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Japanese Kampo Medicine, Ninjinyoeito, Inhibits the Induction of iNOS Gene Expression in Proinflammatory Cytokine-Stimulated Hepatocytes

Yoshito Tanaka[1], Masaki Kaibori[1], Hirokazu Miki[1], Masaharu Oishi[1], Richi Nakatake[1], Katsuji Tokuhara[1], Mikio Nishizawa[2], Tadayoshi Okumura[1,3*] and A-Hon Kwon[1]

[1]Department of Surgery, Kansai Medical University, Hirakata, Osaka, 573-1010, Japan.
[2]Department of Biomedical Sciences, College of Life Sciences, Ritsumeikan University, Kusatsu, Shiga 525-8577, Japan.
[3]Research Organization of Science and Technology, Ritsumeikan University, Kusatsu, Shiga 525-8577, Japan.

Authors' contributions

This work was carried out in collaboration between all authors. Authors YT, HM, MO and RN managed the analyses of the study. Author YT performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors MK, KT, MN, TO and AHK managed the coordination of the study. Authors MK, TO and MN conceived the study and participated in its design. This work was carried out in collaboration between all authors. All authors read and approved the final manuscript.

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**Corresponding author: Email: okumura@hirakata.kmu.ac.jp;*

ABSTRACT

Background: Japanese Kampo medicine, ninjinyoeito (NYT), is used for the treatment of complaints in patients with general fatigue, anorexia and anemia, and is recently applied in patients with chronic hepatitis C. However, there is little scientific evidence to demonstrate the liver-protective effects of NYT. We examined proinflammatory cytokine stimulated hepatocytes as a simple *in vitro* liver injury model to determine liver-protective effects of NYT and to clarify the mechanisms involved in.
Methods: Primary cultured rat hepatocytes were treated with interleukin (IL)-1β in the presence or absence of NYT. Inflammatory biomarkers including inducible nitric oxide synthase (iNOS) and tumor necrosis factor (TNF)-α were analyzed.
Results: IL-1β stimulated the induction of iNOS and enhanced the production of NO. Simultaneous addition of NYT decreased the levels of iNOS protein and its mRNA expression, resulting in the reduction of NO production. NYT also reduced the expression of TNF-α mRNA and enhanced the expression of IL-10 mRNA. NYT inhibited two essential signaling pathways for iNOS induction, the activation of nuclear factor (NF)-κB and the upregulation of type I IL-1 receptor. Experiments with transfection and iNOS gene antisense transcript revealed that NYT reduced the levels of iNOS mRNA at both the promoter activation and mRNA stabilization steps. The delayed administration of NYT after IL-1β addition also inhibited iNOS induction.
Conclusions: Results demonstrate that NYT can influence the induction of inflammatory mediators, such as iNOS and TNF-α, in part through the inhibition of NF-κB activation in hepatocytes. NYT may have therapeutic potential for various acute liver injuries.

Keywords: Inducible nitric oxide synthase; nitric oxide; liver injury; primary cultured hepatocytes; nuclear factor-κB; type I interleukin-1 receptor; tumor necrosis factor-α.

1. INTRODUCTION

In Japan, over one hundred Kampo formulas (Japanese herbal medicine) have been approved as ethical drugs by the Ministry of Health, Welfare and Labor of Japan, and are clinically used for the treatment of a wide variety of diseases. It has been reported that over the 80% of physicians in Japan who have been educated in Western medicine at Japanese medical schools have some experience in using Kampo medicines ethically. Kampo medicine vastly differs from Western medicine, and even from Western herbal medicines, with regard to many points such as in philosophy, the concepts of diseases and the therapeutic approach. Among the differences, the combinatorial use of multiple herbs and extraction by hot water are characteristics of Kampo medicines. The use of a single herb is exceptional.

One such Kampo medicine, ninjinyoeito (NYT), has been used for the treatment of complaints in patients with general fatigue, anorexia and anemia, and has recently been treated in patients with chronic hepatitis C [1]. NYT is an aqueous extract from twelve herbs: *Angelicae radix, Rehmanniae radix, Atractylodis rhizoma, Hoelen, Ginseng radix, Cinnamomi cortex, Paeonic radix, Aurantii nobilis pericarpium, Polygalae radix, Astragali radix, Schisandrae fructus and Glycyrrhizae radix* with a weight ratio of 4:4:4:4:3:2.5:2:2:2:1.5:1:1, which is now manufactured under modern scientific quality controls. Supplementation with NYT has shown a generalized positive effect on the anti-

oxidant and anti-inflammatory effects, with no reported adverse effects. It is reported that NYT exerted anti-oxidative action in oxidative related damage in rat hepatocytes [2] and suppressed porcine serum-induced liver fibrosis in rats [3]. However, there is little scientific evidence to demonstrate the liver-protective effects of NYT.

In hepatic disorders, the inflammatory cells such as macrophages gather around hepatic stellate cells and discharge a variety of cytokines. During inflammation, proinflammatory cytokines and nitric oxide (NO) produced by inducible nitric oxide synthase (iNOS) play an important role as factors in liver injury [4]. However, NO has been reported to exert either detrimental or beneficial effects, depending on the insults and tissues involved.

We have previously reported that the induction of iNOS and NO production is upregulated concomitantly with the production of proinflammatory cytokines in animal liver injury models caused by various insults, such as ischemia-reperfusion, partial hepatectomy and endotoxin shock [5–9]. 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, such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6 and cytokine-induced neutrophil chemoattractant (CINC)-1 (human IL-8 analogue). Furthermore, these drugs also inhibited the induction of iNOS and the production of NO in primary cultured rat hepatocytes [7,10,11]. Thus, the prevention of iNOS induction and NO production is considered to be one of the indicators of liver protection.

In the present study, we examined proinflammatory cytokine (IL-1β)-stimulated cultured hepatocytes as a simple *in vitro* liver injury model. The objective of this study is to examine whether NYT influences iNOS and several inflammatory mediators and provides the beneficial effects in IL-1β-stimulated cultured hepatocytes.

2. MATERIALS AND METHODS

2.1 Materials

Kampon injinyoeito (NYT) was provided by Kracie Co., Ltd. (Osaka, Japan). NYT was dissolved in Williams' medium E (WE) and vortexed for 30 min at room temperature, followed by centrifugation (30,000 *g* for 30 min). The supernatant was filter-sterilized with a 0.45-μm membrane filter (Millipore, Billerica, MA, USA) prior to use in experiments. Recombinant human IL-1β (2×10^7 U/mg protein) was purchased from My Bio Source (San Diego, CA, USA). Male Wistar rats (200–220 g and 6.5 weeks old) were purchased from Charles River (Tokyo, Japan), kept at 22℃ under a 12:12 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.

2.2 Primary Cultures of Hepatocytes

Hepatocytes were isolated from the rats by perfusion with collagenase (Wako Pure Chemicals, Osaka, Japan) [12]. Isolated hepatocytes were suspended in culture medium at 6×10^5 cells/mL, seeded into 35mm plastic dishes (2 mL/dish; Falcon Plastic, Oxnard, CA, USA) and cultured at 37℃ 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 (100 μg/ml), fungisone (0.25

μg/ml), aprotinin (0.1 μg/ml, Roche, Mannheim, Germany), dexamethasone (10 nM) and insulin (10 nM). After 2 h, the medium (1.5 ml/35mm dish) was replaced with fresh serum-free and hormone containing WE. 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 number of cells attached to the dishes was calculated by counting the number of nuclei [13] and using a ratio of 1.37±0.04 nuclei/cell (mean ± SE, n = 7 experiments).

2.3 Treatment of Cells with NYT

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 NYT. The doses of NYT used are indicated in the appropriate figures and their legends.

2.4 Determination of NO Production and Lactate Dehydrogenase (LDH) Activity

Culture medium was used for measurements of nitrite (a stable metabolite of NO) to reflect NO production by the Griess method [14]. Culture medium was also used for measurements of LDH activity to reflect cell viability using a commercial kit (Roche Diagnostics, Mannheim, Germany).

2.5 Western Blot Analysis

Total cell lysates were obtained from cultured cells as described previously with minor modifications [10]. Briefly, cells (1 x 10^6 cells/35mm dish) were lysed with sample buffer for sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) (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, 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 100mm dishes (5 x 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℃, 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).

2.6 Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted from cultured hepatocytes using a guanidinium-phenol-chloroform method [15]. 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 [16], with minor modifications. For iNOS (257bp), TNF-α (275bp), IL-10 (245bp), IL-1R1 (327bp) and elongation factor-1α (EF; internal control) (335 bp) mRNAs, an oligo(dT) primer was used for RT and the primers sets were used for PCR (Table 1). For the antisense transcript of iNOS (211bp), the sense primer 5'-CCTTTGCCTCATACTTCCTCAGA-3' was used for RT and the primer set was used for PCR (Table 1). The PCR protocols for iNOS, TNF-α, IL-10, IL-1RI and EF were: 10 cycles of (94℃, 60 s; 72℃, 120 s); 15 cycles of (94℃, 60 s; 65℃, 90 s; 72℃, 20 s); and 5 (iNOS, EF) or 15 (TNF-α, IL-10, IL-1R1) cycles of (94℃, 60 s; 60℃, 90 s; 72℃, 20 s). The PCR protocol for the antisense transcript was: 10 cycles of (94℃, 60 s; 65℃, 90 s; 72℃, 20 s); 15 cycles of (94℃, 60 s; 60℃, 90 s; 72℃, 20 s); and five cycles of (94℃, 60 s; 55℃, 90 s; 72℃, 20 s). The amplified products were analyzed by 3% agarose gel electrophoresis with ethidium bromide, and the levels of iNOS, TNF-α, IL-10, 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)/Gen Bank under Accession numbers AB250951 and AB250952, respectively.

Table 1. Primers and nucleotide sequence

Primer name	Nucleotide sequence
iNOS F/R	5' CCAACCTGCAGGTCTTCGATG 3'/5' GTCGATGCACAACTGGGTGAAC 3'
iNOS asRNA F/R	5' CCTTTGCCTCATACTTCCTCAGA 3'/5'ATCTTCATCAAGGAATTATACACGG 3'
TNF-α F/R	5' TCCCAACAAGGAGGAGAAGTTCC 3'/5' GGCAGCCTTGTCCCTTGAAGAGA 3'
IL-10 F/R	5' GCAGGACTTTAAGGGTTACTTGG 3'/5' CCTTTGTCTTGGAGCTTATTAAA 3'
IL-1RI F/R	5'-CGAAGACTATCAGTTTTTGGAAC-3'/5'-GTCTTTCCATCTGAAGCTTTTGG-3'
EF F/R	5' TCTGGTTGGAATGGTGACAACATGC 3'/5' CCAGGAAGAGCTTCACTCAAAGCTT 3'

iNOS, inducible nitric oxide synthase; as, antisense transcript; TNF-α, tumor necrosis factor-α; IL-10, interleukin-10; IL-1R1, type I IL-1 receptor; EF, elongation factor-1α; F/R, forward/reverse.

2.7 Electrophoretic Mobility Shift Assay (EMSA)

Nuclear extracts were prepared, and EMSA was performed as previously described [17]. Briefly, nuclear extracts from hepatocytes (4 μg) were mixed with 1 μg of poly (dI-dC) and a probe for 20 min at room temperature (total mixture, 15 μl). To prepare a double-stranded DNA probe, annealed oligonucleotides harbouring a κB site (5'-AGTTGAGGGGACTTTCCCAGGC-3'; only the sense strand is shown) were labelled with [γ-^{32}P]-Adenosine-5′-triphosphate (ATP; DuPont-New England Nuclear Japan, Tokyo, Japan) and T4 polynucleotide kinase (Takara Bio Inc.). Samples were resolved on a 4.8%

polyacrylamide gel, followed by drying and autoradiography. The protein concentration was measured by the method of Bradford [18] with a binding assay kit (Bio-Rad) using bovine serum albumin as a standard.

2.8 Transfection and Luciferase Assay

Transfection of cultured hepatocytes was performed as described previously [19]. Briefly, hepatocytes were cultured at 4 x 10^5 cells/dish (35 x 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 NYT. The luciferase and β-galactosidase activities of cell extracts were measured using PicaGene (Wako Pure Chemicals) and Beta-Glo (Promega) kits, respectively.

2.9 Statistical Analysis

The results shown are representative of three to four independent experiments yielding similar findings. All data were expressed as means ± SD. Differences were analyzed by the Bonferroni-Dunn test, and values of $P<0.05$ were considered to indicate significant differences.

3. RESULTS

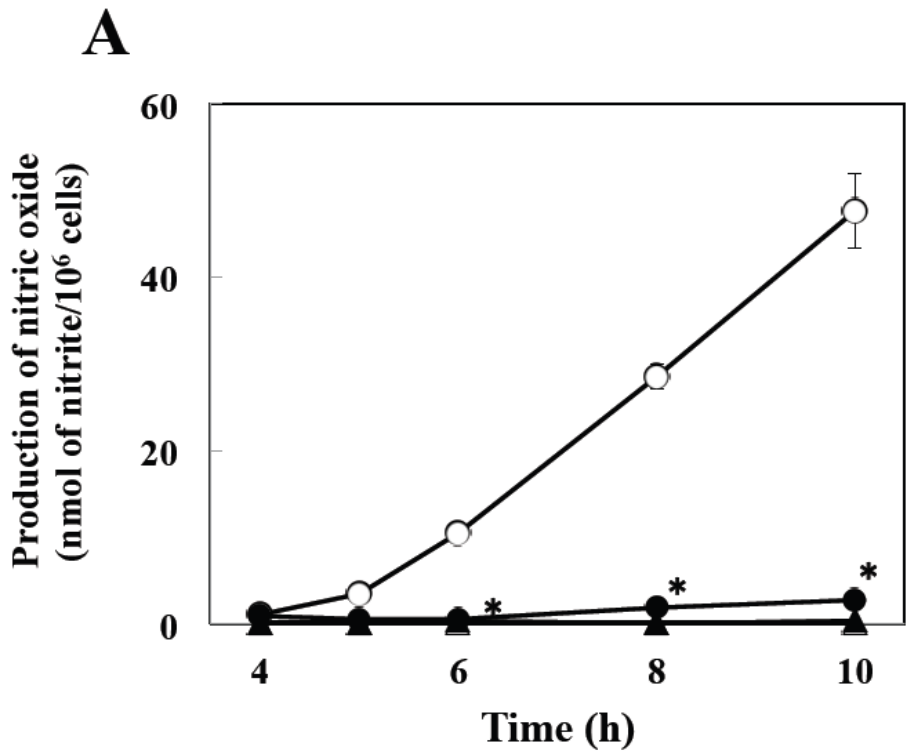
3.1 NYT Inhibits NO Production and iNOS Induction in IL-1β-stimulated Hepatocytes

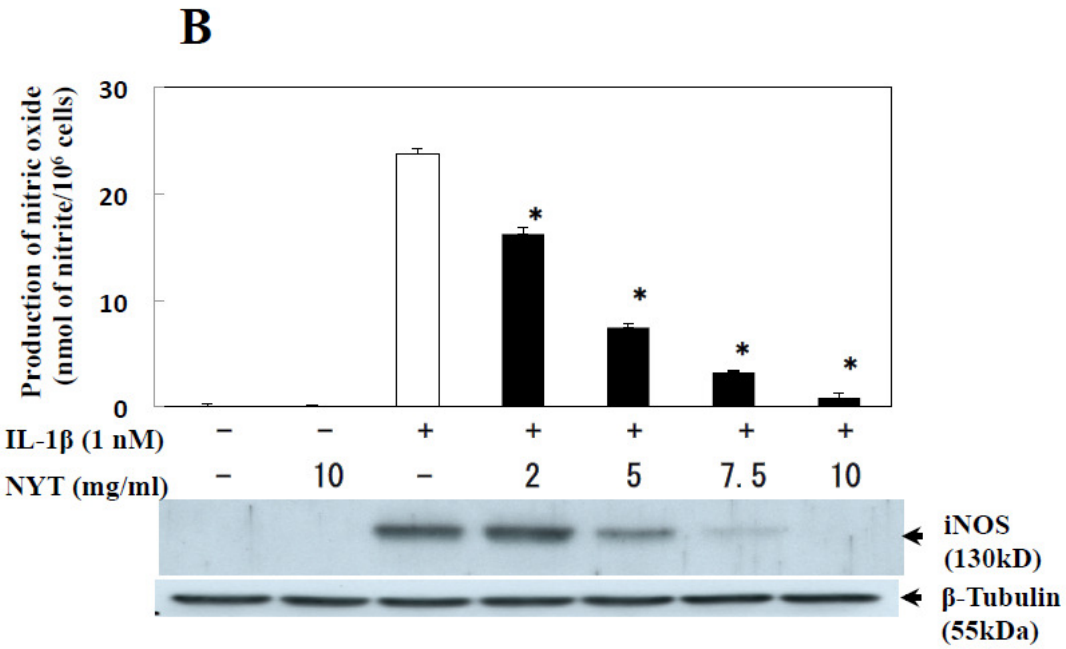
The proinflammatory cytokine IL-1β stimulated the induction of iNOS, which was followed by the production of NO in primary cultured rat hepatocytes [20]. The simultaneous addition of NYT and IL-1β reduced the levels of nitrite (a stable metabolite of NO) time- and dose-dependently in the culture medium (Fig. 1A and 1B). NYT showed more than 90% inhibition at 10 mg/ml. NYT had 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 NYT dose-dependently reduced the expression of the iNOS protein, showing its maximal effect at 10 mg/mL (Fig. 1B). RT-PCR analysis demonstrated that NYT decreased the expression of iNOS mRNA time-dependently (Fig. 1C). These results suggested that NYT inhibited the induction of iNOS gene expression at a transcriptional and/or post-transcriptional step.

3.2 NYT Influences mRNA Expression of Pro- and Anti-inflammatory Cytokines

The expression of other mRNA was examined. IL-1β increased the levels of proinflammatory cytokine, TNF-α mRNA expression [21] and NYT decreased its mRNA expression (Fig. 3, upper). In contrast, NYT enhanced the levels of anti-inflammatory cytokine, IL-10 mRNA expression (Fig. 3, medium).


A
Production of nitric oxide
(nmol of nitrite/10⁶ cells)
60
40
20
0
4
6
8
10
Time (h)
*
*
*


B
Production of nitric oxide
(nmol of nitrite/10⁶ cells)
30
20
10
0
*
*
*
*
IL-1β (1 nM)
– – + + + + +
NYT (mg/ml)
– 10 – 2 5 7.5 10
iNOS
(130kD)
β-Tubulin
(55kDa)

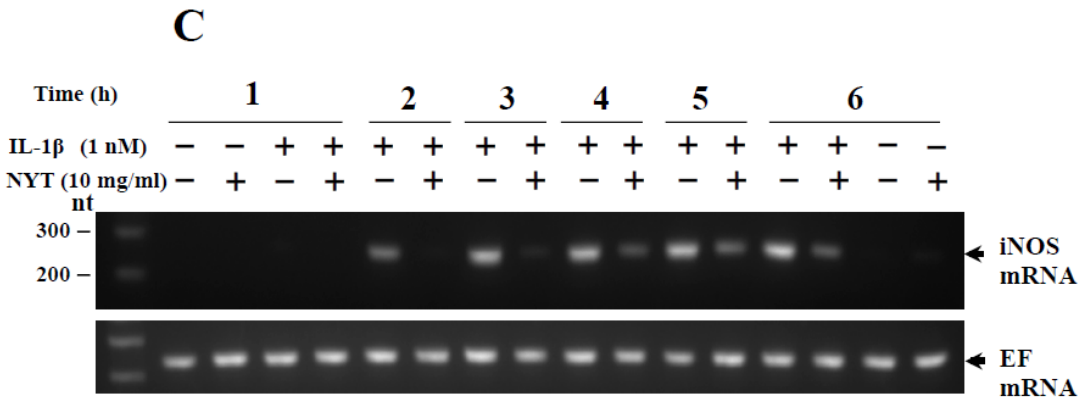


Fig. 1. Effects of NYT on the induction of NO production and iNOS in IL-1β-stimulated hepatocytes

*Cultured hepatocytes were treated with interleukin (IL)-1β (1 nM) in the presence or absence of ninjinyoeito (NYT, 2–10 mg/mL). (A) Effect of NYT (10 mg/mL) treatment for the indicated times on nitric oxide (NO) production (IL-1β, open circles; IL-1β + NYT, filled circles; NYT, filled triangles; controls (without IL-1β and NYT), open triangles). (B) Effects of treatment with various doses of NYT (2–10 mg/mL) for 8 h on NO production (upper) and inducible nitric oxide synthase (iNOS protein, medium). The levels of nitrite were measured in the culture medium (data are means ± SD for n = 3 dishes/point; * P<0.05 versus IL-1β alone). In the western blotting panels, 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. (C) Effects of NYT (10 mg/mL) treatment for the indicated times on the expression of iNOS mRNA. Total RNA was analyzed by strand-specific RT-PCR to detect iNOS mRNA, using EF mRNA as an internal control.*

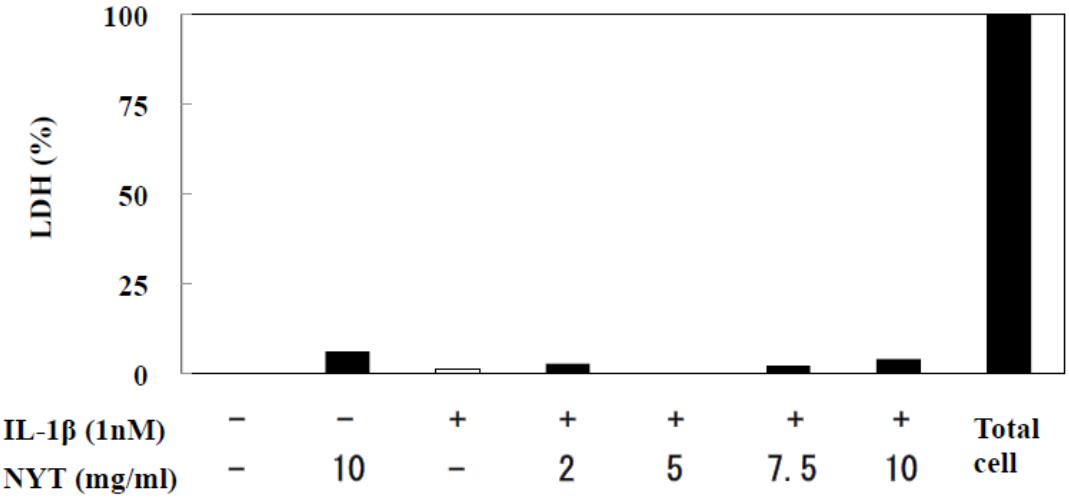


Fig. 2. Effects of NYT on cellular cytotoxicity

The cells were treated with IL-1β (1 nM) in the presence or absence of NYT (2–10 mg/mL) for 8 h. Lactate dehydrogenase (LDH) activity was measured in the culture medium (data are means ± SD, n = 3 dishes/point).

3.3 NYT Decreases iNOS mRNA Synthesis and Stabilization

We examined the mechanisms involved in the inhibition of iNOS induction. The expression of iNOS mRNA is regulated by iNOS promoter transactivation with transcription factors such as NF-κB and by post-transcriptional modifications such as mRNA stabilization [22]. Therefore, we carried out transfection experiments with constructs containing firefly luciferase controlled by the iNOS promoter (pRiNOS-Luc-SVpA and pRiNOS-Luc- 3′UTR) (Fig. 4A), which detect iNOS promoter transactivation (mRNA synthesis) and mRNA stabilization, respectively [23]. IL-1β increased the luciferase activities of these constructs, and these effects were significantly inhibited by NYT (Fig. 4B and 4C). As we have reported previously [24], the iNOS gene antisense transcript (asRNA) is involved in the stability of iNOS mRNA. iNOS asRNA analysis by RT-PCR revealed that IL-1β increased the expression of iNOS asRNA in a time-dependent manner, and that NYT markedly inhibited this effect (Fig. 4D).

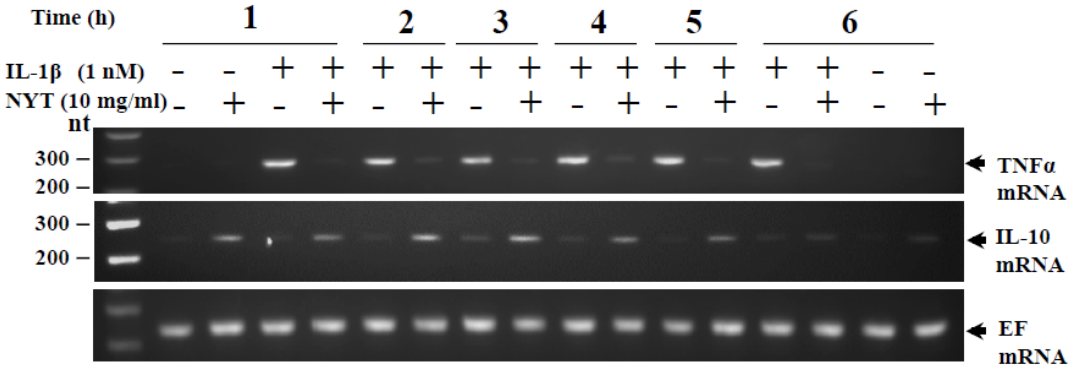
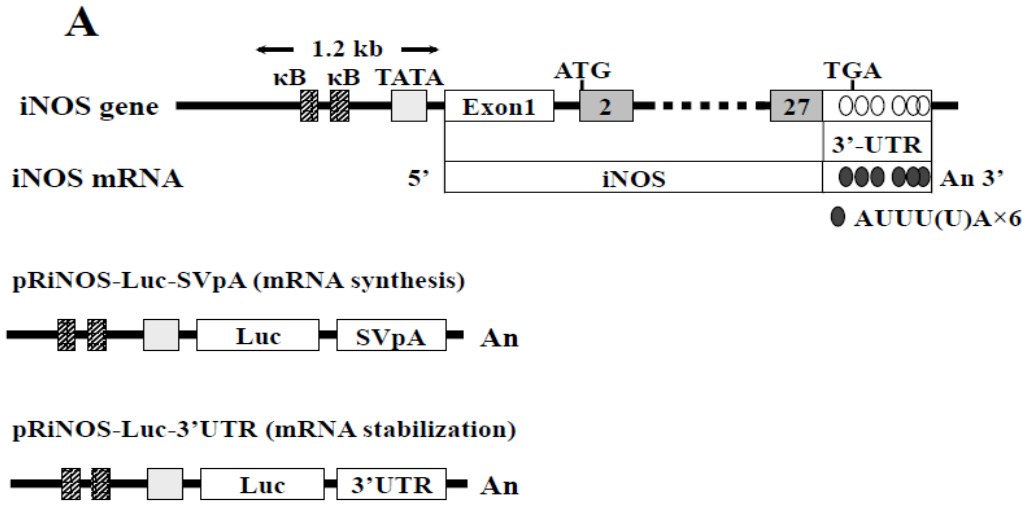


Fig. 3. Effects of NYT on expression of TNF-α and IL-10

The cells were treated with IL-1β (1 nM) in the presence or absence of NYT (10 mg/mL) for the indicated times. Total RNA was analyzed by strand-specific RT-PCR to detect TNF-α and IL-10, using EF mRNA as an internal control.



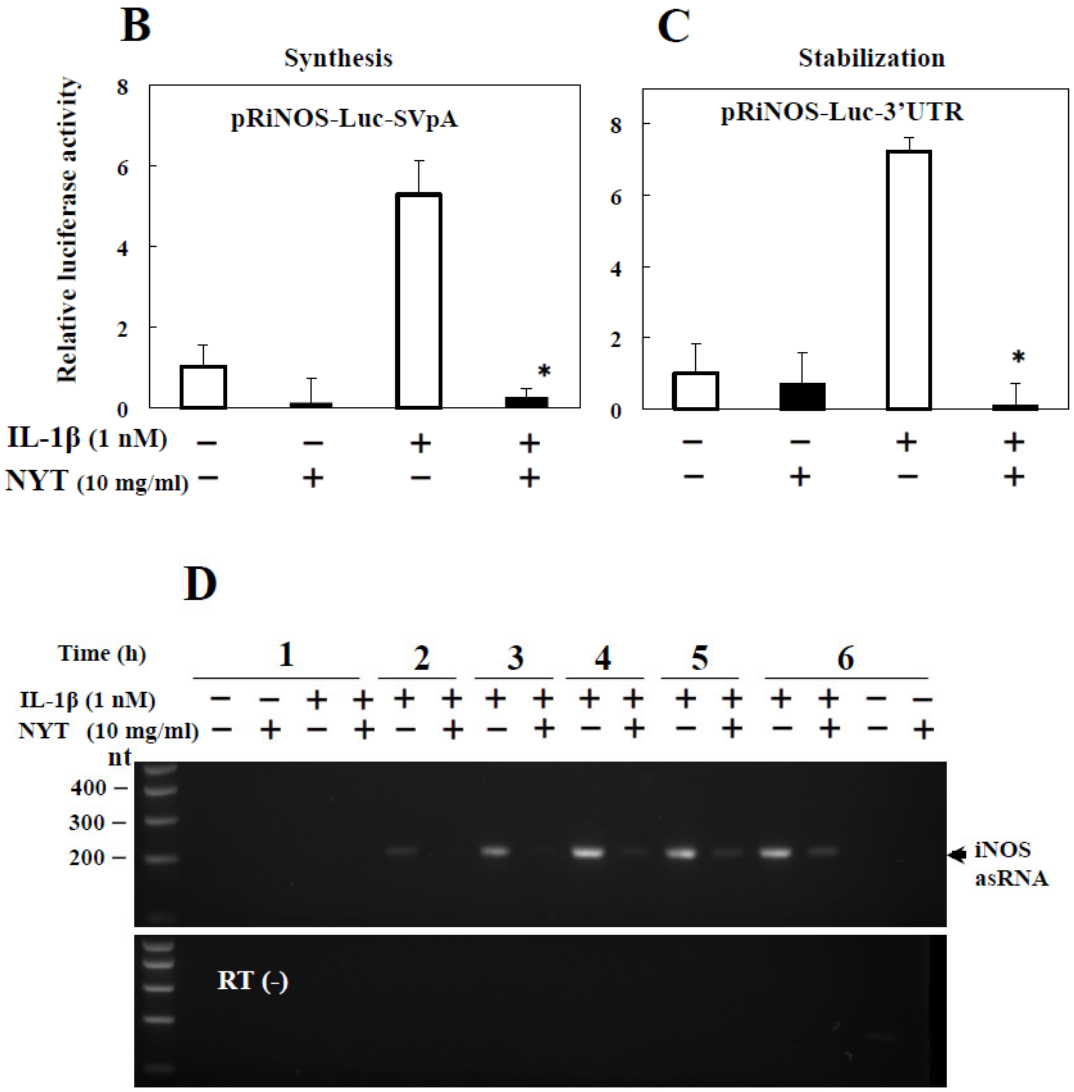


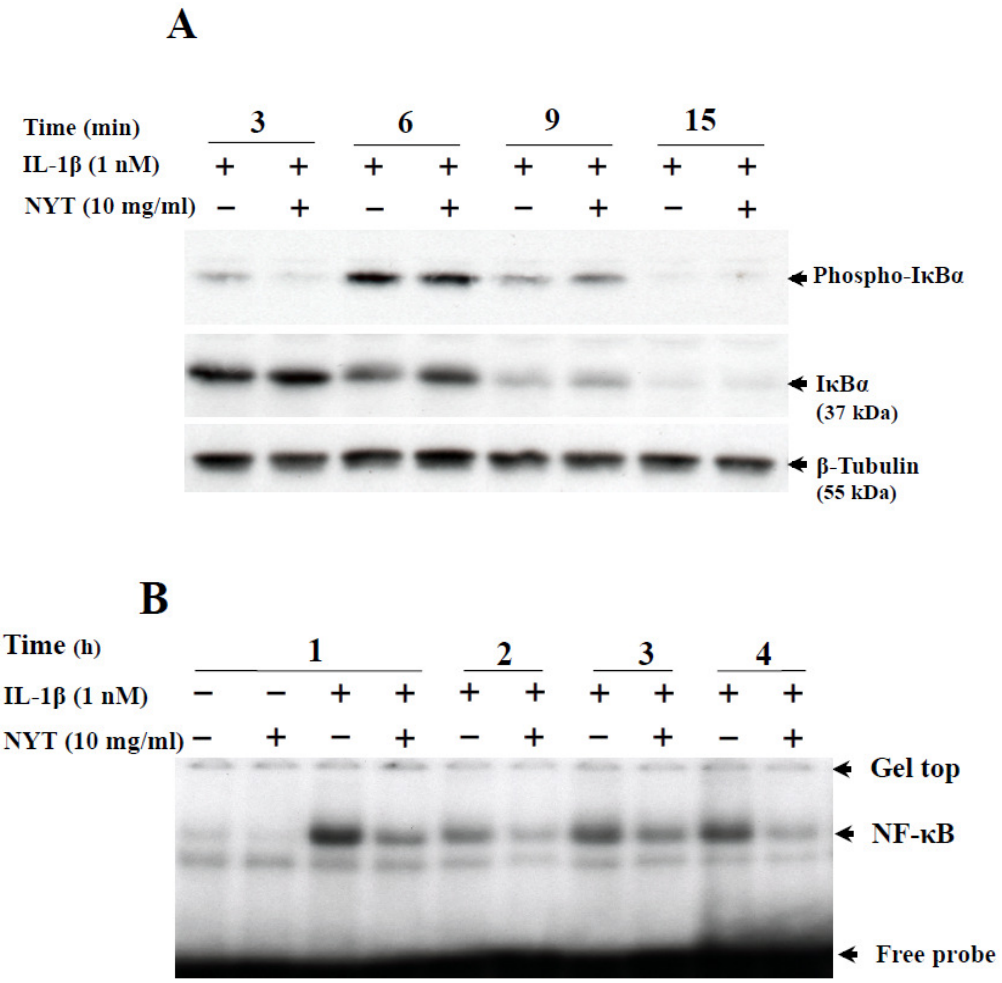
Fig. 4. Effects of NYT on the transactivation of the iNOS promoter and the expression of the iNOS gene antisense transcript

*(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). '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 NYT (10 mg/mL) for 8 h for pRiNOS-Luc-SVpA (B) and 5 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 the control activity (without IL-1β and NYT). Data are means ± SD for n = 4 dishes. *P<0.05 versus IL-1β alone. (D) The cells were treated with IL-1β (1 nM) in the presence or absence of NYT (10 mg/mL) for the indicated times. Total RNA was analyzed by strand-specific RT-PCR to detect the iNOS gene antisense transcript (asRNA). RT(-) denotes a negative control PCR using total RNA without RT.*

3.4 NYT Inhibits NF-κB Activation and IL-1RI Upregulation

There are two essential signaling pathways for iNOS induction, IκB kinase and phosphatidylinositol 3-kinase (PI3K)/Akt pathways [25]. In the former, 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). NYT had no effect on the phosphorylation and degradation of IκBα after IL-1β stimulation (Fig. 5A). In contrast, electrophoretic mobility shift assay with nuclear extracts revealed that NYT inhibited NF-κB activation at 1–4 h (Fig. 5B). Immunoprecipitation and western blotting of nuclear extracts showed that NYT inhibited nuclear translocation of NF-κB subunit p65 (Fig. 5C).

In the latter, IL-1β stimulates the upregulation of IL-1RI through the activation of PI3K/Akt [25]. Immunoprecipitation-western blotting analysis revealed that NYT inhibited the phosphorylation (activation) of Akt at 30 min, a downstream kinase of PI3K (Fig. 6A). RT-PCR and western blotting analyses revealed that NYT reduced both levels of IL-1RI mRNA and protein expression (Fig. 6B and 6C).



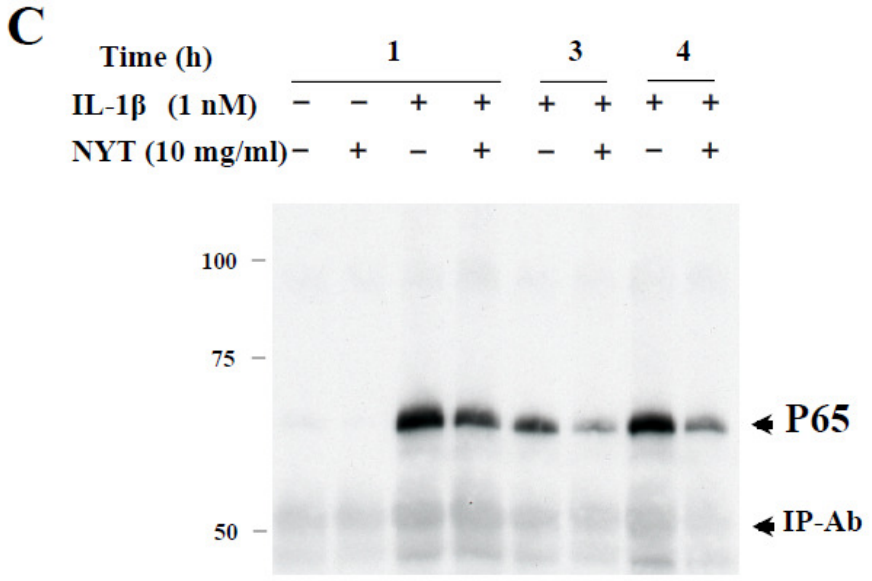
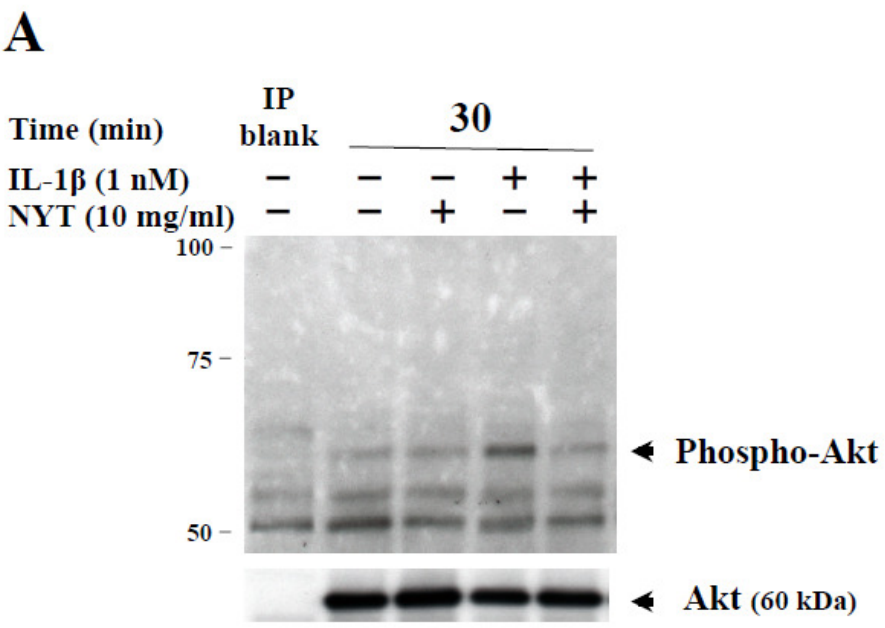


Fig. 5. Effects of NYT on the degradation of IκBα and the activation of NF-κB

The cells were treated with IL-1β (1 nM) in the presence or absence of NYT (10 mg/mL) for the indicated times. (A) Cell lysates (20 μg of protein) were subjected to SDS-PAGE in a 12.5% gel, followed by immunoblotting with an anti-phospho-IκBα, anti-IκBα, or anti-β-tubulin antibody. (B) Activation of NF-κB. Nuclear extracts (4 μg of protein) were analyzed by an electrophoretic mobility shift assay. Representative results of three independent experiments are shown. (C) Nuclear translocation of NF-kB subunit p65. Nuclear extracts were immunoprecipitated, and the immunoprecipitates were analyzed by western blotting with an anti-p65 antibody.



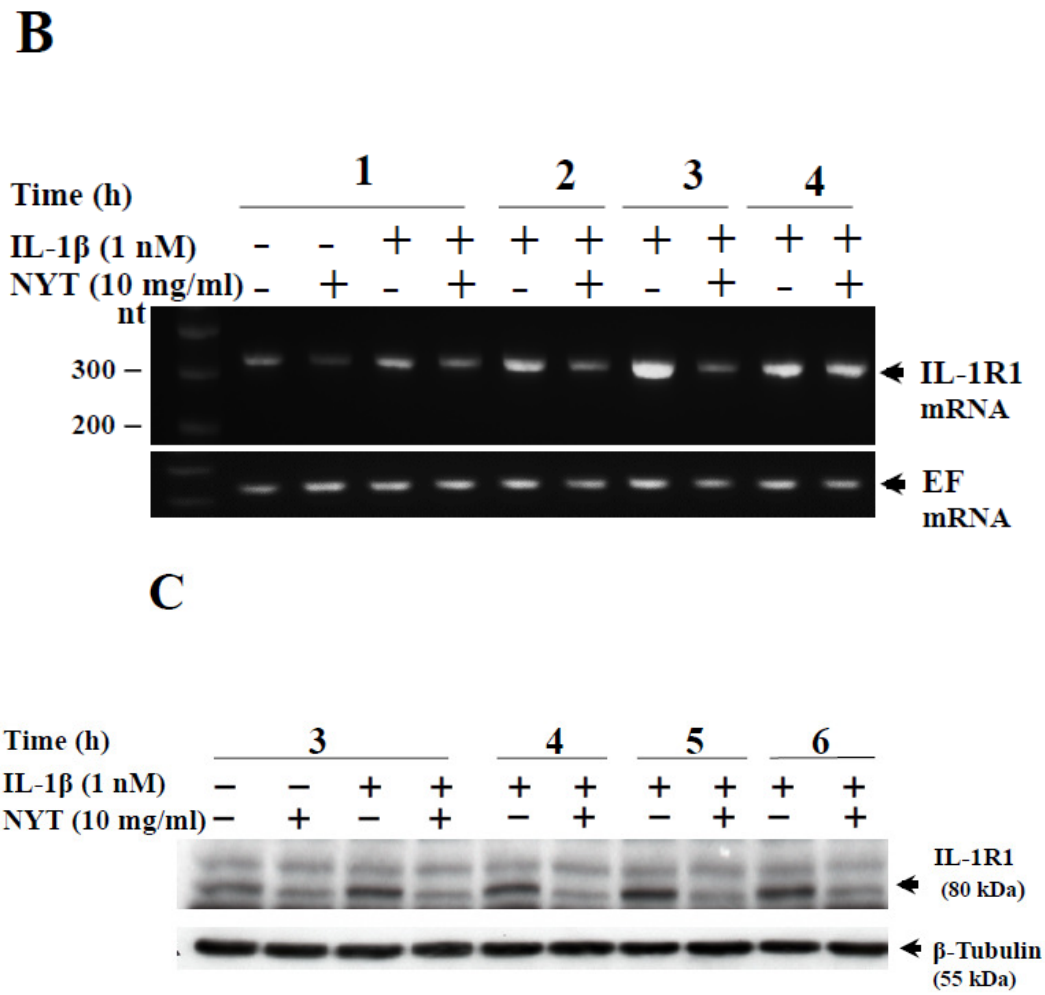


Fig. 6. Effects of NYT on the upregulation of type I IL-1 receptor

The cells were treated with IL-1β (1 nM) in the presence or absence of NYT (10 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 RT-PCR to detect the type I IL-1 receptor (IL-1RI) mRNA, using EF mRNA as an internal control. (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.

3.5 The Delayed Administration of NYT Inhibits iNOS Induction

We examined whether the delayed administration of NYT influences iNOS induction. NYT was added to the medium 0–4 h after the addition of IL-1β. The delayed administration of NYT up to 4 h after IL-1β addition still markedly inhibited NO production, although the magnitude of inhibition decreased in a time-dependent manner (Fig. 7).

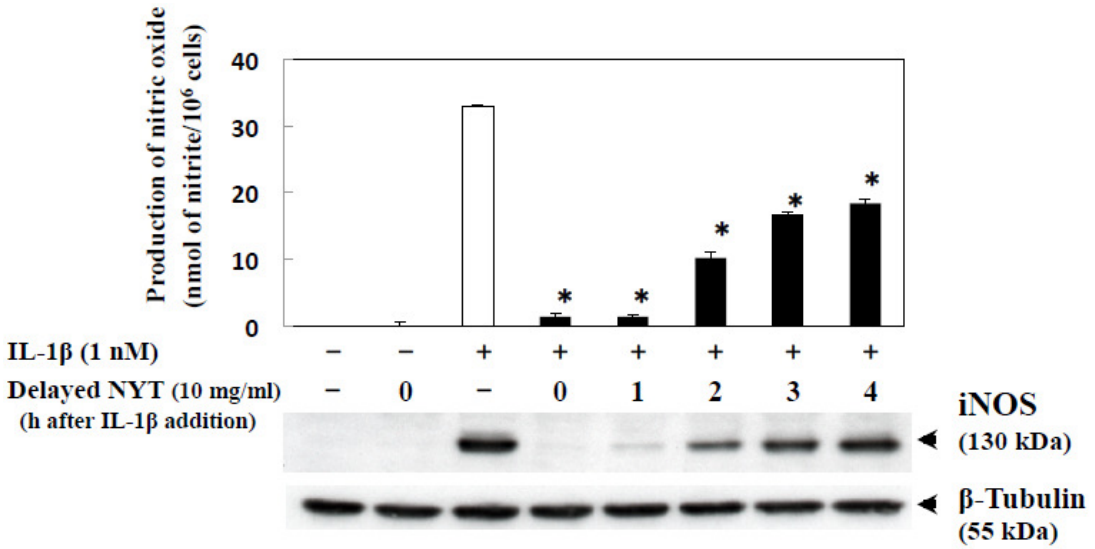


Fig. 7. Effects of delayed NYT administration on the induction of iNOS in hepatocytes

*The cells were treated with NYT (10 mg/mL) at 0–4 h after the addition of IL-1β (1 nM). The effects of NYT on NO production (upper) and iNOS protein (lower) were analyzed at 8 h after IL-1β addition. The levels of nitrite were measured in the culture medium (data are means ± SD, n = 3 dishes/point; *P<0.05 versus IL-1β alone). In the western blotting panels, 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*.

4. DISCUSSION

In this study, we found that NYT inhibited the induction of iNOS and even TNF-α, and enhanced anti-inflammatory cytokine IL-10 mRNA in IL-1β stimulated hepatocytes.

It is known that the induction of iNOS gene expression is regulated by iNOS promoter transactivation and by post-transcriptional modifications [22]. NF-κB plays a key role in inflammation by regulating genes encoding iNOS and proinflammatory cytokines such as TNF-α [26]. 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) the 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 site [27]. NYT inhibited NF-κB activation (Fig. 5B) and the translocation of p65 to the nucleus (Fig. 5C), although NYT had no effect on the phosphorylation and degradation of IκBα (Fig. 5A). Furthermore, in experiments with iNOS promoter constructs, NYT was found to inhibit iNOS induction at both of its mRNA synthesis and stabilization steps (Fig. 4).

In concert with NF-κB activation, the upregulation of IL-1RI through the activation of PI3K/Akt is also essential for iNOS induction [25]. We found that NYT decreased the expression of IL-1RI mRNA and protein (Fig. 6B and6C) through the inhibition of Akt phosphorylation (Fig. 6A), which is presumably 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 adenine/uracil (AU)-rich elements (ARE) that are associated with ARE-binding proteins such

as HuR and heterogeneous nuclear ribonucleoproteins L/I (PTB), which serve to stabilize the mRNA [28]. Recently, we have reported that asRNAs are often transcribed from many inducible genes, such as iNOS and TNF-α [21,29]. The iNOS asRNA interacts with and stabilizes iNOS mRNA [24,30]. We have reported that drugs such as edaravone (free radical scavenger) (11), FR183998 (Na^+/H^+ exchanger inhibitor) [7,9], insulin growth factor I [8], sivelestat (neutrophil elastase inhibitor) [31], kampo inchinkoto (TJ-135) [32] and hochuekito (TJ-41) [33] inhibited iNOS induction partly by suppressing iNOS asRNA production in animal models and/or primary cultured hepatocytes. In this study, NYT also decreased the iNOS asRNA expression (Fig. 4D).

These results demonstrate that NYT inhibits two essential signaling pathways, NF-κB activation and IL-1RI upregulation in the induction of iNOS. NYT probably reduced iNOS mRNA expression through the inhibition of its mRNA synthesis and stabilization, leading to the reduction of iNOS protein and NO production.

During endotoxemia, proinflammatory cytokines such as TNF-α, IL-1β and IL-6 are considered to play an important role in the injury to multiple organs in addition to the liver. TNF-α is a key mediator of the cytokine cascade in sepsis [34,35]. Therefore, the inhibition of TNF-α expression is important in anti-inflammatory therapy. In our *in vitro* model, we have recently reported that IL-1β stimulated the induction of TNF-α in parallel with the induction of iNOS [21]. In this study, NYT decreased the expression of TNF-α mRNA (Fig. 3). In contrast, NYT enhanced the levels of anti-inflammatory cytokine, IL-10 mRNA expression (Fig. 3). IL-10 is an anti-inflammatory cytokine that inhibits the activation of macrophages/monocytes and T cells, thereby causing the inhibition of production of proinflammatory cytokines. It has been reported that IL-10 is involved in the prevention of liver injury in D-galactosamine/lipopolysaccharide (LPS)-treated animals [36,37]. Accordingly, a part of protective effects by NYT probably come from the enhancement of IL-10 expression in cultured hepatocytes.

These observations suggest that NYT can inhibit the induction of inflammatory mediators (iNOS and TNF-α), and enhance the induction of anti-inflammatory cytokine (IL-10) in liver injury, leading to the liver-protective effects of NYT. The inhibition of NO production by NYT seems to be common in other inflammatory cells. Oral administration of NYT had therapeutic effects on NO-induced lung injury in cytomegalovirus-treated mice [38]. NYT decreased the production of NO stimulated by LPS in mouse macrophage-like RAW264.7 [39]. These findings are partly similar to our present findings in hepatocytes, and suggest that the inhibitory effects of NYT are not specific for hepatocytes.

The delayed treatment with NYT after IL-1β addition was found to cause a significant reduction in NO production and iNOS induction (Fig. 7). This observation may be of clinical importance, since the initiation of therapeutic NYT treatment is usually delayed from the onset of diseases. The clinically recommended dose of NYT is 2.5-5 g/day. The concentrations (2-10 mg/ml) of NYT used in this experiment are probably extraordinary large. In this *in vitro* model, high dosages of drugs are often required for inhibition of NO production/iNOS induction, which may be one of limitations. For examples, 1-5 mM of perfenidone (anti-fibrotic drug) [10], 10-30 μM of FR183998 [7,9], 300-600 μM of edaravone [11] and 1-5 mM of sivelestat [31] are required for the inhibition of iNOS induction. Our simple *in vitro* experiment with cultured 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, there are a variety of factors involved in liver injury in addition to iNOS

and proinflammatory cytokines. Thus a liver-protective effect in drugs deduced from this model need to be examined and supported in *in vivo* animal models.

5. CONCLUSION

In conclusion, Kampo NYT can prevent IL-1β-stimulated liver injury in cultured hepatocytes by inhibiting the induction of inflammatory mediators such as iNOS and TNF-α, in part through the inhibition of NF-kB activation. NYT may have therapeutic potential for various acute liver injury including ischemia-reperfusion, endotoxin shock and hemorrhagic shock.

CONSENT

Not applicable.

ETHICAL APPROVAL

All authors hereby declare that "Principles of laboratory animal care" (NIH publication No. 85-23, revised 1985) were followed, as well as specific national laws where applicable. All experiments have been examined and approved by the appropriate ethics committee.

All authors hereby declare that all experiments have been examined and approved by the appropriate ethics committee and have therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Cyong JC, Ki SM, Iijima K, Kobayashi T, Furuya M. Clinical and pharmacological studies on liver diseases treated with Kampo herbal medicine. Am J Chin Med. 2001;28(3-4):351-60.
2. Takayama F, Egashira T, Yamanaka Y. Protective effect of Ninjin-yoei-to on damage to isolated hepatocytes following transient exposure to tert-butyl hydroperoxide. Jpn J Pharmacol. 2001;85(3):227-233.
3. Ochi T, Kawakita T, Nomoto K. Effects of Hochu-ekki-to and Ninjin-youei-to, traditional Japanese medicines, on porcine serum-induced liver fibrosis in rats. Immunopharmacol Immunotoxicol. 2004;26(2):285-98.
4. Colasanti M, Suzuki H. The dual personality of NO. Trends Pharmacol Sci. 2000;21:249–52.
5. Tsuchiya H, Kaibori M, Yanagida H, et al. Pirfenidone prevents endotoxin-induced liver injury after partial hepatectomy in rats. J Hepatol. 2004;40:94–101.

6. Tsuji K, Kwon AH, Yoshida H, et al. Free radical scavenger (edaravone) prevents endotoxin-induced liver injury after partial hepatectomy in rats. J Hepatol. 2005;42:94–101.
7. Tanaka H, Uchida Y, Kaibori M, et al. Na^+/H^+ exchanger inhibitor, FR183998, has protective effect in lethal acute liver failure and prevents iNOS induction in rats. J Hepatol. 2008;48:289–99.
8. Hijikawa T, Kaibori M, Uchida Y, et al. Insulin-like growth factor-I prevents liver injury through the inhibition of TNF-α and iNOS induction in D-galactosamine and lipopolysaccharide-treated rats. Shock. 2008;29:740–7.
9. Ishizaki M, Kaibori M, Uchida Y, et al. Protective effect of FR183998, a Na^+/H^+ exchanger inhibitor, and its inhibition of iNOS induction in hepatic ischemia-reperfusion injury in rats. Shock. 2008;30:311–17.
10. Nakanishi H, Kaibori M, Teshima S, et al. Pirfenidone inhibits the induction of iNOS stimulated byinterleukin-1β at a step of NF-kB DNA binding in hepatocytes. J Hepatol. 2004;41:730–6.
11. Yoshida H, Kwon AH, Kaibori M, et al. Edaravone prevents iNOS expression by inhibiting its promoter transactivation and mRNA stability in cytokine-stimulated hepatocytes. Nitric Oxide. 2008;18:105–12.
12. Seglen PO. Preparation of isolated rat liver cells. Methods Cell Biol. 1976;13:29–83.
13. Horiuti Y, Ogishima M, Yano K, Shibuya Y. Quantification of cell nuclei isolated from hepatocytes by cell lysis with nonionic detergent in citric acid. Cell Struct Funct. 1991;16:203–7.
14. Green LC, Wagner DA, Glogowski J, Skipper PL, Wishnok JS, Tannenbaum SR. Analysis of nitrate, nitrite and [15N] nitrate in biological fluids. Anal Biochem. 1982;126:131–8.
15. Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol chloroform extraction. Anal Biochem. 1987;162:156–9.
16. Nishizawa M, Nakajima T, Yasuda K, et al. Close kinship of human 20 alpha-hydroxysteroid dehydrogenase gene with three aldo-keto reductase genes. Genes Cells. 2000;5:111–25.
17. Oda M, Sakitani K, Kaibori M, Inoue T, Kamiyama Y, Okumura T. Vicinal dithiol-binding agent, phenylarsine oxide, inhibits iNOS gene expression at a step of NF-kB DNA binding in hepatocytes. J Biol Chem. 2000;275:4369–73.
18. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem. 1976;72:248–54.
19. Matsui K, Kawaguchi Y, Ozaki T, et al. Effect of active hexose correlated compound on the production of nitric oxide in hepatocytes. JPEN J Parenter Enteral Nutr. 2007;31:373–81.
20. Kitade H, Sakitani K, Inoue K, et al. Interleukin-1β markedly stimulates nitric oxide formation in the absence of other cytokines or lipopolysaccharide in primary cultured rat hepatocytes, but not in Kupffer cells. Hepatology. 1996;23:797–802.
21. Yoshigai E, Hara T, Inaba H, Hashimoto I, Tanaka Y, Kaibori M, Kimura T, Okumura T, Kwon AH, Nishizawa M. Interleukin 1β induces tumor necrosis factor α secretion from rat hepatocytes. Hepatol Res. 2014;44:571–583.
22. Kleinert H, Pautz A, Linker K, Schwarz PM. Regulation of the expression of inducible nitric oxide synthase. Eur J Pharmacol. 2004;500:255–66.

23. Yamada M, Nishizawa M, Nakatake R, et al. Characterization of alternatively spliced isoforms of the type Iinterleukin-1 receptor on iNOS induction in rat hepatocytes. Nitric Oxide. 2007;17:98–105.
24. Matsui K, Nishizawa M, Ozaki T, Kimura T, Hashimoto I, Yamada M, Kaibori M, Kamiyama Y, Ito S, Okumura T. Natural antisense transcript stabilizes inducible nitric oxide synthase mRNA in rat hepatocytes. Hepatology. 2008;47:686-697.
25. Teshima S, Nakanishi H, Nishizawa M, et al. Up-regulation of IL-1 receptor through PI3K/Akt is essential for the induction of iNOS gene expression in hepatocytes. J Hepatol. 2004;40:616–23.
26. Lawrence T, The nuclear factor NF-κB pathway in inflammation. Cold Spring Harb Perspect Biol. 2009;1:a001651.
27. Akira S, Kishimoto T. NF-IL6 and NF-kB in cytokine gene regulation. Adv Immunol. 1997;65:1–46.
28. Pautz A, Linker K, Hubrich R, Korhonen R, Altenhofer S, Kleivert H. The polypyrimidine tract-binding protein (PTB) is involved in the post-transcriptional regulation of human inducible nitric oxide synthase expression. J Biol Chem. 2006;281:32294–302.
29. Nishizawa M, Okumura T, Ikeya Y, Kimura T. Regulation of inducible gene expression by natural antisense transcripts. Front Biosci. 2012;17:938–958.
30. Yoshigai E, Hara T, Araki Y, Tanaka Y, Oishi M, Tokuhara K, Kaibori M, Okumura T, Kwon AH, Nishizawa M. Natural antisense transcript-targeted regulation of inducible nitric oxide synthase mRNA levels. Nitric Oxide. 2013;30:9-16.
31. Araki Y, Matsumiya M, Matsuura T, et al. Sivelestat suppresses iNOS gene expression in proinflammatory cytokine stimulated hepatocytes. Dig Dis Sci. 2011;56:1672–81.
32. Matsuura T, Kaibori M, Araki Y, Matsumiya M, Yamamoto Y, Ikeya Y, Nishizawa M, Okumura T, Kwon AH. Japanese herbal medicine, inchinkoto, inhibits inducible nitric oxide synthase induction in interleukin-1β-stimulated hepatocytes. Hepatol Res. 2012;42(1):76-90.
33. Matsumiya M, Kaibori M, Araki Y, Matsuura T, Oishi M, Tanaka Y, Nishizawa M, Okumura T, Kwon AH. Japanese herbal medicine hochuekkito inhibits the expression of proinflammatory biomarker, inducible nitric oxide synthase, in hepatocytes. Medicinal Chemistry (Medchem). 2012;2(2):112. DOI: 10.4172/2161-0444.1000112.
34. Suzuki S, Nakamura S, Serizawa A, Sakaguchi T, Konno H, Muro H, Kosugi I, Baba S. Role of Kupffer cells and the spleen in modulation of endotoxin-induced liver injury after partial hepatectomy. Hepatology. 1996;24(1):219-225.
35. Fong Y, Tracey K, Moldawer L, Hesse D, Manogue K, Kenney J, Lee A, Kuo G, Allison A, Lowry S, Cerami A. Antibodies to cachectin/tumor necrosis factor reduce interleukin 1 beta and interleukin 6 appearance during lethal bacteremia. J Exp Med. 1989;170(5):1627-1633.
36. Santucci L, Fiorucci S, Chiorean M, Brunori P, Di Matteo F, Sidoni A, Migliorati G, Morelli A. Interleukin 10 reduces lethality and hepatic injury induced by lipopolysaccharide in galactosamine-sensitized mice. Gastroenterology. 1996;111(3):736-744.
37. Qiu Z, Kwon AH, Tsuji K, Kamiyama Y, Okumura T, Hirao Y. Fibronectin prevents D-galactosamine/lipopolysaccharide-induced lethal hepatic failure in mice. Shock. 2006;25(1):80-87.

38. Tanaka K, Sawamura S. Therapeutic effect of a traditional Chinese medicine, ren-shen-yang-rong-tang (Japanese name: Ninjin'yoeito) on nitric oxide-mediated lung injury in a mouse infected with murine cytomegalovirus. Int Immunopharmacol. 2006;6(4):678-685.
39. Kaneko T, Chiba H, Horie N, Hashimoto K, Satoh K, Kusama K, Sakagami H. Effect of two different groups of Chinese medicines on nitric oxide production by mouse macrophage-like cells. In Vivo. 2004;18(6):771-778.

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