Original Article

Japanese herbal medicine, inchinkoto, inhibits inducible nitric oxide synthase induction in interleukin-1β-stimulated hepatocytes

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Aim: A herbal medicine, kampo inchinkoto (TJ-135), is used to treat jaundice and liver fibrosis in patients with cirrhosis. In the inflamed liver, proinflammatory cytokines stimulate the induction of inducible nitric oxide synthase (iNOS) gene expression. Over-production of nitric oxide (NO) by iNOS has been implicated as a factor in liver injury. We examined interleukin (IL)-1β-stimulated hepatocytes as a simple in vitro injury model to determine liver-protective effects of TJ-135. The objective was to investigate whether TJ-135 influences iNOS induction and to determine its mechanism.

Methods: Primary cultured rat hepatocytes were treated with IL-1β in the presence or absence of TJ-135. The induction of iNOS and its signaling pathway were analyzed.

Results: IL-1β produced increased levels of NO. This effect was inhibited by TJ-135, which exerted its maximal effects at 3 mg/mL. TJ-135 decreased the levels of iNOS protein and its mRNA expression. Experiments with nuclear extracts revealed that TJ-135 inhibited the translocation of nuclear factor-κB (NF-κB) to the nucleus and its DNA binding. TJ-135 also inhibited the activation of Akt, resulting in the reduction of type I IL-1 receptor mRNA and protein expression. Transfection experiments with iNOS promoter-luciferase constructs demonstrated that TJ-135 suppressed iNOS induction by inhibition of promoter transactivation and mRNA stabilization. TJ-135 reduced the expression of an iNOS gene antisense-transcript. Delayed administration or withdrawal of TJ-135 after IL-1β addition also inhibited iNOS induction.

Conclusions: Results indicate that TJ-135 inhibits the induction of iNOS at both transcriptional and post-transcriptional steps, leading to the prevention of NO production. TJ-135 may have therapeutic potential for various liver injuries through the suppression of iNOS induction.

Key words: inducible nitric oxide synthase, interleukin-1β, liver injury, nuclear factor-κB, primary cultured hepatocytes, type I interleukin-1 receptor

INTRODUCTION

Japanese Traditional Herbal medicines (Kampo) have been empirically administered by clinicians to patients with a variety of diseases. One such medicine, inchinkoto (TJ-135), is traditionally used for icteric patients with cirrhosis, and also used as an anti-inflammatory, antipyretic, choleptic and diuretic agent for liver disorders and jaundice. TJ-135 is an aqueous extract from three herbs: Artemisia capillaris spica, Gardenia fructus and Rhei rhizome with a weight ratio of 4:3:1, which is now manufactured under modern scientific quality controls. A. capillaris and G. fructus are effective for liver diseases, and R. rhizome is a laxative. It has been reported that TJ-135 was used to improve acute hepatitis of unknown etiology, but the mechanism is unknown.1,2 TJ-135 is considered as a choleretic and hepatoprotective agent with relevant effects on bile formation,3 hepatic oxidative stress, hepatic fibrogenesis and stellate cell apoptosis.4–6

However, there is little scientific evidence to demonstrate the liver-protective effects of TJ-135. In hepatic disorders, inflammatory cells such as platelets and macrophages gather around hepatic stellate cells and

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discharge cytokines. During inflammation, pro-inflammatory cytokines and nitric oxide (NO) produced by inducible nitric oxide synthase (iNOS) play an important role as factors in liver injury. 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 have 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 pro-inflammatory cytokines in the liver.8–12 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 the production of NO.8–12 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-135 for in vivo animal models. We investigated whether TJ-135 directly influences iNOS induction in cultured hepatocytes and the mechanism involved.

MATERIALS AND METHODS

Materials

INCHINKOTO (TJ-135) was provided by Tsumura Co., Ltd. (Tokyo, Japan). TJ-135 was dissolved in Williams’ medium E (WE) and vortexed for 30 min at room temperature, followed by centrifugation (11 000 g for 15 min). The supernatant was filter-sterilized with a 0.45-μm membrane filter (Millipore, Billerica, MA, USA) prior to use in experiments. TJ-135 components (A. capillaris, G. fructus and R. rhizome) were purchased from Tochimoto Tenkaido Co., Ltd. (Osaka, Japan), and were extracted with water under reflux for one hour. Extracted solutions were freeze-dried to obtain the water extracts; 0.85 g, 2.67 g and 1.61 g from A. capillaris, G. fructus and R. rhizome (each 10 g), respectively.

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). Rats were kept at 22 °C under a 12:12 h light : dark (LD) 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 rats (200–220 g; Charles River, Tokyo, Japan) by perfusion with collagenase (Wako Pure Chemicals, Osaka, Japan).15,16 Isolated hepatocytes were suspended in culture medium at 6 × 10^4 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, Heps (5 mM), penicillin (100 U/mL), streptomycin (0.1 mg/mL), dexamethasone (10 nM) and insulin (10 nM). 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 nuclei17 and using a ratio of 1.37 ± 0.04 nuclei/cell (mean ± SE, n = 7 experiments).

Treatment of cells with TJ-135 and its components

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-135 and its components. The doses of TJ-135 and its components used are indicated in the appropriate figures and their legends.

Determination of NO production and lactate dehydrogenase 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 lactate dehydrogenase (LDH) activity to reflect cell viability using a commercial kit (Wako Pure Chemicals).

Western blot analysis

Total cell lysates were obtained from cultured cells as described previously13 with minor modifications. Briefly, 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 ethylenediaminetetraacetic acid [EDTA], 1 mM ethyleneglycol bis (2-aminoethyl ether) tetraacetic acid (EGTA), phosphatase inhibitor cocktail [Nacalai...
(a) Production of nitric oxide (nmol of nitrite/10^6 cells)

(b) Production of nitric oxide (nmol of nitrite/10^6 cells)

(c) Time (h) | IL-1β (1 nM) | TJ-135 (3 mg/mL) | 4 | 5 | 6 | 7 | 8 | 10
---|---|---|---|---|---|---|---|---
| – | – | – | + | + | + | + | + | +

(d) Time (h) | IL-1β (1 nM) | TJ-135 (3 mg/mL) | 1 | 2 | 3 | 4 | 5
---|---|---|---|---|---|---|---
| – | – | – | + | + | + | + | +

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Inos mRNA. Total RNA was analyzed by strand-specific reverse transcription-polymerase chain reaction (RT-PCR) to detect iNOS mRNA. Anti-iNOS or anti-phospho-IκBα (Ser32/36) antibody (Cell Signaling, Beverly, MA, USA), human IκBα and human IκBβ; mouse type I IL-1 receptor (IL-1RI) (Santa Cruz Biotechnology, Santa Cruz, CA, USA); and rat β-tubulin (internal control; Clone TUB2.1, Sigma Chemical Co., St. Louis, MO, USA), followed by visualization with an enhanced chemiluminescence (ECL) blotting detection reagent (GE Healthcare Biosciences Corp., Piscataway, NJ, USA).

For Akt, total cell lysates prepared from 100-mm dishes (5 × 10⁶ cells/dish) were pre-cleared with Protein A (Sigma Chemical Co.) and then mixed with a mouse monoclonal antibody against human Akt1 (Akt5G3; Cell Signaling) and Protein G-Sepharose (Pharmacia LKB Biotech, Uppsala, Sweden). After incubation overnight at 4°C, immunocomplexes were centrifuged (16 000 g for 5 min). The beads were washed with solubilizing buffer, dissolved in SDS-PAGE sample buffer, and western blotting using rabbit polyclonal antibodies against human Akt and phospho-Akt (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
Total RNA was extracted from cultured hepatocytes using a guanidinium-phenol-chloroform method with Trizol reagent (Invitrogen, Carlsbad, CA, USA) or a phenol-free, filter-based total RNA isolation kit (RNAqueous 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 reverse transcription-polymerase chain reaction (RT-PCR) analysis, cDNAs were synthesized from total RNA with strand-specific primers, and step-down PCR was performed using PCR708 (Astec, Fukuoka, Japan), as previously described, with minor modifications. For iNOS, IL-1RI, p65 and elongation factor-1α (EF; internal control) mRNAs, an oligo(dT) primer was used for RT and the primer sets 5'-CCAACCTTGCAGTCCTTGCAT-3' and 5'-GTCGATGCAACCTGGTGAAC-3' (257-bp product), 5'-CGGACGTATCGTTTTGGACAG-3' and 5'-GTCTTTCTCATCTGAAGCTTTG-3' (327-bp product), 5'-ACCCTTCTTCAGTCCCATAGA-3' and 5'-ACCTCAATGTCTTCTTTCGAG-3' (262-bp product), and 5'-TCTGGTGGAACTTGTGACAC-3' and 5'-CCAGGAAGACCTTCAC-3' (307-bp product) were used for PCR, respectively. For the antisense-transcript of iNOS, the sense primer 5'-CCTTTGCCTCATACTTCCTCAGA-3' was used for RT and the primer set 5'-ACCCAGGGCCATCAGCCGTG-3' and 5'-ATCTTCATCAAGGAATTATACAGG-3' (211-bp product) was used for PCR. The PCR protocols for iNOS, EF and IL-1RI were: 10 cycles of (94°C, 60 s; 65°C, 90 s; 72°C, 120 s); 15 cycles of (94°C, 60 s; 65°C, 90 s; 72°C, 20 s); and five (iNOS, EF) or 15 (IL-1RI) cycles of (94°C, 60 s; 60°C, 90 s; 72°C, 20 s). The PCR protocol for the antisense-transcript was: 10 cycles of (94°C, 60 s; 65°C, 90 s; 72°C, 20 s); 15 cycles of (94°C, 60 s; 60°C, 90 s; 72°C, 20 s); and five cycles of (94°C, 60 s; 55°C, 90 s; 72°C, 20 s). The amplified products were analyzed by 3% agarose gel electrophoresis.
sis with ethidium bromide, and the levels of iNOS, IL-1RI, EF and antisense-transcript were semi-quantified using a UV transilluminator. The cDNAs for the rat iNOS mRNA and antisense-transcript were deposited in the DNA Data Bank of Japan/European Bioinformatics Institute (DDBJ/EMBL)/GenBank under Accession numbers AB250951 and AB250952, respectively.

Electrophoretic mobility shift assay

Nuclear extracts were prepared as reported previously with minor modifications. Briefly, the dishes were placed on ice, washed with Tris-HCl-buffered saline, harvested into the same buffer using a rubber policeman and centrifuged (1840 g for 1 min). The precipitate (2 × 10⁶ cells from two 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 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 (two to three times for 1 min each) and centrifuged (15 000 g for 5 min). The nuclear pellet was resuspended in extraction buffer (10 mM Hepes, pH 7.9, 0.4 M NaCl, 0.1 mM EDTA, 0.1 mM EGTA, 500 U/mL trasyol, 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 −80°C until use.

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. A rat cDNA for the 3′-untranslated region (UTR) of the iNOS mRNA was amplified with the primers 5′-tgctctagAC ATGAGGGGTTCGAGAGA-3′ and 5′-gctgcgatctttaTT CTGATCAAACACTCATTTT-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.

Transfection and luciferase assay

Transfection of cultured hepatocytes was performed as described previously. Briefly, hepatocytes were cultured at 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). After incubation for 15 min on a magnetic plate at room temperature, the medium was replaced with fresh WE containing serum. Cells were cultured overnight, and then treated with IL-1β in the presence or absence of sivelestat. The luciferase and β-galactosidase activities of cell extracts were measured using PicaGene (Wako Pure Chemicals) and Beta-Glo (Promega) kits, respectively.

Statistical analysis

Results shown are representative of three to four independent experiments yielding similar findings.

Figure 2 Effects of kampo inchinkoto (TJ-135) on cellular cytotoxicity. Cells were treated with IL-1β (1 nM) in the presence or absence of TJ-135 (0.5–3.0 mg/mL) for 8 h. Lactate dehydrogenase (LDH) activity was measured in the culture medium (data are means ± standard deviation [SD], n = 3 dishes/point).

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Figure 3 Effects of kampo inchinkoto (TJ-135) on the degradation of IκB proteins and activation of nuclear factor-κB (NF-κB). Cells were treated with interleukin-1β (IL-1β) (1 nM) in the presence or absence of TJ-135 (3 mg/mL) for the indicated times. (a, b) Cell lysates (20 μg of protein) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in a 12.5% gel, followed by immunoblotting with an anti-phospho-IκBα, anti-IκBα, anti-IκBβ or anti-β-tubulin antibody. (c) Activation of NF-κB. Nuclear extracts (4 μg of protein) were analyzed by electrophoretic mobility shift assay (EMSA) (upper). The bands corresponding to NF-κB were quantified by densitometry (lower, means ± standard deviation (SD) for n = 3 experiments; * P < 0.05 vs. IL-1β alone). (d) Nuclear translocation of NF-κB subunit p65. Nuclear extracts were immunoprecipitated, and the immunoprecipitates were analyzed by western blotting with an anti-p65 antibody. (e) Total RNA was analyzed by strand-specific reverse transcription-polymerase chain reaction (RT-PCR) to detect p65 mRNA, using elongation factor (EF) mRNA as an internal control.
Differences were analyzed by the Bonferroni-Dunn test, and values of $P < 0.05$ were considered to indicate statistical significance.

RESULTS

Effects of TJ-135 on the induction of NO production and iNOS in IL-1β-stimulated hepatocytes

The proinflammatory cytokine IL-1β stimulates the induction of iNOS, which is followed by the production of NO in primary cultured rat hepatocytes. Simultaneous addition of TJ-135 with IL-1β time- and dose-dependently reduced the levels of nitrite (a NO metabolite) in the culture medium (Fig. 1a, b). TJ-135 exerted its maximal effects at the concentration of 3 mg/mL, decreasing NO production to near basal levels. TJ-135 showed no cellular cytotoxicity within the indicated concentrations, as evaluated by the release of LDH into the culture medium (Fig. 2) and Trypan blue exclusion by hepatocytes (data not shown). Western blotting analysis revealed that TJ-135 time- and dose-dependently decreased the levels of iNOS protein expression, showing its maximal effect at 3 mg/mL (Fig. 1c). RT-PCR analysis revealed that TJ-135 decreased the levels of iNOS mRNA expression (Fig. 1d). These results suggested that TJ-135 inhibited the induction of iNOS gene expression at a transcriptional and/or post-transcriptional step.

Effects of TJ-135 on NF-κB activation and IL-1RI upregulation

We examined the mechanisms involved in the inhibition of iNOS induction. IL-1β stimulates the degradation of IκB proteins after the phosphorylation by IκB kinase, which is followed by the activation of NF-κB (i.e. translocation from the cytoplasm to the nucleus, and DNA binding). TJ-135 had no effect on the degradation of IκBα at 10–15 min (Fig. 3a, middle), and although TJ-135 reduced IκBα phosphorylation after 5 min of IL-1β stimulation, it had no
effect on phosphorylation levels at 10 min (Fig. 3a, top). In addition, TJ-135 did not inhibit the degradation of IκBα and IκBβ at 0.5 h, and rather decreased their recovery at one hour and thereafter (Fig. 3b). In contrast, electrophoretic mobility shift assays (EMSAs) with nuclear extracts revealed that TJ-135 inhibited NF-κB activation at 1–5 h (Fig. 3c), although the difference at 5 h was not significant. In support of this observation, immunoprecipitation and western blotting of nuclear extracts showed that TJ-135 decreased the levels of NF-κB subunit p65 in the nucleus (Fig. 3d). Furthermore, TJ-135 decreased the levels of p65 mRNA expression (Fig. 3e).

Interleukin-1β also stimulates the upregulation of IL-1RI through the activation of phosphatidylinositol 3-kinase (PI3K)/Akt. Immunoprecipitation-western blotting analysis revealed that TJ-135 inhibited the phosphorylation (activation) of Akt, a downstream kinase of PI3K (Fig. 4a). RT-PCR and western blot analyses revealed that TJ-135 reduced the levels of IL-1RI mRNA and protein expression (Fig. 4b,c).

Figure 4 Effects of kampo inchinkoto (TJ-135) on the upregulation of IL-1RI. Cells were treated with IL-1β (1 nM) in the presence or absence of TJ-135 (3 mg/mL) for the indicated times. (a) Phosphorylation of Akt. Total cell lysates were immunoprecipitated with an anti-Akt antibody, followed by immunoblotting with an anti-phospho-Akt or anti-Akt antibody. (b) Total RNA was analyzed by strand-specific reverse transcription-polymerase chain reaction (RT-PCR) to detect IL-1RI mRNA, using elongation factor (EF) mRNA as an internal control. (c) Cell lysates (50 μg of protein) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in a 7.5% gel, and immunoblotted with an anti-IL-1RI or anti-β-tubulin antibody.

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Effects of TJ-135 on iNOS promoter activation and its mRNA stabilization

Next, 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 detect iNOS promoter transactivation (mRNA synthesis) and mRNA stabilization, respectively.29 IL-1β increased the luciferase activities of these constructs, an effect significantly inhibited by TJ-135 (Fig. 5b,c). Furthermore, iNOS antisense-transcript analysis by RT-PCR revealed that IL-1β
increased the expression of the iNOS gene antisense-transcript in a time-dependent manner, and that TJ-135 markedly inhibited this effect (Fig. 3d).

Effects of delayed administration or withdrawal of TJ-135 on iNOS induction

We examined whether delayed administration of TJ-135 influences iNOS induction. TJ-135 was added to the medium 1–4 h after the addition of IL-1β. Although the magnitude of inhibition decreased time-dependently, delayed administration of TJ-135 up to 4 h after IL-1β addition still markedly inhibited NO production (Fig. 6). We then studied whether TJ-135 is effective even if it is not present in the medium for the entire experimental duration. We compared the time course of IL-1β-stimulated NO production in the absence of TJ-135 with that seen when TJ-135 was added 3 h after IL-1β addition (3 h delay of TJ-135) and when TJ-135 was washed out for 3 h after initial co-administration of TJ-135 and IL-1β (3 h withdrawal of TJ-135). As shown in Figure 7a, even after a 3 h delay prior to addition, TJ-135 inhibited approximately 70% of NO production. Similarly, after withdrawal of TJ-135 for 3 h after co-administration with IL-1β, NO production was inhibited by more than 90% compared with the level of production seen with IL-1β alone. The 3 h delay of TJ-135 decreased the levels of iNOS protein but not as effectively after the 3 h withdrawal of TJ-135 (Fig. 7b). However, both delay and withdrawal of TJ-135 had similar inhibitory effects on the expression of iNOS mRNA and its antisense-transcript (7C and 7D), the activation of NF-κB (Fig. 7e) and the nuclear translocation of NF-κB subunit p65 (Fig. 7f).

Effects of TJ-135 components on NO production and the induction of iNOS

We examined the effects of the three components of TJ-135 on the production of NO and expression of iNOS protein. As shown in Figure 8, the extract of A. capillaris dose-dependently inhibited NO production (ED50 = 0.53 mg/mL) and iNOS protein expression in IL-1β-stimulated hepatocytes. This effect was of similar magnitude as with complete TJ-135. The extract of G. fructus also dose-dependently decreased NO production (ED50 = 1.67 mg/mL) but less effectively than A. capillaris. The extracts of A. capillaris and G. fructus showed no cellular cytotoxicity at the indicated concentrations, as evaluated by LDH release into the medium (data not shown). The extract of R. rhizome had inhibitory effects at 0.25 and 0.5 mg/mL, but showed cytotoxicity at concentrations of 1 mg/mL and above.

DISCUSSION

In the present study, we found that Kampo inchinkoto, TJ-135, inhibited iNOS induction, followed by the reduction of NO production in IL-1β-stimulated hepatocytes (Fig. 1a–d). It is known that the levels of iNOS mRNA are regulated by iNOS promoter transactivation under the control of transcription factors such as NF-κB and by posttranscriptional modifications such as mRNA stabilization.30 In experiments with iNOS promoter constructs, TJ-135 was found to inhibit iNOS induction at both the mRNA synthesis and stabilization phases (Fig. 5). During mRNA synthesis, TJ-135 probably reduced the transactivation of the iNOS promoter (Fig. 5b) through the inhibition of NF-κB activation (Fig. 3c), although TJ-135 had no effect on IκBα and IκBβ degradation (Fig. 3a,b). NF-κB typically exists in the form of p50/65 heterodimers attached to its inhibitory proteins (IκBα, IκBβ and IκBβ) in the cytoplasm of cells. The activation of NF-κB involves (i) proteolytic degradation of IκBα in proteasome after the phosphorylation by IκB kinase (ii) the translocation of NF-κB to the nucleus, and (iii) its binding to the promoter κB
Figure 7 Effects of delayed administration and withdrawal of kampo inchinkoto (TJ-135) on the induction of inducible nitric oxide synthase (iNOS) in hepatocytes. Cultured hepatocytes were treated with TJ-135 (3 mg/mL) at 3 h after the addition of interleukin-1β (IL-1β) (1 nM) (3 h delay) or treated with simultaneous addition of IL-1β and TJ-135, followed by the withdrawal of TJ-135 at 3 h (3 h withdrawal). The effects of TJ-135 on IL-1β-stimulated nitric oxide (NO) production (a), iNOS protein expression (b), iNOS mRNA expression (c), iNOS antisense-transcript expression (d), nuclear factor-kB (NF-kB) levels (e) and nuclear translocation of NF-kB subunit p65 (f) were analyzed at the indicated times after IL-1β addition. (a) The levels of nitrite (IL-1β, ○; IL-1β + TJ-135 (3 h delay), ■; IL-1β + TJ-135 (3 h withdrawal), ▲) were measured in the culture medium (data are means ± standard deviation [SD], n = 3 dishes/point; *P < 0.05 vs. IL-1β alone). (b) Cell lysates (20 μg of protein) from cells stimulated as described above were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in a 7.5% gel, and immunoblotted with an anti-iNOS or anti-β-tubulin antibody. (c, d) Total RNA was analyzed by strand-specific reverse transcription-polymerase chain reaction (RT-PCR) (to detect iNOS mRNA, using EF mRNA as an internal control, and the iNOS gene antisense-transcript (AST)). (e) Nuclear extracts (4 μg of protein) from cells stimulated as described above were analyzed by electrophoretic mobility shift assay (EMSA). (f) Nuclear extracts were immunoprecipitated, and these precipitates were analyzed by western blotting using an anti-p65 antibody. W, 3 h withdrawal of TJ-135; D, 3 h delay of TJ-135.
TJ-135 inhibited the translocation of p65 to the nucleus (Fig. 3d) at least partly by decreasing p65 mRNA expression (Fig. 3e).

In addition to the activation of NF-κB through IκB degradation, the upregulation of IL-1RI through the activation of PI3K/Akt is also essential for iNOS induction. IL-1β stimulates the induction of IL-1RI, which precedes the induction of iNOS. The upregulation of IL-1RI is associated with a second activation of Akt, which accelerates the phosphorylation of the NF-κB p65 subunit and increases the transcriptional activation of the iNOS gene. In the present study, we found that TJ-135 decreased the expression of IL-1RI mRNA and protein (Fig. 4b,c) through the inhibition of Akt phosphorylation (Fig. 4a), which is presumably also involved in the observed decrease in iNOS promoter transactivation activity.

Regarding 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), which serve to stabilize the mRNA. Recently, we found that the antisense strand corresponding to the 3′-UTR of the 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. In our in vitro model, TJ-135 destabilized the iNOS mRNA by inhibiting iNOS gene antisense-transcript expression (Fig. 3d). Drugs such as edaravone (free radical scavenger), FR183998 (Na+/H+ exchanger inhibitor), insulin growth factor I, and sivelestat were found to inhibit iNOS induction partly by suppressing iNOS antisense-transcript.
production in animal models of liver injury and primary cultured hepatocytes.

Delayed treatment with TJ-135 or withdrawal of TJ-135 after IL-1β addition was found to cause a significant reduction in NO production and iNOS protein expression (Figs 6, 7a,b). The fact that a delay in initiating treatment does not abrogate the effects of the drug may be of clinical importance, since TJ-135 treatment is not usually administered at the precise moment of disease onset. In the case of the 3 h withdrawal treatment, TJ-135 reduced the levels of NO production and iNOS protein expression to the same extent as TJ-135 addition without withdrawal. We concluded that delayed treatment with TJ-135, unlike withdrawal treatment, could not influence the IL-1β-stimulated, Akt-driven expression of IL-1RI mRNA, since these events are almost complete at 3 h (Fig. 4a,b). The resultant inhibition of iNOS induction will therefore be smaller. However, we found that both delayed and withdrawal treatments reduced the expression of iNOS mRNA and its antisense-transcript to similar levels (Fig. 7c,d). These treatments were also equi-effective at inhibiting NF-κB activation (Fig. 7e) and nuclear translocation of p65 (Fig. 7f). We cannot therefore rule out the possibility that TJ-135 may affect iNOS induction at a translational step by inhibiting IL-1RI upregulation via the PI3K/Akt pathway.

Recently, Kawai et al. have reported that preoperative administration of inchinkoto exerts beneficial effects in rat liver with ischemia-reperfusion and subsequent hepatectomy, where inchinkoto attenuated ischemia-reperfusion injury-induced mortality. They demonstrated that inchinkoto reduced the upregulation of genes for inflammatory cytokines and iNOS, and increased levels of liver nitrotyrosine. Nitrotyrosine is an oxidative product of peroxynitrite formed by excess NO, and is a marker of NO-dependent damage in vivo. We found that all three components in inchinkoto, A. capillaris, G. fructus and R. rhizome, are involved in the inhibitory effect of TJ-135 on NO production, where A. capillaris contributes most significantly to the effect of TJ-135 (Fig. 8). We also found that genipin, the major ingredient of G. fructus and an aglycone converted in the gut by intestinal bacteria, inhibited the induction of iNOS (T. Matsuura and T. Okumura, unpubl. data, 2010). Genipin was also found to reduce iNOS in a rat model of ischemia-reperfusion injury.

In conclusion, TJ-135 inhibited the induction of iNOS gene expression through the inhibition of its promoter transactivation and mRNA stabilization in pro-inflammatory cytokine-stimulated hepatocytes, a simple in vitro liver injury model. TJ-135 may have therapeutic
REFERENCES


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