

Curcumin protects liver inflammation by suppressing expression of inducible nitric oxide synthase in primary cultured rat hepatocytes

Richi Nakatake^{1*}, Hidehiko Hishikawa¹, Hideyuki Matushima¹, Yusuke Nakamura¹, Morihiko Ishizaki¹, Kosuke Matsui¹, Masaki Kaibori¹, Mikio Nishizawa², Tadayoshi Okumura^{1,3}, and Masanori Kon¹

¹Department of Surgery, Kansai Medical University, Hirakata, Osaka, Japan; ²Department of Biomedical Sciences, College of Life Sciences; and ³Research Organization of Science and Technology, Ritsumeikan University, Kusatsu, Shiga, Japan

Submission Date: May 7th, 2017, Acceptance Date September 27th, 2017, Publication Date: September 30th, 2017,

Citation: Nakatake R., Hishikawa H., Matsuhima H., Nakamura Y., Ishizaki M., Matsui K., Kaibori M., Nishizawa M., Okumura T., Kon M., Curcumin protects liver inflammation by suppressing expression of inducible nitric oxide synthase in primary cultured rat hepatocytes. *Functional Foods in Health and Disease* 2017; 7(9); 716-734

Running title: Curcumin inhibits hepatic iNOS induction

***Correspondence:** Richi Nakatake, M.D. and Ph.D., Department of Surgery, Kansai Medical University, 2-5-1 Shinmachi, Hirakata, Osaka, 573-1010, Japan

ABSTRACT

Background: Curcumin has beneficial effects on organ metabolism. However, there is little evidence that curcumin affects inflammatory mediators, such as tumor necrosis factor (TNF)- α and nitric oxide (NO). In an inflamed liver, proinflammatory cytokines stimulate liver cells, followed by the induction of inducible NO synthase (iNOS). Excessive NO produced by iNOS is one of the factors in liver injury. Therefore, inhibiting iNOS induction for preventing liver injury is important.

Objective: This study aimed to investigate liver protective effects of curcumin by examining interleukin (IL)-1 β -stimulated hepatocytes.

Methods: Primary cultured rat hepatocytes were treated with IL-1 β in the presence or absence of curcumin. Induction of NO production and iNOS, and the signaling pathway of iNOS were analyzed.

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

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

Key words: curcumin, inducible nitric oxide synthase, liver injury, primary cultured hepatocytes, nuclear factor- κ B, type I interleukin-1 receptor, tumor necrosis factor- α .

INTRODUCTION:

Attention is being focused on the new academic discipline of anti-aging medicine, which aims to slow age-related pathological changes. One important research subset in this field involves foods and herbs that are known to be particularly effective for maintaining and enhancing health. These foods of interest have been shown to have remarkable pharmacological effects, comparable with those of existing pharmaceutical products. Therefore, substantial research efforts are currently ongoing to determine the active compounds that are responsible for these positive effects.

Curcumin, a diferuloyl-methane derived from the rhizome of *Curcuma longa*, is the active component of the spice turmeric (Fig. 1) [1]. Turmeric and curcumin have been used for thousands of years in Southeast Asia and India in a variety of medicinal applications [2]. Traditionally, curcumin has been used in Chinese and Indian folk medicine to treat various diseases and health problems, including respiratory conditions, anorexia, rheumatism, liver disease, and cancer. In recent years, some studies have investigated the various biological effects of curcumin [3, 4]. In addition to the reported cytoprotective effect of curcumin, curcumin has an antioxidant effect by removing free radicals and an anti-inflammatory effect by inhibiting activation of nuclear factor kappa B (NF- κ B) [4]. Two clinical trials that investigated curcumin concluded that this compound may be useful in preventing heart failure and effective against a range of diseases, including cancer [4, 5].

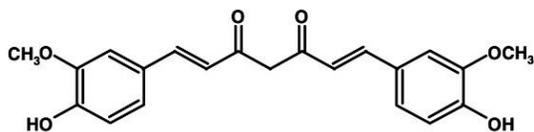


Fig. 1. Structure of curcumin.

In hepatic disorders, inflammatory cells such as macrophages gather around hepatic stellate (Kupffer) cells and discharge a variety of cytokines. During inflammation, proinflammatory cytokines and nitric oxide (NO), which is produced by inducible nitric oxide synthase (iNOS) in Kupffer cells and hepatocytes, play an important role as factors in liver injury [6]. However, NO exerts 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 [7-11] demonstrated how liver-protective effects inhibit induction of iNOS and NO production. These drugs also decrease production of various inflammatory mediators, such as tumor necrosis factor (TNF)- α , interleukin (IL)-1 β , IL-6, and cytokine-induced neutrophil chemoattractant (CINC)-1 (human IL-8 analogue) [7-11].

We previously reported that in hepatocytes that were isolated by the collagen perfusion method from rats and cultured the proinflammatory cytokine IL-1 β stimulated induction of iNOS gene expression in primary cultures of rat hepatocytes [12]. Previous studies on IL-1 β -stimulated hepatocytes have shown that clinical drugs [9, 13, 14] as mentioned above also inhibit induction of iNOS and production of NO. Therefore, by using cultured hepatocytes, prevention of iNOS induction and NO production is considered to be one of the indicators of liver protection [15].

In the present study, we examined IL-1 β -stimulated hepatocytes and investigated the mechanisms underlying liver-protective effects of curcumin that are observed in various animal models. We examined whether curcumin affects induction of iNOS, and if so what mechanisms were involved in the action of curcumin.

MATERIALS AND METHODS:

Materials. Curcumin (Wako Pure Chemicals, Osaka, Japan) was dissolved in dimethyl sulfoxide (DMSO) at 100 mM and stored at -20°C . The final concentration of DMSO in the culture medium was 0.1% or less. Recombinant human IL-1 β (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, and received food and water ad libitum. 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 cultures of hepatocytes. Hepatocytes were isolated from rats by perfusion with collagenase (Wako Pure Chemicals) [16]. Isolated hepatocytes were suspended in a 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 CO₂ incubator under a humidified atmosphere of 5% CO₂ 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 \pm SE, n = 7 experiments).

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

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).

Western blot analysis. Total cell lysates were obtained from cultured cells as described previously with minor modifications [13]. 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 (Affinity BioReagents, Golden, CO, USA), human I κ B α , 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 overnight at 4°C, immunocomplexes were centrifuged ($16,000 \times g$ 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. In the case

of p65, nuclear extracts were immunoprecipitated with an anti-p65 antibody (H286; Santa Cruz Biotechnology). The bands were analyzed by western blotting using an antibody against human NF- κ B p65 (BD Transduction Laboratories, Lexington, KY, USA).

Reverse transcriptase-polymerase chain reaction (RT-PCR). Total RNA was extracted from cultured hepatocytes using the guanidinium-phenol-chloroform method [19]. For strand-specific RT-PCR analysis, cDNA was 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 (257 bp), TNF- α (275 bp), CINC-1 (231 bp), IL-6 (286 bp), IL-1RI (327 bp), and elongation factor-1 α (EF; internal control) (335 bp) mRNA, 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'-TGCCCCTCCCCACATTCTCT-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 the Rotor-Gene Q 2plex HRM (Qiagen, Tokyo, Japan). The 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 the accession numbers AB250951 and AB250952 respectively.

Electrophoretic mobility shift assay. Nuclear extracts were prepared and an electrophoretic mobility shift assay was performed as previously described [21]. Briefly, nuclear extracts from hepatocytes (4 μ g) were mixed with 1 μ g of poly(dI-dC) and a probe for 20 min at room temperature (total mixture, 15 μ l). To prepare a double-stranded DNA probe, annealed oligonucleotides harboring a κ B site (5'-AGTTGAGGGGACTTTCCCAGGC-3'; only the sense strand is shown) were labelled with [γ -³²P]-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 by the method of Bradford [22] with a binding assay kit (Bio-Rad) using bovine serum albumin as a standard.

Transfection and luciferase assay. Transfection of cultured hepatocytes was performed as previously described [23]. Briefly, hepatocytes were cultured at 4×10^5 cells/dish (35 \times 10 mm) in WE 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 μ g) and the CMV promoter-driven β -galactosidase plasmid pCMV-LacZ (1 ng) as an internal control were mixed with MATra-A reagent (1 μ l; 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-1 β in the presence or absence of curcumin. Luciferase and β -galactosidase activities of cell extracts were measured using PicaGene (Wako Pure Chemicals) and Beta-Glo (Promega, Wisconsin, USA) kits respectively.

Statistical analysis. The results shown are representative of three to four independent experiments yielding similar findings. All data are expressed as means \pm SD. Differences were analyzed by the Bonferroni–Dunn test, and a value of $P < 0.05$ was considered to indicate a significant difference.

Primer name	Nucleotide sequence
iNOS F/R	5' CCAACCTGCAGGTCTTCGATG 3'/5' GTCGATGCACAACCTGGGTGAAC 3'
as iNOS F/R	5' CCTTTGCCTCATACTTCCTCAGA 3'/5' ATCTTCATCAAGGAATTATACACGG 3'
TNF- α F/R	5' TCCCAACAAGGAGGAGAAGTTCC 3'/5' GGCAGCCTTGCCCTTGAAGAGA 3'
IL-6 F/R	5' GAGAAAAGAGTTGTGCAATGGCA 3'/5' TGAGTCTTTTATCTCTTGTGTTGAAG 3'
CINC-1 F/R	5' GCCAAGCCACAGGGGCGCCCGT 3'/5' ACTTGGGGACACCCTTTAGCATC 3'
IL-1RI F/R	5'-CGAAGACTATCAGTTTTTGAAC-3'/5'-GTCTTCCATCTGAAGCTTTTGG-3'
EF F/R	5' TCTGTTGGAATGGTGACAACATGC 3'/5' CCAGGAAGAGCTTCACTCAAAGCTT 3'

Table 1. Primers and nucleotide sequences

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-1RI, type I IL-1 receptor; EF, elongation factor-1 α ; F/R, forward/reverse.

RESULTS:

Curcumin inhibits NO production and iNOS induction in IL-1 β -stimulated hepatocytes. The proinflammatory cytokine IL-1 β stimulated induction of iNOS, which was followed by production of NO in primary cultured rat hepatocytes. Simultaneous addition of curcumin and IL-1 β reduced the levels of nitrite (a stable metabolite of NO) time and dose-dependently in the culture medium (Fig. 2A and 2B). Curcumin showed more than 90% inhibition at 25 μ M. Curcumin had no cellular cytotoxicity with the various concentrations, as evaluated by the release of LDH into the culture medium (Fig. 3) and Trypan blue exclusion by hepatocytes (data not shown).

Western blotting analysis showed that curcumin dose-dependently reduced the expression of the iNOS protein, showing its maximal effect at 25 μ M (Fig. 2B). RT-PCR analysis showed that curcumin decreased the expression of iNOS mRNA time-dependently (Fig. 2C). These results suggested that curcumin inhibited the induction of iNOS gene expression at a transcriptional and/or post-transcriptional step.

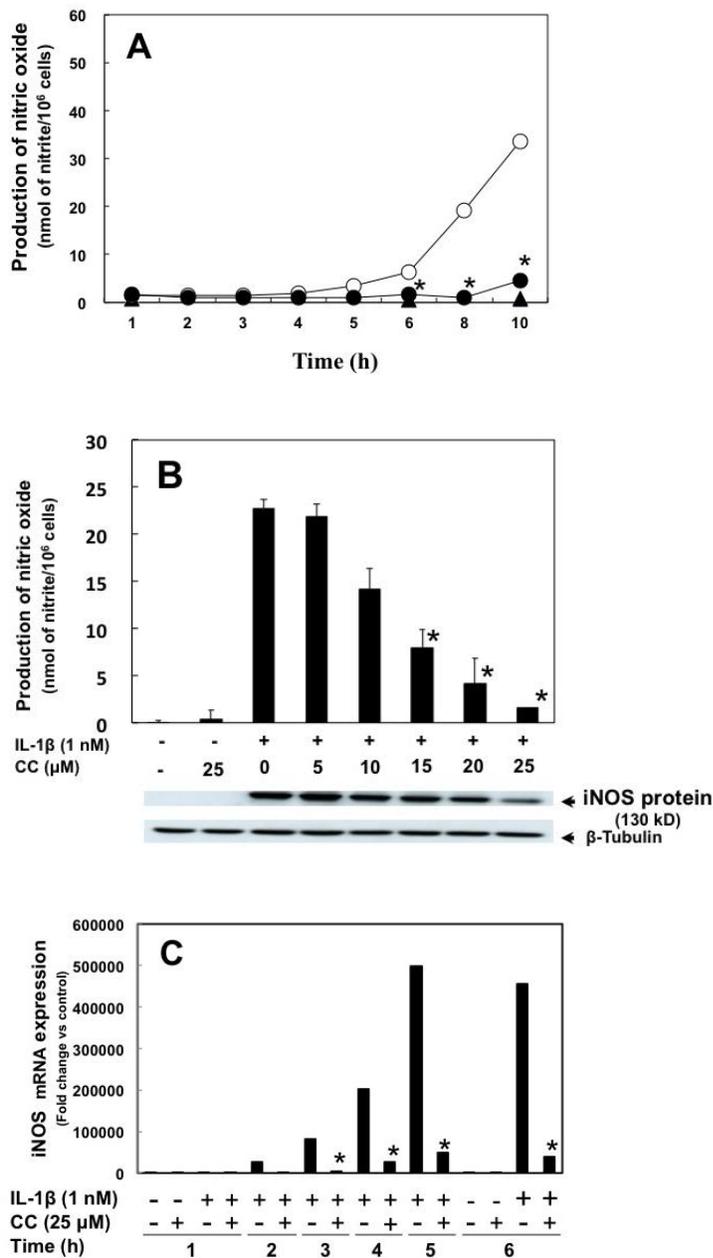


Fig. 2. Effects of curcumin on induction of NO production and iNOS in IL-1 β -stimulated hepatocytes. Cultured hepatocytes were treated with interleukin (IL)-1 β (1 nM) in the presence or absence of curcumin (5–25 μ M). (A) Effect of curcumin (25 μ M) treatment for the indicated times on nitric oxide (NO) production (IL-1 β , open circles; IL-1 β + curcumin, filled circles; curcumin, filled triangles; controls [without IL-1 β and curcumin], open triangles). (B) Effects of treatment with various doses of curcumin (5–25 μ M) for 8 h on NO production (upper panel) and inducible nitric oxide synthase (iNOS) protein (medium). Nitrite levels were measured in culture medium. Data are means \pm SD for n = 3 dishes/point; *P < 0.05 versus IL-1 β alone. In the western blotting panels, cell lysates (20 μ g of protein) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis in a 7.5% gel, and immunoblotted with an anti-iNOS or anti- β -tubulin antibody. (C) Effects of curcumin (25 μ M) treatment for the indicated times on iNOS mRNA expression. Total RNA was analyzed by strand-specific RT-PCR to detect iNOS mRNA, using EF mRNA as an internal control.

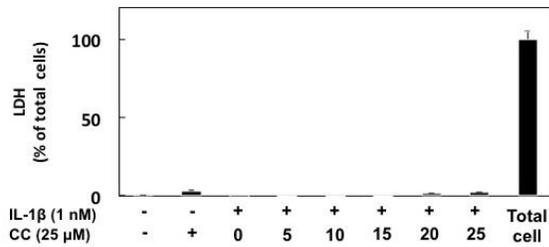


Fig. 3. Effects of curcumin on cellular cytotoxicity. Cells were treated with IL-1β (1 nM) in the presence or absence of curcumin (5–25 μM) for 8 h. Lactate dehydrogenase (LDH) activity was measured in the culture medium (data are means ± SD, n = 3 dishes/point).

Curcumin affects mRNA expression of proinflammatory cytokines. The expression of other mRNAs was examined. IL-1β increased the level of proinflammatory cytokine expression, by increasing TNF-α, CINC-1, and IL-6 mRNA levels, and curcumin decreased TNF-α, CINC-1, and IL-6 mRNA levels (Fig. 4).

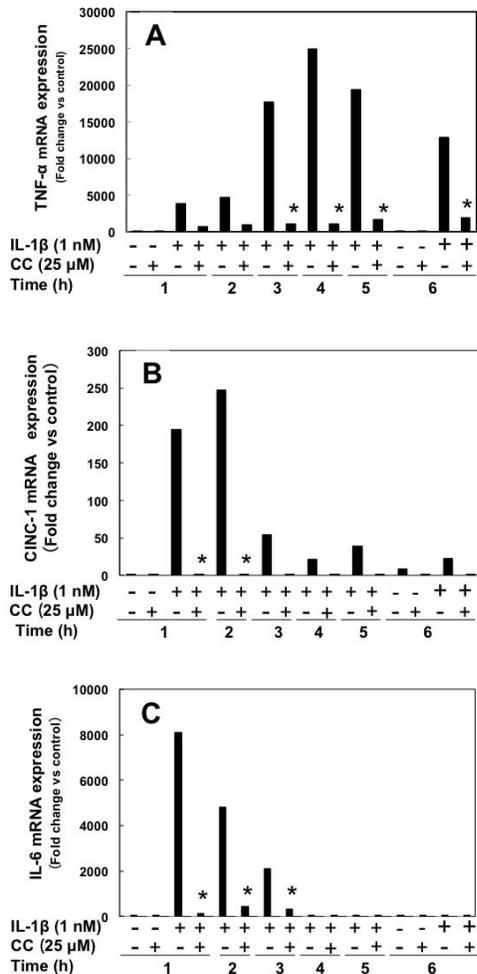


Fig. 4. Effects of curcumin on TNF-α, CINC, and IL-6 mRNA expression. Cells were treated with IL-1β (1 nM) in the presence or absence of curcumin (25 μM) for the indicated times. Total RNA was analyzed by strand-specific RT-PCR to detect (A) TNF-α, (B) CINC-1, and (C) IL-6, using EF mRNA as an internal control.

Curcumin decreases iNOS mRNA synthesis and stabilization. We examined the mechanisms that are involved in inhibiting iNOS induction. Expression of iNOS mRNA is regulated by iNOS promoter transactivation with transcription factors, such as NF- κ B, and by post-transcriptional modifications, such as mRNA stabilization [24]. 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. 5A), which detects iNOS promoter transactivation (mRNA synthesis) and mRNA stabilization respectively. IL-1 β increased the luciferase activity of these constructs, and these effects were significantly inhibited by curcumin (Fig. 5B and 5C). Furthermore, iNOS antisense transcript (asRNA) analysis by real-time RT-PCR showed that IL-1 β increased the expression of iNOS asRNA in a time-dependent manner, and that curcumin markedly inhibited this effect (Fig. 5D).

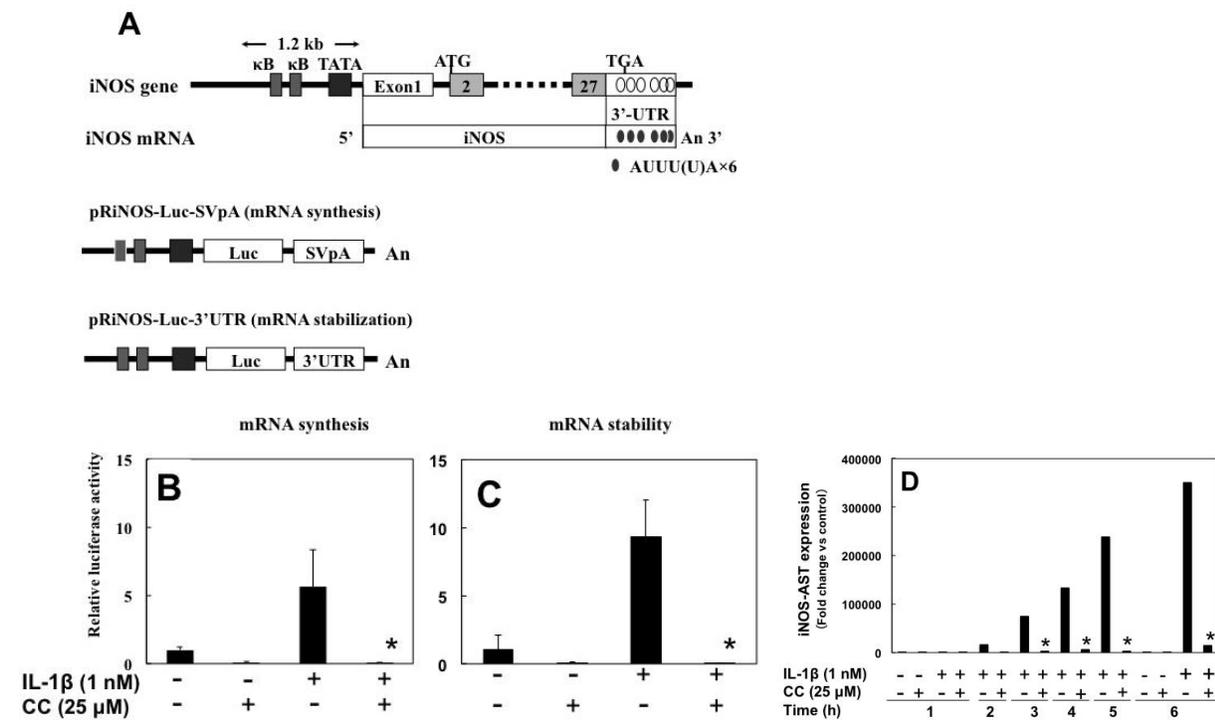


Fig. 5. Effects of curcumin on transactivation of the iNOS promoter and expression of the iNOS gene antisense transcript. (A) Schematic representation of the promoter region of the iNOS gene. Two reporter constructs are shown beneath the iNOS gene and mRNA. The constructs consist of the rat iNOS promoter (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 \times 6), which contribute to mRNA stabilization. (B, C) Each construct was introduced into hepatocytes, and the cells were treated with IL-1 β (1 nM) in the presence or absence of curcumin (25 μ M/mL) for 8 h for pRiNOS-Luc-SVpA (B) and 5 h for pRiNOS-Luc-3'UTR (C). Luciferase activity was normalized by β -galactosidase activity. Fold activation was calculated by dividing luciferase activity by control activity (without IL-1 β and curcumin). Data are means \pm SD for n = 4 dishes. *P < 0.05 versus IL-1 β alone. (D) The cells were treated with IL-1 β (1 nM) in the presence or absence of curcumin (25 μ M/mL) for the indicated times. Total RNA was analyzed by strand-specific RT-PCR to detect the iNOS gene antisense transcript (AST).

Curcumin inhibits activation of NF-κB and upregulation of IL-1RI. There are two essential signaling pathways for induction of iNOS, called the IκB kinase and phosphatidylinositol 3-kinase (PI3K)/Akt pathways. In the former pathway, IL-1β stimulates degradation of IκB proteins after phosphorylation by IκB kinase, which is followed by activation of NF-κB (i.e., translocation from the cytoplasm to the nucleus and DNA binding). Curcumin had no effect on degradation of IκBα after IL-1β stimulation, but rather enhanced its degradation (Fig. 6A). However, an electrophoretic mobility shift assay with nuclear extracts showed that curcumin inhibited NF-κB activation at 2–5 h, which indicated blockade of NF-κB nuclear translocation (Fig. 6B).

In the PI3K/Akt pathway, IL-1β stimulates upregulation of IL-1RI by activation of PI3K/Akt [25,26]. Immunoprecipitation-western blotting analysis showed that curcumin inhibited phosphorylation (activation) of Akt, a downstream kinase of PI3K (Fig. 7A). Real-time RT-PCR and western blotting analyses revealed that curcumin reduced expression levels of IL-1RI mRNA and protein (Fig. 7B and 7C).

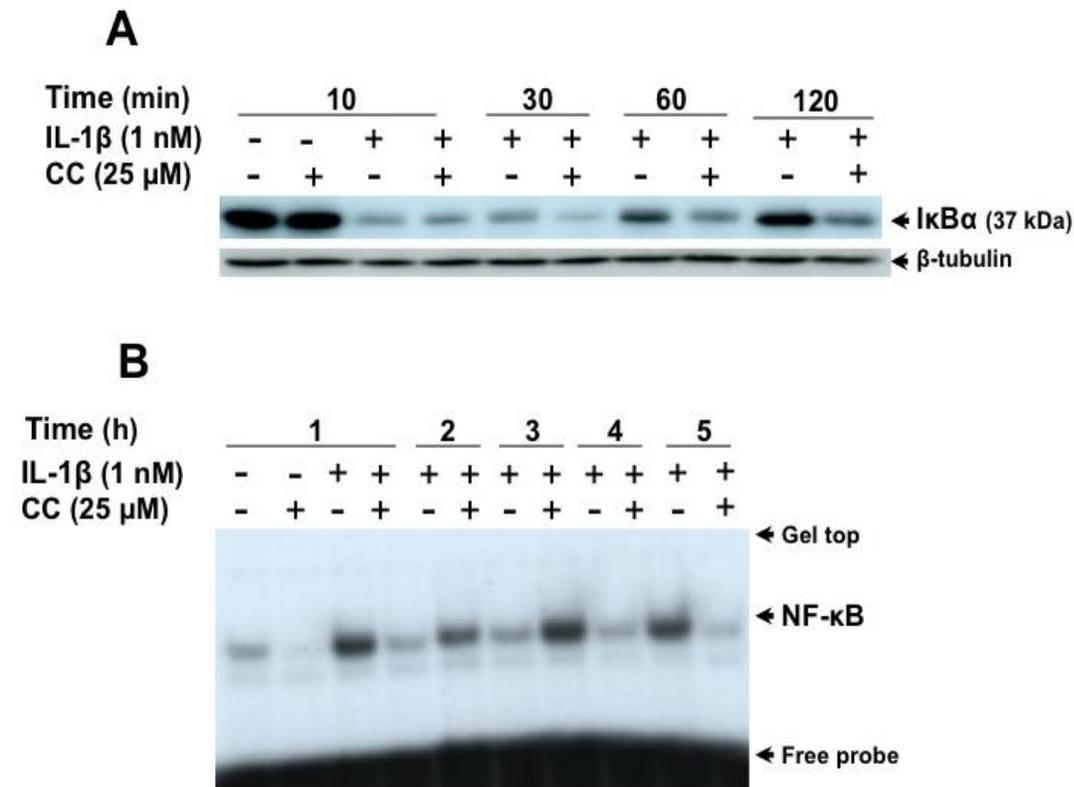


Fig. 6. Effects of curcumin on degradation of IκBα and activation of NF-κB. Cells were treated with IL-1β (1 nM) in the presence or absence of curcumin (25 μM) for the indicated times. (A) Cell lysates (20 μg of protein) were subjected to SDS-PAGE in a 12.5% gel, followed by immunoblotting with an anti-IκBα 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.

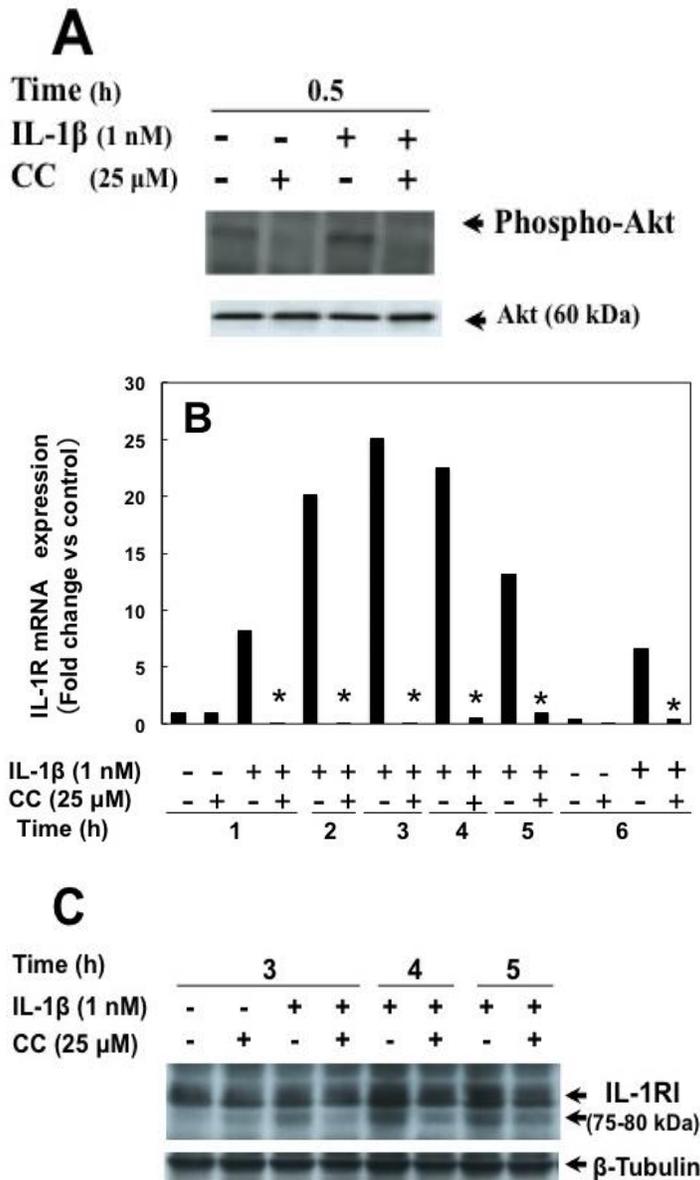


Fig. 7. Effects of curcumin on upregulation of type I IL-1 receptor. Cells were treated with IL-1 β (1 nM) in the presence or absence of curcumin (25 μ M) for the indicated times. (A) Phosphorylation of Akt. Total cell lysates were immunoprecipitated with an anti-Akt antibody, followed by immunoblotting (SDS-PAGE in a gel with a gradient of 6–9%) with an anti-phospho-Akt or anti-Akt antibody. (B) Total RNA was analyzed by strand-specific RT-PCR to detect type I IL-1 receptor (IL-1RI) mRNA, using EF mRNA as an internal control. (C) Cell lysates (50 μ g of protein) were subjected to SDS-PAGE in a 7.5% gel, and immunoblotted with an anti-IL-1RI or anti- β -tubulin antibody.

Delayed administration of curcumin inhibits iNOS induction. We examined whether delayed administration of curcumin affects induction of iNOS. Curcumin was added to the medium 0–5 h after addition of IL-1 β . Delayed administration of curcumin up to 2 h after IL-1 β addition still markedly inhibited NO production, although the magnitude of inhibition decreased in a time-dependent manner (Fig. 8).

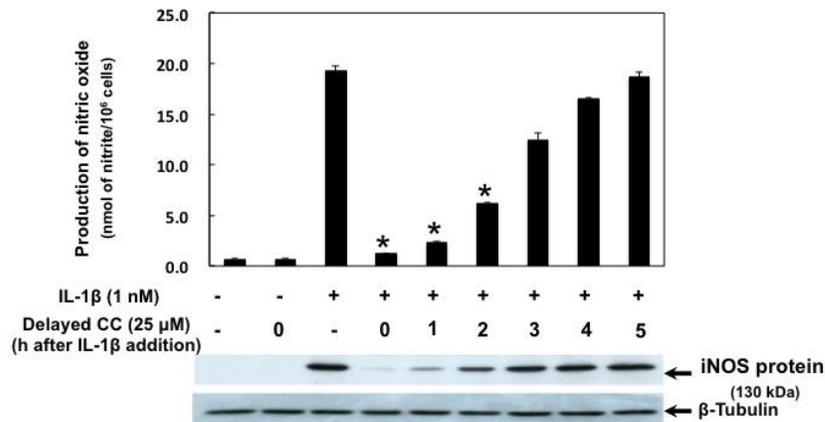


Fig. 8. Effects of delayed glutamic acid administration on induction of iNOS in hepatocytes. Cells were treated with curcumin (25 μM) at 0–4 h after addition of IL-1β (1 nM). The effects of curcumin on NO production (upper panel) and iNOS protein (lower panel) were analyzed at 8 h after addition of IL-1β. Nitrite levels 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 immunoblotted with an anti-iNOS or anti-β-tubulin antibody.

DISCUSSION:

Curcumin is a pharmacologically safe compound with known anti-inflammatory, anticarcinogenic, and free radical scavenger properties [27-32]. Curcumin supplementation in animal models results in an increase in several cytoprotective enzymes in liver and kidney tissues [33-35]. The anti-inflammatory and antioxidant properties of curcumin have been well documented [36-38]. However, the way in which these inhibitory responses are modulated by curcumin is still unclear [39].

In this study, we found that curcumin inhibited induction of iNOS, and even TNF-α and IL-6 in IL-1β-stimulated hepatocytes. This finding indicates that curcumin may have anti-inflammatory effects in the liver. Intervention in NF-κB activation may also be beneficial in suppressing toxic/septic shock, graft versus host reactions, radiation damage, and inflammatory reactions. NF-κB plays a major role in inflammation by regulating genes encoding iNOS and proinflammatory cytokines, such as TNF-α [40]. Induction of iNOS gene expression is regulated by iNOS promoter transactivation and by post-transcriptional modifications [24]. Curcumin was recently reported to inhibit NOS [41, 42]. Ex vivo studies have suggested that the inducibility of NOS activity in macrophages is inhibited by 1–20-μM concentrations of curcumin [43]. Additionally, administration of an aqueous alkaline solution of curcumin in drinking water significantly inhibits murine hepatic lipopolysaccharide-induced iNOS gene expression [44].

Curcumin also displays anticarcinogenic properties in animals as indicated by its ability to inhibit initiation of tumors when induced by phorbol esters [33, 34], which are known to activate

NF- κ B. Because inhibiting NF- κ B activity may represent a mechanism of intervention during carcinogenesis, curcumin's activity could have considerable implications for chemoprevention of cancer. NF- κ B typically exists in the form of p50/65 heterodimers that are attached to its inhibitory proteins (I κ Bs, I κ B α , and I κ B β) in the cytoplasm of cells. Activation of NF- κ B involves i) proteolytic degradation of I κ Bs in the proteasome after phosphorylation by I κ B kinase, ii) translocation of NF- κ B to the nucleus, and iii) its binding to the promoter κ B site [45]. In our study, curcumin in primary cultured rat hepatocytes inhibited NF- κ B activation (Fig. 6B). We anticipated that curcumin would inhibit I κ B α degradation, resulting in inhibition of NF- κ B nuclear translocation as well as in ML-1a (a human myelomonoblastic leukemia cell line) [46]. However, curcumin did not inhibit I κ B α degradation in this condition (Fig. 6A). Our results indicate that curcumin administration results in inhibition at a step in the signal transduction cascade of NF- κ B activation that occurs after I κ B phosphorylation, but before the point at which various signals that are transduced by different stimuli converge. Many in vitro studies have reported that curcumin may inhibit NF- κ B and mediate activation of hepatic stellate cells by stimulating peroxisome proliferator-activated receptor- γ (PPAR- γ) activity [47]. This leads to inhibition of cellular proliferation and induction of apoptosis of stimulated hepatic stellate cells. Further mechanisms involved in the inhibition of NF- κ B activation are under investigation.

In concert with NF- κ B activation, upregulation of IL-1RI by activation of PI3K/Akt is also essential for the induction of iNOS [26]. We found that curcumin decreased IL-1RI mRNA and protein expression (Fig. 7B and 7C) by inhibiting Akt phosphorylation (Fig. 7A). In regards to the activation of Akt, we previously found that inchinkoto [48], hochuekito [49], saireito [50], and genipin [51] inhibited phosphorylation of Akt. These compounds also decreased IL-1RI mRNA and protein expression, similar to curcumin. In contrast, dexamethasone [52], adenosine [53], and glutamic acid [54] inhibit upregulation of IL-1RI, but have no effects on phosphorylation of Akt. Therefore, the mechanisms involved in a reduction of induction of IL-1RI by dexamethasone, adenosine, and glutamic acid are unclear in the present.

Furthermore, in our experiments with iNOS promoter constructs, curcumin was found to inhibit induction of iNOS at the mRNA synthesis and stabilization steps (Fig. 5B and 5C). With regards to iNOS mRNA stabilization, the 3'-UTR of iNOS mRNA in rats has six adenine/uracil-rich elements (AREs). These AREs are associated with ARE-binding proteins, such as HuR and the heterogeneous nuclear ribonucleoprotein L/I (PTB), which serve to stabilize mRNA [55]. Recently, we reported that asRNAs are often transcribed from many inducible genes, such as iNOS and TNF- α [56]. The iNOS asRNA interacts with and stabilizes iNOS mRNA [57, 58]. We have reported that drugs, such as FR183998 (Na⁺/H⁺ exchanger inhibitor) [9, 10], insulin-like growth factor-I [11], edaravone (free radical scavenger) [14], inchinkoto [48], hochuekito [49], saireito [50], genipin [51], glutamic acid [54], and sivelestat [59] inhibit iNOS induction. This was

achieved partly by suppressing iNOS mRNA production in animal models and/or primary cultured hepatocytes. In the current study, curcumin also decreased iNOS mRNA expression (Fig. 5D).

These findings show that curcumin inhibits two essential signaling pathways, NF- κ B activation and IL-1RI upregulation, in the induction of iNOS. Curcumin probably reduces iNOS mRNA expression by inhibition of mRNA synthesis and stabilization, leading to a reduction in iNOS protein and NO production.

Proinflammatory cytokines, such as TNF- α , IL-1 β , and IL-6 play an important role in causing injury to multiple organs, in addition to the liver. Inflammation is a biological response that helps to maintain homeostasis, but excessive inflammation following excessive stress causes organ damage. In our in vitro model, we recently reported that IL-1 β stimulated induction of TNF- α , parallel with the induction of iNOS [60]. In the current study, curcumin decreased TNF- α , CINC-1, and IL-6 mRNA expression (Fig. 4A, 4B, and 4C). This finding suggested that curcumin could inhibit induction of inflammatory mediators (iNOS, TNF- α , CINC-1, and IL-6), which resulted in liver-protective effects.

In our study, delayed treatment with curcumin after addition of IL-1 β caused a significant reduction in NO production and induction of iNOS (Fig. 8). This observation may be of clinical importance because initiation of therapeutic curcumin treatment is usually delayed by the onset of disease. 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.

Many studies ranging from basic studies to clinical studies have been conducted on curcumin and its clinical applications in treating various diseases. Based on the range of effects of curcumin, curcumin by itself may be useful in treating and preventing some diseases, but fully establishing its potential will require further studies and clinical applications. Our simple in vitro experiment with cultured hepatocytes may be adequate for the screening of liver-protective drugs because it is rapid and inexpensive compared with in vivo animal models of liver injury.

CONCLUSIONS:

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

Abbreviations: CC, curcumin; iNOS, inducible nitric oxide synthase; TNF- α , tumor necrosis factor-alpha; NO, nitric oxide; IL, interleukin; NF- κ B, nuclear factor-kappa B; CINC-1, cytokine-induced neutrophil chemoattractant-1; DMSO, dimethyl sulfoxide; LDH, lactate dehydrogenase; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; IL-1RI, the type I interleukin-1 receptor; RT-PCR, reverse transcriptase-polymerase chain reaction.

Disclosure of potential conflicts of interest: The authors declare that they have no conflicts of interest.

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

Funding: This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Culture and Sports of Japan, and by grants from the Science Research Promotion Fund of the Japan Private School Promotion Foundation.

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