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Anti-inflammatory property of the urinary metabolites of nobiletin in mouse

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Abstract—Nobiletin, a major component of polymethoxyflavones in citrus fruits, has a broad spectrum of health beneficial properties including anti-inflammatory and anti-carcinogenic activities. The metabolite identification of nobiletin in mouse urine has concluded that it undergoes mono-demethylation (3′- and 4′-demethylnobiletin) and di-demethylation (3′,4′-didemethylnobiletin) metabolic pathway. Biological screening of nobiletin and its metabolites has revealed that the metabolites possess more potent anti-inflammatory activity than their parent compound. Therefore, this letter reports the identification of nobiletin metabolites and their anti-inflammatory activity against LPS-induced NO production and iNOS, COX-2 protein expression in RAW264.7 macrophage.

The study of health promoting property of nobiletin (I, 5,6,7,8,3′,4′-hexamethoxyflavone, Fig. 1), one of the major components of polymethoxyflavone family in citrus fruits, has made tremendous progress. Both in vitro and in vivo data have shown that nobiletin has anti-inflammatory[1–3] and anti-carcinogenic activities.[4–6] It has been found that the properties of nobiletin are very similar to those of dexamethasone, an anti-inflammatory drug. For instance, nobiletin can interfere with the production of prostaglandin E_2 (PGE_2) in human synovial fibroblasts by selectively down regulating cyclooxygenase-2 (COX-2) activity and also can down-regulate the gene expression of some cytokines like interleukins (IL-1α, IL-1β, IL-6) and TNF-α in mouse macrophages.[1] Molecular biological evidence showed that nobiletin suppresses gene expression and production of some matrix metalloproteinases (MMP-1, MMP-3, and MMP-9) in rabbit articular chondrocytes and synovial fibroblasts.[2] Inhibitory effects on inducible nitric oxide synthase (iNOS) protein production were observed in mouse macrophages.[3] There were many reports to have shown the anti-carcinogenic activity of nobiletin. For example, it can inhibit the proliferation of human prostate cancer cells; inhibit the skin, breast, and colon carcinoma cell lines.[4] Recently, nobiletin was shown to have anti-proliferative and apoptotic

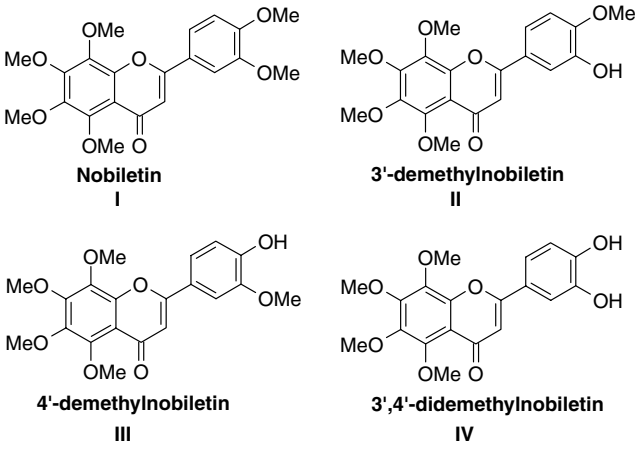

Figure 1. Structures of nobiletin and its metabolites.

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effects on a gastric cancer cell line and have disruptive effect on cell-cycle progression.[5] In another study, nobiletin showed the strongest anti-proliferative activity in a comparative evaluation of 42 flavonoids' activity against six human cancer cell lines including lung, prostate, colon, melanoma, and estrogen receptor positive (ER+) and estrogen receptor negative (ER−) breast cancer.[6] Moreover, another investigation revealed nobiletin being a dual inhibitor of both NO and $O_2^{\cdot -}$ generation in leukocytes, and inhibiting tumor promotion in two-stage mouse skin tumorigenesis.[6]

The biotransformation study of nobiletin has shown that it undergoes demethylation pathway with the formation of mono-demethylated nobiletin as major metabolites. In the in vivo metabolism study of nobiletin from rat urine, 3′-demethylnobiletin (II, 3′-hydroxy-5,6,7,8,4′-pentamethoxyflavone, Fig. 1) has been identified by Murakami et al. as the major metabolite,[7] whereas 4′-demethylnobiletin (III, 4′-hydroxy-5,6,7,8,3′-pentamethoxyflavone, Fig. 1) being identified as the major metabolism product by Ohigashi's research group.[8] Recently, we have identified two nobiletin metabolites in mouse urine, 3′-demethylnobiletin and 4′-demethylnobiletin.[9] In this communication, we report our newly identified nobiletin metabolite, 3′,4′-didemethylnobiletin (IV, 3′,4′-dihydroxy-5,6,7,8-tetramethoxyflavone, Fig. 1), in mouse urine.

Metabolites of bioactive xenobiotics are often associated with certain biological activity. It is of significance to comprehend the biological mode of action of the metabolites which is closely associated with the biological property, application, and usage limitation of the parent compounds. The anti-inflammatory activity screening of nobiletin and its three metabolites identified in our biotransformation study has been performed with the inhibition of LPS-induced NO production and iNOS, COX-2 protein expression in RAW264.7 macrophage. We found that the nobiletin metabolites have more potent activities than their parent compound, nobiletin. Hence, in this letter, we illustrate our findings in the inhibition of aforementioned NO production and gene expression.

In the course of metabolite identification, we reported two nobiletin metabolites, 3′-demethylnobiletin and 4′-demethylnobiletin.[9] By employing same techniques as previously reported, that is, organic synthesis of standard compounds and analysis with HPLC, HPLC–MS, HPLC–MS–MS, and SFC (supercritical fluid chromatography), we report herein the identification of 3′,4′-didemethylnobiletin (IV) as the major di-demethylated metabolite of nobiletin in mouse urine. This is the first time that a di-demethylnobiletin metabolite is identified.

The synthesis of 3′,4′-didemethylnobiletin (IV) as a standard compound followed the same route as that of 3′-demethylnobiletin using 3,4-dibenzyloxybenzaldehyde as the replacement of 3-benzyloxybenzaldehyde in the original synthetic scheme.[10] After multi-step synthesis, the 3′,4′-didemethylnobiletin (IV) was obtained as an off-white solid and characterized by UV, ^{1}H NMR, and MS.[10]

The HPLC–MS–MS profiles of the standard compound (3′,4′-didemethylnobiletin, IV) and mouse urine were obtained and are illustrated in Figure 2.[11] From the HPLC trace of mouse urine (Fig. 2a), the major peak at retention time (RT) of 14.65 min matches the compound IV (RT = 14.58 min, Fig. 2b). The MS/MS spectra of compound IV (Fig. 2a) and the major peak in HPLC with an RT at 14.58 min are identical. Hence, we can deduce from LC–MS–MS that the major di-demethylated metabolite is 3′,4′-didemethylnobiletin (IV), since the standard compound (Fig. 2a) and the major metabolite (Fig. 2b) in mouse urine possess identical MS fragments and have same HPLC retention time under the same experimental conditions. There are at least two other di-demethylnobiletin metabolites also found in the urine metabolite mixture (Fig. 2b, MS/MS not shown). The structures of these two minor di-demethylnobiletin metabolites were not elucidated owing to lack of standard compounds for comparison in

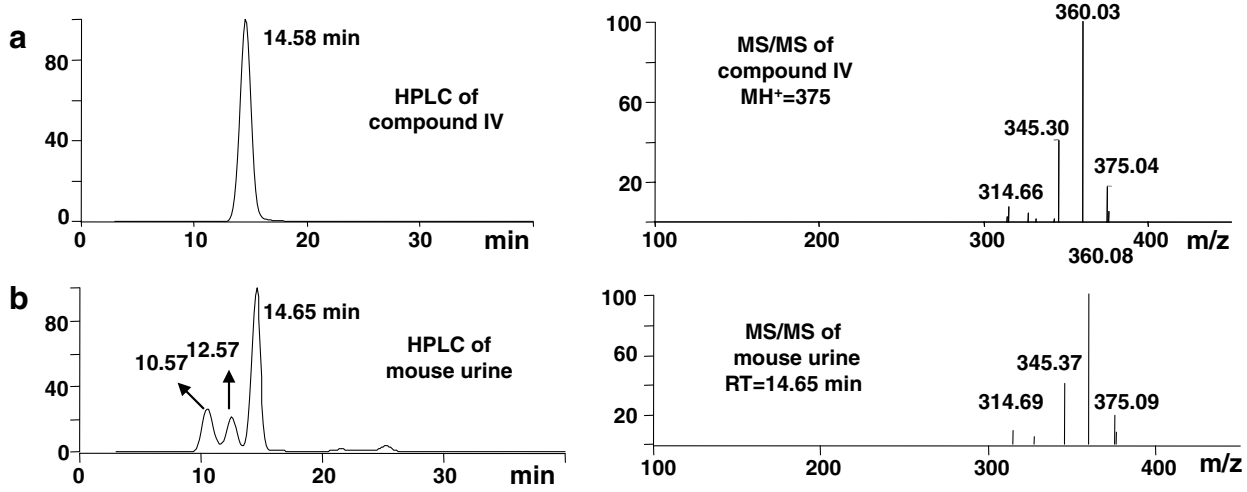

Figure 2. HPLC–MS–MS of (a) standard compound and (b) mouse urine sample.

this study. Thus we can summarize that nobiletin underwent mono-demethylation[9] and di-demethylation as its biotransformation pathway. So far, the identified nobiletin metabolites from mouse urine are: 3′-demethylnobiletin (II), 4′-demethylnobiletin (III), and 3′,4′-didemethylnobiletin (IV).

Inflammation is closely associated with carcinogenesis and acts as a driving force in premalignant and malignant transformation of cells.[12,13] The most prominent molecular mechanisms in the inflammatory processes are (i) NO production by iNOS and (ii) formation of prostaglandins by COX-2.[14,15] NO is produced endogenously by a family of nitric oxide synthases (NOSs) with a wide range of physiological and pathophysiological actions.[16,17] Increased NOS expression and/or activity have been reported in human gynecological and breast tumors as well as tumors in central nervous system.[18–20] Studies have suggested that increased cyclooxygenase activity and levels of prostaglandins may play important roles in multiple epithelial cancers such as colon carcinoma.[21,22] It has been known that COX-2-derived bioactive lipids, such as prostaglandin E_2, are potent inflammatory mediators that promote tumor growth and metastasis by stimulating cell proliferation, invasion, and angiogenesis.[23] High levels of prostaglandins may promote the development of malignancy.[24] Therefore, the inhibition of inflammation and the down-regulation of the activation of inflammatory mediators during the inflammatory process may be useful in preventing and treating inflammation associated diseases like cancer, etc.

To evaluate the anti-inflammatory property of nobiletin metabolites (Fig. 1), the nitrite (nitric oxide) production induced by LPS in RAW264.7 macrophage was investigated by Griess reaction. As shown in Figure 3, after treatment with LPS (100 ng/mL) alone for 24 h, by comparing with the control group, the concentration of nitrite in the medium increased remarkably. When treated with nobiletin and its metabolites (10 and 30 μM) together with LPS for 24 h, a significant inhibition of nitrite production was detected in the presence of compounds II, III, and IV, but not compound I (nobiletin).[25] We also determined the cytotoxicity of compounds I–IV in RAW264.7 macrophage using MTT assay, and the result indicated these four compounds did not affect cell viability even at the 30 μM (data not shown).

To further determine the effects of nobiletin and its three metabolites on LPS-induced iNOS and COX-2 expression, the levels of iNOS and COX-2 proteins were assayed using Western blotting analysis.[25] After treated with different concentrations (5 and 20 μM) of the four compounds and LPS (100 ng/mL) for 24 h, compounds III and IV significantly inhibited iNOS and COX-2 protein expression (Fig. 4). RT-PCR analysis also demonstrated that when co-treated with PMFs and LPS (100 ng/mL), compounds III and IV also markedly decreased LPS-induced *iNOS* and *COX-2* gene expression. However, interestingly, LPS-induced *iNOS* gene expression was not inhibited by compound II even at 20 μM. As shown in Figure 3, compound II inhibited LPS-induced NO production in a concentration-dependent manner, but neither the iNOS mRNA nor protein levels. Hence, we inferred that inhibitory effect of compound II on LPS-induced NO production might be through the inhibition of iNOS enzyme activity.

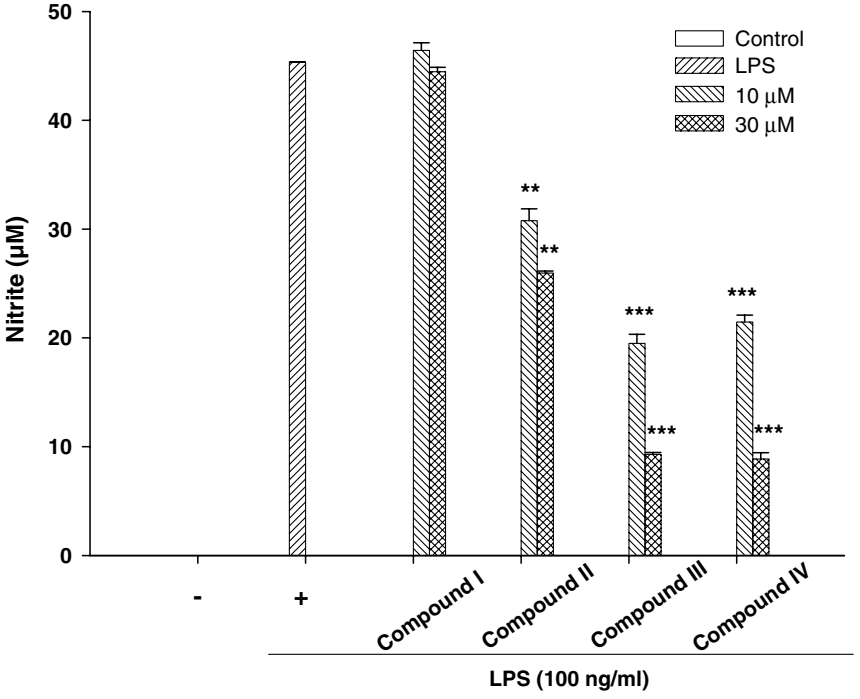

Figure 3. Inhibition effects of nobiletin and its metabolites on LPS-induced nitrite production in RAW264.7 macrophage. (The cells were treated with 100 ng/mL LPS only or with compounds at 10 or 30 μM for 24 h. At the end of incubation time, 100 μL of the culture medium was collected for nitrite assay.[25] $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ indicate statistically significant differences from the LPS-treated group.)

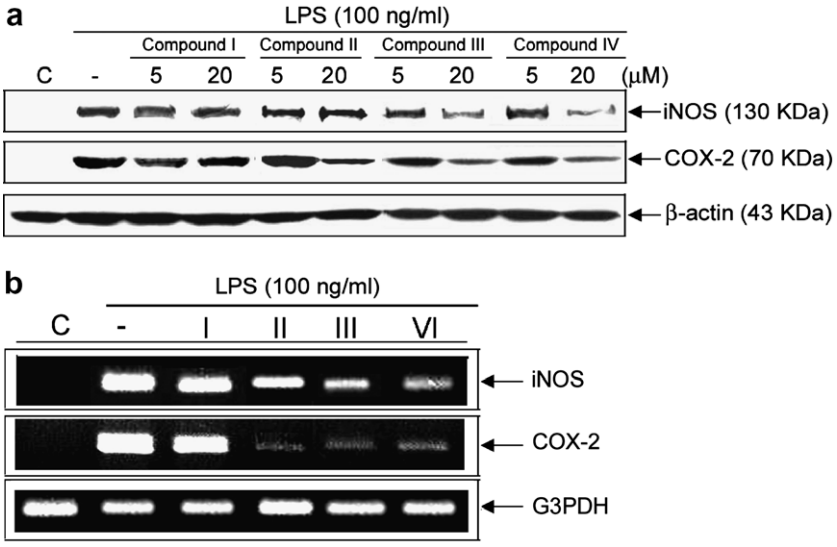

Figure 4. Inhibitory effects of nobiletin and metabolites on LPS-induced iNOS and COX-2 expression. (a) The cells were treated with LPS (100 ng/mL) alone or with compounds (5 or 20 μM) for 24 h. Equal amounts of total proteins (50 μg) were subjected to 10% SDS–PAGE, and expression of iNOS, COX-2 and β-actin protein was detected by Western blot using specific antibodies. (b) The cells were treated with LPS (100 ng/mL) alone or with compounds (20 μM) for 24 h and the total RNA was isolated. Two micrograms of total RNA was transcribed into cDNA using SuperScrip II RNAse H-reverse transcriptase. The mRNA expressions were performed using the RT-PCR.

In this study, we found that compound IV (3′,4′-didemethylnobiletin) strongly inhibited the induction of iNOS in RAW264.7 cells activated with LPS, whereas compound I (nobiletin) has less effect. Nitrate can be microbiologically reduced to nitrite,[26] which can then interact with dietary substrates such as amine or amides to produce *N*-nitroso compounds. The formation of carcinogenic *N*-nitrosoamines resulting from elevated NO formation has been demonstrated in cell cultures and in vivo.[27] Our results demonstrated that compound IV (3′,4′-didemethylnobiletin) was the potent inhibitor of inducible NO synthase protein; therefore, it may block the formation of *N*-nitroso compounds and peroxynitrite or hydroxyl radicals, and could thus inhibit carcinogenesis. Taken together, these results suggest that nobiletin metabolites, especially 4′-demethylnobiletin (III) and 3′,4′-didemethylnobiletin (IV), may exert its anti-inflammatory and anti-carcinogenic properties by suppressing *iNOS* and *COX-2* gene expression.

References and notes

1. Lin, N.; Sato, T.; Takayama, Y.; Mimaki, Y.; Sashida, Y.; Yano, M.; Ito, A. *Biochem. Pharmacol.* **2003**, *65*, 2065.
2. Ishiwa, J.; Sato, T.; Mimaki, Y.; Sashida, Y.; Yano, M.; Ito, A. *J. Rheumatol.* **2000**, *27*, 20.
3. Murakami, A.; Nakamura, Y.; Torikai, K.; Tanaka, T.; Koshiba, T.; Koshimizu, K.; Kuwahara, S.; Takahashi, Y.; Ogawa, K.; Yano, M.; Tokuda, H.; Nishino, H.; Mimaki, Y.; Sashida, Y.; Kitanaka, S.; Ohigashi, H. *Cancer Res.* **2000**, *60*, 5059.
4. Kandaswami, C.; Perkins, E.; Soloniuk, D. S.; Drzewiecki, G.; Middleton, E. *Cancer Lett.* **1991**, *59*, 147.
5. Yoshimizu, N.; Otani, Y.; Saikawa, Y.; Kubota, T. *Aliment. Pharmacol. Ther.* **2004**, *20*, 95.
6. Manthey, J. A.; Guthrie, N. *J. Agric. Food Chem.* **2002**, *50*, 5837.
7. Murakami, A.; Kuwahara, S.; Takahashi, Y.; Ito, C.; Furukawa, H.; Ju-ichi, M.; Koshimizu, K.; Ohigashi, H. *Biosci. Biotechnol. Biochem.* **2001**, *65*, 194.
8. Yasuda, T.; Yoshimura, Y.; Yabuki, H.; Nakazawa, T.; Ohsawa, K.; Mimaki, Y.; Sashida, Y. *Chem. Pharm. Bull.* **2003**, *51*, 1426.
9. Li, S.; Wang, Z.; Sang, S.; Huang, M.-T.; Ho, C.-T. *Mol. Nutr. Food Res.* **2006**, *50*, 291.
10. Li, S.; Pan, M.-H.; Lai, C.-S.; Lo, C.-Y.; Dushenkov, S.; Ho, C.-T. *Bioorg. Med. Chem.* **2007**, *15*, 3381.
11. LC/MS analysis was carried out with a Finnigan Spectra System and a LTQ linear ion trap mass detector (ThermoFinigan, San Jose, CA) incorporated with electrospray ionization (ESI) interface. A 50×2.0 mm i.d., 3 μm Gemini C_{18} column (Phenomenex) was used for separation with a flow rate of 0.2 mL/min. The positive ion polarity mode was set for ESI ion source with the voltage on the ESI interface maintained at approximately 5 kV. The structural information of all the test compounds was obtained by tandem mass spectrometry (MS/MS) through collision-induced dissociation (CID) with a relative collision energy setting of 35%.
12. Balkwill, F.; Coussens, L. M. *Nature* **2004**, *431*, 405.
13. Philip, M.; Rowley, D. A.; Schreiber, H. *Semin. Cancer Biol.* **2004**, *14*, 433.
14. Moncada, S. *J. R. Soc. Med.* **1999**, *92*, 164.
15. Turini, M. E.; DuBois, R. N. *Annu. Rev. Med.* **2002**, *53*, 35.
16. Moncada, S.; Palmer, R. M.; Higgs, E. A. *Pharmacol. Rev.* **1991**, *43*, 109.
17. Nathan, C.; Xie, Q. W. *Cell* **1994**, *78*, 915.
18. Thomsen, L. L.; Lawton, F. G.; Knowles, R. G.; Beesley, J. E.; Riveros-Moreno, V.; Moncada, S. *Cancer Res.* **1994**, *54*, 1352.
19. Thomsen, L. L.; Miles, D. W.; Happerfield, L.; Bobrow, L. G.; Knowles, R. G.; Moncada, S. *Br. J. Cancer* **1995**, *72*, 41.

20. Cobbs, C. S.; Brenman, J. E.; Aldape, K. D.; Bredt, D. S.; Israel, M. A. *Cancer Res.* **1995**, *55*, 727.
21. Levy, G. N. *FASEB J.* **1997**, *11*, 234.
22. Dubois, R. N.; Abramson, S. B.; Crofford, L.; Gupta, R. A.; Simon, S. L.; Van De Putte, L. B.; Lipsky, P. E. *FASEB J.* **1998**, *12*, 1063.
23. Mann, J. R.; Backlund, M. G.; DuBois, R. N. *Nat. Clin. Pract. Oncol.* **2005**, *2*, 202.
24. Yoshimura, R.; Matsuyama, M.; Tsuchida, K.; Takemoto, Y.; Nakatani, T. *Nippon Rinsho* **2005**, *63*, 1839.
25. Pan, M.-H.; Lai, C.-S.; Wang, Y.-J.; Ho, C.-T. *Biochem. Pharmacol.* **2006**, *72*, 1293.
26. Ravindranath, V.; Chandrasekhara, N. *Toxicology* **1981**, *20*, 251.
27. Iyengar, R.; Stuehr, D. J.; Marletta, M. A. *Proc. Natl. Acad. Sci. U.S.A* **1987**, *84*, 6369.