Active Hexose Correlated Compound Inhibits the Expression of Proinflammatory Biomarker iNOS in Hepatocytes


Department of Surgery, Kansai Medical University, Moriguchi, Department of Biomedical Sciences, College of Life Sciences, and Research Organization of Science and Technology, Ritsumeikan University, Kusatsu, Japan

Key Words
Active hexose correlated compound · Interleukin-1β · iNOS · Nuclear factor-κB · Type I interleukin-1 receptor · iNOS gene antisense transcript

Abstract
Background/Aims: Excess production of nitric oxide (NO) by inducible nitric oxide synthase (iNOS) has been implicated as a proinflammatory biomarker in liver injury. The application of active hexose correlated compound (AHCC) as a functional food in complementary and alternative medicine has increased. The possibility that AHCC might inhibit iNOS induction was investigated as a potential liver-protective effect.

Methods: Hepatocytes were isolated from rats by collagenase perfusion and cultured. Primary cultured hepatocytes were treated with interleukin-1β in the presence or absence of AHCC-sugar fraction (AHCC-SF).

Results and Conclusion: AHCC-SF inhibited the production of NO and reduced expressions of iNOS mRNA and its protein. AHCC-SF had no effects on either IκB degradation or nuclear factor-κB (NF-κB) activation. In contrast, AHCC-SF inhibited the upregulation of type I interleukin-1 receptor (IL-1RI) through the inhibition of Akt phosphorylation. Transfection experiments with iNOS promoter-luciferase constructs revealed that AHCC-SF reduced the levels of iNOS mRNA at both promoter transactivation and mRNA stabilization steps. AHCC-SF inhibited the expression of iNOS gene antisense transcript, which is involved in iNOS mRNA stabilization. These findings demonstrate that AHCC-SF suppresses iNOS gene expression through a IκB/NF-κB-independent but Akt/IL-1RI-dependent pathway, resulting in the reduction of NO production. AHCC-SF may have therapeutic potential for various liver injuries.

Introduction
In the liver, nitric oxide (NO) is produced by constitutively expressed endothelial nitric oxide synthase (eNOS) or inducible NOS (iNOS). eNOS is located in vascular sinusoidal endothelial cells, and NO produced by eNOS maintains hepatic circulation and endothelial integrity. iNOS is negligible under physiological conditions, but is expressed in hepatic cells including hepatocytes and Kupffer cells under pathological conditions such as sepsis, hemorrhagic shock, ischemia-reperfusion, hepatitis, and cirrhosis. During infection and inflammation in the liver, excess production of NO by iNOS is thought to be
involved in liver injury. The expression of iNOS is a biomarker in proinflammation, although NO has been reported to exert either detrimental or beneficial effects depending on the insults and tissues involved. In our previous reports, clinical drugs, which showed liver-protective effects in various animal models of liver injury [1–5], prevented iNOS induction in the liver as well as decreased production of various inflammatory mediators. These drugs also inhibited iNOS induction and NO production in proinflammatory cytokine-stimulated cultured hepatocytes of rats [3, 6, 7], which is used as a simple in vitro injury model.

Proinflammatory cytokine interleukin (IL)-1β, or a mixture of IL-1β, tumor necrosis factor (TNF)-α and interferon-γ, induces the expression of iNOS gene in primary cultures of human and rat hepatocytes [8, 9]. The induction of iNOS is regulated by transcriptional activation of the iNOS promoter with transcription factors including nuclear factor (NF)-κB, and by post-transcriptional modifications including mRNA stabilization [10]. There are two essential pathways involved in iNOS induction, IκB kinase/IκB/NF-κB activation and phosphatidylinositol-3 kinase (PI3K)/Akt/type I IL-1 receptor (IL-1RI) upregulation [11]. IL-1β stimulates the degradation of IκB after its phosphorylation by IκB kinase, which is followed by the translocation of NF-κB from cytoplasm to the nucleus and DNA binding (NF-κB activation). IL-1β also stimulates the upregulation of IL-1RI through activation of PI3K/Akt, which is essential for both transcriptional activation and mRNA stabilization in iNOS induction [7, 11–13]. In the case of mRNA stabilization, we have reported that natural iNOS gene antisense transcript interacts with 3′-untranslated region (UTR) containing AU-rich elements (ARE) of iNOS mRNA, leading to iNOS mRNA stabilization in IL-1β-stimulated hepatocytes [14].

The functional food active hexose correlated compound (AHCC) is an extract prepared from cultured mycelium of Basidiomycetes mushrooms. In recent reports [15–20], supplementation with AHCC has shown a generalized positive effect on the immune systems, as well as anti-inflammatory and anti-oxidant effects. AHCC is a mixture of polysaccharides, amino acids, lipids, and minerals, in which oligosaccharides are the major components constituting about 74% of the mixture. These oligosaccharides are believed to account for the biological activities of AHCC [21, 22]. In the liver, we reported that AHCC improved the prognosis of postoperative hepatocellular carcinoma patients [23]. However, the molecular mechanism by which AHCC protects the liver is not fully understood. In the current study, the possibility that AHCC might inhibit NO production was pursued as a possible liver-protecting mechanism. We intended to examine whether AHCC influences the induction of iNOS gene expression in primary cultures of rat hepatocytes, and if so, study the mechanism involved in this process.

**Materials and Methods**

**Materials**

Recombinant human IL-1β (2 × 10^7 U/mg protein) was provided by Otsuka Pharmaceutical Co. Ltd. (Tokushima, Japan). [γ-32P]adenosine-5′-triphosphate (ATP; –222 TBq/mmol) and [α-32P]deoxyguanosine-5′-triphosphate (dCTP; –111 TBq/mmol) were obtained from DuPont-New England Nuclear Japan (Tokyo, Japan). Rats were kept at 22 °C under a 12-h light/12-h dark cycle, and received food and water at 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.

**Preparation of Sugar Fraction of AHCC**

As shown in figure 1, the extract of AHCC (20 g), which was supplied by Amino Up Chemical Co. Ltd (Sapporo, Japan), was dissolved in H2O (80 ml), applied on the column of DIAION HP-20 (5 × 25 cm; Mitsubishi Chemical Co., Japan) and eluted with H2O (1 liter) and methanol. The first eluate (water fraction containing hydrophilic compounds) was concentrated under vacuum, followed by lyophilization (18.1 g of yellowish powder). The water fraction was dissolved in H2O (35 ml), mixed with methanol (180 ml) and centrifuged (1,600 g for 15 min), which was repeated twice. Then the precipitate was dissolved in H2O (100 ml), applied on the DOWEX 50 WX8 (4.4 × 17 cm; The Dow Chemical Company, USA), and eluted with H2O (0.5 liter) and ammonia (3 N). The final water fraction containing AHCC-sugar fraction (AHCC-SF) was lyophilized (4.93 g) and stored at –20°C.

---

**Fig. 1.** Preparation of AHCC-SF. Separation flow of AHCC.
Primary Cultures of Hepatocytes

Hepatocytes were isolated from male Wistar strain rats (200–220 g; Charles River, Tokyo, Japan) by collagenase (Wako Pure Chemicals, Osaka, Japan) and pronase (Wako Pure Chemicals, Osaka, Japan) according to the manufacturer’s instructions. Next, 10 μg of total RNA was fractionated by 1% agarose-formaldehyde gel electrophoresis, transferred to nylon membranes (Nytran; Schleicher and Schuell, Dassel, Germany), immobilized by baking at 80°C for 1 h and hybridized with DNA probes. A cDNA probe for rat iNOS (830 bp) was described previously [29]. cDNAs encoding rat IL-1RI [30] and mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [31] were prepared by RT-PCR [32]. The cDNAs were radiolabeled with [α-32P]dCTP by the random priming method.

For strand-specific RT-PCR analysis, cDNAs were synthesized from total RNA with strand-specific primers, and step-down PCR was performed as previously described [32, 33]. For iNOS and elongation factor (EF)-α (internal control) mRNAs, an oligo(dT) primer was used for RT and primer sets 5'-CCAACCGTGAGGCTCTGAAG-3' and 5'-GTGACGCTCAACCTGGGTGAAAC-3' (257 bp product) and 5'-TCTGGTGGAATGTTGTAACATGTCGTG-3' and 5'-CCAGGAAGAGCTTCACTCTCAAG-3' (307 bp product) were used for PCR, respectively. For the antisense transcript of iNOS gene, sense primer 5'-TGCCCCTTCATCAGTCTC-3' was used for RT and the primer set 5'-ACAGGGAGGCCATCCCCTGGGC-3' and 5'-CTGTACCATACACATCTTTATATAA-3' (186 bp product) were used for PCR. The iNOS mRNA and antisense transcript levels were measured in triplicate by real-time PCR using an iCycler System (Bio-Rad Laboratories). SYBR Green I (Roche Diagnostics) was included in the reaction mixture, and the following touchdown protocol was applied: 1 cycle of 94°C for 1 min; and 50 cycles of 94°C for 30 s, (72–0.3 × n)° C for 1 min where n is the number of cycles, and 72°C for 30 s. Rat cDNAs for the iNOS mRNA and antisense transcript were deposited in DDBJ/EMBL/GenBank under accession No. AB250951 and AB250952, respectively.

Electrophoretic Mobility Shift Assay (EMSA)

Nuclear extracts were prepared according to Schreiber et al. [34] with minor modifications [35]. Briefly, the dishes were placed on ice, washed with Tris–HCl-buffered saline, harvested with the same buffer using a rubber policeman and centrifuged (1,840 g for 1 min). The precipitate (2 × 106 cells from two 100-mm dishes) was suspended in 400 μl of lysis buffer (10 mM Hepes, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 500 U/ml trasyol, 0.5 mM PMSF and 1 mM dithiothreitol) and incubated on ice for 15 min. After addition of Nonidet P-40 (final: 0.625%), the cells were lysed by vortexing (2–3 times for 1 min each) and centrifuged (15,000 g for 1 min). The nuclear pellet was resuspended with extraction buffer (10 mM Hepes, pH 7.9, 0.4 mM NaCl, 0.1 mM EDTA, 0.1 mM EGTA, 500 U/ml trasyol, 0.5 mM PMSF and 1 mM dithiothreitol) and incubated on ice for 15 min. After addition of cold probes as a competitor, the dried gels were treated with 1 ml of 100 μg/ml 32P-labeled competitor DNA and run on polyacrylamide gel electrophoresis. The dried gels were analyzed by autoradiography. An NF-kB consensus oligonucleotide (5'-AGTTGAGGGGA-CTTCCAGGGGC-3') from the mouse immunoglobulin κ light chain was purchased (Promega, Madison, WI, USA).
Madison, Wisc., USA) and labeled with $[^{32}]P$ATP and T4 polynucleotide kinase. The protein concentration was measured by the method of Bradford [36] with a binding assay kit (Bio-Rad Laboratories) using bovine serum albumin as a standard.

**Construction of Luciferase Reporter Plasmids and Expression Plasmids**

The 1.2-kb 5'-flanking region including the TATA box of the rat iNOS gene was inserted into the pGL3-Basic vector (Promega) to create pRiNOS-Luc-SVpA [35]. A rat cDNA for the 3'-UTR of the iNOS mRNA was amplified with the primers 5'-tgctcattagcgttgaggggtttgagaga-3' and 5'-gggattcttattttctttgatcaaactcttatttt-3', and the resultant cDNA was digested with BamH I and Xba I. This cDNA for the iNOS 3'-UTR (submitted to DDBJ/EMBL/GenBank under accession No. AB250951) was used to replace the SV40 polyadenylation signal (SVpA) of pRiNOS-Luc to create pRiNOS-Luc-3'-UTR [12].

**Transfection and Luciferase Assay**

Transfection of cultured hepatocytes was performed as described previously [37, 38]. Briefly, hepatocytes were cultured at $4 \times 10^5$ cells/dish (35 × 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. The cells were cultured overnight, and then treated with IL-1β in the presence or absence of AHCC-SF. The luciferase and β-galactosidase activities of cell extracts were measured using PicaGene (Wako Pure Chemicals) and Beta-Glo (Promega) kits, respectively.
Statistical Analysis
The results shown in the figures are representative of 3–4 independent experiments yielding similar findings. Differences were analyzed by the Bonferroni-Dunn test, and values of \( p \leq 0.05 \) were considered to indicate statistical significance.

Results

AHCC-SF Inhibits iNOS Induction in IL-1β-Stimulated Hepatocytes
The proinflammatory cytokine IL-1β stimulates the induction of iNOS gene expression in primary cultures of rat hepatocytes [9, 37]. Simultaneous addition of AHCC-SF (1–8 mg/ml) with IL-1β decreased the production of NO time- and dose-dependently (fig. 2A, B). AHCC-SF had a maximal effect (over 80% inhibition) at 8 mg/ml, but showed no cellular cytotoxicity as evaluated by release of lactate dehydrogenase into the culture medium and Trypan blue exclusion in hepatocytes (data not shown). Western and northern blot analyses revealed that AHCC-SF decreased the levels of iNOS protein (fig. 2B, C, upper panel) and iNOS mRNA (fig. 2C, lower panel), suggesting that it inhibited the induction of iNOS gene expression at a transcriptional and/or post-transcriptional step.

AHCC-SF Has No Effects on IκB Degradation and NF-κB Activation
We examined the mechanism involved in the inhibition of iNOS induction. AHCC-SF did not influence the degradation of IκBα and IκBβ proteins at 0.5 h and their recovery at 1–4 h (fig. 3), EMSAs with the nucleus revealed that AHCC-SF had no effect on the activation of NF-κB (fig. 4, left). Furthermore, supershift experiments showed that AHCC-SF also had no effect on the components of NF-κB subunits (p50 and p65) (fig. 4, right), since the NF-κB bands stimulated by IL-1β disappeared similarly in the presence of antibodies against p50 and p65, irrespective of the presence of AHCC-SF.
AHCC-SF Decreases Upregulation of IL-1RI

IL-1β stimulates the upregulation of IL-1RI through activation of PI3K/Akt [11], which is essential for the induction of iNOS gene expression in addition to NF-κB activation in hepatocytes. AHCC-SF reduced the phosphorylation of Akt (fig. 5A), which is a downstream kinase of PI3K. AHCC-SF also inhibited the increased expressions of IL-1RI mRNA and its protein (fig. 5B, C).

These observations suggest that AHCC-SF can influence the downstream events of IL-1RI upregulation, but not through IκB degradation and NF-κB activation.

AHCC-SF Affects iNOS mRNA Synthesis and Stabilization

Next, we carried out transfection experiments with iNOS promoter-firefly luciferase constructs, namely
pRiNOS-Luc-SVpA and pRiNOS-Luc-3’-UTR (fig. 6A), which detect the activities of iNOS promoter transactivation (iNOS mRNA synthesis) and iNOS mRNA stabilization, respectively [12, 39]. IL-1β increased the luciferase activities of these constructs, and AHCC-SF significantly reduced both of these luciferase activities (fig. 6B, C). The luciferase activities were normalized by the β-galactosidase activity. The fold activation was calculated by dividing the luciferase activity by that of the control (without IL-1β and AHCC-SF). Data are means ± SD (n = 4 dishes). * p < 0.05 versus control, # p < 0.05 versus IL-1β alone.

Discussion

In the present study, AHCC-SF was found to inhibit iNOS induction at the steps of both its promoter transactivation (mRNA synthesis) and mRNA stabilization in proinflammatory cytokine-stimulated hepatocytes (fig. 6). In the former, although AHCC-SF reduced the activities of iNOS promoter transactivation, AHCC-SF had no effects on IκB degradation (fig. 3) and NF-κB activation (fig. 4), indicating that AHCC-SF cannot influence the nuclear translocation of NF-κB and its DNA binding in IκB kinase signaling. In concert with IκB degradation/NF-κB activation, the upregulation of IL-1RI, which stimulates the phosphorylation of NF-κB subunit p65, is required for transcriptional activation of the iNOS gene, as we reported previously [11]. In the present study,
we found that AHCC-SF decreased the expression of IL-1β mRNA and protein (fig. 5B, C) through the inhibition of Akt phosphorylation (fig. 5A) in PI3K/Akt signaling, presumably leading to the inhibition of p65 phosphorylation and resulting in decreased activities of iNOS promoter transactivation (fig. 6B).

Regarding the iNOS mRNA stabilization, the 3'-UTR of the iNOS mRNA in rats has six AREs (AUUU(U)A), which are associated with ARE-binding proteins such as HuR and heterogeneous nuclear ribonucleoproteins L/I (PTB), thus contributing to the stabilization of the mRNA [40]. Recently, we found that the antisense strand corresponding to the 3'-UTR of iNOS mRNA is transcribed from the iNOS gene, and that the iNOS mRNA antisense transcript plays a key role in stabilizing the iNOS mRNA by interacting with the 3'-UTR and ARE-binding proteins [14]. In our in vitro model, AHCC-SF prevented the stabilization of iNOS mRNA (fig. 6C) by decreasing the iNOS gene mRNA antisense transcript expression (fig. 7A). Drugs such as edaravone (free radical scavenger) [7], FR183998 (Na+/H+ exchanger inhibitor) [3, 5], insulin-like growth factor I [4] and dexamethasone [41] were found to inhibit iNOS induction partly by suppressing iNOS antisense transcript production in primary cultured hepatocytes (our in vitro model) and in animal models of liver injury. In the case of dexamethasone, it
had no effects on either NF-κB activation or IL-1RI up-regulation as compared with AHCC-SF. Dexamethasone inhibited the stabilization of iNOS mRNA but had no effect on the iNOS promoter transactivation [41], suggesting that IL-1RI up-regulation as well as NF-κB activation is involved in transcriptional activation of the iNOS gene as mentioned before.

Our in vitro results suggest that AHCC-SF might inhibit the induction of iNOS expression and NO production in liver injury, which leads to liver-protective effects. Although such liver-protective effects derived from our in vitro model need to be examined and supported in vivo animal models of liver injury, our simple model with cultured hepatocytes may be adequate for the screening of liver-protective drugs, because it is rapid and inexpensive compared with animal models. In conclusion, AHCC-SF inhibited iNOS gene expression at transcriptional and post-transcriptional steps in cultured hepatocytes in an in vitro liver injury model. AHCC may have liver-protective effects for various liver injuries.

Acknowledgements

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.

References


