Biochemical and Biophysical Research Communications 439 (2013) 54–59

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Citrus nobiletin suppresses inducible nitric oxide synthase gene expression in interleukin-1β-treated hepatocytes

Emi Yoshigai [a,b,1], Toru Machida [a,1], Tetsuya Okuyama [b], Masatoshi Mori [a], Hiromitsu Murase [a], Ryota Yamanishi [a], Tadayoshi Okumura [c,d], Yukinobu Ikeya [e], Hoyoku Nishino [b,f], Mikio Nishizawa [a,*]

[a] Department of Biomedical Sciences, College of Life Sciences, Kusatsu, Shiga, Japan
[b] Ritsumeikan Global Innovation Research Organization (R-GIRO), Kusatsu, Shiga, Japan
[c] Research Organization of Science and Technology, Ritsumeikan University, Kusatsu, Shiga, Japan
[d] Department of Surgery, Kansai Medical University, Hirakata, Osaka, Japan
[e] Department of Pharmacy, College of Pharmaceutical Sciences, Ritsumeikan University, Kusatsu, Shiga, Japan
[f] Department of Biochemistry, Kyoto Prefectural University of Medicine, Kyoto, Japan

ARTICLE INFO

Article history:
Received 31 July 2013
Available online 16 August 2013

Keywords:
Chinpi
Flavonoid
Nitric oxide
Inflammation
Flavone
Plant polyphenol

ABSTRACT

Background: Nobiletin is a polymethoxylated flavone that is abundant in the peels of citrus fruits, such as *Citrus unshiu* (Satsuma mandarin) and *Citrus sinensis*. The dried peels of *C. unshiu* (*chinpi*) have been included in several formulae of Japanese Kampo medicines. Nobiletin may suppress the induction of inducible nitric oxide synthase (iNOS), which synthesizes the inflammatory mediator nitric oxide (NO) in hepatocytes.

Methods: A *C. unshiu* peel (CUP) extract was prepared. Primary cultured rat hepatocytes were treated with the CUP extract or nobiletin in the presence of interleukin 1β (IL-1β), which induces iNOS expression. NO production and *iNOS* gene expression were analyzed.

Results: High-performance liquid chromatography analyses revealed that the nobiletin content in the CUP extract was 0.14%. Nobiletin dose-dependently reduced the NO levels and decreased iNOS expression at the protein, mRNA and antisense transcript levels. Flavone, which does not contain any methoxy groups, also suppressed iNOS induction. Nobiletin reduced the transcriptional activity of iNOS promoter-luciferase constructs and the DNA-binding activity of nuclear factor κB (NF-κB) in the nuclei.

Conclusions: The suppression of iNOS induction by nobiletin suggests that nobiletin may be responsible for the anti-inflammatory effects of citrus peels and have a therapeutic potential for liver diseases.

1. Introduction

Many plants contain flavonoids, which are often biologically active [1]. For example, an extract of the milk thistle (*Silybum marianum*) is a mixture of flavones and thought to be beneficial for hepatoprotection [2]. Citrus peels have been used as spices in hot wine in Europe, and dried peels of the *Citrus unshiu* Markovich (Satsuma mandarin), known as *chinpi*, have been used in several formulae of Japanese herbal (Kampo) medicines, such as *rikkunshito* and *kososan*. The peels of *C. unshiu* contain a variety of flavonoids: flavanone glycosides (*e.g.*, hesperidin and narirutin), flavone glycosides (*e.g.*, diosmin), flavonol glycosides (*e.g.*, rutin) and polymethoxylated flavones, which are exclusively present in citrus fruits [3]. Nobiletin (5,6,7,8,3′,4′-hexamethoxyflavone), a polymethoxylated flavone, is abundantly present in the peels of *C. unshiu*, *C. sinensis* (orange) and *Citrus depressa* (shiikuwasa) [3,4]. When nobiletin is administered to mice or rats, it exerts antitumor and anti-inflammatory effects [5,6]. However, whether nobiletin exhibits anti-inflammatory activity in hepatocytes remains unknown.

The inflammatory mediator nitric oxide (NO) plays a pivotal role in various diseases [7], and NO is synthesized by inducible nitric oxide synthase (iNOS). The *iNOS* gene is induced by the proinflammatory cytokine interleukin 1β (IL-1β) in primary cultured rat hepatocytes, and this induction mimics liver injury, such as hepatitis [8,9]. In contrast, macrophages treated by lipopolysaccharide (LPS) have a different mechanism of iNOS induction. We demonstrated that NO is a sensitive marker that can be used to monitor inflammatory responses to Japanese herbal medicines, such as rhubarb (Rhei rhizoma) [9] and *kinginka* (Flos Lonicerae japonicae) [10]. These hepatoprotective herbal medicines suppress the IL-1β mediated induction of NO and iNOS protein in the hepatocytes.

* Corresponding author. Address: Department of Biomedical Sciences, College of Life Sciences, Ritsumeikan University, 1-1-1 Nojihigashi, Kusatsu, Shiga 525-8577, Japan. Fax: +81 77 561 2876.
E-mail address: nishizaw@sk.ritsumei.ac.jp (M. Nishizawa).
[1] These authors contributed equally to this work.

0006-291X/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbrc.2013.08.029

Two transcription factors are involved in IL-1β-mediated iNOS induction: nuclear factor κB (NF-κB) and CCAAT/enhancer-binding protein β (C/EBPβ) [8]. The *iNOS* gene promoter contains NF-κB and C/EBPβ binding sites, and NF-κB and C/EBPβ synergistically activate *iNOS* transcription through their binding sites and are involved in *iNOS* gene expression [8]. NF-κB plays a key role in inflammation by regulating genes encoding iNOS, inflammatory cytokines and chemokines [11]. iNOS induction is also regulated via a post-transcriptional mechanism [12,13]. The antisense transcripts (asRNAs) are transcribed from human, rat and mouse *iNOS* genes and interact with the 3′-untranslated region of iNOS mRNA to stabilize iNOS mRNA [13–15]. Recently, herbal constituents (*e.g.*, chlorogenic acid and gomisin N) and Kampo formulae (*e.g.*, *inchinkoto*) have been reported to decrease the levels of iNOS asRNA, leading to the inhibition of iNOS expression [9,10,16].

In the present study, we analyzed the *C. unshiu* peel (CUP) extract and examined whether the CUP extract suppressed iNOS expression in IL-1β-treated hepatocytes. Next, we examined whether nobiletin suppressed iNOS expression and NO induction. We clarified whether the flavone skeleton of nobiletin was responsible for the suppression of iNOS expression by comparing the effects of nobiletin with the effects of flavone, which is not a naturally occurring flavonoid [17]. Finally, the contribution of nobiletin to the anti-inflammatory activity of the CUP extract was examined.

2. Materials and methods

2.1. Materials

Nobiletin and flavone were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan) and had purities greater than 95% and 98%, respectively. Dimethyl sulfoxide (DMSO) was purchased from Wako Pure Chemical Industries Ltd. and was used to dissolve these compounds and the CUP extract.

2.2. Plant materials and extraction

Peels of *C. unshiu* Markovich, which were collected in Huwei Province, China, and identified and authenticated by Dr. Yutaka Yamamoto (Tochimoto Tenkaido Co. Ltd., Osaka, Japan), were purchased from Tochimoto Tenkaido Co. Ltd. A voucher specimen was deposited in the Ritsumeikan Herbarium of Pharmacognosy, Ritsumeikan University, under code number RIN-CU-016. The dried peels of *C. unshiu* (100.9 g) were pulverized and extracted twice using absolute methanol under reflux. The solvent was evaporated under reduced pressure to yield the methanol extract (38.60 g).

2.3. Identification and analysis of nobiletin using high-performance liquid chromatography (HPLC)

Quantitative analyses of nobiletin in the *C. unshiu* methanol extract were performed on a Shimadzu LC-20A system with a UV–VIS detector (Shimadzu Corporation, Kyoto, Japan). Samples were separated using a Cosmosil $5C_{18}$-AR-II column (Nacalai Tesque Inc., Kyoto, Japan) with a mobile phase of absolute acetonitrile: 0.5% (v/v) phosphoric acid–water (40:60 to 50:50 over a 40 min period) at 30 °C. The detection wavelength was set at 254 nm.

2.4. Preparation of primary cultured rat hepatocytes

Male Wistar rats were purchased from Charles River Laboratories Japan Inc. (Yokohama, Japan), housed at 21–23 °C and acclimatized. Hepatocytes were isolated from the rat livers by collagenase perfusion [18]. Isolated cells were resuspended in Williams' E medium (Sigma–Aldrich Corp., St. Louis, MO, USA), seeded at 1.2×10^6 cells per dish, incubated at 37 °C for 2 h, and the medium was replaced. The hepatocytes were incubated at 37 °C overnight and analyzed on the next day. All animal care and experimental procedures were carried out in accordance with the guidelines and laws of the Japanese government and were approved by the Animal Care Committee of Ritsumeikan University, Biwako-Kusatsu Campus.

2.5. Determination of NO levels and LDH activity

Hepatocytes were treated with the CUP extract, nobiletin or flavone in the presence of 1 nM rat IL-1β (PeproTech, Rocky Hill, NJ, USA) for 8 h. Nitrite (a stable metabolite of NO) levels in the medium were measured using the Griess method [19], and the half-maximal inhibitory concentrations (IC_{50}) were determined [10]. To monitor cytotoxicity, the LDH activities in the medium were measured using LDH Cytotoxicity Detection Kits (Takara Bio Inc., Otsu, Shiga, Japan).

2.6. Western blot analyses

Hepatocytes were treated with 1 nM IL-1β and nobiletin or flavone for 8 h, and whole-cell lysates were prepared [16]. Briefly, hepatocytes were lysed, subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and immunoblotted onto a Sequi-Blot membrane (Bio-Rad, Hercules, CA, USA). Immunostaining was performed using primary antibodies raised against rat iNOS (Thermo Fisher Scientific, Waltham, MA, USA) and rat β-tubulin (Cell Signaling Technology Inc., Danvers, MA, USA), followed by visualization with an Enhanced Chemiluminescence Blotting Detection Reagent (GE Healthcare Biosciences Corp., Piscataway, NJ, USA).

2.7. Reverse transcription-polymerase chain reactions (RT-PCR)

Hepatocytes were treated with 1 nM IL-1β and/or nobiletin or flavone for 4 h, and total RNA was prepared from the hepatocytes [14]. The cDNA was reverse-transcribed in a strand-specific manner with an oligo(dT) primer for mRNAs and a sense primer for iNOS asRNA, and PCR was performed with paired primers [14]. Primer sequences CCAACCTGCAGGTCTTCGATG and GTCGATGCACAACTGGGTGAAC (5′ → 3′) were used to detect the iNOS mRNA by cDNA amplification. The primer sequences used for the iNOS asRNA detection were TGCCCCTCCCCCACATTCTCT (RT), ACCAGGAGGCGCCATCCCGCTGC and CAAGGAATTATACACGGAAGGGCC (PCR). PCR primers for the elongation factor 1α (EF) mRNA, TCTGGTTGGAATGGTGACAACATGC and CCAGGAAGAGCTTCACTCAAAGCTT, were used as internal controls. mRNA levels were estimated in triplicate by real-time PCR with the Thermal Cycler Dice Real Time System (Takara Bio Inc.) [14]. The values obtained were normalized to EF mRNA.

2.8. Firefly luciferase assays

Hepatocytes (3.0×10^5 cells per dish) were transfected with plasmid DNA using MATra-A Reagent (IBA GmbH, Göttingen, Germany) [14]. Two plasmids were used: pRiNOS-Luc-SVpA, a luciferase reporter plasmid, and pCMV-LacZ, an internal control plasmid expressing β-galactosidase under the cytomegalovirus enhancer/promoter [20]. The cells were treated with 1 nM IL-1β for 8 h. The luciferase and β-galactosidase activities were measured using the PicaGene (Wako Pure Chemical Industries Ltd.) and Beta-Glo kits (Promega Corporation, Madison, WI, USA), respectively.

2.9. Electrophoretic mobility shift assays (EMSA)

EMSAs were performed as previously described [8,16]. Briefly, hepatocyte nuclear extracts (4.0 μg) were mixed with 1.0 μg of poly(dI-dC). To prepare a double-stranded DNA probe, annealed oligonucleotides harboring an NF-κB-binding site (5′-AGTTGAGGGGACTTTCCCAGGC-3′; only the sense strand is shown) were labeled with [γ-^{32}P]ATP (PerkinElmer Inc., Waltham, MA, USA). The probe was added to the nuclear extracts, incubated for 20 min and resolved on a polyacrylamide gel. The gel was dried and subjected to autoradiography.

2.10. Statistical analyses

The results presented in the figures are representative of at least three independent experiments that yielded similar results. The values are presented as the means ± standard deviation (SD). Differences were analyzed using Student's *t*-test. Statistical significance was set at $P < 0.05$ and $P < 0.01$.

3. Results

3.1. The content of nobiletin in the methanol extract of C. unshiu peels

We extracted the dried peels of *C. unshiu* using absolute methanol, and this methanol extract (CUP extract) was used in the subsequent experiments. The CUP extract was analyzed using HPLC and compared with the chromatogram of pure nobiletin (standard), which had a retention time of 13.2 min (Fig. 1A–C). A prominent peak at the same retention time was identified in the chromatogram of the CUP extract, suggesting that nobiletin is one of the major constituents of the CUP extract. The quantitative analyses using HPLC indicated that the nobiletin content in the CUP extract was 0.14% by weight.

3.2. The CUP extract suppresses the induction of NO production in hepatocytes

Next, we investigated the effects of the CUP extract on NO induction in IL-1β-treated rat hepatocytes. The CUP extract was dissolved in DMSO and added to the medium with IL-1β. The final concentrations of DMSO were adjusted to less than 1.0% (v/v) in the medium, because DMSO concentrations greater than 2.0% (v/v) significantly reduced NO and iNOS protein expression (data not shown).

The CUP extract significantly decreased the levels of NO production in a dose-dependent fashion (Fig. 1D). Because the LDH activity in the medium was very low (data not shown), the CUP extract appeared to not be toxic to the hepatocytes at the concentration indicated in the figure. These data imply that the CUP extract effectively suppressed NO induction in hepatocytes.

3.3. Nobiletin suppresses NO and iNOS induction in hepatocytes

To examine whether nobiletin, one of the major constituents in the CUP extract, affects *iNOS* gene expression, we added nobiletin to the medium of rat hepatocytes. Nobiletin suppressed NO induction in the presence of IL-1β in a dose-dependent manner (Fig. 2A). Nobiletin effectively suppressed NO production with an IC_{50} value of 51 μM. When evaluating LDH release into the medium, nobiletin displayed no cytotoxicity at the indicated concentrations (data not shown).

Western blot analyses indicated that nobiletin decreased iNOS protein expression in hepatocytes, showing a maximal effect at 100 μM (Fig. 2A). Furthermore, quantitative RT-PCR analyses revealed that nobiletin reduced iNOS mRNA levels in a time-dependent manner (Fig. 2B), suggesting that nobiletin significantly suppressed iNOS induction at the mRNAlevel. In addition, nobiletin decreased iNOS expression at the mRNA and protein levels, as well as NO production. Taken together, these results imply that

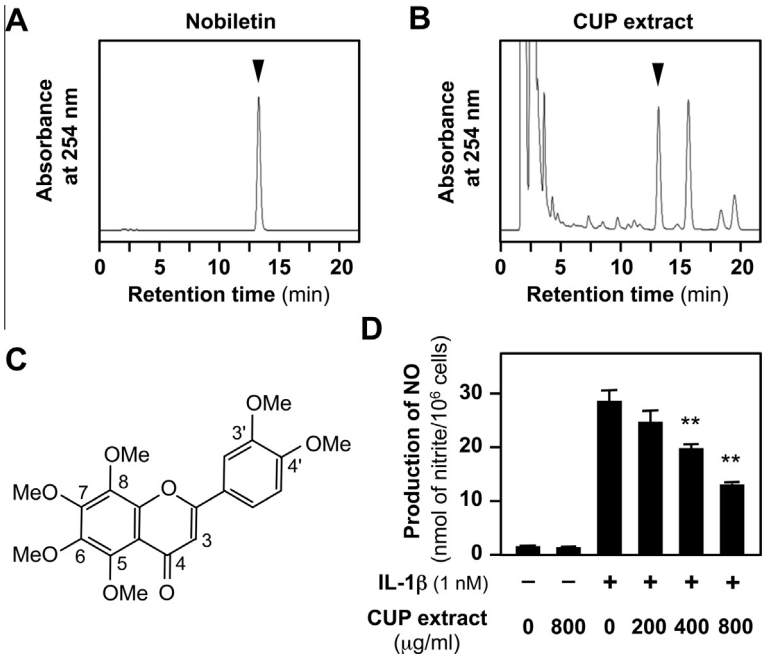

Fig. 1. Nobiletin is present in the methanol extract of *C. unshiu* peels. (A) HPLC chromatogram of nobiletin. Commercially available pure nobiletin was separated using HPLC and was used as a standard. (B) HPLC chromatogram of the CUP extract. The CUP extract was separated using HPLC under the same conditions as in (C). A single peak (arrowhead) corresponding to nobiletin was used to estimate its content in the CUP extract. (C) The chemical structure of nobiletin. (D) The CUP extract suppresses the induction of NO production. Hepatocytes were treated with 1 nM IL-1β in the presence of the CUP extract for 8 h. The NO levels in the medium were measured in triplicate (means ± SD). $^{**}P < 0.01$ versus IL-1β alone.

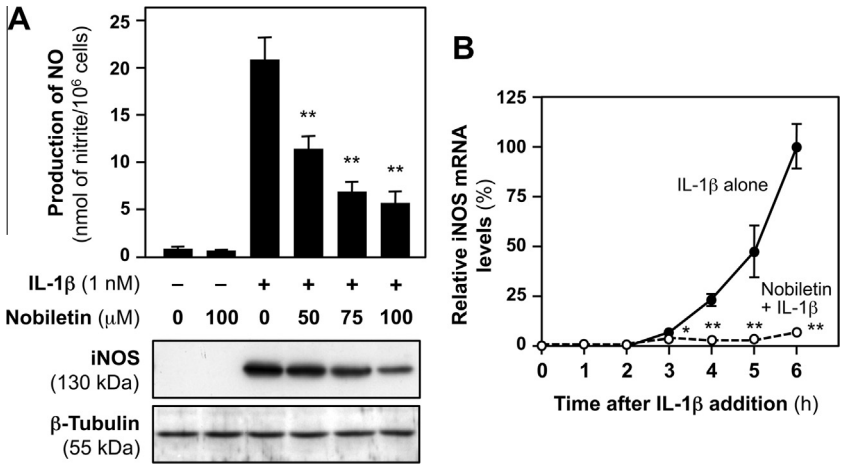

Fig. 2. The effects of nobiletin on the induction of *iNOS* gene expression in hepatocytes. (A) Nobiletin suppresses the induction of NO production and iNOS protein expression. Hepatocytes were treated with IL-1β and/or nobiletin for 8 h. The NO levels in the medium were measured in triplicate (means ± SD), and cell extracts were immunoblotted with an anti-iNOS or anti-β-tubulin antibody (internal control). $^{**}P < 0.01$ versus IL-1β alone. (B) Nobiletin decreases iNOS mRNA expression. Hepatocytes were treated with 1 nM IL-1β and/or 100 μM nobiletin, and total RNA from the cells was analyzed using quantitative RT-PCR to estimate the levels of iNOS mRNA and EF mRNA (internal control). The values obtained were normalized to EF mRNA, and the value at 6 h in the presence of IL-1b alone was set at 100%. $^{*}P < 0.05$ and $^{**}P < 0.01$ versus IL-1β alone.

nobiletin inhibited the induction of *iNOS* gene expression at the transcriptional level.

3.4. Nobiletin decreases iNOS promoter activity

iNOS gene expression is transcriptionally regulated by transcription factors, including NF-κB, and is post-transcriptionally regulated by iNOS asRNA, which interacts with iNOS mRNA [14]. To investigate how nobiletin suppressed *iNOS* gene expression, we performed reporter assays using an iNOS promoter–firefly luciferase construct, pRiNOS-Luc-SVpA (Fig. 3A). Because luciferase transcription is driven by the *iNOS* gene promoter, luciferase activity represents the promoter activity corresponding to iNOS mRNA synthesis [20]. IL-1β increased luciferase activity, whereas nobiletin significantly reduced luciferase activity in the presence of IL-1β, demonstrating that nobiletin reduced iNOS promoter activity.

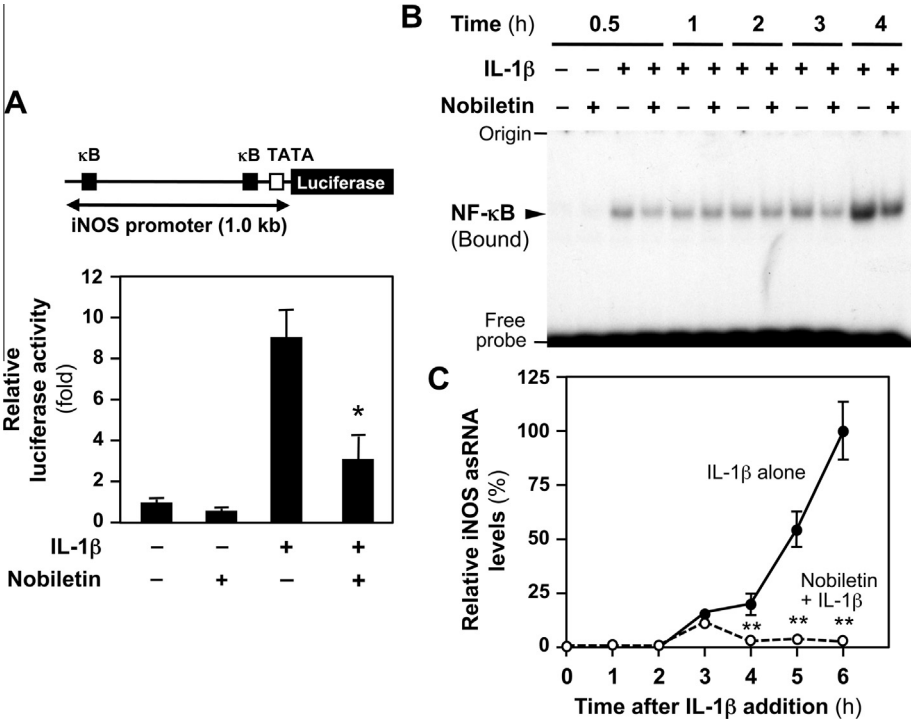

Fig. 3. The effects of nobiletin on *iNOS* gene transcription and iNOS asRNA expression. (A) Nobiletin decreases the iNOS promoter activity. Hepatocytes were transfected with an iNOS promoter-luciferase construct (pRiNOS-Luc-SVpA; top) and pCMV-LacZ (internal control) and subsequently treated with 1 nM IL-1β and/or 100 μM nobiletin. κB: NF-κB-binding site; TATA: TATA box. The luciferase activity in the transfected cells was normalized to the β-galactosidase activity, and the fold activation was calculated by dividing the normalized luciferase activity by the luciferase activity in the presence of IL-1β alone. The data represent the means ± SD ($n = 3$). $^{*}P < 0.05$ versus IL-1β alone. (B) Nobiletin decreases the DNA-binding activity of NF-κB. Hepatocytes were treated with IL-1β and/or nobiletin for the indicated times. Nuclear extracts were prepared from the cells and analyzed using EMSA to detect NF-κB that was bound to a DNA probe harboring an NF-κB-binding site. (C) Nobiletin decreases the iNOS asRNA expression. Hepatocytes were treated with IL-1β and/or nobiletin. Total RNA was analyzed using quantitative RT-PCR to detect iNOS asRNA. A negative control PCR using total RNA without RT did not give amplification (data not shown). The value at 6 h in the presence of IL-1β was set at 100%. $^{**}P < 0.01$ versus IL-1β alone.

To confirm the nobiletin-mediated transcriptional suppression, we estimated the DNA-binding activity of NF-κB, which is highly involved in iNOS induction. Nuclear extracts of the hepatocytes were analyzed using EMSA with a probe harboring an NF-κB-binding site (Fig. 3B). The results showed decreased band signals at 3–4 h, suggesting that nobiletin decreased the DNA-binding activity of NF-κB. These data support the nobiletin-mediated transcriptional suppression of iNOS expression.

3.5. Nobiletin decreases iNOS asRNA levels

The possibility that nobiletin post-transcriptionally suppresses iNOS expression was investigated. We measured the expression levels of iNOS asRNA, which interacts with and stabilizes iNOS mRNA [13,14]. Quantitative RT-PCR revealed that nobiletin significantly reduced iNOS asRNA levels at 4–6 h (Fig. 3C). Because decreases in iNOS asRNA lead to decreased iNOS mRNA stability, these results imply that nobiletin may post-transcriptionally regulate iNOS mRNA levels by reducing iNOS asRNA stability.

3.6. Flavone suppresses NO and iNOS protein induction

Nobiletin is a flavone that naturally occurs in citrus fruits and possesses six methoxy groups (Fig. 1C). Although a variety of hydroxylated flavones are synthesized from cinnamoyl-CoA and malonyl-CoA in many plants [17], flavone does not possess any methoxy and hydroxyl groups and is not biosynthesized by most plants. We examined flavone to clarify the contribution of the flavone skeleton to the nobiletin-mediated suppression of iNOS expression.

The addition of flavone to the medium significantly decreased NO production in IL-1β-treated hepatocytes (Fig. 4A). The IC_{50} value of flavone was 2.1 μM, which was much lower than that of nobiletin. When LDH activity was evaluated in the medium, flavone displayed no obvious cytotoxicity (data not shown). Flavone decreased both iNOS protein and mRNA levels in a similar manner to nobiletin (Fig. 4A and B). These results imply that the mechanism underlying the suppression of iNOS expression by flavone may be closely related to that of nobiletin.

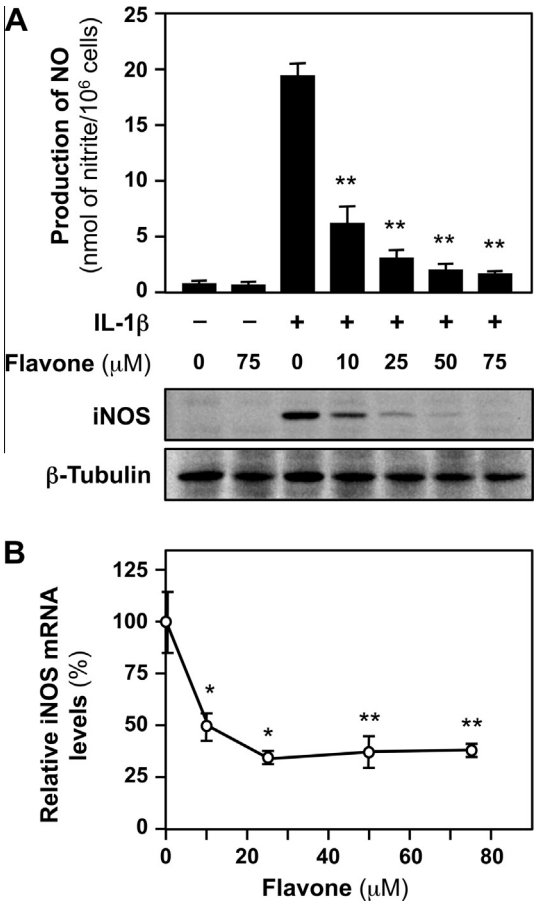

Fig. 4. Effects of flavone on iNOS induction. (A) Flavone decreases NO production and iNOS protein expression. Hepatocytes were treated with IL-1β and/or flavone. The NO levels in the medium were measured in triplicate (means ± SD), and the cell extracts were resolved using SDS–PAGE and immunoblotted with anti-iNOS or anti-β-tubulin antibodies. $^{**}P < 0.01$ versus IL-1β alone. (B) Flavone decreases iNOS mRNA expression. Hepatocytes were treated with IL-1β and flavone for 4 h, and total RNA was analyzed using quantitative RT-PCR to estimate the iNOS mRNA levels. The values obtained were normalized to EF mRNA, and the value at 0 nM was set at 100%. $^{*}P < 0.05$ and $^{**}P < 0.01$ versus 0 nM.

4. Discussion

The results of the present study clearly demonstrate that the methanol extract of CUP and nobiletin suppressed the induction of NO production and iNOS expression in IL-1β-treated hepatocytes (Figs. 1D and 2). The inflammatory mediator NO is produced in response to IL-1β, and the primary cultured hepatocyte system that was used in our study mimics liver injury [7–9]. Therefore, the suppression of iNOS induction suggests that the CUP extract and nobiletin have anti-inflammatory effects. Because our HPLC analyses demonstrated that nobiletin is one of the major constituents of the CUP extract (Fig. 1A and B), nobiletin was determined to be at least partially responsible for the anti-inflammatory effects of the CUP extract.

Nobiletin suppressed NO production, with an IC_{50} value of 51 μM, in rat hepatocytes (Fig. 2A). In another NO assay system using an LPS-treated mouse macrophage cell line, RAW264.7, nobiletin displayed IC_{50} values of 27 μM [4] and 80 μM [5], which were comparable to the values measured in rat hepatocytes. In contrast, rutin, which is a flavonol glycoside that is abundant in *C. unshiu*, displayed a much higher IC_{50} value (>300 μM) in the hepatocytes (data not shown). Other major constituents in *C. unshiu* peels, such as hesperidin (flavanone glycoside) and diosmin (flavone glycoside), did not cause significant decreases in NO production in RAW264.7 cells [4,21]. These data suggest that nobiletin is responsible for the anti-inflammatory effects of the CUP extract and the dried peels of *C. unshiu*.

The IC_{50} value of nobiletin was compared with those of other herbal constituents, which were determined using the hepatocyte system. The IC_{50} value of nobiletin was much lower than that of chlorogenic acid (652 μM), the main constituent of *kinginka*, and that of caffeic acid (226 μM), which is a phenylpropanoid metabolized from chlorogenic acid [10]. The IC_{50} value of nobiletin was lower than that of gomisin N (72 μM), which is a dibenzocyclooctadiene lignan of *gomishi* [16]. These data suggest that nobiletin is more effective at suppressing NO production than these herbal constituents. Therefore, nobiletin has potential values to treat inflammatory diseases, such as viral hepatitis, alcoholic liver diseases and non-alcoholic steatohepatitis (NASH).

Flavone possesses a flavone skeleton and is not a naturally occurring flavonoid in plants [17]. In IL-1β-treated hepatocytes, flavone was revealed to have a very low IC_{50} value of 2.1 μM. Flavone treatment also decreased iNOS protein levels in a similar manner to nobiletin (Fig. 4A). These data imply that flavone suppresses iNOS expression via a mechanism that is similar to that of nobiletin and that the flavone skeleton is essential for the suppression of NO and iNOS.

Our results suggest that nobiletin regulates iNOS expression at both the transcriptional and post-transcriptional levels. NF-κB is a

key regulator of iNOS induction [8]. The results of the luciferase assay demonstrated that nobiletin decreased the promoter activity of the *iNOS* gene (Fig. 3A), and the EMSA that was conducted using nuclear extracts indicated that nobiletin reduced the DNA-binding activity of NF-κB (Fig. 3B). Similarly, the Japanese Kampo formula *inchinkoto* decreased the iNOS promoter activity by affecting the nuclear translocation of NF-κB and preventing NF-κB from binding to the *iNOS* gene promoter [9]. Therefore, nobiletin may inhibit these steps of NF-κB activation.

Nobiletin may also inhibit iNOS mRNA–asRNA interaction, thereby suppressing iNOS expression [12]. Recently, it was reported that asRNAs are often transcribed from many inducible genes, such as *iNOS* and tumor necrosis factor α [12,22]. The iNOS asRNA interacts with and stabilizes iNOS mRNA [13,14]. Nobiletin-mediated decreases in iNOS mRNA levels were correlated with iNOS asRNAlevels (Figs. 2B and 3C). Because similar correlations were observed with other herbal constituents, such as chlorogenic acid and gomisin N [10,16], nobiletin may reduce the expression of iNOS asRNA and suppress iNOS induction. Furthermore, hydroxylated flavones, such as apigenin, have been reported to bind directly to RNA with high affinity [23]. Therefore, we believe that nobiletin may interfere with the iNOS mRNA–asRNA interaction by binding to these RNAs.

In rat astrocytes, nobiletin also suppresses iNOS expression [24]. Additionally, nobiletin decreases prostaglandin E_2 production in RAW264.7 cells and exhibits anti-tumor-promoting activity in mouse skin [5]. Because inflammation has been associated with neurodegeneration and cancer, anti-inflammatory effects of nobiletin may enhance neuroprotection and prevent tumorigenesis, including hepatocellular carcinoma. Similarly to herbal medicines for prostate cancer [25], future studies are necessary to examine whether nobiletin is used as a complementary and alternative medicine for an anti-inflammatory therapy for liver diseases.

Conflicts of interest

M. Nishizawa and T. Okumura received research grants from the Amino Up Chemical Co., Ltd. (Sapporo, Japan). E. Yoshigai, T. Okuyama, M. Nishizawa and T. Okumura received research grants from the R-GIRO. T. Machida, M. Mori, H. Murase and R. Yamanishi performed this study as graduate students of the Graduate School of Science and Engineering, Ritsumeikan University.

Acknowledgments

We wish to thank Ms. Noriko Kanazawa for her secretarial assistance and Miss Mana Kano for her technical assistance. This work was supported in part by research grants from the Amino Up Chemical Co., Ltd. and the R-GIRO.

References

[1] O. Benavente-García, J. Castillo, Update on uses and properties of citrus flavonoids: new findings in anticancer, cardiovascular, and anti-inflammatory activity, J. Agric. Food Chem. 56 (2008) 6185–6205.
[2] J. Féher, G. Lengyel, Silymarin in the prevention and treatment of liver diseases and primary liver cancer, Curr. Pharm. Biotechnol. 13 (2012) 210–217.
[3] Y. Nogata, K. Sakamoto, H. Shiratsuchi, et al., Flavonoid composition of fruit tissues of citrus species, Biosci. Biotechnol. Biochem. 70 (2006) 178–192.
[4] S.Y. Choi, H.C. Ko, S.Y. Ko, et al., Correlation between flavonoid content and the NO production inhibitory activity of peel extracts from various citrus fruits, Biol. Pharm. Bull. 30 (2007) 772–778.
[5] A. Murakami, Y. Nakamura, K. Torikai, et al., Inhibitory effect of citrus nobiletin on phorbol ester-induced skin inflammation, oxidative stress, and tumor promotion in mice, Cancer Res. 60 (2000) 5059–5066.
[6] R. Suzuki, H. Kohno, A. Murakami, et al., Citrus nobiletin inhibits azoxymethane-induced large bowel carcinogenesis in rats, Biofactors 22 (2004) 111–114.
[7] M. Colasanti, H. Suzuki, The dual personality of NO, Trends Pharmacol. Sci. 13 (2000) 249–252.
[8] K. Sakitani, M. Nishizawa, K. Inoue, et al., Synergistic regulation of inducible nitric oxide synthase gene by CCAAT/enhancer-binding protein β and nuclear factor κB in hepatocytes, Genes Cells 3 (1998) 321–330.
[9] T. Matsuura, M. Kaibori, Y. Araki, et al., Japanese herbal medicine, inchinkoto inhibits inducible nitric oxide synthase induction in interleukin-1β-stimulated hepatocytes, Hepatol. Res. 42 (2012) 76–90.
[10] N. Ohno, E. Yoshigai, T. Okuyama, et al., Chlorogenic acid from the Japanese herbal medicine *kinginka* (Flos Lonicerae japonicae) suppresses the expression of inducible nitric oxide synthase in rat hepatocytes, HOAJ Biol. 1 (2012) 2.
[11] T. Lawrence, The nuclear factor NF-κB pathway in inflammation, Cold Spring Harb. Perspect. Biol. 1 (2009) a001651.
[12] M. Nishizawa, T. Okumura, Y. Ikeya, T. Kimura, Regulation of inducible gene expression by natural antisense transcripts, Front. Biosci. 17 (2012) 938–958.
[13] E. Yoshigai, T. Hara, Y. Araki, et al., Natural antisense transcript-targeted regulation of inducible nitric oxide synthase mRNA levels, Nitric *O*xide 30 (2013) 9–16.
[14] K. Matsui, M. Nishizawa, T. Ozaki, et al., Natural antisense transcript stabilizes inducible nitric oxide synthase messenger RNA in rat hepatocytes, Hepatology 47 (2008) 686–697.
[15] H. Yoshida, A.H. Kwon, K. Habara, et al., Edaravone inhibits the induction of iNOS gene expression at transcriptional and posttranscriptional steps in murine macrophages, Shock 30 (2008) 734–739.
[16] Y. Takimoto, H.Y. Qian, E. Yoshigai, et al., Gomisin N in herbal drug gomishi (*Schisandra chinesis*) suppresses inducible nitric oxide synthase gene via C/EBPβ and NF-κB in rat hepatocytes, Nitric *O*xide 28 (2013) 47–56.
[17] P.M. Dewick, Medicinal Natural Products: A Biosynthetic Approach, second ed., John Wiley & Sons, Chishester, England, 2001.
[18] T. Kanemaki, H. Kitade, Y. Hiramatsu, et al., Stimulation of glycogen degradation by prostaglandin E_2 in primary cultured rat hepatocytes, Prostaglandins 45 (1993) 459–474.
[19] L.C. Green, D.A. Wagner, J. Glogowski, et al., Analysis of nitrate, nitrite, and [^{15}N]nitrate in biological fluids, Anal. Biochem. 126 (1982) 131–138.
[20] M. Yamada, M. Nishizawa, R. Nakatake, et al., Characterization of alternatively spliced isoforms of the type I interleukin-1 receptor on iNOS induction in rat hepatocytes, Nitric Oxide 17 (2007) 98–105.
[21] O.A. Harasstani, S. Moin, C.L. Tham, et al., Flavonoid combinations cause synergistic inhibition of proinflammatory mediator secretion from lipopolysaccharide-induced RAW 264.7 cells, Inflamm. Res. 59 (2010) 711–721.
[22] E. Yoshigai, T. Hara, H. Inaba et al., Interleukin 1β induces tumor necrosis factor α secretion from rat hepatocytes, *Hepatol. Res.*, in press. http://dx.doi.org/10.1111/hepr.12157.
[23] S. Nafisi, A. Shadaloi, A. Feizbakhsh, H.A. Tajmir-Riahi, RNA binding to antioxidant flavonoids, J. Photochem. Photobiol. B 94 (2009) 1–7.
[24] H. Ihara, H. Yamamoto, T. Ida, et al., Inhibition of nitric oxide production and inducible nitric oxide synthase expression by a polymethoxyflavone from young fruits of *Citrus unshiu* in rat primary astrocytes, Biosci. Biotechnol. Biochem. 76 (2012) 1843–1848.
[25] S.J. Klempner, G. Bubley, Complementary and alternative medicines in prostate cancer: from bench to bedside?, Oncologist 17 (2012) 830–837