Effects of medicinal plant ipe on expression of inducible nitric oxide synthase in inerleukin-1β-stimulated Hepatocytes

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ABSTRACT

Background*:* The traditional medicine ipe is thought to have various pharmacological actions including anticancer and anti-inflammatory activities. However, there is little scientific evidence to demonstrate the organ-protective effects of ipe. The prevention of nitric oxide (NO) production in inflamed livers by inducible NO synthase (iNOS) is an indicator of liver protection. We examined proinflammatory cytokine-stimulated hepatocytes as a simple "*in vitro* liver injury model" to determine ipe's liver-protective effects of ipe and clarify its mechanisms. This study aims to examine whether ipe influences iNOS induction and NO production, and if so, the mechanisms involved in its action.

Methods: Primary cultured hepatocytes were treated with interleukin (IL)-1β in the presence or absence of ipe. The induction of iNOS and its signal pathway were analyzed.

Results: Ipe inhibited the production of NO stimulated by IL-1β and showed the greatest effect (more than 90% inhibition) at 2 mg/ml. Ipe decreased iNOS protein and mRNA expression. Ipe decreased NF-κB activation (its translocation to the nucleus and DNA binding), although there was no effect on IκBα degradation. Ipe inhibited Akt activation, followed by decreased the type I IL-1 receptor mRNA and protein levels. Transfection experiments revealed that ipe decreased both activities of iNOS promoter transactivation and mRNA stability. In support of the latter

observation, ipe inhibited the expression of the antisense transcript of the iNOS gene.

Conclusion: Ipe blocked IκB kinase and phosphatidylinositol 3-kinase/Akt signal pathways, which caused the reduction of iNOS mRNA synthesis and its stability. This resulted in the inhibition of iNOS induction and NO production. Ipe may have a potent beneficial effect against NO-mediated injury in organs including the liver.

Key words: ipe, inducible nitric oxide synthase, liver injury, primary cultured hepatocytes, nuclear factor-κB, the type I interleukin-1 receptor, iNOS antisense transcript

INTRODUCTION

Recent accumulated evidence indicates that traditional medicines, including domestic Kampo (Japanese herbal) and imported foreign medicines, and functional foods are clinically used to treat various diseases in Japan. Traditional medicines are used for pre- and post-operative treatments in patients with digestive diseases. Over 80% of physicians in Japan have some experience in using such medicines. One such medicine, ipe (ipe roxo, taheebo tea), is a traditional medicine extracted from the dried inner bark of *Tabebuia avellanedae/ipe* (lapacho tree), which is a popular tree distributed throughout the tropical rain forests of Central and South America. Ipe and its constituents, such as lapachol, are thought to have various pharmacological actions such as antiulcer, antibacterial, antioxidant, anti-inflammatory and anticancer activities [1-5].

In hepatic disorders, proinflammatory cytokines and nitric oxide (NO) produced by inducible nitric oxide synthase (iNOS) in Kupffer cells and hepatocytes play important roles as factors in liver injury [6], although NO exerts either detrimental or beneficial effects, depending on the insults and tissues involved. In animal liver injury models caused by various insults, such as ischemia-reperfusion, partial hepatectomy and endotoxin shock, we have previously reported that drugs showing liver-protective effects inhibited the induction of iNOS and NO production and 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) [7-11]. Furthermore, in *in vitro* experiments with primary cultured rat hepatocytes stimulated by IL-1β, these drugs also inhibited iNOS induction and NO production [9,12,13]. Thus, by using our cultured hepatocytes, the prevention of iNOS induction and NO production is an indicator of liver protection. Recently Awale et al. reported that a water extract of the inner bark of taheebo from Brazil showed significantly inhibited NO production in lipopolysaccharide-activated J774.1 macrophage-like cells [14]. However, they did not find the mechanisms involved in the extract's action or its isolated constituents.

In the present study, we examined IL-1β-stimulated cultured hepatocytes as a simple *in vitro* liver injury model.

MATERIALS AND METHODS
Materials
Ipe roxo powder (IkedaYakuso Co., Ltd., Osaka, Japan) and was dissolved in Williams' Medium E (WE), vortexed for 30 min at room temperature, followed by centrifugation (9,000 rpm (13,000

×g) for 30 min at 4ºC, JA-12 rotor/Avanti HP-30I, Beckman Coulter Co., Ltd., USA). The supernatant (ipe extract) 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 MyBioSource (San Diego, CA, USA). [γ-^{32}P] Adenosine-5'-triphosphate(ATP;-222TBq/mmol)and [α-^{32}P]deoxycytidine-5'-triphosphate (dCTP; -111 TBq/mmol) were obtained from DuPont-New England Nuclear Japan (Tokyo, Japan). Male Wistar rats (200–250 g, 6-7 weeks old) were purchased from Charles River (Tokyo, Japan), kept at 22°C under a 12:12 h light:dark cycle, and given 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 hepatocyte cultures
Hepatocytes were isolated from rats using collagenase (Wako Pure Chemicals, Osaka, Japan) perfusion [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 units/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 [17] and using a ratio of 1.37 ± 0.04 nuclei/cell (mean ± SE, n=7 experiments).

Cell treatment with ipe extract
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 ipe extract. The ipe extract doses used are indicated in the appropriate figures and their legends.

Determining NO production and lactate dehydrogenase (LDH)
Culture medium was used for nitrite (stable metabolite of NO) for NO production measurements using the Griess method [18] and LDH activity for cellular viability using a commercial kit (Wako Pure Chemicals).

Western blot analysis
Total cell lysates were obtained from cultured cells [19], 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, CA, USA). Immunostaining was performed using primary antibodies against mouse iNOS (Affinity BioReagents, Golden, CO, USA), human IκBα, mouse IL-1RI (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and rat b-tubulin (internal control; Clone TUB2.1; Sigma Chemical Co.),

followed by visualization with an ECL blotting detection reagent (GE Healthcare Biosciences Corp., Piscataway, NJ, USA).

For Akt, total cell lysates prepared from 100-mm 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°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 using western blotting (SDS-PAGE in a gradient 6-9% gel, Multigel II mini (Cosmo Bio Co., Ltd., Tokyo, Japan)) 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 using western blotting (SDS-PAGE in a gradient 6%-9% gel, Multigel II mini (Cosmo Bio Co., Ltd.)) using an antibody against human NF-kB p65 (BD Transduction Laboratories, Lexington, KY, USA).

Northern blot analysis and reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was extracted from cultured hepatocytes using a guanidinium-phenol-chloroform method [20] with Trizol reagent (Invitrogen, Carlsbad, CA, 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 [21]. cDNAs encoding rat IκBα, p65, IL-1RI [22] and mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [23] were prepared using RT-PCR [24]. The cDNA was radiolabeled with [α-^{32}P]dCTP usig the random priming method.

For strand-specific RT-PCR analysis, cDNA was synthesized from total RNA with strand-specific primers and step-down PCR was performed as previously described [24,25]. 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 iNOS antisense-transcript, 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 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, $(72 - 0.3 \times n)$°C for 1 min where n is 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 Nos. AB250951 and AB250952, respectively.

Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared according to Schreiber *et al.* [26] with minor modifications [27]. Binding reactions 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, 1 mg of poly (dI-dC)) with the probe (approximately 40,000 cpm) for 20 min at room temperature. The products were electrophoresed at 100 V in a 4.8% polyacrylamide gel in high ionic strength buffer (50 mM Tris-HCl, 380 mM glycine, 2 mM EDTA, pH 8.5) and the dried gels were analyzed using autoradiography. An NF-κB consensus oligonucleotide (5'-AGTTGAGGGGA-CTTTCCCAGGC-3') from mouse immunoglobulin k light chain was purchased from Promega (Madison, WI, USA) and labeled with $[\gamma-^{32}P]$ATP and T4 polynucleotide kinase. The protein concentration was measured using the Bradford method [28] with a dye binding assay kit (Bio-Rad Laboratories).

Construction of luciferase reporter plasmids and expression plasmids

The 1.2-kb 5'-flanking region including the TATA box of the rat iNOS gene was inserted into the pGL3-Basic vector (Promega) to create pRiNOS-Luc-SVpA [27]. A rat cDNA for the 3'-UTR of the iNOS mRNA was amplified with the primers
5'-tgctctaGACAGTGAGGGGTTTGGAGAGA-3' and
5'-gcggatcctttaTTCTTGATCAAACACTCATTTT-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 [29,30]. 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 dexamethasone. The cell extracts' luciferase and β-galactosidase activities were measured using PicaGene (Wako Pure Chemicals) and Beta-Glo (Promega) kits, respectively.

Statistical analysis

The results shown in the figures are representative of 3-4 independent experiments yielding similar findings. Differences were analyzed using the Bonferroni-Dunn test; values of *P*<0.05 were considered to indicate statistical significance.

RESULTS

Ipe inhibits NO production and iNOS induction in hepatocytes

The proinflammatory cytokine IL-1β stimulates iNOS induction, which was followed by NO production of in primary cultured rat hepatocytes [31]. The simultaneous addition of ipe 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, upper). Ipe showed more than 90% inhibition at 2 mg/ml. Ipe 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).
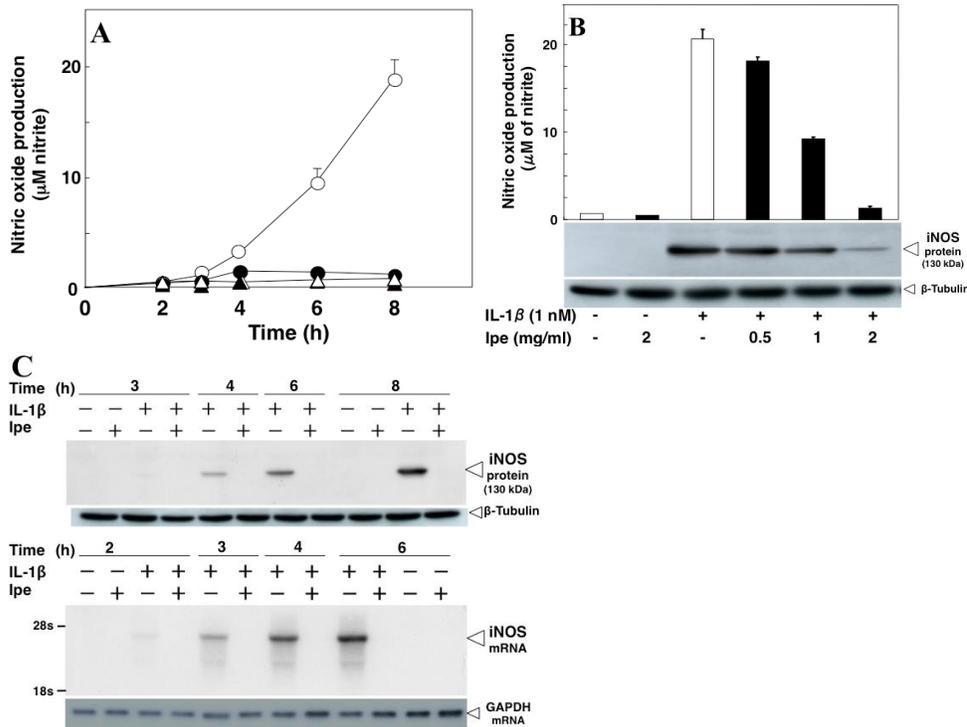

Figure 1. Effects of ipe extract on the induction of NO production and iNOS in hepatocytes. Cultured hepatocytes were treated with IL-1β (1 nM) in the presence or absence of ipe extract (ipe; 0.5-2 mg/ml). (A) Effects of ipe extract (2 mg/ml) for the indicated times on NO production (IL-1β, open circles; IL-1β+ipe, closed circles; ipe, closed triangles; controls (without IL-1β and ipe), open triangles). (B) Effects of various doses of ipe extract (0.5-2 mg/ml) over 8 h on NO production (top) and iNOS protein (middle). (C) Effects of ipe extract (2 mg/ml) for the indicated times on the expression of iNOS protein and mRNA. The nitrite levels were measured in culture medium (data are means ± SD with n=3 dishes/point; *P<0.05 vs. 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 using northern blotting and the filters were probed with labeled inserts for iNOS or GAPDH cDNA.
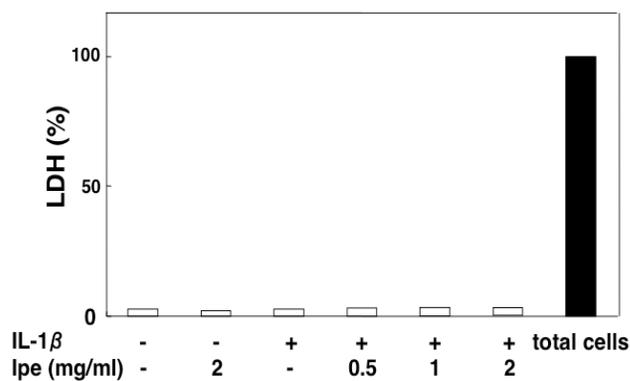

Figure 2. Effects of ipe extract on cellular cytotoxicity. Cultured hepatocytes were treated with IL-1β (1 nM) in the presence or absence of ipe extract (ipe; 0.5-2 mg/ml) for 8 h. Lactate dehydrogenase (LDH) activities were measured in culture medium (data are means ± SD with n=3 dishes/point).

Western blotting analysis revealed that ipe dose-dependently reduced iNOS protein expression, with the greatest effect at 2 mg/mL (Fig. 1B, middle). Western and northern blotting analyses demonstrated that ipe decreased the expression of iNOS protein and mRNA time-dependently (Fig. 1C). These results suggested that ipe inhibited iNOS gene expression induction at a transcriptional and/or post-transcriptional step.

Ipe 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 [32]. In the former pathway, IL-1β stimulates IκB protein degradation after the phosphorylation by IκB kinase, which is followed by NF-κB activation (that is, translocation from the cytoplasm to the nucleus and DNA binding). Ipe had no effect on IκBα degradation and recovery after IL-1β stimulation (Fig. 3, upper), whereas it tended to increase IκBα mRNA expression levels at 1-3 h (Fig. 3, lower).
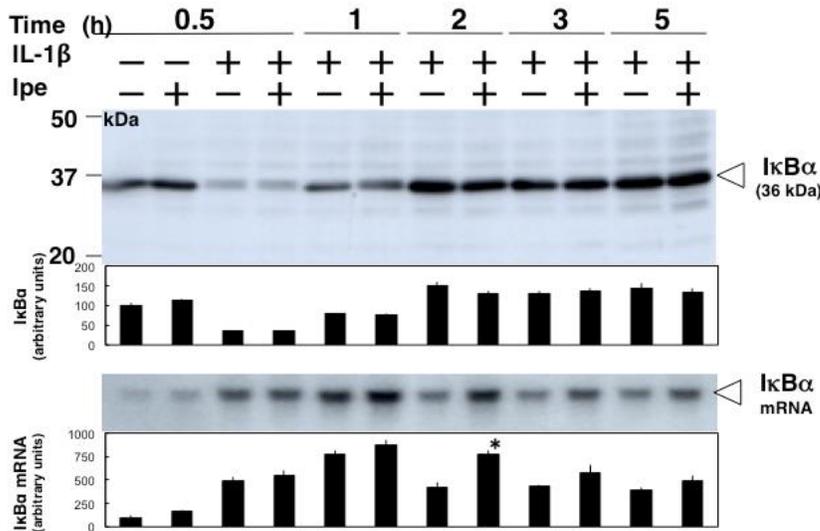

Figure 3. Effects of ipe extract on the degradation of IκBα. Cells were treated with IL-1β (1 nM) in the presence or absence of ipe extract (2 mg/ml) for the indicated times. Cell lysates (20 μg of protein) were subjected to SDS-PAGE in a 12.5% gel, followed by immunoblotting with an anti-IκBα antibody. Total RNA (10 μg) was analyzed by northern blotting and the filters were probed with labeled inserts for IκBα cDNA. Representative results of three independent experiments are shown. The bands corresponding to IκB protein or mRNA were quantitated using densitometry (mean ± SD; n= 3 experiments). *P < 0.05 versus IL-1β alone

Conversely, the electrophoretic mobility shift assay with nuclear extracts revealed that ipe inhibited NF-κB activation at 4 h and thereafter (Fig. 4A, left), although ipe had no effect at 1-3 h. In support of this observation, immunoprecipitation and western blotting experiments with nuclear extracts showed that ipe also had no effect on the nuclear translocation of NF-κB subunit p65 at 1 h, but decreased nuclear p65 levels at 5 h (Fig. 4B, upper). Ipe also reduced p65 mRNA expression levels at 2-3 h (Fig. 4B, lower). Supershift experiments revealed that ipe did not influence the components of NF-κB (subunits p50 and p65) (Fig. 4A, right) because the NF-κB bands stimulated by IL-1β disappeared similarly in the presence of antibodies against p50 and p65, irrespective of the presence of ipe.
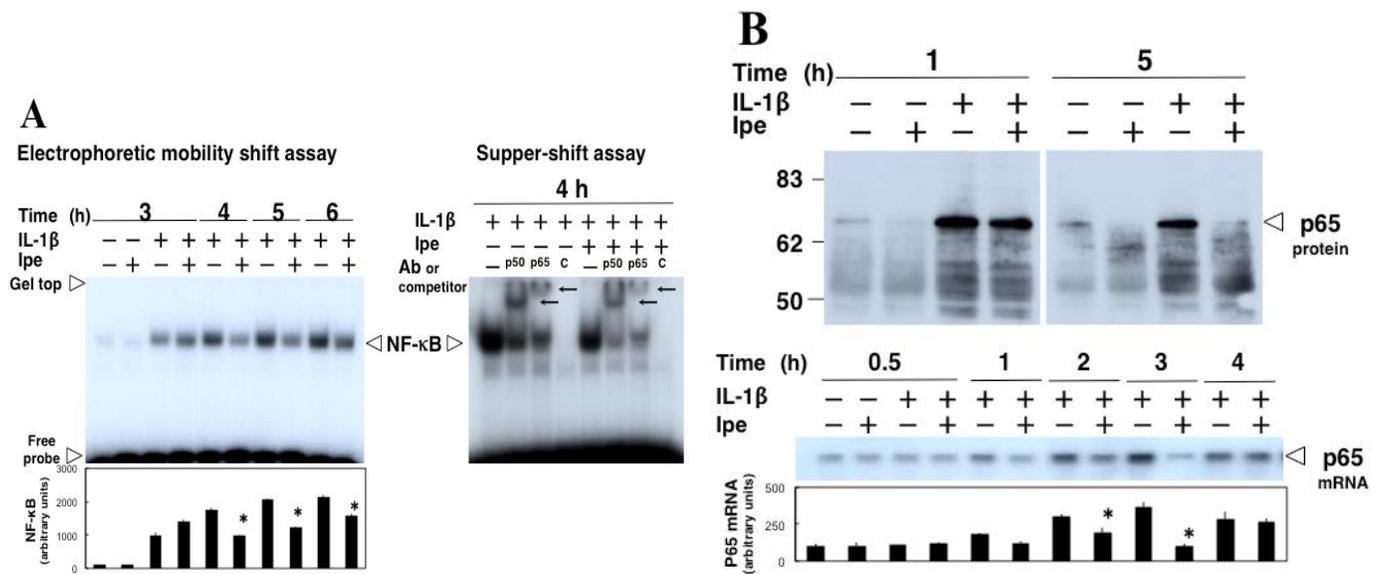
Figure. 4. Effects of ipe extract on the activation of NF-κB. Cells were treated with IL-1β (1 nM) in the presence or absence of ipe extract (ipe; 2 mg/ml) for the indicated times. (A) Activation of NF-κB; nuclear extracts (4 µg of protein) were analyzed by EMSAs (left). Supershift assay (right); nuclear 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. (B) Nuclear translocation of NF-κB subunit p65 and its mRNA expression; nuclear extracts were immunoprecipitated and the immunoprecipitates were analyzed by western blotting with an anti-p65 antibody (upper). Total RNA (10 µg) was analyzed by northern blotting and the filters were probed with labeled inserts for p65 cDNA (lower). The bands corresponding to NF-κB or p65 mRNA were quantitated using densitometry (mean ± SD; n= 3 experiments). *P < 0.05 versus IL-1β alone

In the latter pathway, IL-1β stimulates IL-1RI upregulation through PI3K/Akt activation [32]. Immunoprecipitation-western blotting analysis revealed that ipe inhibited Akt (a downstream kinase of PI3K) phosphorylation (activation) at 0.5 h (Fig. 5A). Northern and western blotting analyses revealed that ipe reduced both IL-1RI mRNA and protein expression levels (Fig. 5B and 5C).
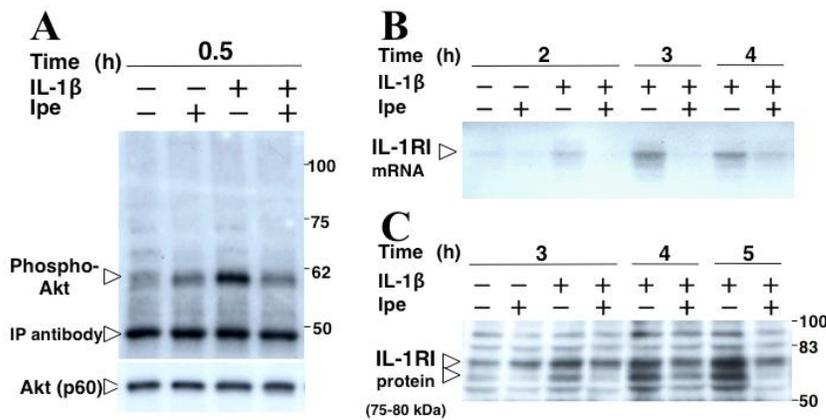

Figure 5. Effects of ipe extract on IL-1RI upregulation. Cells were treated with IL-1β (1 nM) in the presence or absence of ipe extract (ipe; 2 mg/ml) for the indicated times. (A) Phosphorylation of Akt. Total cell lysates were immunoprecipitated with an anti-Akt antibody, followed by immunoblotting with an anti-phospho-Akt or anti-Akt antibody. (B) Total RNA (10 µg) was analyzed by northern blotting and the filters were probed with labeled inserts for IL-1RI 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 antibody.

Ipe decreases iNOS mRNA synthesis and stabilization

We examined the mechanisms involved in the inhibition of iNOS induction. iNOS mRNA expression is regulated by iNOS promoter transactivation with transcription factors such as NF-κB and by post-transcriptional modifications such as mRNA stabilization [33]. Therefore, we conducted transfection experiments with constructs containing firefly luciferase controlled by the iNOS promoter (pRiNOS-Luc-SVpA and pRiNOS-Luc- 3′UTR), which detect iNOS promoter transactivation (mRNA synthesis) and mRNA stabilization, respectively [34]. IL-1β increased these constructs' luciferase activities; these effects were significantly inhibited by ipe (Fig. 6).
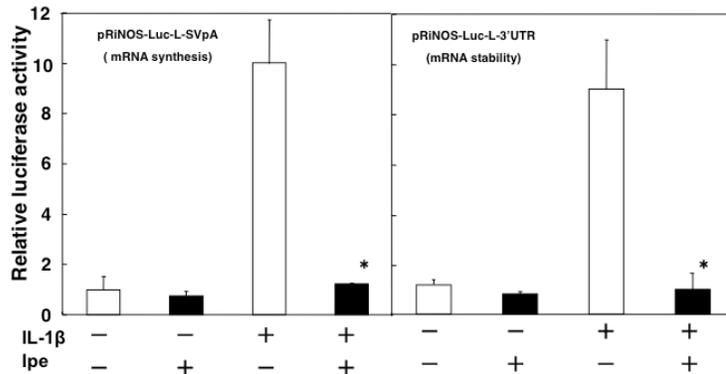

Figure 6. Effects of ipe extract on the transactivation of the iNOS promoter. The constructs consist of the rat iNOS promoter (1.2 kb), luciferase gene and SV40 poly(A) region (pRiNOS-Luc-SVpA) or iNOS 3'-UTR (pRiNOS-Luc-3'UTR). The iNOS 3'-UTR contains AU-rich elements (AUUU(U)A x 6), which contribute to mRNA stabilization. Each construct was introduced into hepatocytes and the cells were treated with IL-1β (1 nM) in the presence or absence of ipe extract (ipe; 2 mg/ml) for 8 h for pRiNOS-Luc-SVpA (left) and 4 h for pRiNOS-Luc-3'UTR (right). 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 ipe). Data are means ± SD (n = 4 dishes). *P<0.05 vs. IL-1β alone.

We have recently reported that the natural antisense-transcript of the iNOS gene upregulates iNOS mRNA stability in IL-1β-stimulated hepatocytes [35]. RT-PCR and quantitative real-time PCR experiments revealed that IL-1β time-dependently increased the iNOS gene antisense-transcript expression with increased iNOS mRNA levels and that ipe decreased both antisense-transcript and iNOS mRNA levels (Fig. 7).
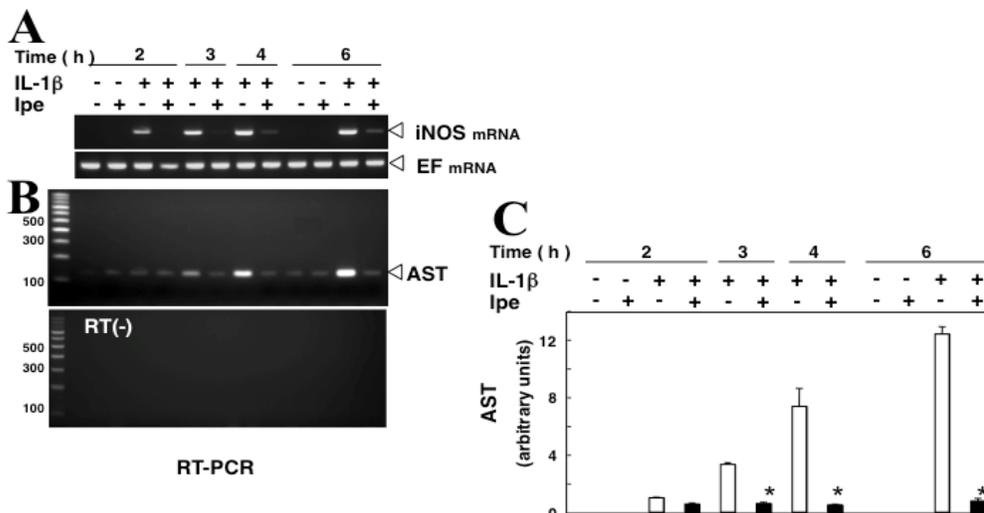

Figure 7. Effects of ipe extract on the expression of the iNOS gene antisense-transcript in hepatocytes. Cells were treated with IL-1β (1 nM) in the presence or absence of ipe extract (ipe; 2 mg/ml) for the

indicated times. Total RNA was analyzed by strand-specific RT-PCR to detect (A) iNOS with EF-1α (EF) as an internal control and (B) the iNOS gene antisense-transcript (AST). (C) Quantitative RT-PCR was conducted for the iNOS gene AST and the copy number of the iNOS gene AST was normalized by that of a negative PCR control using total RNA without RT (RT(-)). Data are means ± SD (n = 3 experiments). *P<0.05 vs. IL-1β alone.

DISCUSSION

In this study, we found that ipe inhibited iNOS induction in IL-1β stimulated hepatocytes, which indicates that ipe may have an anti-inflammatory effect in the liver. 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 various 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.

    iNOS gene expression induction is regulated by iNOS promoter transactivation and by post-transcriptional modifications [36]. NF-κB plays a key role in inflammation by regulating genes encoding iNOS and proinflammatory cytokines such as TNF-α [37]. 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. NF-κB activation involves i) the proteolytic degradation of IκBs in proteosome after phosphorylation by IκB kinase, ii) the translocation of NF-κB to the nucleus and iii) its binding to the promoter κB site [38]. Ipe inhibited NF-κB activation (Fig. 4A) and p65 translocation to the nucleus (Fig. 4B), although it had no effect on IκB αdegradation (Fig. 3A). In concert with NF-κB activation, IL-1RI upregulation through PI3K/Akt activation is also essential for iNOS induction [32]. Ipe inhibited Akt activation (phosphorylation) (Fig. 5A), up-stream kinase in this signal, which resulted in decreased IL-1RI mRNA and protein expression (Fig. 5B and 5C).

    Furthermore, in experiments with iNOS promoter constructs, ipe inhibited iNOS induction at both of its mRNA synthesis and stabilization steps (Fig. 6). 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 [39]. Recently, we reported that antisense (as)RNAs are often transcribed from many inducible genes, such as iNOS and TNF-α [40]. The iNOS asRNA interacts with and stabilizes iNOS mRNA [35,41]. We reported that drugs such as edaravone (free radical scavenger) [42], FR183998 (Na^+/H^+ exchanger inhibitor) [9,11], insulin growth factor I [10], sivelestat [43], kampo inchinkoto (TJ-135) [44], kampo saireito (TJ-114) [45] and kampo hochuekito (TJ-41) [46] inhibited iNOS induction partly by suppressing iNOS asRNA production in animal models and/or primary cultured hepatocytes. In this study, ipe also decreased the iNOS asRNA expression (Fig. 7).

    These results demonstrate that ipe inhibits two essential signaling pathways, NF-κB activation and IL-1RI upregulation in the induction of iNOS. Ipe probably reduced iNOS mRNA expression by inhibiting its mRNA synthesis and stabilization, which decreased iNOS protein and NO production. In the clinical study, we would like to examine the Ipe treatment in patients with sepsis in future. Lapachol, one of Ipe ingredients, will be examined to our *in vitro* (IL-1β treated culture hepatocytes) and *in vivo* (animal models of liver injury) studies.

CONCLUSION

Ipe can prevent IL-1β-stimulated liver injury in cultured hepatocytes by inhibiting the induction of inflammatory mediator iNOS gene expression, in part by inhibiting NF-kB activation. Ipe may have therapeutic potential for liver injury.

Abbreviations: iNOS, inducible nitric oxide synthase; NO, nitric oxide; IL-1β, interleukin-1β; NF-κB, nuclear factor-kappa B; IL-1RI, the type I interleukin-1 receptor; TNF-α, tumor necrosis factor-alpha; CINC-1, cytokine-induced neutrophil chemoattractant-1; WE, Williams' Medium E

Competing Interest: The authors declare that they have no conflicts of interest.

Authors' Contributions: T. Ozaki participated in data collection, statistical analysis and manuscript drafting. Y. Kawaguchi and M. Nishizawa assisted in the design of the study and in supervision. K. Matsui, M. Kotsuka and H. Iida participated in data collection. T. Okumura gave significant advice regarding protocol development and participated in manuscript drafting. M. Kaibori and M. Sekimoto assisted in the design of the study and in financial support.

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