Japanese Herbal Medicine Hochuekkito Inhibits the Expression of Proinflammatory Biomarker, Inducible Nitric Oxide Synthase, in Hepatocytes

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Abstract

Hochuekkito (TJ-41) is used for the treatment of complaints in patients with general fatigue. However, there is little scientific evidence to demonstrate the liver-protective effects of TJ-41. During inflammation, proinflammatory cytokines stimulate the induction of inducible nitric oxide synthase (iNOS). Over-production of NO by iNOS has been implicated as a factor in liver injury. We examined proinflammatory cytokine-stimulated hepatocytes as a simple in vitro injury model to determine liver-protective effects of TJ-41. The objective was to investigate whether TJ-41 influences iNOS induction and to determine its mechanism. Primary cultured rat hepatocytes were treated with interleukin (IL)-1β in the presence or absence of TJ-41. The induction of iNOS and its signaling pathway were analyzed. IL-1β produced increased levels of NO. This effect was inhibited by TJ-41, which exerted its maximal effects at 6 mg/ml. TJ-41 decreased the levels of iNOS protein and its mRNA expression. Experiments with nuclear extracts revealed that TJ-41 inhibited the induction of iNOS by the inhibition of promoter transactivation and mRNA stabilization. TJ-41 reduced the expression of an iNOS gene antisense-transcript, which is involved in iNOS mRNA stability. Results indicate that TJ-41 inhibits the induction of iNOS at both transcriptional and post-transcriptional steps, leading to the prevention of NO production. TJ-41 may have therapeutic potential for various liver injuries through the suppression of iNOS induction.

Keywords: Inducible nitric oxide synthase; Interleukin-1β; Liver injury; Nuclear factor-xB; Primary cultured hepatocytes; Type I interleukin-1 receptor

Introduction

Hochuekkito (TJ-41) is a traditional Japanese herbal medicine (Kampo), which is composed of ten species of herb (Astragalii Radix, Atractyloidis Lanceae Rhizoma, Ginseng Radix, Angelicae Radix, Bupleuri Fructus, Aurantii Nobilis Pericarpium, Glycyrrhizae Radix, Comificuae Rhizoma and Zingiberis Rhizoma). TJ-41 has been used for the treatment of complaints of general fatigue caused by common cold and severe weakness. TJ-41 activated a variety of immune functions in elderly persons [1]. Preoperative treatment of TJ-41 in patient undergone gastrointestinal surgery prevented immunosuppression [2]. In experiments with animal models, TJ-41 protected immunosuppressed mice from lethal Candida infection [3]. TJ-41 had protective effects on influenza virus- and herpes simplex virus type-1-infected mice [4,5], and inhibited rhinovirus infection in human tracheal epithelial cells [6]. Moreover, TJ-41 has been reported to have anti-cancer effects in a variety of organs and cells [7-10]. However, there is little scientific evidence to demonstrate the liver-protective effects of TJ-41. During inflammation, proinflammatory cytokines and nitric oxide (NO) produced by inducible nitric oxide synthase (iNOS) play an important role as factors in liver injury [11]. However, definition of the role of NO is confounded by reports that it can exert either detrimental or beneficial effects depending on the insults and tissues involved.

We previously reported that in animal liver injury models caused by various insults, such as ischemia-reperfusion, partial hepatectomy and endotoxin shock, the induction of iNOS and NO production is upregulated concomitantly with the production of proinflammatory cytokines in the liver [12-16]. In these studies, drugs showing liver-protective effects inhibited the induction of iNOS and NO production as well as the decreased production of various inflammatory mediators. Furthermore, in vitro experiments with primary cultured rat hepatocytes revealed that these drugs also inhibited the induction of iNOS and NO production [14,17,18]. Thus, downregulating NO production is considered to be an indicator of liver protection.

In this study, we used interleukin (IL)-1β-stimulated cultured hepatocytes as a simple in vitro injury model to investigate the liver-protective effects of TJ-41 for in vivo animal models. We investigated whether TJ-41 directly influences iNOS induction in cultured hepatocytes and the mechanism involved.

Materials and Methods

Materials

Hochuekkito (TJ-41) was generously provided by Tsumura Co. Ltd. (Tokyo, Japan). TJ-41 was dissolved in culture medium, followed by extraction with shaking for 30 min at room temperature and centrifugation (11,000×g for 15 min). The supernatant was used for the experiments. 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) was obtained from DuPont-New England Nuclear Japan (Tokyo, Japan).

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Rats were kept at 22°C under a 12-h/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.

**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) perfusion [19,20]. Isolated hepatocytes were suspended in culture medium at 6×10^5 cells/ml, seeded into 35-mm plastic dishes (2 ml/dish; Falcon Plastic, Oxnard, CA, USA) and cultured at 37°C in a CO₂ incubator under a humidified atmosphere of 5% CO₂ in air.

The presence or absence of TJ-41. The doses of TJ-41 used are indicated in the methods. Culture medium was also used for measurements of LDH activity (lactate dehydrogenase), a metabolite of NO, to reflect NO production by the Griess method ([22]). Culture medium was also used for measurements of LDH activity.

**Treatment of cells with TJ-41**

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 TJ-41. The doses of TJ-41 used are indicated in the appropriate figures and their legends.

**Determinations of NO production and lactate dehydrogenase (LDH)**

Culture medium was used for measurements of nitrite (a stable metabolite of NO) to reflect NO production by the Griess method [22]. 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 [17] with minor modifications as follows. Cells (1×10^6 cells/35-mm dish) were lysed in 100-200 μl of solubilizing buffer (10 mM Tris-HCl, pH 7.4, containing 1% Triton X-100, 0.5% Nonidet P-40, 1 mM EDTA, 1 mM EGTA, phosphatase inhibitor cocktail (Nacalai Tesque, Kyoto, Japan), 1 mM phenylmethylsulfonylfluoride (PMSF) and protease inhibitor cocktail (Roche)), passed through a 26-gauge needle, allowed to stand on ice for 30 min and then centrifuged (16,000× g for 15 min). The supernatant (total cell lysate) was mixed with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (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 IkBa, mouse type I IL-1 receptor (IL-1R1) (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 ECL blotting detection reagent (GE Healthcare Biosciences Corp., Piscataway, NJ, USA).

In the case of Akt, total cell lysates prepared from 100-mm dishes (5×10^6 cells/dish) were precleared 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, the immunocomplexes were centrifuged (16,000× g for 5 min). The beads were washed with solubilizing buffer, dissolved in SDS-PAGE sample buffer, and analyzed by western blotting using rabbit polyclonal antibodies against human Akt and phospho-(Ser473) Akt (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-κB p65 (BD Transduction Laboratories, Lexington, KY, USA).

**Reverse transcriptase-polymerase chain reaction (RT-PCR)**

Total RNA was extracted from cultured hepatocytes using a guanidinium-phenol-chloroform method [23] with Trizol reagent (Invi- trogen, Carlsbad, CA, USA) or a phenol-free, filter-based total RNA isolation kit (RNaseQue Kit; Ambion, Austin, TX, USA) according to the manufacturer’s instructions, and then treated with a TURBO DNA-free Kit (Ambion) if necessary. 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 [24,25] with minor modifications. For iNOS, IL-1RI and elongation factor-1a (EF; internal control) mRNAs, an oligo(dT) primer was used for RT and the primer sets 5'-CCACCTGCGGTTCTTGATG-3' and 5'-GTGCAGT- GACAAGCTGGAAC-3' (257-bp product), manufacturer’s instructions, and then treated with a TURBO DNA-free Kit (Ambion) if necessary. 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 [24,25] with minor modifications. For iNOS, IL-1RI and elongation factor-1a (EF; internal control) mRNAs, an oligo(dT) primer was used for RT and the primer sets 5'-CCACCTGCGGTTCTTGATG-3' and 5'-GTGCAGTGCACAACCTGGTGAC-3' (257-bp product), manufacturer’s instructions, and then treated with a TURBO DNA-free Kit (Ambion) if necessary. 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 [24,25] with minor modifications.

**Electrophoretic mobility shift assay (EMSA)**

Nuclear extracts were prepared according to Schreiber et al. [26] with minor modifications [27]. 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 (2×10^6 cells from three 35-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 μM trysloyl, 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 M NaCl, 0.1 mM EDTA, 0.1 mM EGTA, 500 μM trysloyl, 0.5 mM PMSF and 1 mM dithiothreitol), followed by continuous mixing for 20 min and centrifugation (15,000×g for 5 min). Aliquots of the supernatant (nuclear extract) were frozen in liquid nitrogen and stored at -80ºC until use.

Binding reactions (total: 15 μl) were performed by incubating nuclear extract aliquots (4 μg of protein) in reaction buffer (20 mM Hepes, pH 7.9, 1 mM EDTA, 60 mM KCl, 10% glycerol and 1 μg of poly[dI-dC]) with the probe (approximately 40,000 cpm) for 20 min at room temperature. The products were electrophoresed at 100 V in a 4.8% polyacrylamide gel in high ionic strength buffer (50 mM Tris-HCl, 380 mM glycerol, 2 mM EDTA, pH 8.5) and the dried gels were analyzed by autoradiography.

An NF-κB consensus oligonucleotide (5’-AGTTGAGGGGAGCTTCCCAGGC-3’) from the mouse immunoglobulin κ light chain was purchased (Promega, Madison, WI, USA) and labeled with [γ-32P]ATP and T4 polynucleotide kinase. The protein concentration was measured by the method of Bradford [28] with a binding assay kit (Bio-Rad) 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 [27]. A rat cDNA for the 3’-untranslated region (UTR) of the iNOS mRNA was amplified with the primers 5’-tgctctGATGAGGGGTGGAGAGA-3’ and 5’-gcggatcctttaTTCTTGATCAAACACTCATTTT-3’, and the resultant CDNAs was digested with BamHI and XbaI. This CDNAs 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.

Transfection and luciferase assay

Transfection of cultured hepatocytes was performed as described previously [29,30]. Briefly, hepatocytes were cultured at 3-4×10⁵ 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) in 0.2 ml of WE without supplements and incubated for 20-30 min at room temperature, followed by the addition to cultured hepatocytes. 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 TJ-41. 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<0.05 were considered to indicate statistical significance.

Results

TJ-41 reduces the production of NO and induction of iNOS in IL-1β-stimulated hepatocytes. The proinflammatory cytokine IL-1β stimulates iNOS induction, which is followed by the production of NO in primary cultured rat hepatocytes [31]. Simultaneous addition of TJ-41 with IL-1β inhibited the levels of nitrite (an NO metabolite) in the culture medium in time- and dose-dependent manners (Figure 1A and 1B, upper). TJ-41 exerted its maximal effects at the concentration of 6 mg/ml, decreasing more than 80% of NO production with IL-1β. Western blotting analysis revealed that TJ-41 inhibited the expression of iNOS protein in a dose-dependent manner, having maximal effects at 6 mg/ml (Figure 1B, lower). RT-PCR analysis revealed that TJ-41 reduced the levels of iNOS mRNA.
in a time-dependent manner (Figure 1C), suggesting that TJ-41 inhibited the induction of iNOS gene expression at a transcriptional and/or post-transcriptional step. TJ-41 showed no cellular cytotoxicity within the indicated concentrations, as evaluated by the release of LDH into the culture medium (Figure 2) and Trypan blue exclusion by hepatocytes (data not shown).

**Effects of TJ-41 on the degradation of IκB and activation of NF-κB**

IL-1β stimulates the degradation of IκBα protein, which is followed by the activation of NF-κB (its translocation to the nucleus and DNA binding). TJ-41 did not inhibit the degradation of IκBα at 0.5-2 h (Figure 3A). However, EMSA experiments with nuclear extracts revealed that TJ-41 suppressed NF-κB activation at 1-3 h (Figure 3B). In support of this observation, immunoprecipitation-western blotting of nuclear extracts revealed that TJ-41 reduced the levels of NF-κB subunit p65 in the nucleus (Figure 3C).

**Effects of TJ-41 on the upregulation of IL-1RI**

Two signaling pathways, NF-κB activation through IκB degradation and IL-1RI upregulation through phosphatidylinositol 3-kinase (PI3K)/Akt, are essential for iNOS induction [17,18,32]. Immunoprecipitation western blotting analysis revealed that IL-1β stimulated the phosphorylation of Akt, which was inhibited by TJ-41 (Figure 4A). RT-PCR and western blot analyses showed that TJ-41 reduced the expressions of IL-1RI mRNA and its protein, respectively (Figure 4B and 4C).

**Effects of TJ-41 on iNOS promoter activation and iNOS mRNA stabilization**

We examined the mechanisms involved in the inhibition of iNOS mRNA expression. It is known that the levels of iNOS mRNA are regulated by iNOS promoter transactivation with transcription factors such as NF-κB and by posttranscriptional modifications such as mRNA stabilization [33]. Therefore, we carried out transfection experiments with iNOS promoter-firefly luciferase constructs, namely pRiNOS-Luc-SVpA and pRiNOS-Luc-3’ untranslated region (UTR) (Figure 5A), which detect iNOS promoter activation (mRNA synthesis) and its mRNA stability, respectively [34,35]. IL-1β increased the luciferase activities of these constructs, and TJ-41 significantly reduced both of these luciferase activities (Figure 5B and 5C). In support of the latter, RT-PCR analysis revealed that IL-1β increased the expression of the iNOS gene antisense-transcript, which is involved in iNOS mRNA stability, and that TJ-41 markedly reduced the levels of the antisense-transcript (Figure 6).
Discussion

In the present study, we demonstrated that TJ-41 inhibited iNOS gene expression at transcriptional and posttranscriptional steps in IL-1β-stimulated hepatocytes. In the experiments with iNOS promoter constructs, TJ-41 was found to inhibit iNOS induction at the steps of both its mRNA synthesis and stabilization (Figure 5B and 5C). In the former, TJ-41 probably reduced the transactivation of the iNOS promoter through the inhibition of NF-kB activation (Figure 3B), especially by the inhibition of p65 nuclear translocation (Figure 3C). However, TJ-41 did not inhibit IκBα degradation (Figure 3A).

In concert with NF-κB translocation, the upregulation of IL-1RI is required for transcriptional activation of the iNOS gene as reported previously [32]. We found that TJ-41 decreased the expression of IL-1RI mRNA and protein (Figure 4B and 4C) through the inhibition of Akt phosphorylation (Fig. 4A), presumably leading to the inhibition of nuclear translocation and decreased DNA binding of NF-κB and resulting in decreased activities of iNOS promoter transactivation.

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 [36]. We recently reported the expression of an iNOS gene antisense-transcript in primary cultured hepatocytes. We previously reported the expression of an iNOS gene antisense-transcript that interacts with the 3'-UTR containing AU-rich elements (AREs) of iNOS mRNA and its ARE-binding proteins, thereby leading to iNOS mRNA stabilization in cytokine-stimulated hepatocytes [37]. In our in vitro model, TJ-41 destabilized the iNOS mRNA through the inhibition of iNOS gene antisense-transcript expression (Figure 6). In our recent study, not only TJ-41 but also drugs such as edaravone (free radical scavenger) [18], FR183998 (Na+/H+ exchanger inhibitor) [14] and sivelestat [38] were found to inhibit iNOS induction by suppressing both NF-kB activation and iNOS antisense-transcript production in primary cultured hepatocytes. Active hexasome correlated compound (AHCC), which is a functional food extracted from mushrooms (Basidiozymyla), has anti-cancer effect and enhance NK cell activity. We previously reported that AHCC
improved the prognosis of postoperative hepatocellular carcinoma patients [39]. AHCC also inhibited iNOS induction by suppressing iNOS antisense-transcript production, but not NF-κB activation in primary cultured hepatocytes [34]. In this point, TJ-41 is manifestly different from AHCC. Kaneko et al. [40] reported that TJ-41 markedly reduced extracellular concentration of NO at higher concentration in lipopolysaccharide-stimulated mouse macrophage-like RAW264.7 cells, while TJ-41 slightly enhanced NO at moderate concentration due to the co-existence of both the inhibitor and stimulator for NO production. In contrast, Liu et al. [41] reported that TJ-41 increased NO in serum and pancreas tissue, leading to improve islet damage in diabetic rats. Taken together, the influence of TJ-41 on NO production may be dependent on dose of TJ-41, type of cells and tissues, and insults involved. NK cell activation and NO suppression may be partly involved in the protective effects of TJ-41 against surgical stress or infection, even in the anti-cancer effects.

Ochi et al. [42] reported that TJ-41 suppressed liver fibrosis by inhibiting the productions of fibrogenic cytokines such as TGF-β1 and IL-13 in rats. However, to our knowledge, the effect of TJ-41 on acute liver diseases had never been investigated. Our simple in vitro experiment with primary cultured rat 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, the liver-protective effects of drugs deduced from this model need to be examined and supported. Considering the present results, TJ-41 is expected to have liver protective effect and iNOS inhibition in animal models of acute liver injury. Since major constituents of TJ-41 had already been elucidated by three-dimensional high-performance chromatography [4], this in vitro model may be suitable to investigate what constituent or combination of constituents is effective to inhibit iNOS induction. In conclusion, TJ-41 inhibited NO production and iNOS expression at transcriptional and posttranscriptional steps in proinflammatory cytokine-stimulated hepatocytes. TJ-41 may have therapeutic potential for various acute liver injuries via iNOS suppression.

References


