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Effect of Active Hexose Correlated Compound on the Production of Nitric Oxide in Hepatocytes

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ABSTRACT. *Background:* Active hexose correlated compound (AHCC) is a "complex compound" containing polysaccharides. AHCC has been reported to improve the prognosis of postoperative hepatocellular carcinoma patients. However, the molecular mechanism of this improvement is not fully understood. In the diseased liver, nitric oxide (NO) generated by inducible nitric oxide synthase (iNOS) is considered to be a causal factor for various hepatopathies. In this study, the possibility of AHCC regulation of NO production by iNOS was pursued as a potential liver-protecting mechanism. *Methods:* Primary cultured rat hepatocytes were treated with interleukin-1β (IL-1β) in the presence or absence of AHCC. NO production, iNOS induction, and iNOS signal were analyzed. *Results:* IL-1β stimulated iNOS induction through the activation of nuclear factor κB (NFκB), leading to NO production. The addition of AHCC inhibited NO production, showing >80% inhibition at 8 mg/mL. AHCC also decreased the levels of iNOS protein and mRNA. However, AHCC influenced neither the degradation of inhibitory protein κB (IκB) nor the activation of NFκB stimulated by IL-1β. Transfection experiments with an iNOS promoter-luciferase construct (iNOS-Luc) revealed that AHCC had no effect on the transactivation activity of the iNOS promoter. By contrast, AHCC inhibited the activity of iNOS-Luc containing a 3′untranslated region (UTR) with adenosine and uridine (AU)–rich elements, which shows the stabilizing activity of iNOS mRNA. *Conclusions:* Results indicated that AHCC inhibits the induction of iNOS at the level of transcription, causing a decrease in NO production in hepatocytes. AHCC seems to decrease the levels of iNOS mRNA by reducing mRNA stabilization rather than inhibiting its synthesis. (*Journal of Parenteral and Enteral Nutrition* **31:**373–381, 2007)

Nitric oxide (NO), a short-lived free radical, mediates a variety of physiologic functions, including vascular tone regulation, neurotransmission, and immune response mediation.[1] In the liver, NO is generated from D-arginine by constitutively expressed endothelial nitric oxide synthase (eNOS) or inducible nitric oxide synthase (iNOS). eNOS is located in vascular endothelial cells and plays important roles in microvascular homeostasis. By contrast, iNOS is not present under physiologic conditions but is induced transcriptionally under pathologic conditions such as endotoxin shock, warm ischemia-reperfusion, hepatitis, and liver cirrhosis. In liver injury, lipopolysaccharide and proinflammatory cytokines such as tumor necrosis factor (TNF)-α and interleukin (IL)-1β stimulate the induction of iNOS gene expression, which is followed by excess production of NO. A relatively large amount of NO production influences metabolism and various hepatic functions.

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NO has cytoprotective effects in the liver during endotoxemia and other types of fulminant hepatic failure[2–4] and is a potent antimalarial effector molecule in hepatocytes.[5] By contrast, Thiemerman et al[6] found that an iNOS-selective inhibitor attenuated liver damage and dysfunction in lipopolysaccharide-treated rats. NO inhibits Krebs cycle enzymes in the mitochondria within hepatocytes,[7] resulting in a decrease in hepatic ATP levels in rat models of partial hepatectomy,[8] obstructive jaundice,[9] and sepsis.[10] Thus, the production of NO is implicated in diverse functions associated with cytoprotection or injury in the liver.[11] Whether NO protects or injures probably depends on the type of insult, the source and amount of NO production, and the cellular redox status of the liver.[12]

Active hexose correlated compound (AHCC), which is extracted from mushrooms (*Basidiomycetes*), was developed by the Amino Up Chemical Co Ltd (Sapporo, Japan) in 1989. AHCC is a "complex compound" containing a variety of polysaccharides and other components, in which acetylated α-1,4 glucan is one of the major components. The application of AHCC has been rapidly increased in complementary and alternative medicine as a functional food.[13,14] In clinical studies, AHCC has been reported to improve the prognosis of postoperative hepatocellular carcinoma patients.[15]

Furthermore, it was reported that AHCC protects the liver from carbon tetrachloride (CCl_4)–induced liver damage in mice.[16] However, the molecular mechanism by which AHCC protects the liver is not fully understood.

In this study, the possibility of AHCC regulation of NO production by IL-1β–stimulated iNOS was pursued as a possible liver-protecting mechanism. We examined whether AHCC influences the induction of iNOS stimulated by proinflammatory cytokines in primary cultures of rat hepatocytes.

MATERIALS AND METHODS

Materials

Recombinant human IL-1β (2×10^7 units/mg protein) was provided by Otsuka Pharmaceutical Co (Tokushima, Japan). [γ-^{32}P]Adenosine-5′-triphosphate (ATP, −222 TBq/mmol) and [α-^{32}P]deoxycytidine-5′-triphosphate (dCTP, −111 TBq/mmol) were from DuPont-New England Nuclear Japan (Tokyo, Japan). AHCC was provided by the Amino Up Chemical Co Ltd and dissolved in Williams' medium E. All other chemicals were of reagent grade. Rats were kept at 22°C under a 12-hour light-dark cycle and received food and water *ad libitum*. Animal experiments were approved by the Animal Care Committee of Kansai Medical University.

Primary Cultures of Hepatocytes

Hepatocytes were isolated from male Wistar-strain rats (200–250 g; Charles River, Boston, MA) by collagenase (Wako Pure Chemical, Osaka, Japan) perfusion, as described previously.[17] The isolated hepatocytes were suspended in culture medium at 6×10^5 cells/mL, seeded into plastic dishes (2 mL/35 mm; Falcon Plastic, Oxnard, CA), 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' medium E supplemented with 10% newborn calf serum, Hepes (5 mmol/L), penicillin (100 units/mL), streptomycin (0.1 mg/mL), dexamethasone (10 nmol/L), and insulin (10 nmol/L). After 7 hours, the medium was replaced with fresh serum- and hormone-free medium; cells were cultured overnight and used in experiments. The purity of isolated hepatocytes was >99% by microscopic observation.[18] The number of cells attached to the dishes was calculated by counting the nuclei[19] and using a ratio of 1.37 ± 0.04 nuclei per cell (mean ± SE; n = 7 independent experiments).

Treatment of Cells With AHCC

On day 1, cultured hepatocytes were washed with fresh serum- and hormone-free medium, treated with AHCC at various concentrations (1–8 mg/mL) 30 minutes before experiments, and then incubated with IL-1β (1 nmol/L) in the same medium for the indicated times.

Determinations of NO Production and Lactate Dehydrogenase (LDH)

Culture medium was used for the measurements of nitrite (a stable metabolite of NO) as an indicator of the level of NO by the method of Griess,[20] and LDH activity for a measure of cellular viability using a commercial kit (Wako Pure Chemical).

Western Blot Analysis

Cells (1×10^6 cells/dish, 35 × 10 mm) were lysed in 100–200 μL of solubilizing buffer (10 mmol/L Tris-HCl, pH 7.4, containing 1% Triton X-100, 0.5% Nonidet P-40, 1 mmol/L sodium orthovanadate, 1 mmol/L phenylmethylsulfonylfluoride [PMSF], and a protease inhibitor cocktail; Roche Diagnostics, Mannheim, Germany), passed through a 26-gauge needle, and incubated on ice for 30 minutes, followed by centrifugation (16,000 × *g* for 15 minutes). The supernatant (total cell lysate) was mixed with sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (final concentrations: 125 mmol/L Tris-HCl buffer, 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). Immunostaining was performed using a rabbit polyclonal antibody directed against mouse iNOS (Affinity BioReagents, Golden, CO) as the primary antibody and an enhanced chemiluminescence (ECL) blotting detection reagent (GE Healthcare Biosciences Corp, NJ).

Northern Blot Analysis

Total RNA was extracted from cultured hepatocytes using the acid guanidinium–phenol–chloroform method.[21] Total RNA (10 μg) was fractionated by 1% agarose-formaldehyde gel electrophoresis, transferred to nylon membranes (Nytran; Schleicher & Schuell, Dassel, Germany), and immobilized by baking at 80°C for 1 hour before hybridization with DNA probes. A cDNA probe for rat iNOS (830 base pairs) was provided.[22] A cDNA encoding mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH)[23] was prepared by polymerase chain reaction (PCR).[24] These cDNAs were radiolabeled with [α-^{32}P]dCTP by the random priming method.

Electrophoretic Mobility Shift Assay (EMSA)

Nuclear extracts were prepared at 4°C according to the method of Schreiber et al,[25] with minor modifications,[26] unless otherwise stated. Briefly, approximately 2×10^6 cells (2 35-mm dishes) were placed on ice, washed with Tris-buffered saline, harvested in the same buffer, and centrifuged (1840 × *g* for 1 minute). Cell pellets were resuspended in 400 μL of lysis buffer (10 mmol/L Hepes, pH 7.9, 10 mmol/L KCl, 0.1 mmol/L EDTA, 0.1 mmol/L EGTA, 500 U/mL aprotinin, 0.5 mmol/L PMSF, and 1 mmol/L dithiothreitol), and cells were allowed to swell on ice for 15 minutes. Twenty-five microliters of 10% Nonident P-40 was added to the lysis buffer and the tube was vigorously vortexed for 1 minute at room temperature and then centrifuged

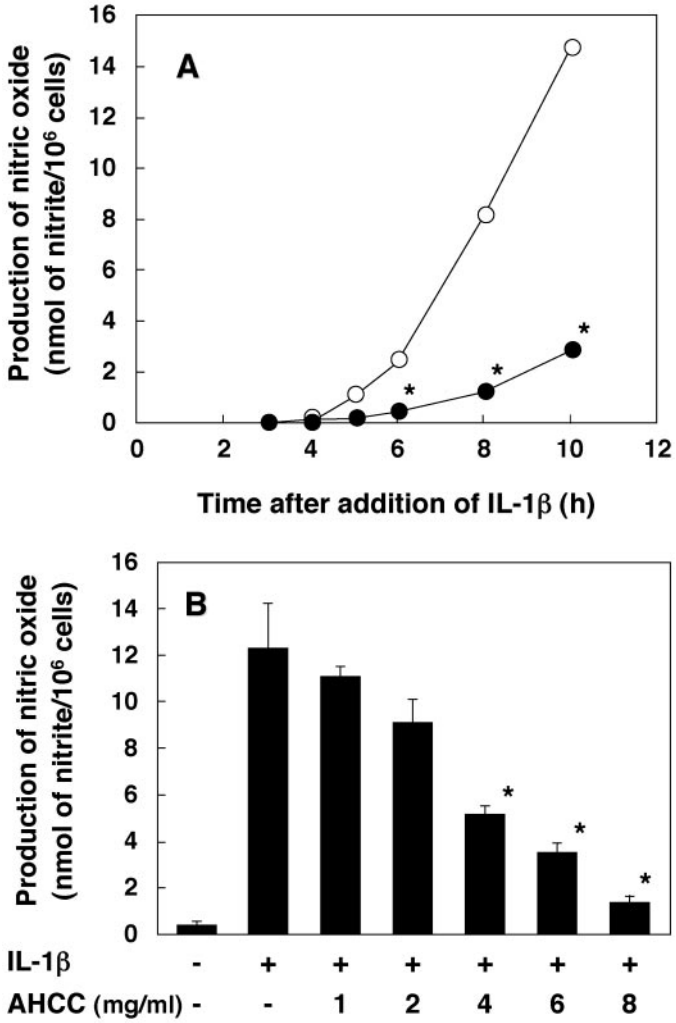

FIGURE 1. Effect of AHCC on the production of nitric oxide in hepatocytes. A, Cells were treated with IL-1β (1 nmol/L) in the presence or absence of AHCC (8 mg/mL) for the indicated times; IL-1β (○) and IL-1β+AHCC (●). B, Cells were treated with IL-1β in the presence of AHCC at various concentrations (1–8 mg/mL) for 8 hours. NO production was measured as nitrite in the culture medium. Data represent mean ± SD (n = 3). *$p < .05$ *vs* IL-1β alone.

(15,000 × *g* for 1 minute). After removal of the supernatant, the nuclear pellet was resuspended in 75 μL of nuclear extraction buffer (20 mmol/L Hepes, pH 7.9, 0.4 M NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 500 U/mL aprotinin, 1 mmol/L PMSF, and 1 mmol/L dithiothreitol). The tube was incubated on ice for 20 minutes with continuous mixing and then centrifuged (15,000 × *g* for 5 minutes). Aliquots of the supernatant were frozen with liquid nitrogen and stored at −80°C until use.

Binding reactions (15 μL total) were performed by incubating an amount of nuclear extract containing 4 μg of protein with reaction buffer (20 mmol/L Hepes, pH 7.9, 1 mmol/L EDTA, 60 mmol/L KCl, 10% glycerol, 1 mg of poly (dI-dC)) in the absence or presence of anti-p50 and anti-p65 antibodies (against nuclear factor κB [NFκB] p50 [NLS] and NFκBp65 [H286]; Santa Cruz Biotech, Santa Cruz, CA) or cold probe as a competitor (250-fold excess), for 30 minutes, followed by a 20-minute incubation at room temperature with the probe (approximately 40,000 cpm). Products were electrophoresed at 100 V on a 4.8% polyacrylamide gel in high-ionic-strength buffer (50 mmol/L Tris-HCl, 380 mmol/L glycine, 2 mmol/L EDTA, pH 8.5), and dried gels were analyzed by autoradiography. An NF κB consensus oligonucleotide (5′-AGTTGAGGGGA-CTTTCCCAGGC) from mouse immunoglobulin k light chain was purchased (Promega, Madison, WI) and labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase. The protein concentration was measured by the method of Bradford[27] with a dye-binding assay kit (Bio-Rad Laboratories), using bovine serum albumin as a standard.

Construction of Luciferase (Luc) Reporter Plasmids and Expression Plasmids

The 1.2-kb 5′-flanking region of the rat iNOS gene (deposited to the DDBJ/EMBL/GenBank under accession No. AB290142), including a TATA box, was inserted into pGL3-Basic vector (Promega) to create pRiNOS-Luc.[26] Rat cDNA for the 3′untranslated region (UTR) of iNOS mRNA was amplified using specific primers (5′-tg*ctctaGA*CAGTGAGGGGTTTG-GAGAGA-3′ and 5′-g*cggatcc*tttaTTCTTGATCAAACA-CTCATTTT-3′), and the resultant cDNA was digested with BamH I and Xba I. This cDNA for the iNOS 3′UTR (deposited under accession No. AB250951) replaced the SV40 polyadenylation signal of pRiNOS-Luc to create pRiNOS-Luc-3′UTR.

Transfection and Luc Assay

Transfection of cultured hepatocytes was performed according to published methods.[26,28] Briefly, hepatocytes were cultured at 4 × 10^5 cells per dish (35 × 10 mm) in Williams' medium E supplemented with serum, dexamethasone, and insulin for 7 hours. Then, cells were subjected to magnet-assisted transfection (MATra). Reporter plasmid pRiNOS-Luc or pRiNOS-Luc-3′UTR (1 μg) and cytomegalovirus (CMV) promot-

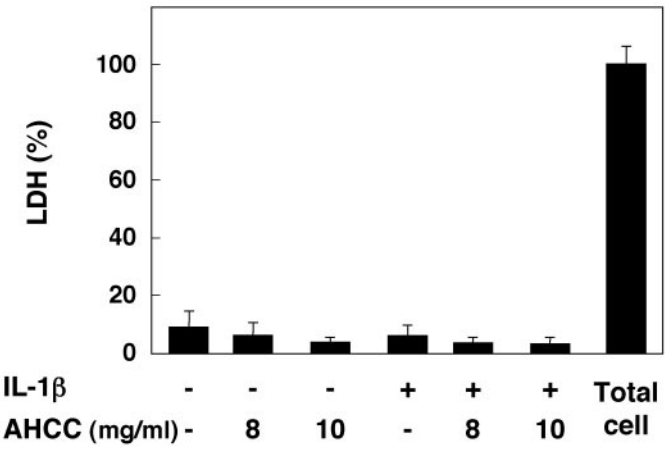

FIGURE 2. Effect of AHCC on cellular viability. Cells were treated with IL-1β (1 nmol/L) in the presence or absence of AHCC (8 or 10 mg/mL) for 8 hours. The culture medium was used to measure the activities of lactate dehydrogenase (LDH). The activity obtained from the supernatant of total cell lysate (10^6 cells/dish) was calculated as 100% (total cell). Data represent mean ± SD (n = 3).

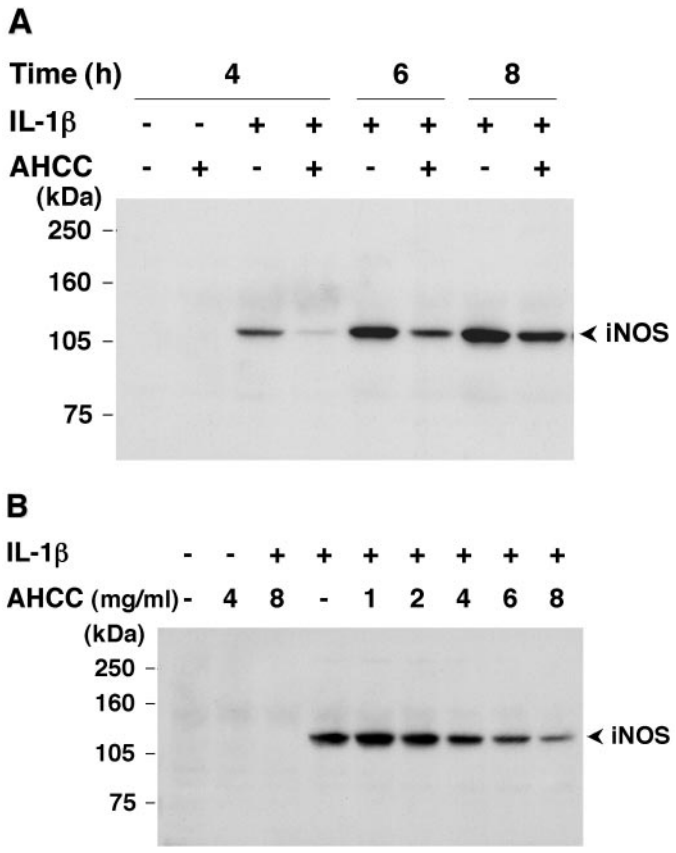

FIGURE 3. Effect of AHCC on the induction of iNOS protein in hepatocytes. Cells were treated with IL-1β (1 nmol/L) in the presence or absence of AHCC (8 mg/mL) for the indicated times (A), or in the presence of AHCC at various concentrations (1–8 mg/mL) for 8 hours (B). Cell lysates (50 μg of protein) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 7.5% polyacrylamide gels, and immunoblotted with an anti-iNOS antibody as described. Molecular mass markers are shown in kDa on the left. Representative results of 4 independent experiments are shown.

er-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 a 15-minute incubation on a magnetic plate at room temperature, the medium was replaced by fresh medium with serum. Then, cells were cultured overnight, treated with or without IL-1β, and the Luc and β-galactosidase activities of the cell extracts were measured using PicaGene (Wako Pure Chemical) and β-Glo kits (Promega), respectively.

Statistical Analysis

Results in the figures are representative of 2–4 independent experiments yielding similar findings. Differences were analyzed by the Bonferroni/Dunn test, and $p < .05$ was considered to indicate statistical significance.

RESULTS

AHCC Decreases the Levels of NO Production in Hepatocytes

The proinflammatory cytokine IL-1β induced the gene expression of iNOS and increased the production

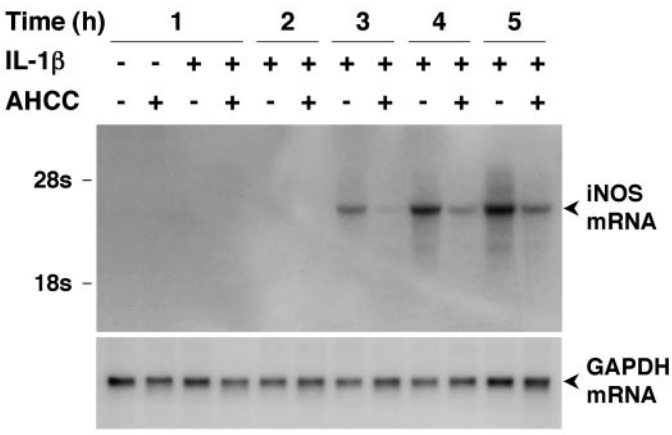

FIGURE 4. Effect of AHCC on the induction of iNOS mRNA in hepatocytes. Cells were treated with IL-1β (1 nmol/L) in the presence or absence of AHCC (8 mg/mL) for the indicated times. Total RNA (10 μg) was analyzed by Northern blot. The filters were hybridized with radiolabeled iNOS and glyceraldehyde-3-phosphate dehydrogenase cDNAs. Representative results of 3 independent experiments are shown.

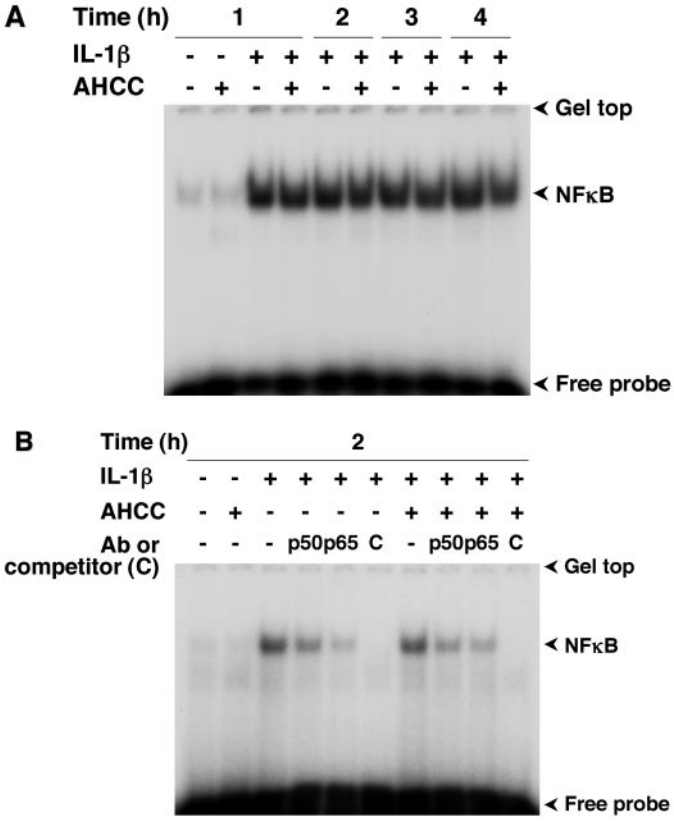

FIGURE 5. Effect of AHCC on the activation of NFκB in hepatocytes. Cells were treated with IL-1β (1 nmol/L) in the presence or absence of AHCC (8 mg/mL) for the indicated times. A, Nuclear extracts (4 μg of protein) were analyzed by an electrophoretic mobility shift assay. B, Nuclear extracts were incubated with the labeled NFκB consensus oligonucleotide in the presence of anti-p50 antibody, anti-p65 antibody, or cold probe (250-fold excess) as a competitor. Representative results of 3 independent experiments are shown.

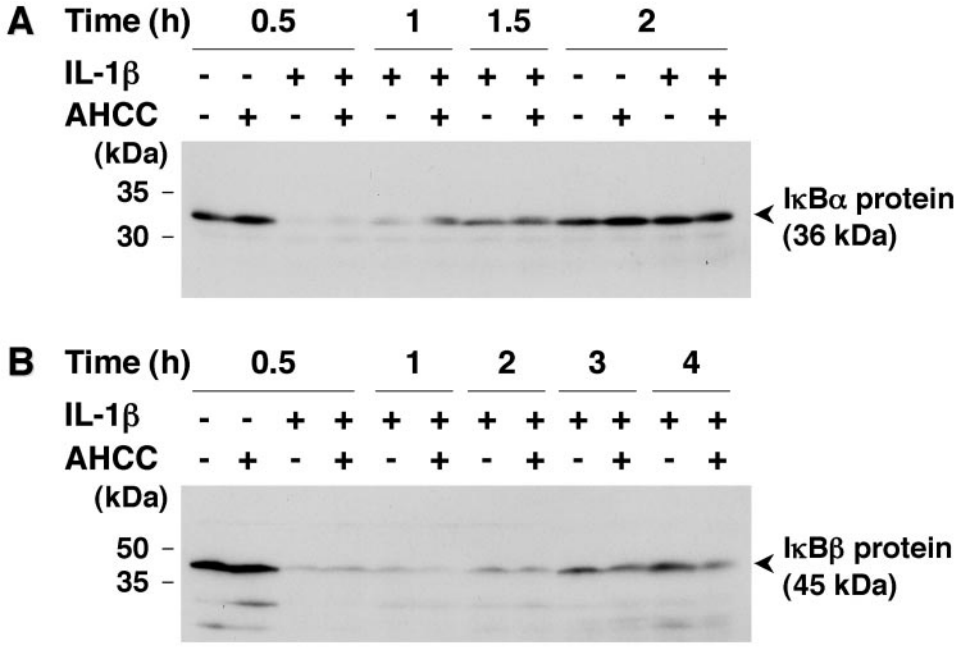

FIGURE 6. Effect of AHCC on the degradation of IκB in hepatocytes. Cells were treated with IL-1β (1 nmol/L) in the presence or absence of AHCC (8 mg/mL) for the indicated times. Cell lysates (50 μg of protein) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) through 12.5% gels, followed by immunoblotting with anti-IκBα (A) or anti-IκBβ (B) antibodies. Representative results of 3 independent experiments are shown.

of NO in primary cultures of rat hepatocytes, as reported previously.[29,30] Pretreatment of rat hepatocytes with AHCC inhibited NO production in a time-dependent fashion (Figure 1A). Figure 1B shows the concentration dependence of the effect of AHCC in the presence of IL-1β for 8 hours. AHCC decreased the level of NO production in a dose-dependent manner to the maximum concentration tested, showing approximately 80% inhibition at a concentration of 8 mg/mL. AHCC (8–10 mg/mL) alone had little effect on the production of NO. AHCC was not toxic to cells within the incubation periods, irrespective of the presence of IL-1β, as tested by the release of LDH (Figure 2) and trypan blue exclusion (data not shown).

AHCC Inhibits the Induction of iNOS Protein and mRNA

We further investigated the mechanisms of the AHCC effect on NO production. Western blot analysis revealed that pretreatment of cells with AHCC decreased the levels of iNOS protein (130 kDa) both time and dose-dependently compared with IL-1β alone (Figure 3A and B). The level of iNOS mRNA was also decreased time-dependently in the presence of AHCC (Figure 4).

AHCC Has No Effect on the Activation of NFκB and the Degradation of Inhibitory Protein κB (IκB)

The promoters of the murine and human genes encoding iNOS contain a consensus sequence for the binding of the transcription factor NFκB,[31–33] which is necessary to confer inducibility by cytokines. NFκB is typically found in the form of a p50/p65 heterodimer attached to the inhibitory molecule IκB in the cytoplasm of cells.[34] IL-1β stimulates the degradation of IκB proteins, followed by the activation of NFκB, that is, its nuclear translocation and DNA binding. An EMSA revealed that AHCC had no effect on NFκB activation (Figure 5A). The supershift assays with antibodies against the NFκB subunits p50 and p65 revealed that AHCC also had no effect on the component subunits of NFκB (Figure 5B). Furthermore, Western blot analysis of IκB proteins showed that AHCC had no effect on the degradation of IκBα and IκBβ stimulated by IL-1β (Figure 6). These results suggested that AHCC had no significant effect on the translocation of NFκB from the cytoplasm to the nucleus or on its DNA binding.

AHCC Inhibits the Activity of the iNOS Promoter Construct Containing 3′UTR

In transfection experiments, we used 2 iNOS promoter firefly Luc constructs: pRiNOS-Luc and pRiNOS-Luc-3′UTR (Figure 7A). The former harbored the 1.2-kb promoter of the rat iNOS gene, the Luc gene and an SV40 polyadenylation signal (SVpA). The SVpA has no AU-rich element (ARE) and is known to stabilize mRNA. The latter harbored the 3′UTR of the rat iNOS mRNA, downstream of the Luc gene, instead of the SVpA. The rat iNOS 3′UTR contains 6 AREs (5′-AUUUA-3′ or 5′-AUUUUA-3′), which are usually found in unstable mammalian mRNAs encoding acute phase proteins and cytokines and are involved in the stabilization of mRNA.[35,36] When the iNOS promoter-Luc-SVpA construct (pRiNOS-Luc-SVpA) was introduced into hepatocytes, IL-1β increased Luc activity with time, showing a maximal effect (6- to 8-fold of control) at 8 hours (data not shown). AHCC had no effect on the transactivation of the pRiNOS-Luc pro-

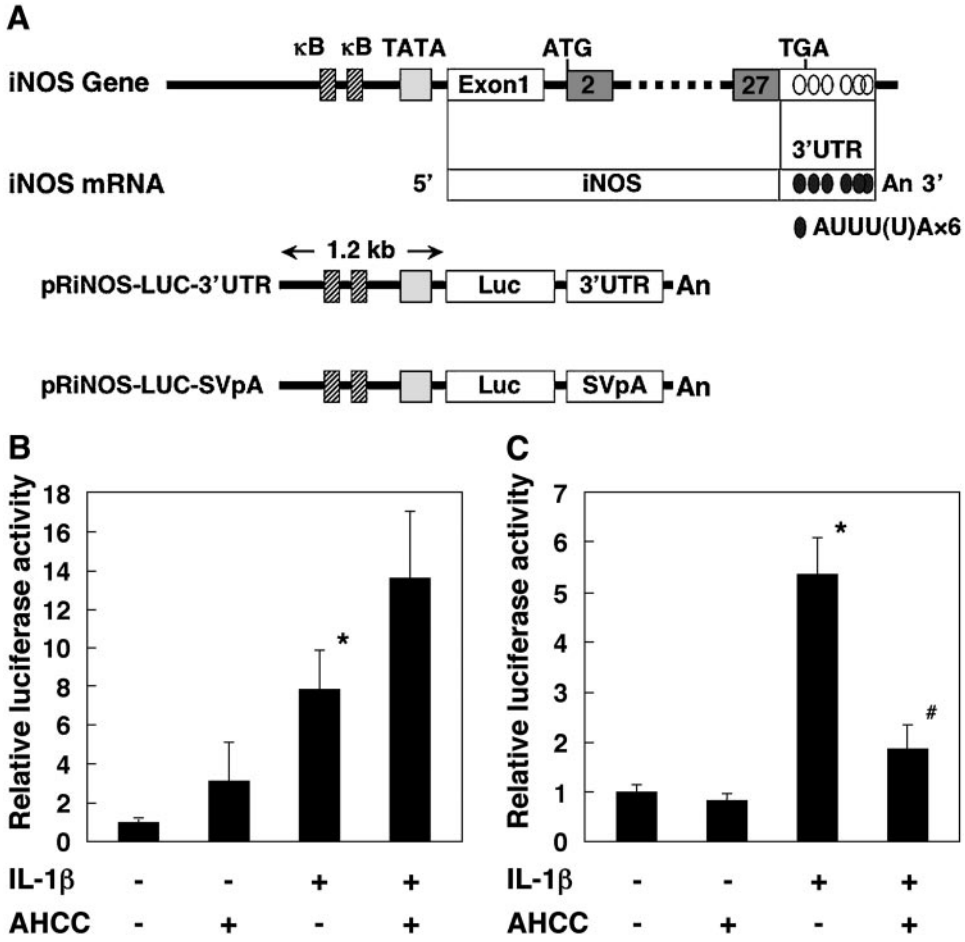

FIGURE 7. Effect of AHCC on iNOS promoter activation in hepatocytes. A, Schematic representation of the promoter region of the iNOS gene. Two reporter constructs are shown beneath the iNOS gene and mRNA. The constructs consist of the rat iNOS promoter (1.2 kb), the luciferase gene, and either the iNOS 3′UTR (pRiNOS-Luc-3′UTR) or an SV40 poly(A) region (pRiNOS-Luc-SVpA), in which An is a poly(A) tail. Each construct was introduced to hepatocytes, and cells were treated with IL-1β (1 nmol/L) in the presence or absence of AHCC (8 mg/mL) for the indicated times; (B) pRiNOS-Luc-SVpA for 8 h and (C) pRiNOS-Luc-3′UTR for 3 h. Luciferase activity was normalized to β-galactosidase activity. Fold activation is calculated by dividing luciferase activity by that of the control, ie, IL-1β (−), or AHCC (−). Data represent mean ± SD (n = 4 dishes). *$p < .05$ *vs* control, #$p < .05$ *vs* IL-1β without AHCC. Representative results of 4 independent experiments are shown.

moter (Figure 7B). When the iNOS promoter-Luc-3′UTR construct (pRiNOS-Luc-3′UTR)—which responded to IL-1β faster than pRiNOS-Luc did—was introduced into heptocytes, a maximal effect (4- to 6-fold of control) was seen at 3 hours; however, AHCC significantly inhibited the activity of the pRiNOS-Luc-3′UTR promoter (Figure 7C). These results suggested that AHCC inhibited the stabilization of iNOS mRNA.

DISCUSSION

In the present study, we found that AHCC markedly inhibited the induction of iNOS mRNA and protein (Figures 3 and 4) and NO production (Figure 1) stimulated by the proinflammatory cytokine IL-1β in primary cultures of rat hepatocytes. AHCC could not influence the IκB/NFκB pathway, which is stimulated by IL-1β through the activation of IκB kinase, as it had no effect on the degradation of the IκB proteins IκBα and IκBβ (Figure 6) or on the activation of NFκB (its nuclear translocation and DNA binding; Figure 5). This suggests that the transcriptional activation of the iNOS gene by the transcription factor NFκB remains unchanged.

In support of these observations, transfection experiments with an iNOS promoter-luciferase construct (pRiNOS-Luc), which detects the transcriptional activity of iNOS mRNA (synthesis of mRNA), showed that AHCC had no inhibitory effect (Figure 7B). By contrast, experiments with an iNOS promoter construct containing its 3′UTR (pRiNOS-Luc-3′UTR) revealed that AHCC significantly reduced Luc activity (Figure 7C), suggesting that the 3′UTR of iNOS mRNA is involved in its stability. These results indicate that AHCC presumably destabilizes iNOS mRNA at a post-transcriptional step, as shown in Figure 8.

Recent accumulated evidence suggests that post-transcriptional mechanisms such as mRNA stabilization are critically involved in the regulation of iNOS expression.[35,36] The 3′UTR of iNOS mRNA, which contains 6 AREs (AUUU(U)A), is associated with ARE-binding proteins such as HuR and hnRNPI/L, resulting

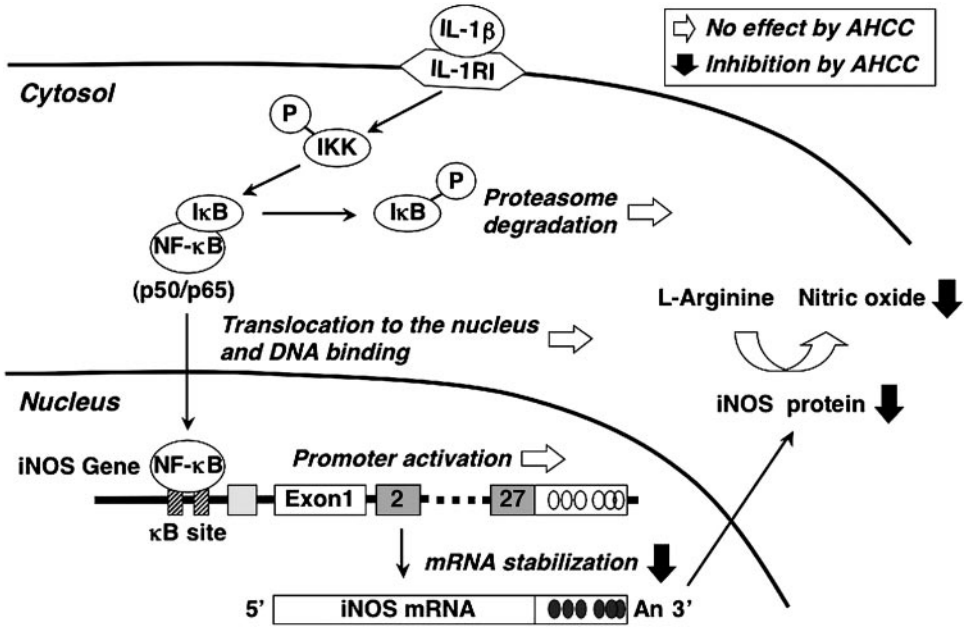

FIGURE 8. Schematic model for the effect of AHCC on the induction of iNOS gene expression in hepatocytes. IL-1β stimulates an essential signal, which is the degradation of IκB and the activation of NFκB, through the phosphorylation of IκB kinase (IKK), resulting in the induction of iNOS. In the presence of IL-1β, AHCC inhibits the stabilizing activity of iNOS mRNA, leading to decreases in the levels of iNOS protein and NO production. An is a poly(A) tail.

in the regulation of mRNA stabilization. This is not only the case for iNOS but also for a variety of inflammatory genes, including cytokines.

It is possible that the inhibitory effect of AHCC on the production of NO through the inhibition of iNOS induction is associated with AHCC-induced protection against liver failure. Further investigation is needed to delineate the mechanism by which AHCC acts on iNOS expression in hepatocytes, as well as to perform an *in vivo* study with an animal model of liver injury. The fractionation and purification of the complex compound AHCC are also needed to identify the effective component associated with the inhibition of iNOS induction and the prevention of liver injury. Such studies may provide a foundation for novel pharmacologic approaches to prevent liver dysfunction.

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Discussant

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1. Conceptually, do the authors believe that this (inducible nitric oxide [iNOS], NO reduction) is the clinically relevant mechanism? Did you postulate that reduction in iNOS would be protective in chemotherapy-induced hepatotoxity? There was little background discussion to support the experimental protocol. Or was this study done to evaluate whether this substance would be another effective iNOS inhibitor like L-NMME or L-NAME, according to its reported clinical performance?
2. Because the substance is thought to have multiple different potential effects on immune function, including enhanced NK activity, the question naturally arises whether the investigators' dosing would be considered analogous to the amounts recommended in clinical practice (dosing is about 1000 mg 3 times per day on the websites; eg, we often attribute different effects to differing doses of a "medication"). Is this at play here?
3. For protocol, my first question also related to the choice of dosing. Did you try logarithmic increases in dosing, or how did you determine that you would see dose dependence in the 0–10 mg/mL range? It would be interesting to know whether you were able to inhibit IL-1β-stimulated NO production completely at any dose. It would be interesting to see whether higher doses had effects on cell viability.
4. As the authors allude to, further experiments are essential. Certainly the next experiment might involve "injured" and "noninjured" animals treated with AHCC who then undergo hepatocyte isolation and are tested for responsiveness to IL-1β in terms of NO production. Do you have any preliminary data?

Author's Response

1. Here is our background to support the experimental protocol for this AHCC study.

As we previously reported in rat models of liver injury,[1–5] we have demonstrated that various insultssuch as hepatic ischemia-reperfusion, hepatectomy/LPS, and D-galactosamine/LPS caused liver damage and increased the mortality rate in concert with the induction of inflammatory mediators, including cytokines and iNOS/NO in the liver. We also have found the molecular mechanisms involved in the induction of iNOS gene expression in hepatocytes by proinflammatory cytokine IL-1β.[6–11] It seems likely that NO produced by iNOS under pathologic conditions as mentioned above has detrimental effects in the liver, although the duality of NO is well documented in a variety of organs, including the liver, intestine, and brain. Finally, we have found that clinical drugs and chemical regents inhibited the induction of iNOS/NO production, as well as proinflammatory cytokines such as TNF-α, IL-1β, interferon-γ, and CINC-1, resulting in the prevention of liver injury.[12–17]

These accumulated observations prompted us to investigate whether AHCC inhibits the induction of iNOS gene expression stimulated by proinflammatory cytokine IL-1β. The possibility of AHCC regulation of NO production by iNOS was pursued as a potential liver-protecting mechanism. As mentioned in this paper, it seems that AHCC inhibits the induction of iNOS at the posttranscriptional step, that is, the destabilization of iNOS mRNA through the 3′-untranslated region (3′UTR), which is a different mechanism where iNOS inhibitors such as L-NMME and L-NAME act on the activities of iNOS protein. We believe such AHCC effect is a clinically relevant mechanism because mRNAs from a variety of inflammatory genes have 3′UTR containing AU-rich elements, which are responsible for the stabilization of mRNA.

2. The concentrations of AHCC used in our experiments were 1–8 mg/mL in cultured hepatocytes. In animal models of cancers and other diseases, AHCC was treated at 100–1000 mg/kg, which corresponds to approximately 1–10 mg/mL of blood.

3. In our preliminary experiment, the maximal concentration of AHCC dissolved in culture medium (Williams' medium E; WE) was about 10 mg/mL, although the small insoluble pellet was precipitated by centrifugation (1500 × *g* for 10 minutes) after AHCC powder was stirred with WE for 30 minutes at room temperature. So we never used doses >10 mg/mL. AHCC (8–10 mg/mL) decreased levels of NO production by 70%–85% but not 100%. The same doses of AHCC were not toxic but rather protective, even in the presence of IL-1β, as shown in Figure 3 of our manuscript.

4. Animal experiments with liver injury are required to examine whether AHCC inhibits the induction of iNOS and NO production in the liver *in vivo* and has protective effects on hepatic dysfunction, as you mentioned in your comment. However, the experiments are under investigation and we have no preliminary data at present.

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