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 [24, 25]. 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, Calif., USA) 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 (WE) supplemented with 10% newborn calf serum, HEPES (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 nuclei [26] and using a ratio of 1.37 ± 0.04 nuclei/cell (mean ± SE, n = 7 experiments).

Treatment of Cells with AHCC-SF

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 AHCC-SF.

Determinations of NO Production

Culture media were used for measurements of nitrite (stable metabolites of NO) for NO production by the Griess method [27].

Western Blot Analysis

Total cell lysates were obtained from cultured cells [6], mixed with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (final concentrations: 125 mM Tris-HCl, pH 6.8, 5% glycerol, 2% SDS and 1% 2-mercaptoethanol), subjected to SDS-PAGE and transferred onto polyvinylidene difluoride membranes (Bio-Rad Laboratories, Hercules, Calif., USA). Immunostaining was performed using primary antibodies against mouse iNOS (Affinity BioReagents, Golden, Colo., USA), human IκBα, human IκBβ, mouse IL-1RI (Santa Cruz Biotechnology, Santa Cruz, Calif., 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, N.J., 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, Mass., 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.

Northern Blot Analysis and RT-PCR

Total RNA was extracted from cultured hepatocytes using a guanidinium-phenol-chloroform method [28] with Trizol reagent (Invitrogen, Carlsbad, Calif., USA) 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-^{32}P]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)-1α (internal control) mRNAs, an oligo(dT) primer was used for RT and primer sets 5′-CCAACCTGCAGGTCTTCGATG-3′ and 5′-GTCGATGCACAACTGGGTGAAC-3′ (257-bp product) and 5′-TCTGGTTGGAATGGTGACAACATGC-3′ and 5′-CCAGGAAGAGCTTCACTCAAAGCTT-3′ (307-bp product) were used for PCR, respectively. For the antisense transcript of iNOS gene, sense primer 5′-TGCCCCTCCCCCACATTCTCT-3′ was used for RT and the primer set 5′-ACCAGGAGGCGCCATCCCGCTGC-3′ and 5′-CTTGATCAAACACTCATTTTATTAAA-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 (2×10^6 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 trasylol, 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 U/ml trasylol, 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.

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 mg of poly(dI-dC)) with the probe (approximately 40,000 c.p.m.) for 20 min at room temperature. In the case of supershift assays, the nuclear extracts were incubated in the presence of anti-p50 and anti-p65 antibodies (NF-κBp50 (NLS) and NF-κBp65 (H286); Santa Cruz Biotechnology) or cold probes as a competitor (250-fold excess) for 30 min at 4°C, followed by incubation with the labeled probe. The products were electrophoresed at 100 V in a 4.8% polyacrylamide gel in high ionic strength buffer (50 mM Tris-HCl, 380 mM glycine, 2 mM EDTA, pH 8.5) and the dried gels were analyzed by autoradiography. An NF-κB consensus oligonucleotide (5′-AGTTGAGGGGA-CTTTCCCAGGC-3′) from the mouse immunoglobulin κ light chain was purchased (Promega,

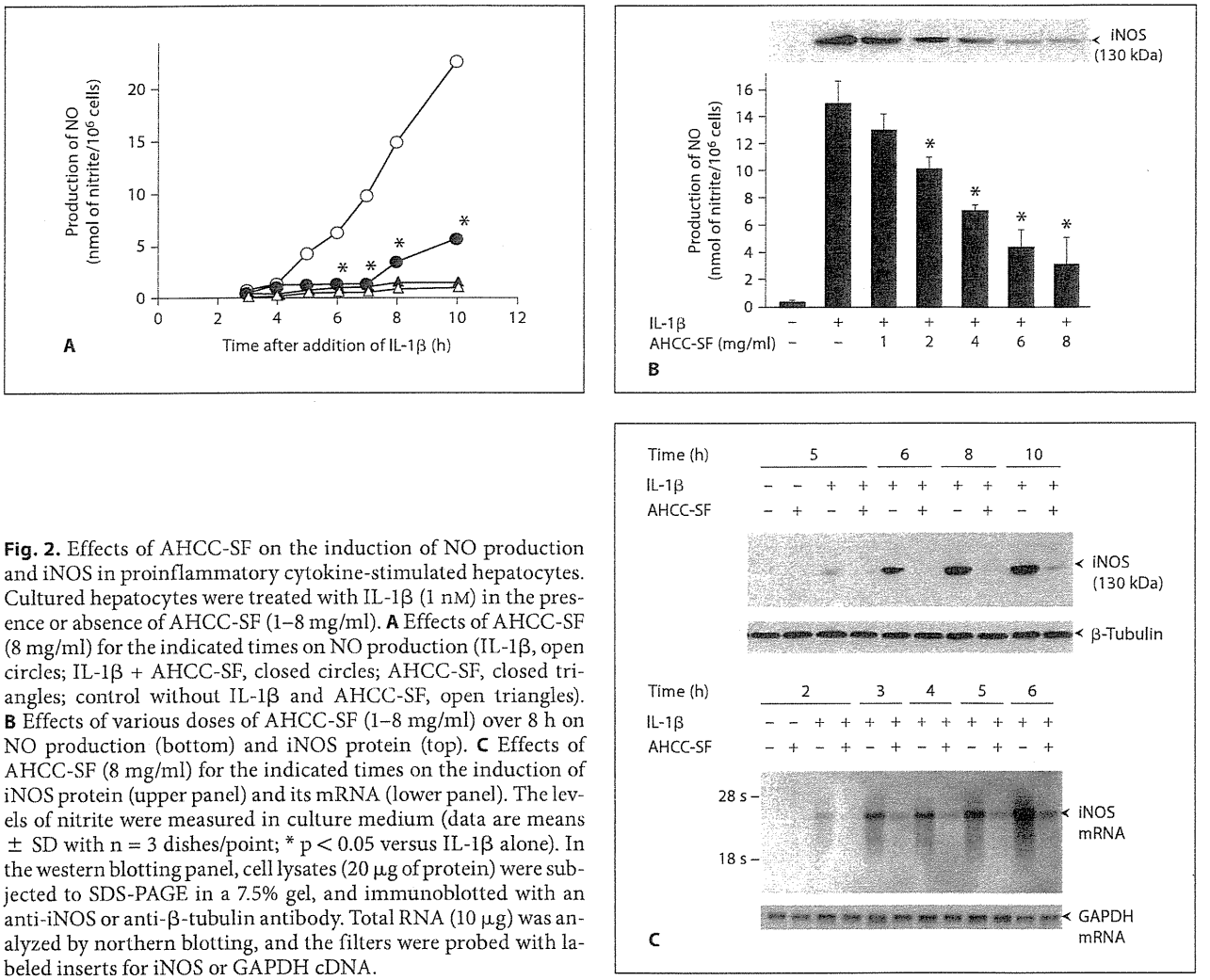

Fig. 2. Effects of AHCC-SF on the induction of NO production and iNOS in proinflammatory cytokine-stimulated hepatocytes. Cultured hepatocytes were treated with IL-1β (1 nM) in the presence or absence of AHCC-SF (1–8 mg/ml). **A** Effects of AHCC-SF (8 mg/ml) for the indicated times on NO production (IL-1β, open circles; IL-1β + AHCC-SF, closed circles; AHCC-SF, closed triangles; control without IL-1β and AHCC-SF, open triangles). **B** Effects of various doses of AHCC-SF (1–8 mg/ml) over 8 h on NO production (bottom) and iNOS protein (top). **C** Effects of AHCC-SF (8 mg/ml) for the indicated times on the induction of iNOS protein (upper panel) and its mRNA (lower panel). The levels of nitrite were measured in culture medium (data are means ± SD with n = 3 dishes/point; * $p < 0.05$ versus IL-1β alone). In the western blotting panel, cell lysates (20 μg of protein) were subjected to SDS-PAGE in a 7.5% gel, and immunoblotted with an anti-iNOS or anti-β-tubulin antibody. Total RNA (10 μg) was analyzed by northern blotting, and the filters were probed with labeled inserts for iNOS or GAPDH cDNA.

Madison, Wisc., USA) and labeled with [γ-^{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′-tgctctaGACAGTGAGGGGTTTGGAGAGA-3′ and 5′-gcggatccttaTTCTTGATCAAACACTCATTTT-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 × 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.

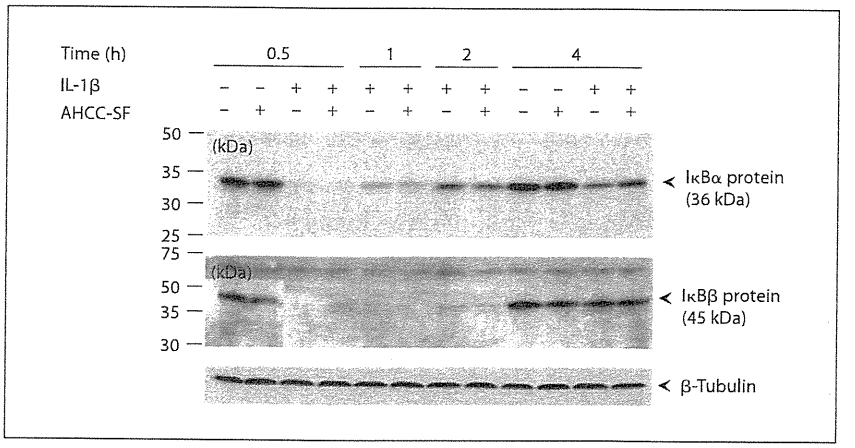

Fig. 3. Effects of AHCC-SF on the degradation of IκB proteins. Cells were treated with IL-1β (1 nM) in the presence or absence of AHCC-SF (8 mg/ml) for the indicated times. Cell lysates (20 μg of protein) were subjected to SDS-PAGE in a 12.5% gel, followed by immunoblotting with an anti-IκBα, anti-IκBβ or anti-β-tubulin antibody.

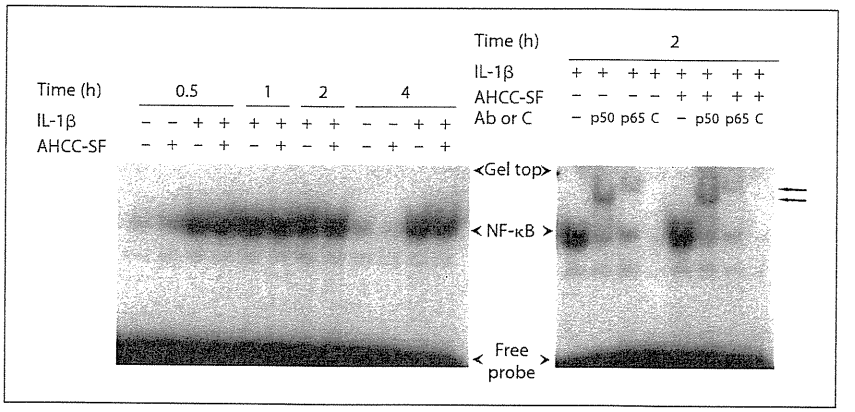

Fig. 4. Effects of AHCC-SF on the activation of NF-κB. Cells were treated with IL-1β (1 nM) in the presence or absence of AHCC-SF (8 mg/ml) for the indicated times. Activation of NF-κB (left): nuclear extracts (4 μg of protein) were analyzed by EMSAs. Supershift assay (right): nuclear extracts were incubated with a labeled NF-κB consensus oligonucleotide in the presence of an anti-p50 antibody, anti-p65 antibody or cold probe as a competitor (C, 250-fold excess). Closed arrows show supershifted bands.

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

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.

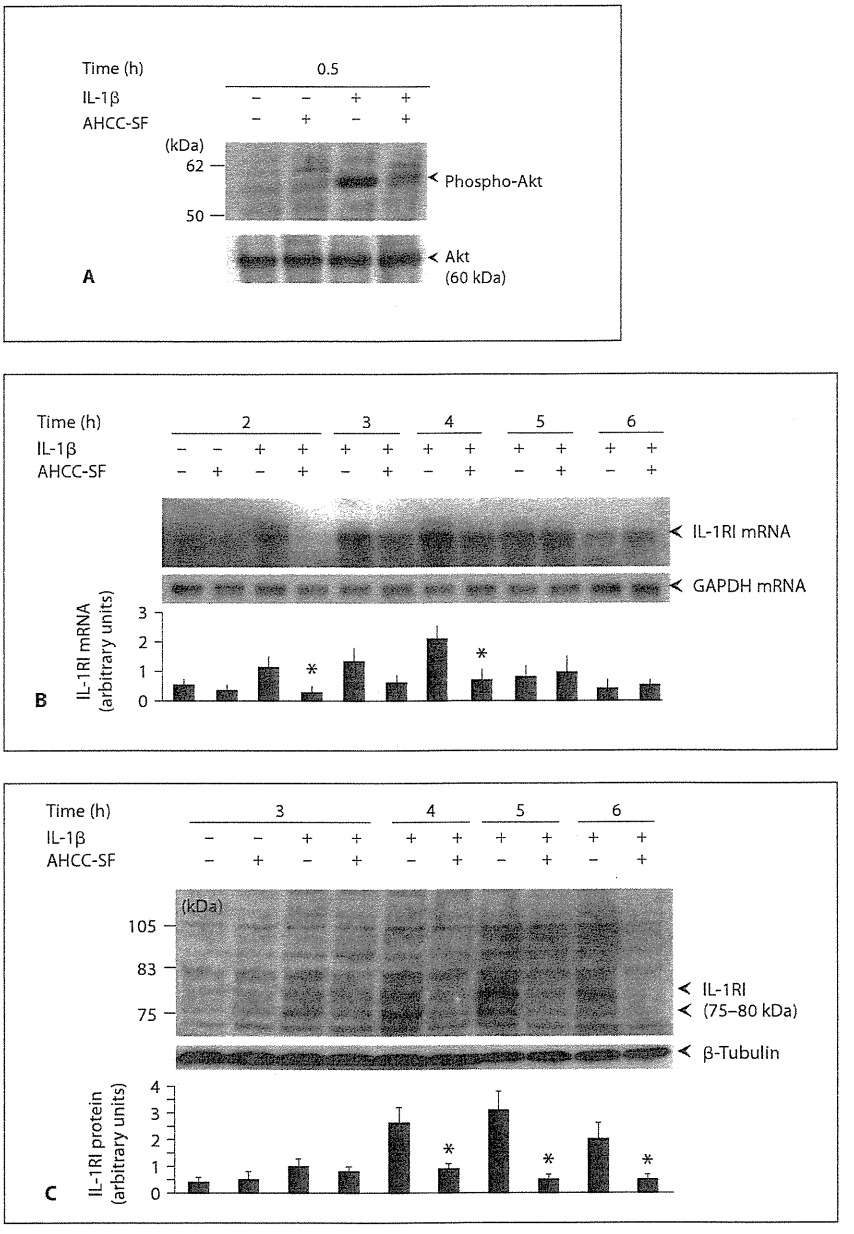

Fig. 5. Effects of AHCC-SF on the upregulation of IL-1RI. Cells were treated with IL-1β (1 nM) in the presence or absence of AHCC-SF (8 mg/ml) for the indicated times. **A** Activation of Akt: total cell lysates were immunoprecipitated with an anti-Akt antibody, followed by immunoblotting with an anti-phospho-Akt or anti-Akt antibody. **B** Total RNA (10 μg) was analyzed by northern blotting, and the filters were probed with labeled inserts for IL-1RI or GAPDH cDNA. **C** Cell lysates (50 μg of protein) were subjected to SDS-PAGE in a 7.5% gel, and immunoblotted with an anti-IL-1RI or anti-β-tubulin antibody. The bands corresponding to IL-1RI mRNA or protein were quantified by densitometry (lower panels: means ± SD for n = 3 experiments; * $p < 0.05$ vs. IL-1β alone).

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

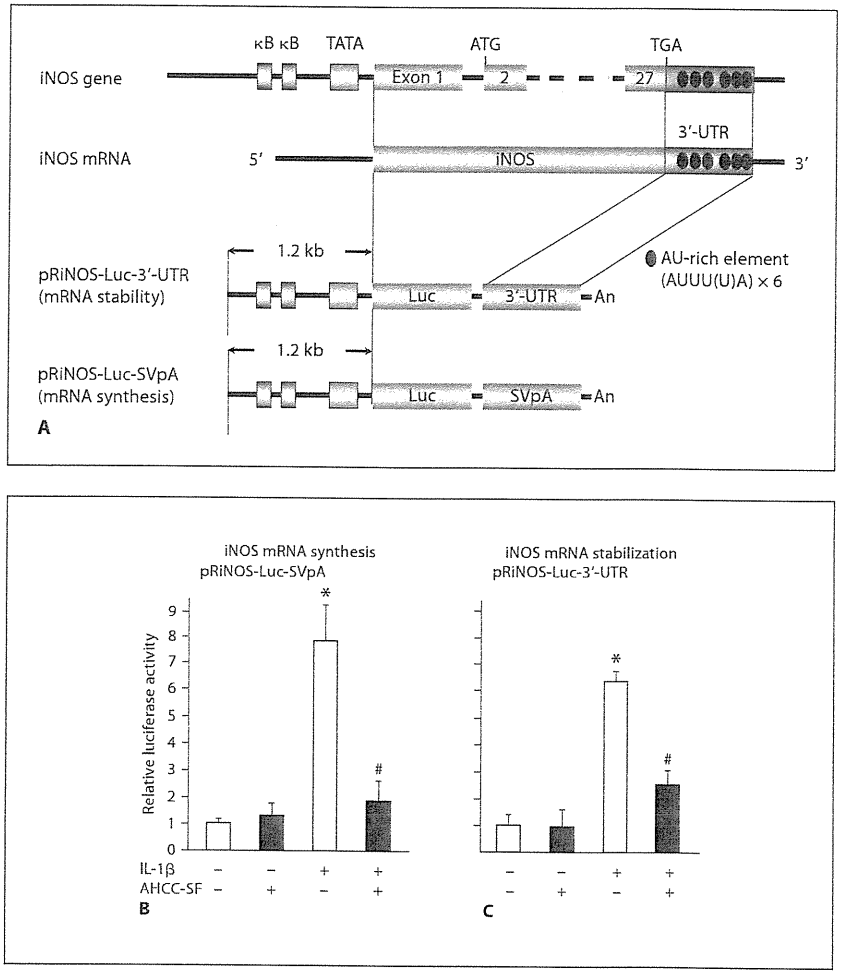

Fig. 6. Effects of AHCC-SF on the transactivation of the iNOS promoter. **A** Schematic representation of the promoter region of the iNOS gene. Two reporter constructs are shown beneath the iNOS gene and mRNA. The constructs consist of the rat iNOS promoter (1.2 kb), luciferase gene and SV40 poly(A) region (pRiNOS-Luc-SVpA) or iNOS 3′-UTR (pRiNOS-Luc-3′-UTR). 'An' indicates the presence of a poly(A) tail. The iNOS 3′-UTR contains AREs (AUUU(U)A × 6), which contribute to mRNA stabilization. **B**, **C** Each construct was introduced into hepatocytes, and the cells were treated with IL-1β (1 nM) in the presence or absence of AHCC-SF (8 mg/ml) for 8 h for pRiNOS-Luc-SVpA (**B**) and 4 h for pRiNOS-Luc-3′UTR (**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.

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). Recently, we found that the natural antisense transcript of the iNOS gene is expressed and involved in the stabilization of iNOS mRNA [14]. RT-PCR and quantitative real-time PCR experiments revealed that IL-1β time-dependently increased the expression of the iNOS gene antisense transcript (fig. 7A) with increased levels of iNOS mRNA (fig. 7B), and that AHCC-SF decreased the levels of both the antisense transcript and iNOS mRNA in a similar manner.

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,

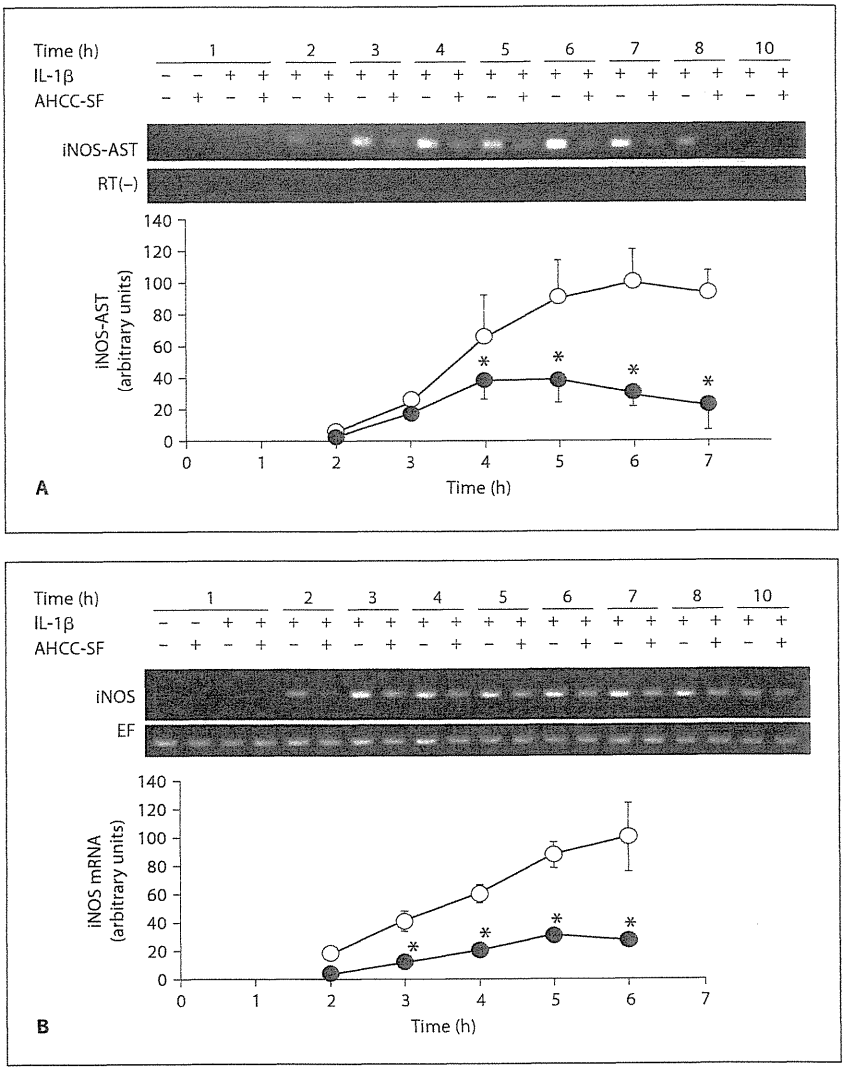

Fig. 7. Effects of AHCC-SF on the expression of the iNOS gene antisense transcript in hepatocytes. Cells were treated with IL-1β (1 nM) in the presence (closed circles) or absence (open circles) of AHCC-SF (8 mg/ml) for the indicated times. Total RNA was analyzed by strand-specific RT-PCR to detect (**A**) the iNOS gene antisense transcript (AST) and (**B**) iNOS with elongation factor-1α (EF) as an internal control. Quantitative RT-PCR was carried out for the iNOS gene AST or iNOS, and the copy number of the iNOS gene AST or iNOS was normalized by that of a negative PCR control using total RNA without RT (RT(–)) or by that of internal control EF. Data are means ± SD (n = 3 experiments). * $p < 0.05$ versus IL-1β alone.

we found that AHCC-SF decreased the expression of IL-1RI 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 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 upregulation 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 upregulation 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 deduced from our in vitro model need to be examined and supported in 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

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Original Article

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

Takashi Matsuura,[1] Masaki Kaibori,[1] Yoshiro Araki,[1] Miho Matsumiya,[1] Yuka Yamamoto,[2] Yukinobu Ikeya,[3] Mikio Nishizawa,[4] Tadayoshi Okumura[1,5] and A-Hon Kwon[1]

[1]*Department of Surgery, Kansai Medical University, Moriguchi, Osaka, and* [2]*Graduate School of Science and Engineering, and* [3]*Departments of Pharmacy, College of Pharmaceutical Sciences and* [4]*Biomedical Sciences, College of Life Sciences, and* [5]*Research Organization of Science and Technology, Ritsumeikan University, Kusatsu, Shiga, Japan*

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, choletic 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

Correspondence: Dr Tadayoshi Okumura, Department of Surgery, Kansai Medical University, 10-15 Fumizonocho, Moriguchi, Osaka 570-8506, Japan. Email: okumura@takii.kmu.ac.jp
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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.[7] 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.[10,13,14] 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-^{32}P]-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 6×10^5 cells/mL, seeded into 35-mm plastic dishes (2 mL/dish; Falcon Plastic, Oxnard, CA, USA) and cultured at 37°C in a CO_2 incubator under a humidified atmosphere of 5% CO_2 in air. The culture medium was WE supplemented with 10% newborn calf serum, Hepes (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 nuclei[17] 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 previously[13] 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

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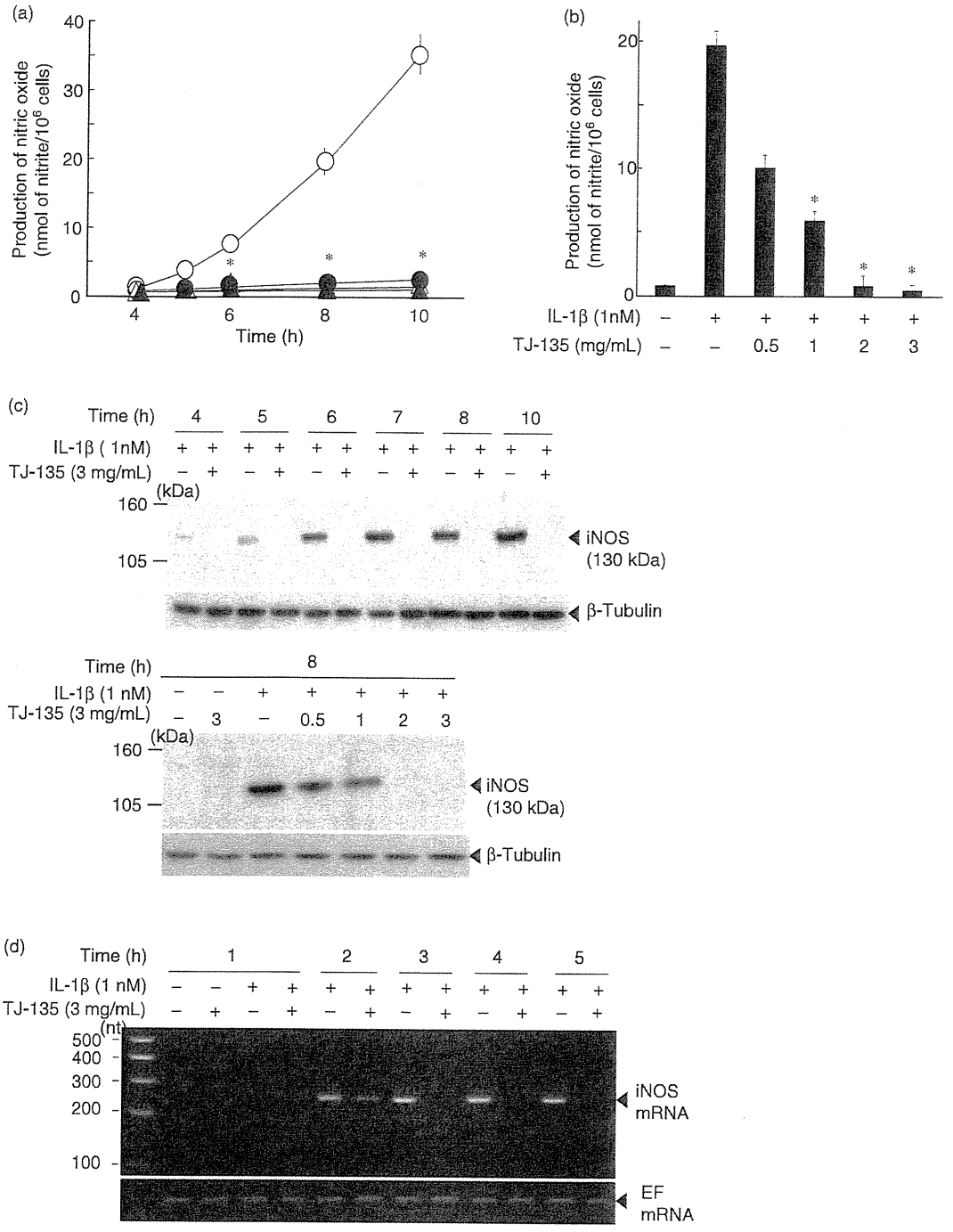

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Tesque, Kyoto, Japan], 1 mM phenylmethylsulfonyl fluoride [PMSF] and protease inhibitor cocktail [Roche Diagnostics, Mannheim, Germany]), passed through a 26-gauge needle, allowed to stand on ice for 30 min before being centrifuged (16 000 *g* for 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 phospho-IκBα (Ser32/36 [5A5]; 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 (5×10^6 cells/dish) were pre-cleared with Protein A (Sigma Chemical Co.) and then mixed with a mouse monoclonal antibody against human Akt1 (Akt5G3; Cell Signaling) 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 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

Total RNA was extracted from cultured hepatocytes using a guanidinium-phenol-chloroform method[19] 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 PC708 (Astec, Fukuoka, Japan), as previously described,[20,21] 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′-CCAACCTGCAGGTCTTCGATG-3′ and 5′-GTCGATG CACAACTGGGTGAAC-3′ (257-bp product), 5′-CGAA GACTATCAGTTTTTGGAAC-3′ and 5′-GTCTTTCCATCT GAAGCTTTTGG-3′ (327-bp product), 5′-ACCCCTTTC AAGTTCCCATAGA-3′ and 5′-ACCTCAATGTCTTCTTTC TGCAC-3′ (262-bp product), and 5′-TCTGGTTGGAA TGGTGACAACATGC-3′ and 5′-CCAGGAAGAGCTTCA CTCAAAGGTT-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′-ACCAGGAGGC GCCATCCCGCTGC-3′ and 5′-ATCTTCATCAAGGAATT ATACACGG-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; 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-1R1) 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 electrophore-

Figure 1 Effects of kampo inchinkoto (TJ-135) on the induction of nitric oxide (NO) production and inducible nitric oxide synthase (iNOS) in pro-inflammatory cytokine-stimulated hepatocytes. Cultured hepatocytes were treated with interleukin-1β (IL-1β) (1 nM) in the presence or absence of TJ-135 (0.5–3.0 mg/mL). (a) Effect of TJ-135 (3 mg/mL) treatment (for the indicated times) on NO production (IL-1β, ○; IL-1β + TJ-135, ●; TJ-135, ▲; controls (without IL-1β and TJ-135), △). (b) Effects of treatment with various doses of TJ-135 (0.5–3.0 mg/mL) for 8 h on NO production. The levels of nitrite were measured in the culture medium (data are means ± standard deviation [SD], $n = 3$ dishes/point; $^*P < 0.05$ *vs.* IL-1β alone). (c) Cell lysates (20 μg of protein) 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. (d) Effects of TJ-135 (3 mg/mL) treatment (for the indicated times) on the expression of iNOS mRNA. 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.

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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[22] with minor modifications.[23] 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 (2×10^6 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 trasylol, 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 1 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 trasylol, 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.

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 mg of poly[dI-dC]) with the probe (approximately 40 000 cpm) for 20 min at room temperature. Products were electrophoresed at 100 V in a 4.8% polyacrylamide gel in high ionic strength buffer (50 mM Tris-HCl, 380 mM glycine, 2 mM EDTA, pH 8.5). Dried gels were analyzed by autoradiography. A NF-κB consensus oligonucleotide (5′-AGTTGAGGGGA-CTTTCCCAGGC-3′) from the mouse immunoglobulin κ light chain was purchased (Promega, Madison, WI, USA) and labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase. The protein concentration was measured by the method of Bradford[24] 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.[23] A rat cDNA for the 3′-untranslated region (UTR) of the iNOS mRNA was amplified with the primers 5′-tgctctaGAC AGTGAGGGGTTTGGAGAGA-3′ and 5′-gcggatcctttaTT CTTGATCAAACACTCATTTT-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.[25,26] Briefly, hepatocytes were cultured at 4×10^5 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. 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.

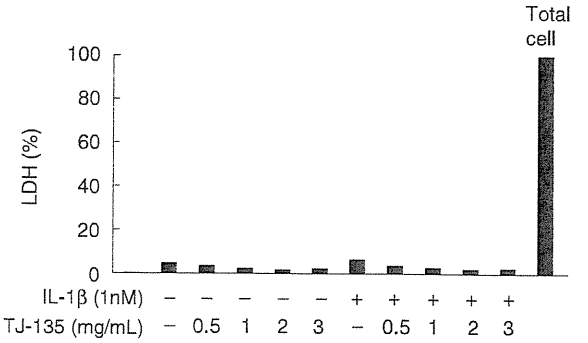

Figure 2 Effects of kampo inchinkoto (TJ-135) on cellular cytotoxicity. Cells were treated with IL-1β (1 nM) in the presence or absence of TJ-135 (0.5–3.0 mg/mL) for 8 h. Lactate dehydrogenase (LDH) activity was measured in the culture medium (data are means ± standard deviation [SD], $n = 3$ dishes/point).

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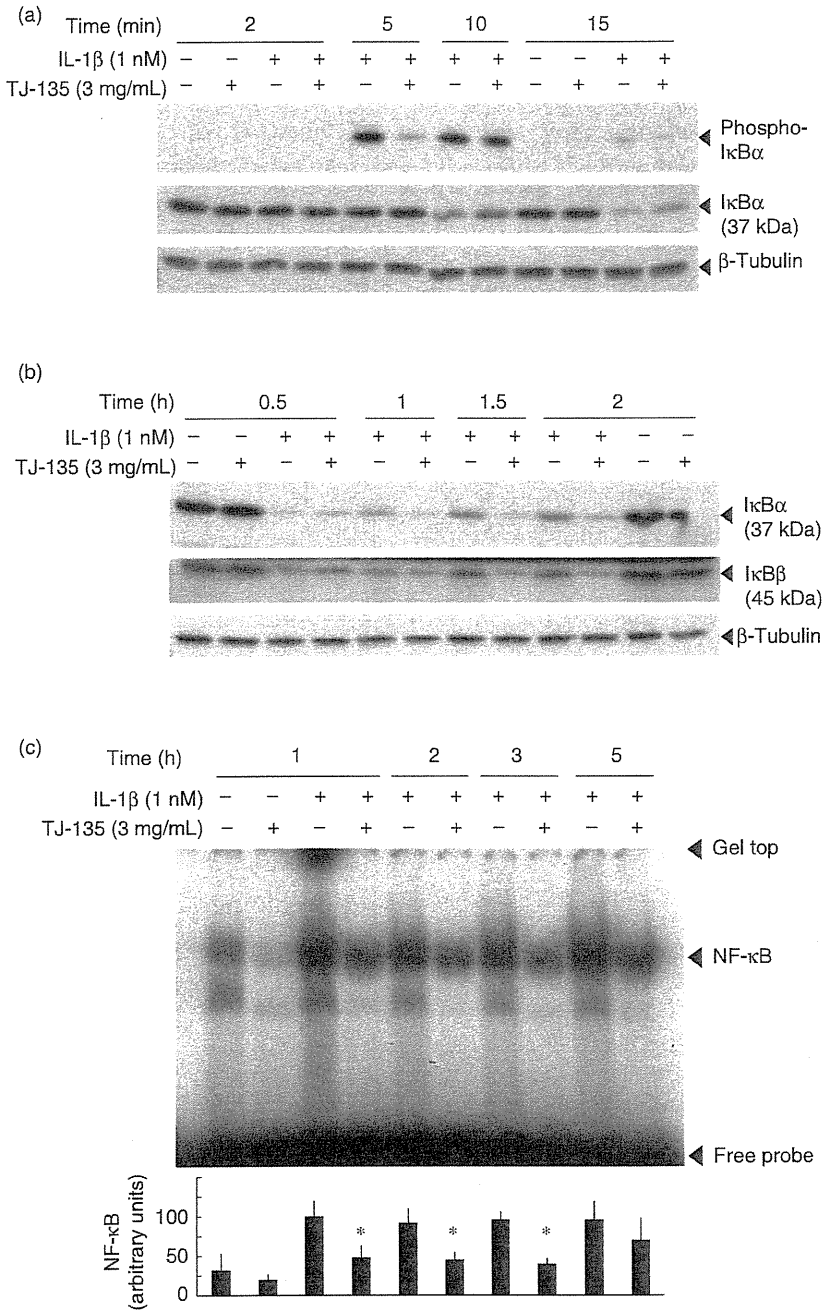

Figure 3 Effects of kampo inchinkoto (TJ-135) on the degradation of IκB proteins and activation of nuclear factor-κB (NF-κB). Cells were treated with interleukin-1β (IL-1β) (1 nM) in the presence or absence of TJ-135 (3 mg/mL) for the indicated times. (a, b) Cell lysates (20 μg of protein) were subjected 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.

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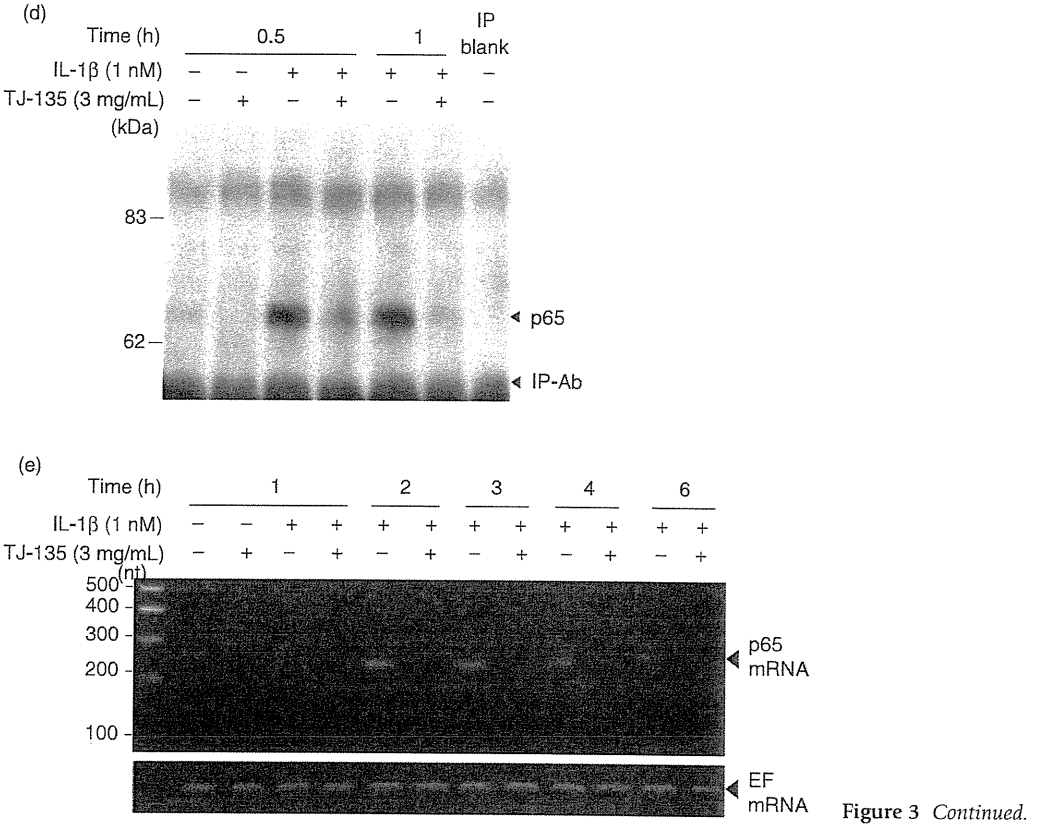

Figure 3 *Continued.*

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.[27,28] 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

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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).

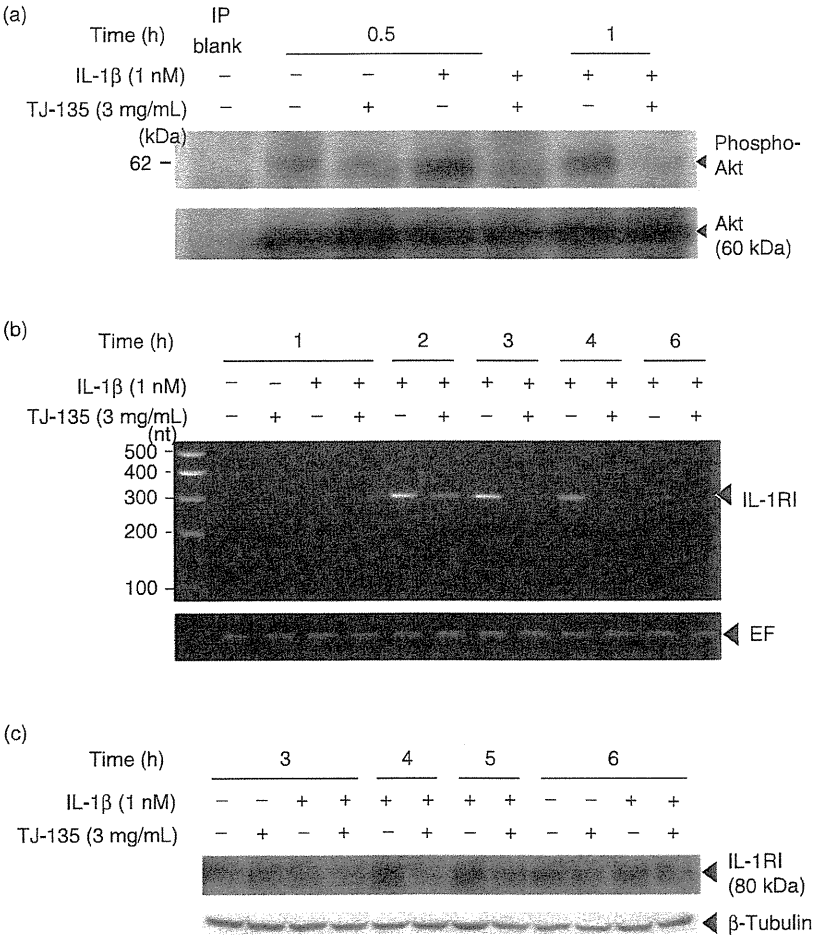

Figure 4 Effects of kampo inchinkoto (TJ-135) on the upregulation of IL-1RI. Cells were treated with IL-1β (1 nM) in the presence or absence of TJ-135 (3 mg/mL) for the indicated times. (a) Phosphorylation of Akt. Total cell lysates were immunoprecipitated with an anti-Akt antibody, followed by immunoblotting 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

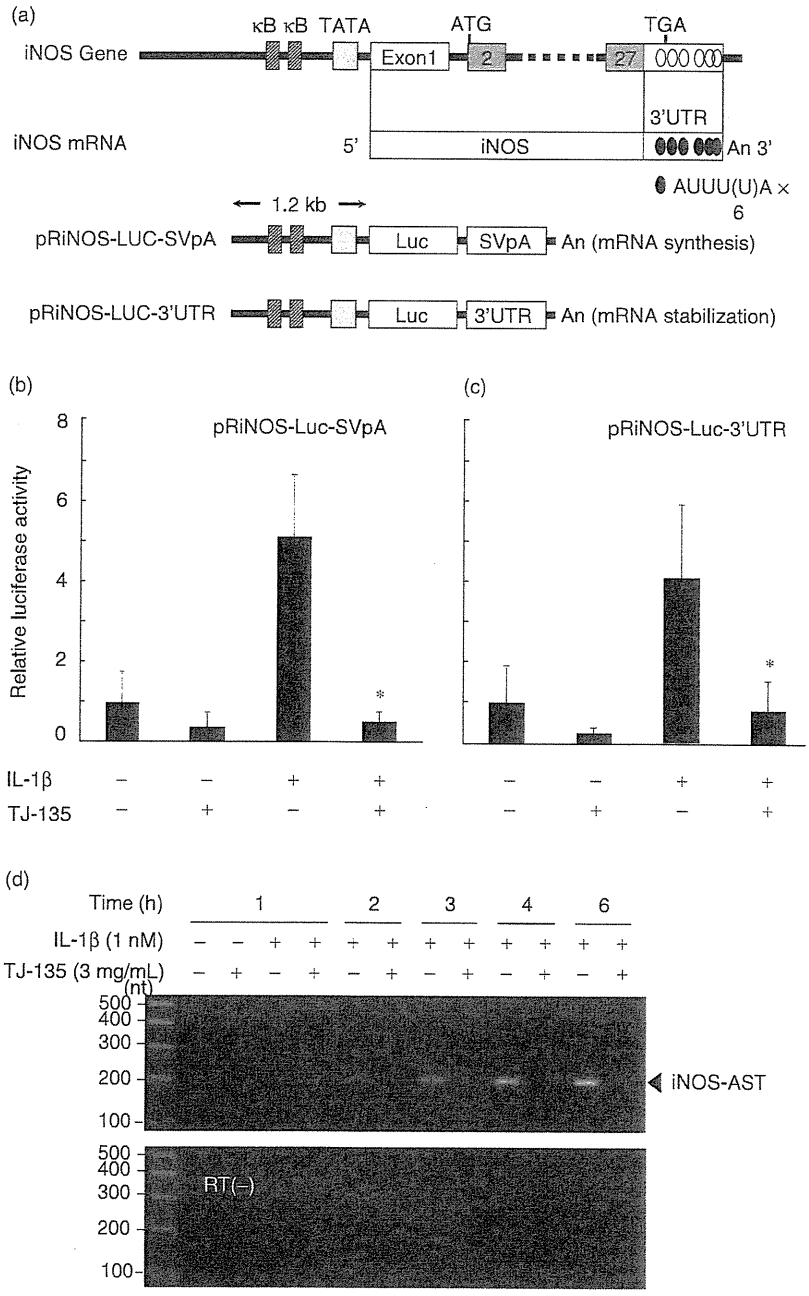

Figure 5 Effects of kampo inchinkoto (TJ-135) on the transactivation of the inducible nitric oxide synthase (iNOS) promoter and the expression of the iNOS gene antisense-transcript. (a) Schematic representation of the promoter region of the iNOS gene. Two reporter constructs are shown beneath the iNOS gene and mRNA. The constructs consist of the rat iNOS promoter (1.2 kb), a luciferase gene and the SV40 poly(A) region (pRiNOS-Luc-SVpA) or iNOS 3′-UTR (pRiNOS-Luc-3′UTR). "A_n" indicates the presence of a poly(A) tail. The iNOS 3′-UTR contains AREs (AUUU(U)A × 6), which contribute to mRNA stabilization. (b, c) Each construct was introduced into hepatocytes, and the cells were treated with interleukin-1β (IL-1β) (1 nM) in the presence or absence of TJ-135 (3 mg/mL) for 8 h for pRiNOS-Luc-SVpA (B) and 4 h for pRiNOS-Luc-3′UTR (C). The luciferase activities were normalized to β-galactosidase activity. The fold activation was calculated by dividing the luciferase activity by the control activity (without IL-1β and TJ-135). Data are means ± standard deviation [SD], $n = 4$ dishes. $^*P < 0.05$ *vs.* IL-1β alone. (d) Cells were treated with IL-1β (1 nM) in the presence or absence of TJ-135 (3 mg/mL) for the indicated times. Total RNA was analyzed by strand-specific reverse transcription-polymerase chain reaction (RT-PCR) to detect the iNOS gene antisense-transcript (AST). RT(−) denotes a negative control PCR using total RNA without RT.

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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).

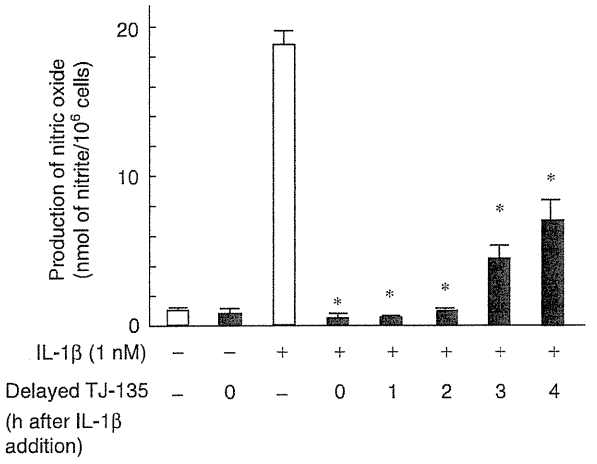

Figure 6 Effects of delayed kampo inchinkoto (TJ-135) administration on the production of nitric oxide (NO) in hepatocytes. Cultured hepatocytes were treated with TJ-135 (3 mg/mL) at 0–4 h after the addition of interleukin-1β (IL-1β) (1 nM). The effects of TJ-135 on NO production were analyzed at 8 h after IL-1β addition. The levels of nitrite were measured in the culture medium (data are means ± standard deviation [SD], $n = 3$ dishes/point; $^*P < 0.05$ *vs.* IL-1β alone).

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 ($ED_{50} = 0.53$ 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 ($ED_{50} = 1.67$ 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 (IκBs, IκBα and IκBβ) in the cytoplasm of cells. The activation of NF-κB involves (i) proteolytic degradation of IκBs in proteosome after the phosphorylation by IκB kinase (ii) the translocation of NF-κB to the nucleus, and (iii) its binding to the promoter κB

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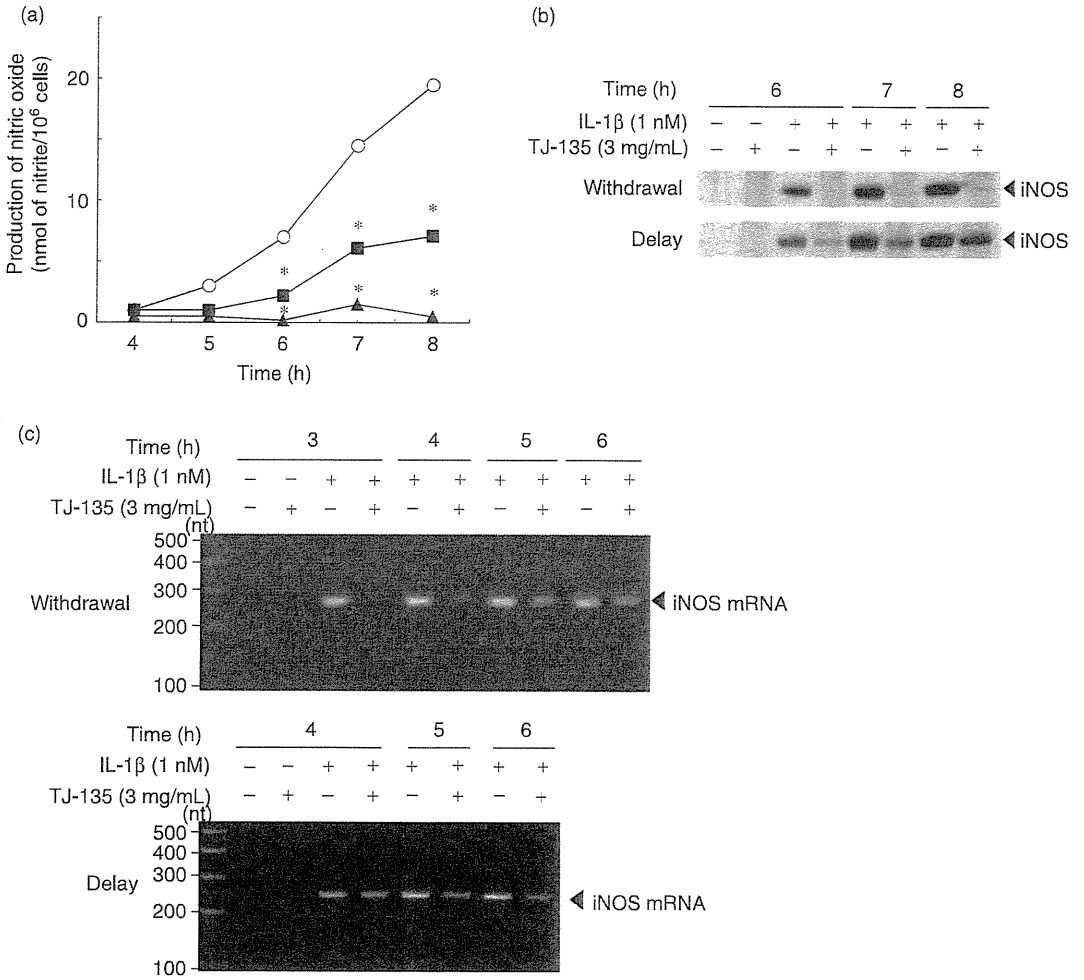

Figure 7 Effects of delayed administration and withdrawal of kampo inchinkoto (TJ-135) on the induction of inducible nitric oxide synthase (iNOS) in hepatocytes. Cultured hepatocytes were treated with TJ-135 (3 mg/mL) at 3 h after the addition of interleukin-1β (IL-1β) (1 nM) (3 h delay) or treated with 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-κB (NF-κB) levels (e) and nuclear translocation of NF-κB 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.

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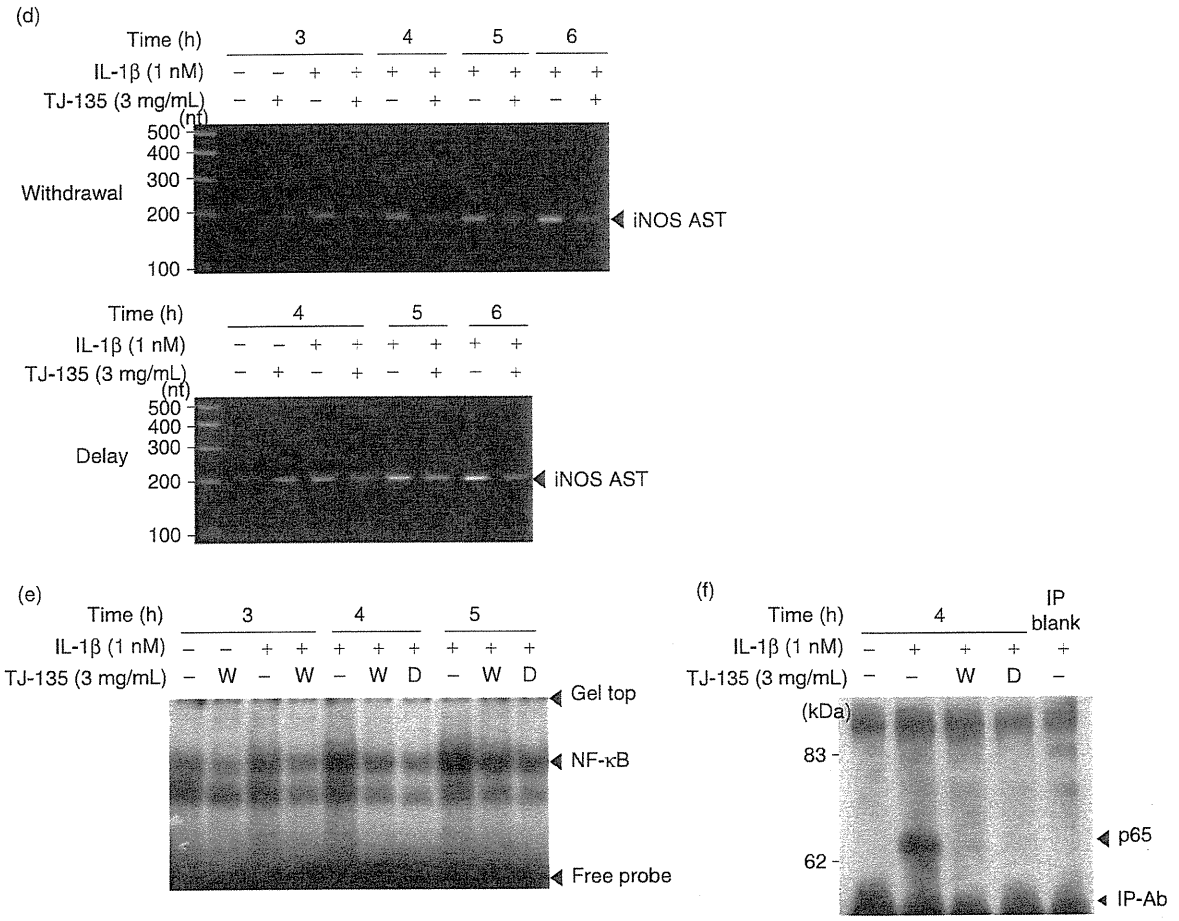

Figure 7 *Continued.*

site.[31] 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.[32] 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.[33] 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.[34] 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),[14] FR183998 (Na^+/H^+ exchanger inhibitor),[10,12] insulin growth factor I[11] and sivelestat[35] were found to inhibit iNOS induction partly by suppressing iNOS antisense-transcript

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