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Glutamic Acid Has a Liver-Protective Effect through the Suppression of Inducible Nitric Oxide Synthase in Primary Cultured Rat Hepatocytes

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Abstract:

Background: Theanine, which is hydrolyzed into glutamic acid (Glu), excerts beneficial effects on organ metabolism. However, there is little scientific evidence that Glu influences inflammatory mediator such as tumor necrosis factor (TNF)-α and nitric oxide (NO).In inflamed liver, proinflammatory cytokines stimulate liver cells, followed by the induction of inducible NO synthase (iNOS). Excessive NO produced by iNOS has been implicated as one of the factors in liver injury. Thus it is essential to inhibit iNOS induction for the prevention of liver injury. In this study, we examined interleukin (IL)-1β-stimulated hepatocytes as a simple "in vitro injury model" to investigate liver protective effects of Glu.

Methods: Primary cultured rat hepatocytes were treated with IL-1β in the presence or absence of Glu. Inflammatory biomarkers including iNOS and TNF-α were analyzed.

Results: Simultaneous addition of IL-1β and Glu decreased the expression levels of iNOS protein and its mRNA, resulting in the inhibition of NO production. Glu also reduced RNA expressions of TNF-α and IL-6. Glu inhibited two essential signaling pathways for iNOS induction, IκB/NF-κB activation and type I IL-1 receptor upregulation. Transfection experiments revealed that Glu reduced the levels of iNOSmRNA at the promoter activation and mRNA stabilization steps. Delayed administration of Glu after IL-1β addition also inhibited iNOS induction.

Conclusions: Glu influenced the induction of pro-inflammatory mediators, such as iNOS and TNF-α, in part through the inhibition of NF-κB activation in hepatocytes. Glu may have therapeutic potential for organ injuries including liver.

Keywords: *theanine, glutamic acid, inducible nitric oxide synthase, liver injury, primary cultured hepatocytes, nuclear factor-kB, type I interleukin-1 receptor, tumor necrosis factor-α.*

1. INTRODUCTION

It is important to prevent organ dysfunction and immune suppression through the reduction of excess inflammation in perioperative surgical management. Recent accumulated evidence indicates that

amino acids and their analogues are used for pre- and post-operative treatments in patients with digestive diseases. Oral administration of amino acids, cystine and theanine, has been shown to suppress increases in C-reactive protein (CRP) levels and neutrophil counts and decreases in the number of lymphocytes after excessive exercise, resulting in a reduction in inflammation and immune dysfunction [1]. Perioperative oral administration of cystine and theanine enhanced the recovery after distal gastrectomy[2].

Theanine (γ-ethylamino-L-glutamic acid) is a unique amino acid analogue of the proteinogenic amino acids, L-glutamic acid (Glu) and L-glutamine, and is also related to glutathione biosynthesis. Theanine is found almost exclusively in green tea that exerts beneficial effects on a variety of organ metabolism including brain [3,4].As an analog of Glu and glutamine, theanine is absorbed in the small intestine after oral ingestion; its hydrolysis to Glu and ethylamine occur both in the intestine and liver[5-7].

In hepatic disorders, the inflammatory cells such as macrophages gather around hepatic stellate (Kupffer) cells and discharge a variety of cytokines. During inflammation, pro-inflammatory cytokines and nitric oxide (NO) produced by inducible nitric oxide synthase (iNOS) in Kupffer cells and hepatocytes play an important role as factors in liver injury [8], although NO has been reported to exert either detrimental or beneficial effects, depending on the insults and tissues involved.

In animal liver injury models caused by various insults, such as ischemia-reperfusion, partial hepatectomy and endotoxin shock, we have previously reported that drugs showing liver-protective effects inhibited the induction of iNOS and NO production as well as decreased production of various inflammatory mediators, such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6 and cytokine-induced neutrophil chemo attractant (CINC)-1 (human IL-8 analogue) [9-13]. Furthermore, in *in vitro* experiments with primary cultured rat hepatocytes, these drugs also inhibited the induction of iNOS and the production of NO [11,14,15].

Thus, by using our cultured hepatocytes, the prevention of iNOS induction and NO production is considered to be one of the indicators of liver protection. In the present study, we examined IL-1β-stimulated cultured hepatocytes as a simple *in vitro* liver injury model. The objective of this study is to examine whether theanine hydrolyzed product, Glu, influences iNOS, and if so, the mechanisms involved in the action of Glu.

2. MATERIALS AND METHODS

2.1. Materials

Glu was dissolved in Williams' medium E (WE) and vortexed for 10 min at room temperature. The supernatant was filter-sterilized with a 0.45-μm membrane filter (Millipore, Billerica, MA, USA) prior to use in experiments. Recombinant human IL-1β (2×10^7 U/mg protein) was purchased from My Bio Source (San Diego, CA, USA). Male Wistar rats (200–250g and 6-7 weeks old)were purchased from Charles River (Tokyo, Japan), kept at 22°C under a 12:12 h light: dark cycle, and received food and water *ad libitum*. All animal experiments were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals of the National Institutes of Health, and approved by the Animal Care Committee of Kansai Medical University.

2.2. Primary Cultures of Hepatocytes

Hepatocytes were isolated from the rats by perfusion with collagenase (Wako Pure Chemicals, Osaka, Japan)[16]. Isolated hepatocytes were suspended in culture medium at 6 x 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 WE supplemented with 10% newborn calf serum, 10 nM insulin and 10 nM dexamethasone. After 5 h, the medium was replaced with fresh serum- and hormone-free WE, and the cells were cultured overnight before use in experiments. The numbers of cells attached to the dishes were calculated by counting the number of nuclei [17] and using a ratio of 1.37 ± 0.04 nuclei/cell (mean ± SE, n = 7 experiments).

2.3. Treatment of Cells with Glutamic Acid

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

2.4. Determination of NO Production and Lactate Dehydrogenase (LDH) Activity

Culture medium was used for measurements of nitrite (a stable metabolite of NO) to reflect NO production by the Griess method [18]. Culture medium was also used for measurements of LDH activity to reflect cell viability using a commercial kit (Roche Diagnostics, Mannheim, Germany).

2.5. Western Blot Analysis

Total cell lysates were obtained from cultured cells as described previously with minor modifications [14]. Briefly, cells (1 x 10^6 cells/35mm dish) were lysed with sample buffer for sodium dodecyl sulphate–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 electro blotted onto a polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA, USA). Immunostaining was performed using primary antibodies against mouse iNOS (Affinity Bio Reagents, Golden, CO, USA), human phospho-IkBα (Ser32/36 [5A5]; Cell Signaling, Beverly, MA, USA), human IkBα, 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), followed by visualization with an enhanced chemiluminescence (ECL) blotting detection reagent (GE Healthcare Biosciences Corp., Piscataway, NJ, USA).

For Akt, total cell lysates prepared from 100 mm dishes (5 x 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) and Protein G-Sepharose (Pharmacia LKB Biotech, Uppsala, Sweden). After incubation overnight at 4°C, immune complexes 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 (SDS-PAGE in a gradient 6-9% gel, Multigel II mini (Cosmo Bio Co., Ltd., Tokyo, Japan)) using rabbit polyclonal antibodies against human Akt and phospho- (Ser473) Akt (Cell Signaling) as primary antibodies. In the case of p65, nuclear extracts were immune precipitated with an anti-p65 antibody (H286; Santa Cruz Biotechnology). The bands were analyzed by western blotting (SDS-PAGE in a gradient 6-9% gel, Multi gel II mini (Cosmo Bio Co., Ltd.)) using an antibody against human NF-kB p65 (BD Transduction Laboratories, Lexington, KY, USA).

2.6. Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted from cultured hepatocytes using a guanidinium-phenol-chloroform method [19]. For strand-specific RT-PCR analysis, cDNAs were synthesized from total RNA with strand-specific primers, and step-down PCR was performed using PC708 (Astec, Fukuoka, Japan), as

previously described [20], with minor modifications. For iNOS (257bp), TNF-α (275bp), CINC-1 (231 bp), IL-6 (286 bp), IL-1RI (327bp) and elongation factor-1α (EF; internal control) (335 bp) mRNAs, an oligo (dT) primer was used for RT and the indicated primers sets were used for PCR (Table 1). For the antisense transcript of iNOS (211 bp), the sense primer (5'-TGCCCCTCCCCCACATTCTCT -3') was used for RT and the indicated primer set was used for PCR (Table 1). These mRNAs and antisense transcript levels were measured by real-time PCR using a Rotor-Gene Q 2plex HRM (QIAGEN, Tokyo, Japan). Rotor-Gene SYBR Green PCR Kit (QIAGEN) was included in the reaction mixture, and the following touchdown protocol was applied: 1 cycle at 95°C for 5 min and 45 cycles at 95°C for 5 s and 60°C for 10 s. The cDNAs for the rat iNOS mRNA and antisense transcript were deposited in the DNA Data Bank of Japan/European Bioinformatics Institute (DDBJ/EMBL)/GenBank under Accession numbers AB250951 and AB250952, respectively.

Table1. *Primers and nucleotide sequences*

Primer name	Nucleotide sequence
iNOS F/R	5' CCAACCTGCAGGTCTTCGATG 3'/5' GTCGATGCACAACTGGGTGAAC 3'
as iNOS F/R	5' CCTTTGCCTCATACTTCCTCAGA 3'/5'ATCTTCATCAAGGAATTATACACGG 3'
TNF-α F/R	5' TCCCAACAAGGAGGAGAAGTTCC 3'/5' GGCAGCCTTGTCCCTTGAAGAGA 3'
IL-6 F/R	5' GAGAAAAGAGTTGTGCAATGGCA 3'/5' TGAGTCTTTTATCTCTTGTTTGAAG 3'
CINC-1 F/R	5' GCCAAGCCACAGGGGCGCCCGT 3'/5' ACTTGGGGACACCCTTTAGCATC 3'
IL-1RI F/R	5'-CGAAGACTATCAGTTTTTGGAAC-3'/5'-GTCTTTCCATCTGAAGCTTTTGG-3'
EF F/R	5' TCTGGTTGGAATGGTGACAACATGC 3'/5' CCAGGAAGAGCTTCACTCAAAGCTT 3'

iNOS, inducible nitric oxide synthase; as, antisense transcript; TNF-α, tumor necrosis factor-alpha; IL-6, interleukin-6; CINC-1, cytokine induced-neutrophil chemoattractant-1; IL-1R1, type I IL-1 receptor; EF, elongation factor-1α; F/R, forward/reverse.

2.7. Electrophoretic Mobility Shift Assay (EMSA)

Nuclear extracts were prepared, and EMSA was performed as previously described [21]. 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 harbouring a κB site (5'-AGTTGAGGGGACTTTCCCAGGC-3'; only the sense strand is shown) were labelled with [γ-^{32}P]-Adenosine-5′-triphosphate (ATP; DuPont-New England Nuclear Japan, Tokyo, Japan) and T4 polynucleotide kinase (Takara Bio Inc.). Samples were resolved on a 4.8% polyacrylamide gel, followed by drying and autoradiography. The protein concentration was measured by the method of Bradford [22]with a binding assay kit (Bio-Rad) using bovine serum albumin as a standard.

2.8. Transfection and Luciferase Assay

Transfection of cultured hepatocytes was performed as described previously [23]. Briefly, hepatocytes were cultured at 4 x 10^5 cells/dish (35 x 10 mm) in WE supplemented with serum, dexamethasone and insulin for 7 h, before being subjected to magnet-assisted transfection (MATra). Reporter plasmids 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 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 containing serum. Cells were cultured overnight, and then treated with IL-1β in the presence or absence of Glu. The luciferase and β-galactosidase activities of cell extracts were measured using PicaGene (Wako Pure Chemicals) and Beta-Glo (Promega) kits, respectively.

2.9. Statistical analysis

The results shown are representative of three to four independent experiments yielding similar findings. All data were expressed as means ± SD. Differences were analyzed by the Bonferroni-Dunn test, and values of $P<0.05$ were considered to indicate significant differences.

3. RESULTS

3.1. Glu Inhibits NO Production and iNOS Induction in IL-1β-Stimulated Hepatocytes

The pro-inflammatory cytokine IL-1β stimulated the induction of iNOS, which was followed by the production of NO in primary cultured rat hepatocytes [24]. The simultaneous addition of Glu and IL-1β reduced the levels of nitrite (a stable metabolite of NO) time- and dose-dependently in the culture medium (Fig. 1A and 1B). Glu showed more than 90% inhibition at 10 mg/ml. Glu had no cellular cytotoxicity within the indicated concentrations, as evaluated by the release of LDH into the culture medium (Fig. 2) and Trypan blue exclusion by hepatocytes (data not shown).

Western blotting analysis revealed that Glu dose-dependently reduced the expression of the iNOS protein, showing its maximal effect at 10 mg/mL (Fig. 1B). RT-PCR analysis demonstrated that Glu decreased the expression of iNOS mRNA time-dependently (Fig. 1C). These results suggested that Glu inhibited the induction of iNOS gene expression at a transcriptional and/or post-transcriptional step.

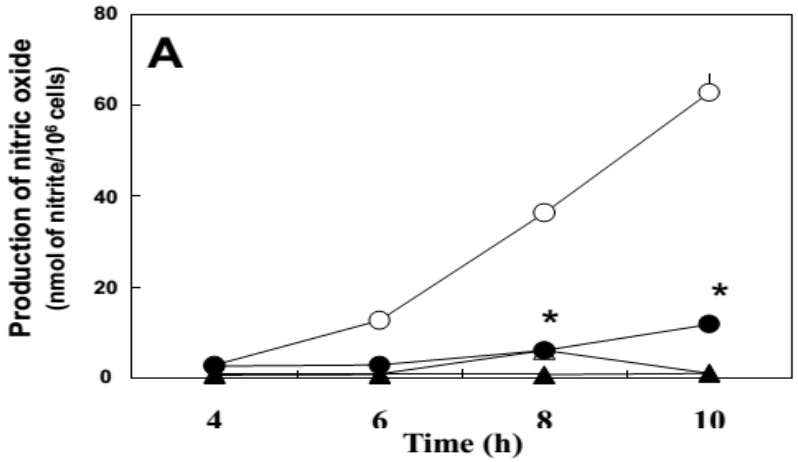

Fig1A. *(Nakatake)*

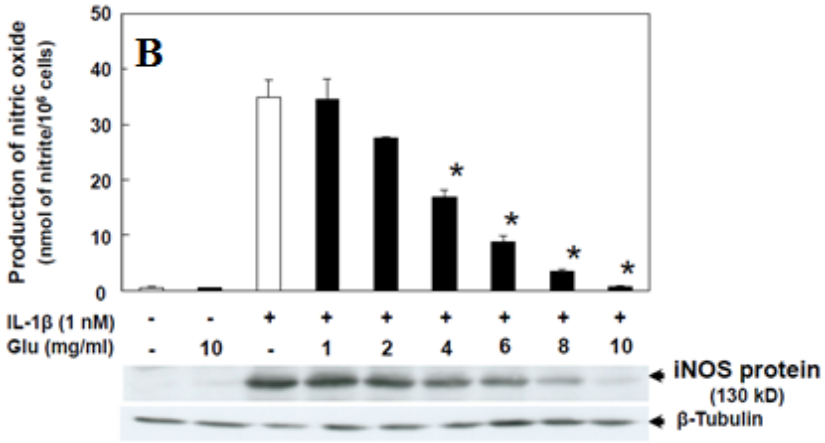

Fig1B. *(Nakatake)*

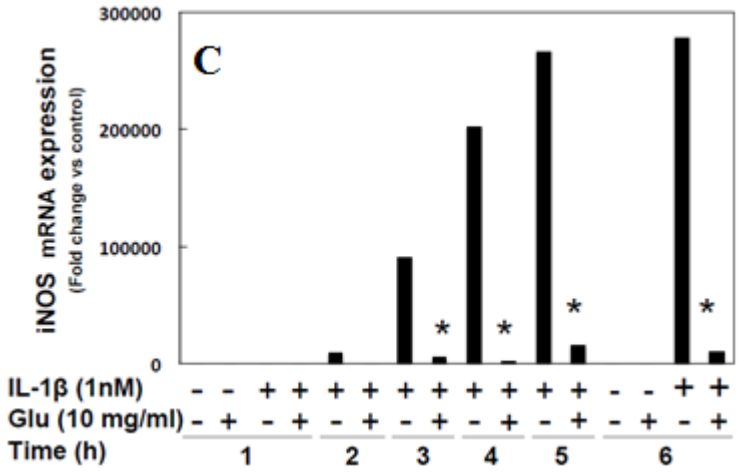

Fig1C. *(Nakatake)*

Fig1. *Effects of glutamic acid on the induction of NO production and iNOS in IL-1β-stimulated hepatocytes.*

*Cultured hepatocytes were treated with interleukin (IL)-1β (1 nM) in the presence or absence of Glu (1–10 mg/mL). (A) Effect of Glu (10 mg/mL) treatment for the indicated times on nitric oxide (NO) production (IL-1β, open circles; IL-1β + Glu, filled circles; Glu, filled triangles; controls (without IL-1β and Glu, open triangles). (B) Effects of treatment with various doses of Glu (1–10 mg/mL) for 8 h on NO production (upper) and inducible nitric oxide synthase (iNOS protein, medium). The levels of nitrite were measured in the culture medium (data are means ± SD for n = 3 dishes/point; * P<0.05 versus IL-1β alone). In the western blotting panels, cell lysates (20 μg of protein) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in a 7.5% gel, and immunoblotted with an anti-iNOS or anti-β-tubulin antibody. (C) Effects of Glu (10 mg/mL) treatment for the indicated times on the expression of iNOS mRNA. Total RNA was analyzed by strand-specific RT-PCR to detect iNOS mRNA, using EF mRNA as an internal control.*

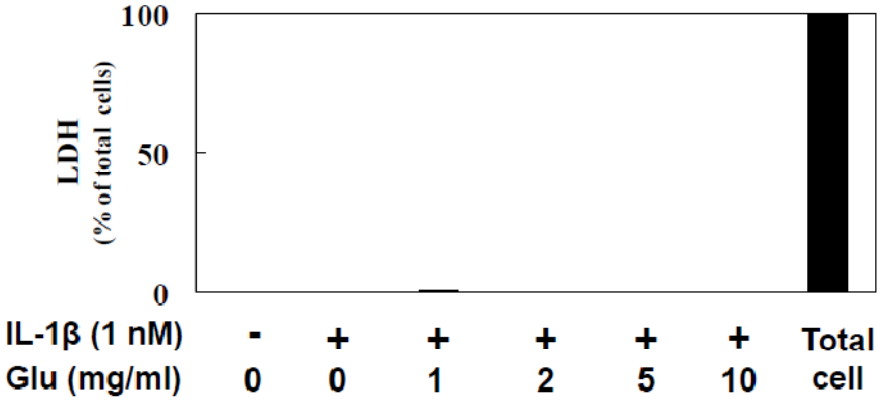

Fig2. *(Nakatake)*

Fig2. *Effects* of glutamic acid on cellular cytotoxicity.

The cells were treated with IL-1β (1 nM) in the presence or absence of Glu (1–10 mg/mL) for 8 h. Lactate dehydrogenase (LDH) activity was measured in the culture medium (data are means ± SD, n = 3 dishes/point).

3.2. Glu Influences mRNA Expression of Proinflammatory Cytokines

The expression of other mRNA was examined. IL-1β increased the levels of pro-inflammatory cytokine expression, TNF-α [25], CINC-1 and IL-6, and Glu decreased the expression of TNF-α and IL-6 mRNA, but not of CINC-1 (Fig. 3).

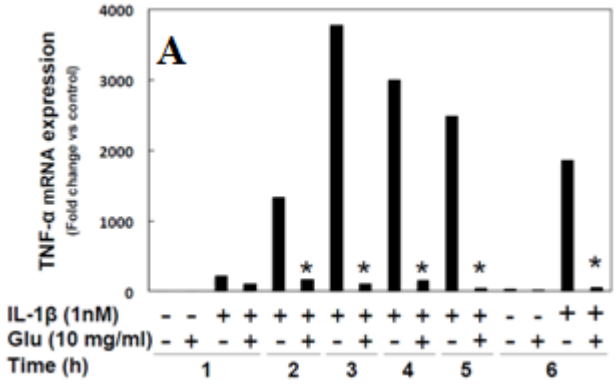

Fig3A. *(Nakatake)*

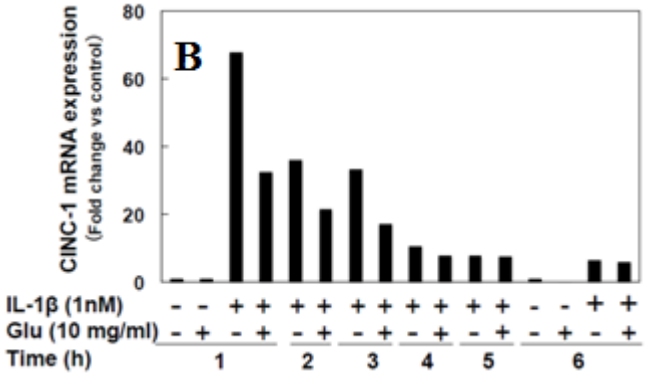

Fig3B. *(Nakatake)*

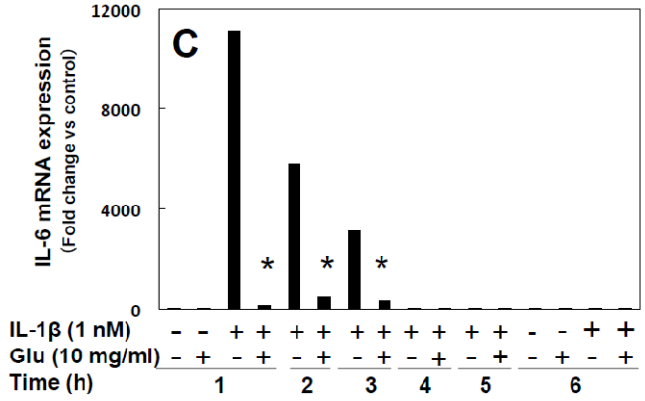

Fig3C. *(Nakatake)*

Fig3. *Effects of glutamic acid on expression of TNF-α, CINC and IL-6 mRNA.*

The cells were treated with IL-1β (1 nM) in the presence or absence of Glu (10 mg/mL) for the indicated times. Total RNA was analyzed by strand-specific RT-PCR to detect TNF-α, CINC-1 and IL-6, using EF mRNA as an internal control.

3.3. Glu Decreases iNOS mRNA Synthesis and Stabilization

We examined the mechanisms involved in the inhibition of iNOS induction. The expression of iNOS mRNA is regulated by iNOS promoter transactivation with transcription factors such as NF-κB and by post-transcriptional modifications such as mRNA stabilization [26]. Therefore, we carried out transfection experiments with constructs containing firefly luciferase controlled by the iNOS promoter (pRiNOS-Luc-SVpA and pRiNOS-Luc- 3′UTR) (Fig. 4A), which detect iNOS promoter transactivation (mRNA synthesis) and mRNA stabilization, respectively [27]. IL-1β increased the

luciferase activities of these constructs, and these effects were significantly inhibited by Glu (Fig. 4B and 4C). Furthermore, iNOS antisense transcript (asRNA) analysis by RT-PCR revealed that IL-1β increased the expression of iNOS asRNA in a time-dependent manner, and that Glu markedly inhibited this effect (Fig. 4D).

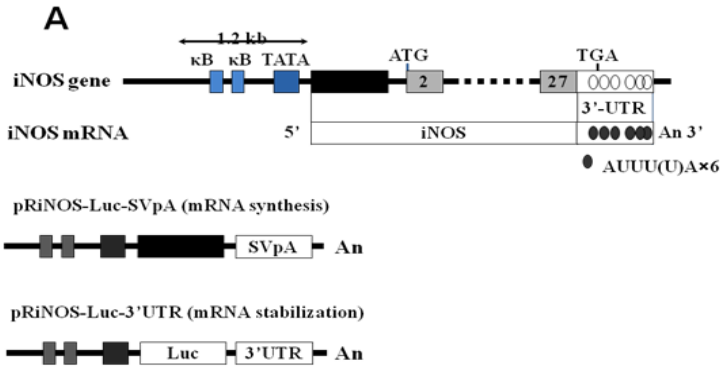

Fig4A. *(Nakatake)*

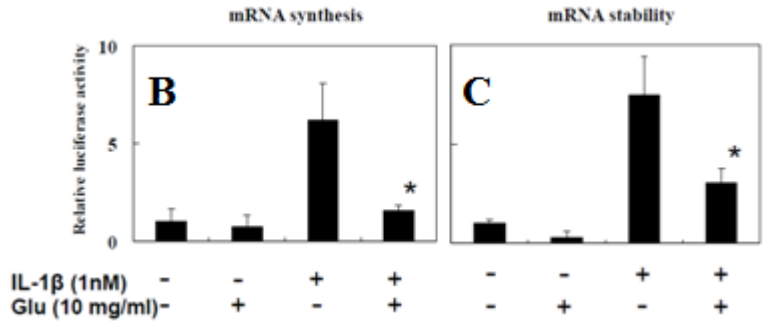

Fig4B & 4C. *(Nakatake)*

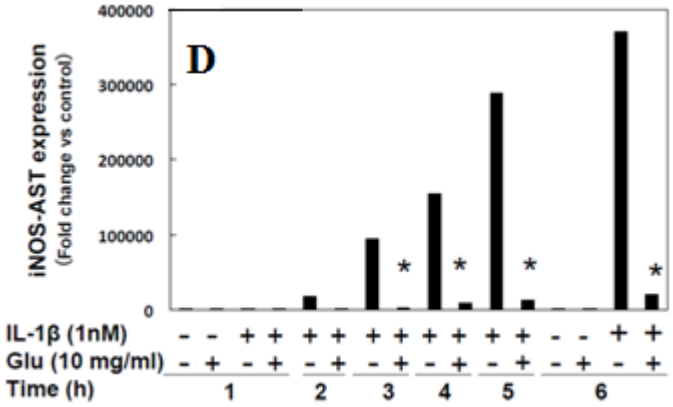

Fig 4D.

Fig4. *Effects of glutamic acid on the transactivation of the iNOS promoter and the expression of the iNOS gene antisense transcript.*

(A) Schematic representation of the promoter region of the iNOS gene. Two reporter constructs are shown beneath the iNOS gene and mRNA. The constructs consist of the rat iNOS promoter (1.2 kb), a luciferase gene and the SV40 poly(A) region (pRiNOS-Luc-SVpA) or iNOS 3'-UTR (pRiNOS-Luc-3'UTR). 'An' indicates the presence of a poly(A) tail. The iNOS 3'-UTR contains AREs (AUUU(U)A × 6), which contribute to mRNA stabilization. (B, C) Each construct was introduced into hepatocytes, and the cells were treated with IL-1β (1 nM) in the presence or absence of Glu (10 mg/mL) for 8 h for pRiNOS-Luc-SVpA (B) and 5 h for pRiNOS-Luc-3'UTR (C). The luciferase activities were normalized by the β-galactosidase activity. The fold activation was

*calculated by dividing the luciferase activity by the control activity (without IL-1β and Glu). Data are means ± SD for n = 4 dishes. *P<0.05 versus IL-1β alone. (D) The cells were treated with IL-1β (1 nM) in the presence or absence of Glu (10 mg/mL) for the indicated times. Total RNA was analyzed by strand-specific RT-PCR to detect the iNOS gene antisense transcript (asRNA).*

3.4. Glu Inhibits NF-κB Activation and IL-1RI Upregulation

There are two essential signaling pathways for iNOS induction, IκB kinase and phosphatidylinositol 3-kinase (PI3K)/Akt pathways [28]. In the former, IL-1β stimulates the degradation of IκB proteins after the phosphorylation by IκB kinase, which is followed by the activation of NF-κB (i.e. translocation from the cytoplasm to the nucleus and DNA binding). Glu enhanced the degradation of of IκBα after IL-1β stimulation, rather than its inhibition of IκBα degradation (Fig. 5A). However, electro phoretic mobility shift assay with nuclear extracts revealed that Glu inhibited NF-κB activation at 2–4 h, indicating the blockade of NF-κB nuclear translocation (Fig. 5B). In support of this observation, immune precipitation and western blotting of nuclear extracts showed that Glu inhibited nuclear translocation of NF-κB subunit p65 (Fig. 5C).

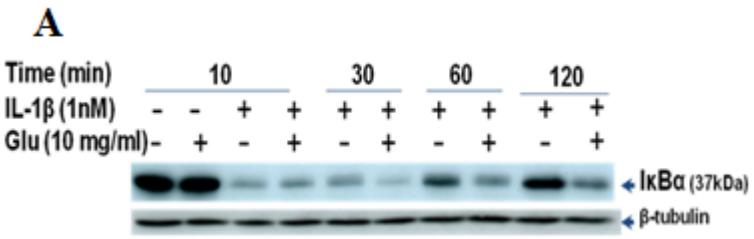

Fig5A. *(Nakatake)*

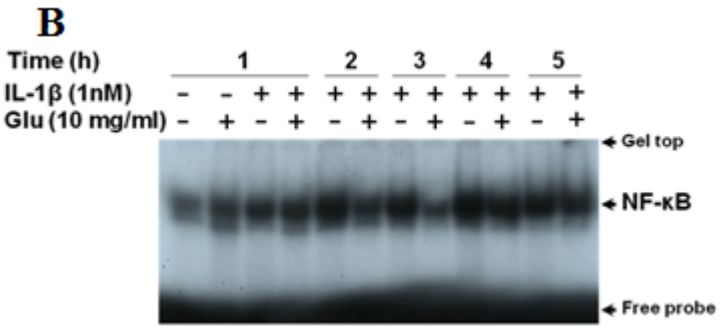

Fig5B. *(Nakatake)*

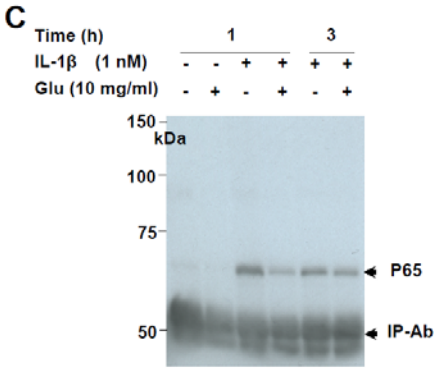

Fig5C. *(Nakatake)*

Fig5. *Effects of glutamic acid on the degradation of IκBα and the activation of NF-κB.*

The cells were treated with IL-1β (1 nM) in the presence or absence of Glu (10 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 immune blotting with an anti-IκBα or anti-β-tubulin antibody. (B) Activation of NF-κB. Nuclear extracts (4 μg of protein) were analyzed by an electrophoretic mobility shift assay. Representative results of three independent experiments are shown. (C) Nuclear translocation of NF-kB subunit p65. Nuclear extracts were immune precipitated, and the immune precipitates were analyzed by western blotting (SDS-PAGE in a gradient 6-9% gel) with an anti-p65 antibody.

In the latter, IL-1β stimulates the upregulation of IL-1RI through the activation of PI3K/Akt[28]. Immunoprecipitation-western blotting analysis revealed that Glu didn't inhibit the phosphorylation (activation) of Akt, a downstream kinase of PI3K (Fig. 6A). In contrast, RT-PCR and western blotting analyses revealed that Glu reduced both levels of IL-1RI mRNA and protein expression (Fig. 6B and 6C).

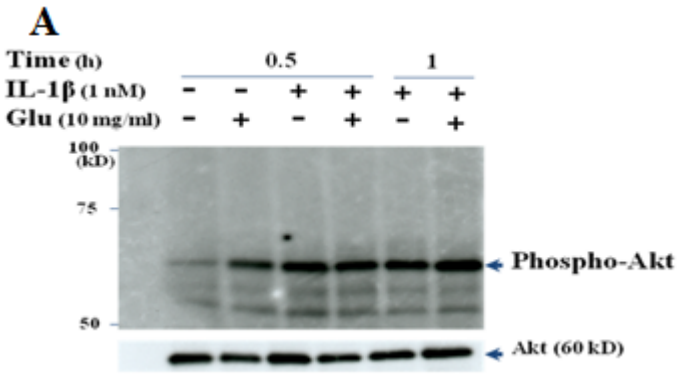

Fig6A. *(Nakatake)*

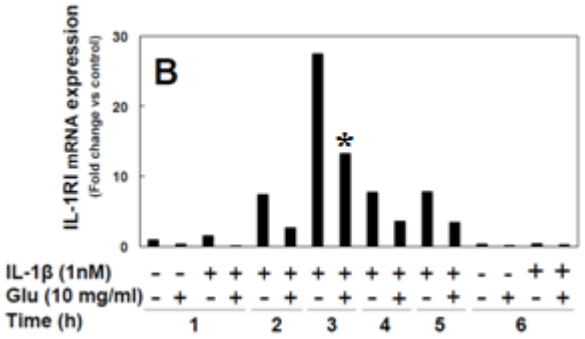

Fig6B. *(Nakatake)*

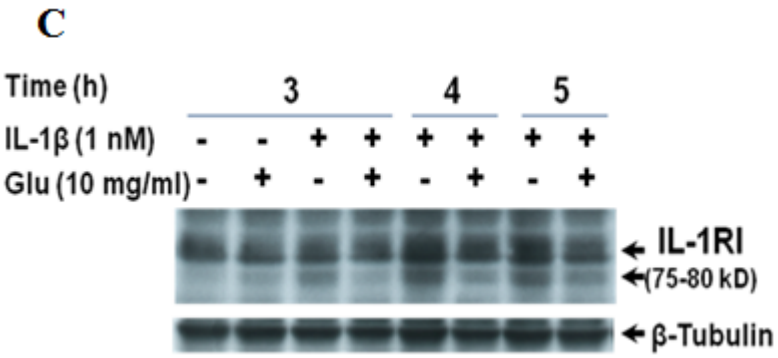

Fig6C. *(Nakatake)*

Fig6. *Effects of glutamic acid on the upregulation of type I IL-1 receptor.*

The cells were treated with IL-1β (1 nM) in the presence or absence of Glu (10 mg/mL) for the indicated times. (A) Phosphorylation of Akt. Total cell lysates were immune precipitated with an anti-Akt antibody, followed by immune blotting (SDS-PAGE in a gradient 6-9% gel)with an anti-phospho-Akt or anti-Akt antibody. (B) Total RNA was analyzed by strand-specific RT-PCR to detect the type I IL-1 receptor (IL-1RI) mRNA, using EF mRNA as an internal control. (C) Cell lysates (50 μg of protein) were subjected to SDS-PAGE in a 7.5% gel, and immune blotted with an anti-IL-1RI or anti-β-tubulin antibody.

3.5. The Delayed Administration of Glu Inhibits iNOS Induction

We examined whether the delayed administration of Glu influences iNOS induction. Glu was added to the medium 0–4 h after the addition of IL-1β. The delayed administration of Glu up to 3 h after IL-1β addition still markedly inhibited NO production, although the magnitude of inhibition decreased in a time-dependent manner (Fig. 7).

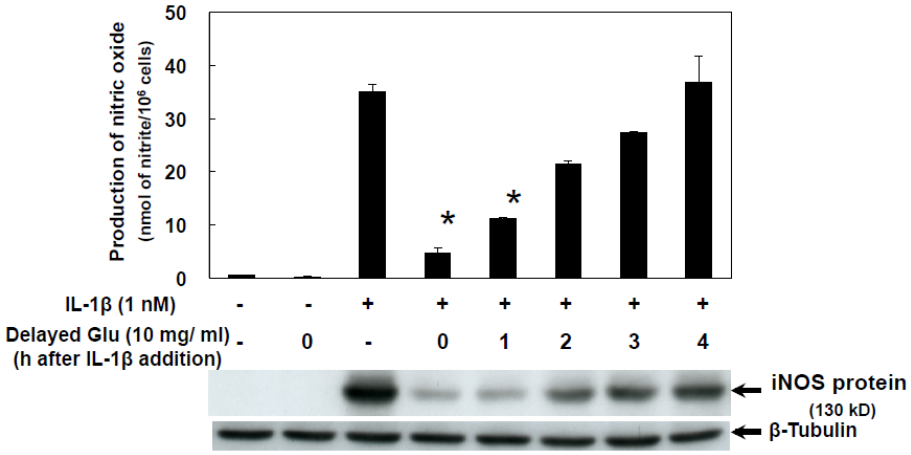

Fig7. *(Nakatake)*

Fig7. *Effects of delayed glutamic acid administration on the induction of iNOS in hepatocytes.*

*The cells were treated with Glu (10 mg/mL) at 0–4 h after the addition of IL-1β (1 nM). The effects of Glu on NO production (upper) and iNOS protein (lower) were analyzed at 8 h after IL-1β addition. The levels of nitrite were measured in the culture medium (data are means ± SD, n = 3 dishes/point; *P<0.05 versus IL-1β alone). In the western blotting panels, cell lysates (20 μg of protein) were subjected to SDS-PAGE in a 7.5% gel, and immune blotted with an anti-iNOS or anti-β- tubulin antibody.*

4. DISCUSSION

In this study, we found that Glu inhibited the induction of iNOS and even TNF-α and IL-6 in IL-1β stimulated hepatocytes, indicating that Glu may have the anti-inflammatory effects in the liver.

It is known that the induction of iNOS gene expression is regulated by iNOS promoter transactivation and by post-transcriptional modifications [26]. NF-κB plays a key role in inflammation by regulating genes encoding iNOS and pro-inflammatory cytokines such as TNF-α [29]. NF-κB typically exists in the form of p50/65 heterodimers attached to its inhibitory proteins (IκBs, IκBα and IκBβ) in the cytoplasm of cells. The activation of NF-κB involves i) the proteolytic degradation of IκBs in proteosome after the phosphorylation by IκB kinase, ii) the translocation of NF-κB to the nucleus and iii) its binding to the promoter κBsite [30]. Glu inhibited NF-κB activation (Fig. 5B) and the translocation of p65 to the nucleus (Fig. 5C), although Glu had no effect on the degradation of IκBα (Fig. 5A). In concert with NF-κB activation, the upregulation of IL-1RI through the activation of PI3K/Akt is also essential for iNOS induction [28]. We found that Glu decreased the expression of IL-

1RI mRNA and protein (Fig. 6B and 6C). However, Glu had no effect on the activation of Akt, up-stream kinase in this signal.

By our primary cultures of rat hepatocytes, we have found that dexamethasone [31], sivelestat (neutrophil elastase inhibitor) [32], adenosine [33], Japanese Kampo medicines (inchinkoto (TJ-135) [34], hochuekito (TJ-41) [35] and saireito (TJ-114) [36]) inhibited NF-κB activation, resulting in the inhibition of iNOS induction and NO production. We expected that these drugs and Kampo medicines might inhibit IκBα degradation, leading to the inactivation of NF-κB nuclear translocation. However, they had no effects on the degradation of IκBα as similar to the effect of Glu. The mechanisms involved in the inhibitory action of Glu on NF-κB activation remained unclear. It cannot negate the possibility that Glu and other drugs may influence the modification of NF-κB molecules (p50 and p65) including their phosphorylation, leading to the reduction of NF-κB activation. In the case of Akt activation, we have found that sivelestat [32], inchinkoto[34], hochuekito [35] and saireito [36] reduced the phosphorylation of Akt, decreasing the expression of IL-1RI mRNA and its protein. In contrast, dexamethasone [31] and adenosine [33] had no effects on Akt phosphorylation as similar to Glu. Thus the mechanisms involved in the depression of IL-1RI induction by dexamethasone, adenosine and Glu are unclear at present.

In experiments with iNOS promoter constructs, Glu was found to inhibit iNOS induction at both of its mRNA synthesis and stabilization steps (Fig. 4). Regarding iNOS mRNA stabilization, the 3′-UTR of the iNOS mRNA in rats has six adenine/uracil (AU)-rich elements (ARE) that are associated with ARE-binding proteins such as HuR and heterogeneous nuclear ribonucleoproteins L/I (PTB), which serve to stabilize the mRNA[37]. Recently, we have reported that antisense (as)RNAs are often transcribed from many induciblegenes, such as iNOS and TNF-α [28,38]. The iNOS asRNA interacts with and stabilizes iNOS mRNA [39, 40]. We have reported that drugs such as edaravone (free radical scavenger) [15], FR183998 (Na^+/H^+ exchanger inhibitor) [11,13], insulin growth factor I[12], sivelestat [32], inchinkoto [34], hochuekito [35] and saireito [36] inhibited iNOS induction partly by suppressing iNOS asRNA production in animal models and/or primary cultured hepatocytes. In this study, Glualso decreased the iNOS asRNA expression (Fig. 4D).

These results demonstrate that Glu inhibits two essential signaling pathways, NF-κB activation and IL-1RI upregulation in the induction of iNOS. Glu probably reduced iNOS mRNA expression through the inhibition of its mRNA synthesis and stabilization, leading to the reducton of iNOS protein and NO production.

During endotoxemia, pro-inflammatory cytokines such as TNF-α, IL-1β and IL-6 are considered to play an important role in the injury to multiple organs in addition to the liver. TNF-α is a key mediator of the cytokine cascade in sepsis [41,42]. Inflammation is a biological response that helps to maintain homeostasis, but excessive inflammation following excessive stress causes organ damage and compromised immune function. Therefore, the inhibition of TNF-α expression is important in anti-inflammatory therapy. IL-6 is a representative pro-inflammatory cytokine that is produced by macrophages and lymphocytes in response to invasion, and its level indicates the strength of a systemic inflammatory reaction. In addition, IL-6 itself promotes the production of acute-phase proteins in the liver, thereby acting as a mediator and promoting further inflammatory reactions [43].

In our *in vitro* model, we have recently reported that IL-1β stimulated the induction of TNF-α in parallel with the induction of iNOS [25]. In this study, Glu decreased the mRNA expression of TNF-α and IL-6 (Fig. 3A and 3C). This observation suggested that Glu could inhibit the induction of inflammatory mediators (iNOS, TNF-α and IL-6), resulting in the liver-protective effects.

The delayed treatment with Glu after IL-1β addition was found to cause a significant reduction in NO production and iNOS induction (Fig. 7). This observation may be of clinical importance, since the initiation of therapeutic Glu treatment is usually delayed from the onset of diseases. Appropriate regulation of inflammatory reactions during the perioperative period is important in preventing the onset of organ damage and infectious complications, achieving a stable postoperative course and early recovery. From this perspective, an immune modulating diet, including ω-3 fatty acids, that aims to reduce excessive inflammation during the perioperative period has been used in clinical settings [44].

Our simple *in vitro* experiment with cultured hepatocytes may be adequate for screening of liver-protective drugs, because it is rapid and inexpensive compared with *in vivo* animal models of liver injury. However, there are a variety of factors involved in liver injury in addition to iNOS and pro inflammatory cytokines. Thus a liver-protective effect in drugs deduced from this model need to be examined and supported in *in vivo* animal models.

Cystine and theanine are both involved in the synthesis of glutathione (GSH), which is a tripeptide consisting of Glu, cysteine and glycine. Hepatic GSH concentrations increased after cystine and theanine administration in mice [45-47]. GSH is the important member of immune system as reported previously. In addition to its strong antioxidant effect, GSH plays crucial role, particularly in the maintenance and regulation of the thiolredox status of the cell and these appear to correlate with proliferation or differentiation of lymphocytes, macrophages, and dendritic cells [48-53]. Cystine is a sulfur-containing amino acid that consists of 2 cysteine molecules joined by a disulfide bond. Transporters on cell membranes (including those of neutrophils, macrophages and dendritic cells) mediate the uptake of cystine into cells and then thioredoxin rapidly reduces it to produce cysteine [54].

Several studies on the effects of cystine and theanine administration in humans have been reported [1,2, 55-58]. These results suggested that cystine and theanine could suppress excessive inflammation and increase the immune response after invasive processes, such as exercise or surgery. Miyachi T et al. reported that perioperative oral administration of cystine and theanine partly suppressed increases in REE after surgery and promoted rapid recovery of body temperature, IL-6 levels, CRP levels, neutrophil count and total lymphocyte fraction in patients with gastric cancer treated by distal gastrectomy [2].

In conclusion, Glu can prevent IL-1β-stimulated liver injury in cultured hepatocytes by inhibiting the induction of inflammatory mediators such as iNOS and TNF-α, in part through the inhibition of NF-kB activation. Glu may have therapeutic potential for liver injury.

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