and serotonin and melatonin production, while also being a potent endogenous free radical scavenger and antioxidant [1, 2, 3]. Tryptophan demonstrated antioxidant activity in rats under experimental endotoxic shock and had protective effects on neuro-inflammation in rats after administering lipopolysaccharide [4, 5]. Surplus dietary tryptophan had limited effects on stress, immunology, behavior, and N retention in a pig model of systemic endotoxemia [6].

Inflammation is known to modify tryptophan metabolism which could impact tryptophan requirement for growth. Dietary tryptophan helped preserve tryptophan homeostasis in pigs suffering from lung inflammation [7]. Ulcerative colitis is the typical progression of chronic inflammatory bowel disease and amino acids have a therapeutic potential [8]. Tryptophan exhibited therapeutic function in a porcine model of dextran sodium sulfate-induced colitis [9]. Dietary tryptophan alleviated dextran sodium sulfate-induced colitis through aryl hydrocarbon receptor in mice [10]. Accumulated evidence indicates that amino acids, particularly tryptophan, exert a protective effect against a variety of organ inflammation and injuries. However, there are few scientific reports about the mechanisms involved in anti-inflammatory action of tryptophan on organ damage, including the liver.

In the injured liver, the production of nitric oxide (NO) by inducible nitric oxide synthase (iNOS) and inflammatory cytokines such as tumor necrosis factor (TNF)-α and interleukin (IL)-1β have been implicated as factors in liver injury [11, 12]. We have previously reported that IL-1β stimulated the gene expressions of iNOS, TNF-α and cytokine-induced neutrophil chemoattractant-1 (CINC-1)/chemokine (C-X-C motif) ligand 1 (CXCL1) (human IL-8 analogue) in primary rat hepatocyte cultures, which was followed by excess NO production (in vitro liver injury model) [13]. Using the “in vitro liver injury model,” a liver-protective effect of clinical drugs, traditional medicines, and functional foods may be determined. The
prevention of iNOS induction and NO production, as well as the inhibition of TNF-α expression, is an indicator of liver protection.

In the current study, we examined whether tryptophan inhibits iNOS induction and NO production in IL-1β-stimulated hepatocytes and, if so, what the mechanisms underlying the NO-inhibiting action (liver-protective effect) of tryptophan were.

MATERIALS AND METHODS

Materials
L-Tryptophan (nacalai tesque, Kyoto, Japan) was dissolved in the culture medium (0.5–2.5 mg/mL) before use. Recombinant human IL-1β (2 × 10^7 U/mg protein) was purchased from MyBioSource (San Diego, CA, USA). Male Wistar rats (200–250 g and 6–7 weeks old) were purchased from Charles River (Tokyo, Japan), kept at 22°C under a 12:12 h light:dark cycle with ad libitum access to food and water. All experiments on rats were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals of the National Institutes of Health. The experiments were approved by the Animal Care Committee of Kansai Medical University.

Primary hepatocyte culture
Hepatocytes were isolated from rats by perfusion with collagenase (Wako Pure Chemicals) [14], suspended in culture medium at 6 × 10^5 cells/mL, seeded into 35-mm plastic dishes (2 mL/dish; Falcon Plastic, Oxnard, CA, USA), and cultured at 37°C in a CO_2 incubator under a humidified atmosphere of 5% CO_2 in air. The culture medium was Williams’ E (WE) medium supplemented with 10% newborn calf serum, Hepes (5 mM), penicillin (100 U/mL), streptomycin (100 μg/mL), fungison (0.25 μg/mL), aprotinin (0.1 μg/mL; Roche, Mannheim, Germany), 10 nM dexamethasone, and 10 nM insulin. After 2 h, the medium (1.5 mL/dish) was replaced with fresh serum-free and hormone-containing WE medium (first medium change). After incubation for 5 h, the medium was replaced with fresh serum and hormone-free WE medium (second medium change) and the cells were cultured overnight. The number of cells attached to the dishes was calculated by counting the number of nuclei [15] and using a ratio of 1.37 ± 0.04 nuclei/cell (mean ± SE, n = 7 experiments).

Treatment of cells with tryptophan
On day 1, the cells were washed with fresh serum and hormone-free WE medium and incubated with IL-1β (1 nM) in the same medium in the presence or absence of tryptophan. The doses of tryptophan used are indicated in the appropriate figures and their legends.

Determination of NO production and lactate dehydrogenase activity
Culture medium was used for measurements of nitrite (a stable metabolite of NO) to reflect NO production by the Griess method [16]. Culture medium was also used for measurements
of lactate dehydrogenase (LDH) activity to reflect cell viability using a commercial kit (Roche Diagnostics, Mannheim, Germany).

**Western blot analysis**

Total cell lysates were obtained from cultured cells, as described previously [17] with minor modifications. Briefly, cells (1 × 10^6 cells/35 mm dish) were lysed with sample buffer for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (final: 125 mM Tris-HCl, pH 6.8; containing 5% glycerol, 2% SDS, and 1% 2-mercaptoethanol), subjected to SDS-PAGE, and electroblotted onto a polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA, USA). Immunostaining was performed using primary antibodies against mouse iNOS (Thermo Fisher Scientific (PA1-036), Rockford, IL, USA), human IκBα, mouse type I IL-1 receptor (IL-1RI) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and rat β-tubulin (internal control; Clone TUB2.1; Sigma Chemical Co., St. Louis, MO, USA). This was followed by visualization with an enhanced chemiluminescence blotting detection reagent (GE Healthcare Biosciences Corp., Piscataway, NJ, USA).

For Akt, total cell lysates prepared from 100-mm dishes (5 × 10^6 cells/dish) were pre-cleared with protein A (Sigma Chemical Co.) and then mixed with a mouse monoclonal antibody against human Akt1 (Akt5G3; Cell Signaling, Beverly, MA, USA) and protein G-Sepharose (Pharmacia LKB Biotech, Uppsala, Sweden). After incubation for 2 h at 4°C, immunocomplexes were centrifuged (16,000 g for 5 min). The beads were washed with solubilizing buffer, dissolved in SDS-PAGE sample buffer, and analyzed by western blotting using rabbit polyclonal antibodies against human Akt and phospho-Akt (Ser473) (Cell Signaling) as primary antibodies.

**Reverse transcriptase-polymerase chain reaction**

Total RNA was extracted from cultured hepatocytes using the guanidinium-phenol-chloroform method [18]. cDNA was synthesized from 1 μg total RNA of each sample with Oligo(dT)20 Primer (25 ng), 5×RT Buffer (5 μl), 10 mM dNTPs mixture (2.5 μl), RNase Inhibitor (0.5 μl), Rever Tra Ace (100 U/μl), and Ultra Pure distilled water (Invitrogen, USA): total 25 μl. The condition of thermal cycler (iCycler, Bio-Rad, CA, USA) were 42°C for 60 min and 95°C for 5 min.

Real-time PCR was performed using SYBR Green (Qiagen, Tokyo, Japan) and primers for each gene. Primer sequences were synthesized by Eurofins Genomics (Tokyo, Japan), which are shown in Table 1. The condition of thermal cycler (Rotor-Gene Q, Qiagen) were 95°C for 5 minutes followed by 40 cycles of 95°C for 5 seconds and 60°C for 10 seconds. Data collection and analyses were performed using the software included with the system. mRNA levels of each gene were measured as CT threshold levels and normalized to those of elongation factor-1α (EF; internal control). The cDNA for the rat iNOS mRNA was deposited in the DNA Data Bank of Japan (DDBJ)/European Bioinformatics Institute (EMBL-EBI)/GenBank under the accession numbers AB250951.
Table 1. Primers and nucleotide sequences for RT-PCR

<table>
<thead>
<tr>
<th>Primer</th>
<th>PCR forward primer/reverse primer</th>
<th>Amplification (bp)</th>
</tr>
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<tbody>
<tr>
<td>EF</td>
<td>5'-TCTGGTTGGAATGGTGACAACATGC-3'</td>
<td>332</td>
</tr>
<tr>
<td></td>
<td>5'-CCAGGAAGAGCTTCACCTCAAAGCTT-3'</td>
<td></td>
</tr>
<tr>
<td>iNOS</td>
<td>5'-CCAACCTGCAGGTCTTCGATG-3'</td>
<td>257</td>
</tr>
<tr>
<td></td>
<td>5'-GTCGATGCACAAACTGGGTGAAC-3'</td>
<td></td>
</tr>
<tr>
<td>TNF-α</td>
<td>5'-TCCCACAAGGAGGAAAGTTCC-3'</td>
<td>275</td>
</tr>
<tr>
<td></td>
<td>5'-GGCAGCCTTGCTCCCTGAAGAGA-3'</td>
<td></td>
</tr>
<tr>
<td>CINC-1</td>
<td>5'-GCCAAGCAGGCGGCGCCCGT-3'</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5'-ACTTGGGGGACCCTTTGACAT-3'</td>
<td>231</td>
</tr>
</tbody>
</table>

EF, elongation factor-1alpha; iNOS, inducible nitric oxide synthase; TNF-α, tumor necrosis factor-alpha; CINC-1, cytokine-induced neutrophil chemoattractant-1.

Transfection and luciferase assay

Transfection of cultured hepatocytes was performed as previously described [19,20,21]. Briefly, hepatocytes were cultured at $4 \times 10^5$ cells/dish (35 × 10 mm) in WE medium supplemented with serum, dexamethasone, and insulin for 7 h, before being subjected to magnet-assisted transfection (MATra). The reporter plasmid pRiNOS-Luc-SVpA or pRiNOS-Luc-3'UTR (1 μg) and the CMV promoter-driven β-galactosidase plasmid pCMV-LacZ (1 ng) as an internal control were mixed with the MATra-A reagent (1 μL; IBA GmbH, Göttingen, Germany). After incubation for 15 min on a magnetic plate at room temperature, the medium was replaced with fresh WE medium containing serum. Cells were cultured overnight and then treated with IL-1β in the presence or absence of tryptophan. Luciferase and β-galactosidase activities of cell extracts were measured using PicaGene (Wako Pure Chemicals) and Beta-Glo (Promega, Wisconsin, USA) kits respectively. The sequence of iNOS gene promoter was deposited in the DDBJ/EMBL-EBI/GenBank under the accession number AB290142.

Electrophoretic mobility shift assay

Nuclear extracts were prepared and an electrophoretic mobility shift assay (EMSA) was performed as previously described [22]. Briefly, nuclear extracts from hepatocytes (4 μg) were mixed with 1 μg of poly(dI-dC) and a probe for 20 min at room temperature (total mixture, 15 μL). To prepare a double-stranded DNA probe, annealed oligonucleotides harboring a κB site (5'-AGTTGAGGGACTTCCCAGGC-3'; only the sense strand is shown) were labelled with [$\gamma$$^{32}$P]-adenosine-5'-triphosphate (ATP; DuPont-New England Nuclear Japan, Tokyo, Japan) and T4 polynucleotide kinase (Takara Bio Inc., Shiga, Japan). Samples were resolved on a 4.8% polyacrylamide gel, followed by drying and autoradiography. Protein concentrations were measured using the method of Bradford [23] with a binding assay kit (Bio-Rad) using bovine serum albumin as a standard.
Statistical analysis: The results shown are representative of three to four independent experiments yielding similar findings. All data are expressed as the mean ± standard deviation (SD). Differences were analyzed by the Bonferroni–Dunn test. A value of $P < 0.05$ was considered to indicate a significant difference.

RESULTS

Tryptophan inhibits induction of iNOS gene expression in hepatocytes

The pro-inflammatory cytokine IL-1β stimulated the induction of iNOS gene expression, followed by iNOS mRNA and protein expressions and NO production. Simultaneous addition of tryptophan and IL-1β inhibited the expression of iNOS protein and production of NO in time and dose-dependent manners, showing the maximal effects at 2.5 mg/ml (Fig. 1A and 1B). Tryptophan reduced both expression of iNOS and TNF-α mRNA, but not of CINC-1 mRNA (Fig. 1C, 1D, and 1E). There were no cellular cytotoxic effects of tryptophan in the concentrations used as evaluated by the release of LDH into the culture medium (Fig. 2).

Figure 1. Effects of tryptophan on NO production, iNOS protein expression, and iNOS, TNF-α and CINC-1 mRNA expressions in hepatocytes. Cells were treated with interleukin (IL)-1β (1 nM) in the presence or absence of tryptophan (Trp). (A) Effects of Trp treatment (2.5 mg/mL) for the indicated times on nitric oxide (NO) production (IL-1β, open circles; IL-1β + Trp, filled circles; Trp, filled triangles; controls (without IL-1β and Trp), open triangles). (B) Effects of Trp treatment with various doses (0.5–2.5
mg/mL) for 8 h on NO production (upper) and iNOS protein expression (medium). Nitrite levels were measured in culture medium. Data are presented as the mean ± SD for n = 3 dishes/point. In the western blot panels, cell lysates (20 μg of protein) were subjected to SDS-PAGE in a 7.5% gel, and immunoblotted with an anti-iNOS or anti-β-tubulin antibody. (C-E) Total RNA was analyzed using strand-specific RT-PCR to detect (C) iNOS, (D) TNF-α and (E) CINC-1, using EF mRNA as an internal control. *P < 0.05 versus IL-1β alone.

**Figure 2.** Effects of tryptophan on cellular cytotoxicity. Cells were treated with IL-1β (1 nM) in the presence or absence of tryptophan (Try, 0.5–2.5 mg/mL) for 8 h. Lactate dehydrogenase (LDH) activity was measured in the culture medium (data are presented as the mean ± SD, n = 3 dishes/point).

Results indicated that tryptophan inhibited the expression of iNOS mRNA at the transcriptional or/and post-transcriptional steps. It is known that the mRNA levels of iNOS were regulated by promoter transactivation via transcriptional factors, such as nuclear factor (NF)-κB, and post-transcriptional modifications, such as mRNA stabilization [24]. Transfection experiments with iNOS promoter luciferase constructs revealed that tryptophan reduced the activities of both promoter transactivation (mRNA synthesis) and iNOS mRNA stability (Fig. 3).

**Figure 3.** Effects of tryptophan on iNOS promoter transactivation (iNOS mRNA synthesis and its stability) in hepatocytes. Two reporter constructs consist of the rat iNOS promoter (1.0 kb), a luciferase gene and the SV40 poly(A) region (pRiNOS-Luc-SVpA) or iNOS 3′-UTR (pRiNOS-Luc-3′UTR). The iNOS 3′-UTR contains AREs (AUUU(U)A × 6), which contribute to mRNA stabilization. Each construct was introduced into hepatocytes, and the cells were treated with IL-1β (1 nM) in the presence or absence of tryptophan (Trp, 2.5 mg/mL) for 8 h for pRiNOS-Luc-SVpA (A) and 4 h for pRiNOS-Luc-3′ UTR (B). Luciferase activity was normalized to β-galactosidase activity. Fold activation was calculated by dividing
Tryptophan inhibits activation of NF-κB and upregulation of type I IL-1 receptor (IL-1RI)

In the induction of iNOS, there are two essential signaling pathways, the IkB degradation/NF-κB activation through IkB kinase and IL-1RI upregulation through phosphatidylinositol 3-kinase (PI3K)/Akt [25].

Tryptophan had no effects on the degradation of IkB (Fig. 4A), but reduced NF-κB activation, its nuclear translocation from cytoplasm and DNA binding, at 3 h and afterwards (Fig. 4B). Tryptophan also inhibited the phosphorylation (activation) of Akt (Fig. 5A), down stream kinase of PI3K, which was followed by the reduction of IL-1RI protein expression (Fig. 5B).

**Figure 4.** Effects of tryptophan on degradation of IkBα and activation of NF-κB. Cells were treated with IL-1β (1 nM) in the presence or absence of tryptophan (Trp, 2.5 mg/mL) for the indicated times. (A) Cell lysates (20 μg of protein) were subjected to SDS-PAGE in a 12.5% gel, followed by immunoblotting with an anti-IkBα or anti- β-tubulin antibody. (B) NF-κB activation. Nuclear extracts (4 μg of protein) were analyzed using an electrophoretic mobility shift assay. Representative results of three independent experiments are shown. The bands corresponding to NF-κB were quantitated using densitometry (mean ± SD; n= 3 experiments). *P < 0.05 versus IL-1β alone.
**Figure 5.** Effects of tryptophan on upregulation of type I IL-1 receptor. Cells were treated with IL-1β (1 nM) in the presence or absence of tryptophan (2.5 mg/mL) for the indicated times. (A) Phosphorylation of Akt. Total cell lysates were immunoprecipitated with an anti-Akt antibody, followed by SDS-PAGE in a gel with a gradient of 6–9% and immunoblotting with an anti-phospho-Akt or anti-Akt antibody. (B) Cell lysates (40 μg of protein) were subjected to SDS-PAGE in a 7.5% gel, and immunoblotted with an anti-IL-1RI or anti-β-tubulin antibody. The bands corresponding to IL-1RI were quantitated using densitometry (mean ± SD; n= 3 experiments). *P < 0.05 versus IL-1β alone.

**Delayed administration of tryptophan inhibits iNOS induction**

Delayed administration of tryptophan at 1-4 h after IL-1β treatment significantly inhibited iNOS protein expression and NO production (Fig. 6).

**Figure 6.** Effects of delayed tryptophan administration on iNOS induction in hepatocytes. Cells were treated with tryptophan (Trp, 2.5 mg/mL) at 0-4 h after addition of IL-1β (1 nM). The effects of Trp on NO production (upper panel) and iNOS protein (middle panel) were analyzed at 8 h after addition of IL-1β. Nitrite levels were measured in the culture medium. Data are presented as the mean ± SD, n = 3 dishes/point; *P < 0.05 versus IL-1β alone. In the western blot panels, cell lysates (20 μg of protein) were subjected to SDS-PAGE in a 7.5% gel, and immunoblotted with an anti-iNOS or anti-β-tubulin antibody.
DISCUSSION

Our current study revealed that, by using pro-inflammatory cytokine IL-1β stimulated hepatocytes (“in vitro liver injury model”)[13], tryptophan inhibited the expression of iNOS gene expression, leading to the depression of iNOS mRNA and protein levels, resulting in the reduction of NO production, which is one of injury markers in the liver. Tryptophan blocked both of two essential signaling pathways in the induction of iNOS (Figs. 4 and 5) and decreased levels of iNOS mRNA, as well as TNF-α (Fig. 1D), through the inhibitions of mRNA synthesis and its stability (Fig. 3). Experiments with delayed administration of tryptophan demonstrated a significant decrease of NO production and iNOS induction (Fig. 6). The observation may be of clinical importance, since the initiation of therapeutic tryptophan treatment is usually delayed from the onset of diseases.

Inflammatory bowel disease (IBD), which includes both ulcerative colitis and Crohn's disease, is a chronic relapsing inflammation of the gastrointestinal tract that is difficult to treat. Animal models of IBD can be chemically induced and are used to study etiology and to evaluate potential treatments of IBD. Dextran sodium sulfate (DSS)-induced rat colitis is widely used as an experimental model for elucidating the etiology of ulcerative colitis. In our previous report, we demonstrated that it was important to elucidate the temporal and spatial dependence of inflammatory biomarkers, including iNOS and TNF-α, in the DSS colitis model to obtain a precise drug effect for ulcerative colitis [26]. Furthermore, we discovered that a mixture of lysine, tryptophan, histidine, and arginine markedly decreased mRNA expression of iNOS and TNF-α through the inhibition of NF-κB activation and increased survival rate in liver injury rats [27]. These results suggest that amino acids such as tryptophan have therapeutic potential for organ injuries, including the liver. Although recent studies in animal models have identified dietary amino acids which improve IBD amino acid supplementation may not be adequate to replace conventional therapy [8]. Further study with amino acids is needed to have a conclusion on the improvement of IBD.

These previous reports, in addition to our study, prompted us to examine whether amino acids including tryptophan supplementation in the diet is useful in patients with various diseases including liver and intestine.

CONCLUSION

Tryptophan inhibited iNOS gene expression at transcriptional and posttranscriptional steps in hepatocytes in an in vitro injury model. Tryptophan may have therapeutic potential not only for liver injury but also for a variety of organ diseases.

List of Abbreviations: iNOS, inducible nitric oxide synthase; NO, nitric oxide; IL-1β, interleukin-1β; NF-κB, nuclear factor-kappa B; IL-1RI, type I interleukin-1 receptor; TNF-α, tumor necrosis factor-alpha; CINC-1, cytokine-induced neutrophil chemoattractant-1.
Competing Interests: The authors declare that they have no conflicts of interest.

Authors’ Contributions: HH and TO participated in data collection, statistical analysis, and drafting of the manuscript. HM, RN, TS, and MH assisted in data collection and provided advice regarding development of the protocol for the study. MN, MK, and TO assisted in the design of the study, participated in supervision, and provided oversight when the manuscript was being drafted.

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