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Nobiletin and its colonic metabolites suppress colitis-associated colon carcinogenesis by down-regulating iNOS, inducing antioxidative enzymes and arresting cell cycle progression

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Abstract

Nobiletin (NOB) is a major citrus polymethoxyflavone (PMF) with various beneficial biological activities. We reported previously that dietary NOB significantly inhibited colitis-associated colon carcinogenesis in azoxymethane (AOM)/dextran sulfate sodium (DSS)-treated mice, and the chemopreventive effects were associated with NOB metabolites found in the mouse colonic tissues. In this study, to better understand the role of colonic metabolites of NOB, we determined the anti-inflammation and anticancer effects of a mixture of NOB and its major metabolites (NOB-Met) at the concentrations equivalent to those found in colonic tissues of NOB-fed mice. The results demonstrated that NOB-Met effectively decreased the expression level of inducible nitric oxide synthase (iNOS), increased the level of heme oxygenase-1 (HO-1) and NADH quinone oxidoreductase 1 (NQO1) and up-regulated nuclear factor erythroid 2-related factor (Nrf2) signaling pathway in lipopolysaccharide (LPS)-stimulated macrophages. NOB-Met also caused a significant cell cycle arrest in human colon cancer cells. Validation study confirmed that dietary NOB led to the effects similar to those described above in the colon of AOM/DSS-treated mice. Specifically, dietary NOB significantly reduced the level of iNOS, up-regulated Nrf2-dependent enzymes and profoundly modulated key signaling proteins resulting in decreased cell cycle progression in the colonic tissue of AOM/DSS-treated mice. Overall, our findings demonstrated that dietary NOB led to the presence of NOB and its metabolites in the colonic tissue, which suppressed colitis-associated colon carcinogenesis *via* down-regulating iNOS, inducing antioxidative enzymes and arresting cell cycle progression.

Keywords: Chemoprevention; Colon carcinogenesis; Nobiletin; Metabolites; iNOS; Nrf2; Cell cycle

1. Introduction

Nobiletin (5,6,7,8,3′,4′-hexamethoxyflavone, NOB) is one of the major polymethoxyflavones (PMFs) mostly found in citrus fruits [1]. In recent years, there has been increasing interest in the investigation of the biological properties, biotransformation and bioavailability of PMFs, due to their wide range of health-promoting effects, including anticancer [2–5], anti-inflammation [6,7], neuroprotection [8] and antiobesity [9]. Previously, we and others have investigated the anticarcinogenic effects of NOB against colon cancer. It was found that NOB inhibited azoxymethane (AOM)-induced colon carcinogenesis in rats, which was associated with increased apoptotic activities and decreased prostaglandin E_2 (PGE_2) in the colon [5]. Recently, we demonstrated that dietary NOB inhibited AOM/dextran sulfate sodium (DSS)-induced colitis-associated colon carcinogenesis in mice [2].

Compelling evidence has indicated that biotransformation plays important roles in the *in vivo* biological properties of orally administered compounds. It has been shown that some PMFs undergo similar patterns of metabolism in rodent models, which result in the production of demethylated metabolites of PMFs [2,10–13]. In particular, we and others have reported three major metabolites of NOB in mice after oral consumption of NOB [2,12,13]. The chemical structures of the three metabolites are shown in Fig. 1, and they are 3′-demethylnobiletin (M1), 4′- demethylnobiletin (M2) and 3′,4′ -didemethylnobiletin (M3). More importantly, we found that the total colonic level of these three metabolites was about 20-fold higher than that of NOB in mice after long-term feeding of NOB [2]. Specifically, feeding of CD-1 mice with 500 ppm of NOB in diet for 20 weeks resulted in 2.0, 3.3, 24.1 and 12.0 nmol/(gram of tissue) of NOB, M1, M2 and M3 in colonic mucosa, respectively [2]. Due to their

Abbreviations: NOB, nobiletin; PMF, polymethoxyflavone; AOM, azoxymethane; DSS, dextran sulfate sodium; LPS, lipopolysaccharide; iNOS, inducible nitric oxide synthase; COX-2, cyclooxygenase-2; Nrf2, nuclear factor erythroid 2-related factor 2; HO-1, heme oxygenase-1; NQO1, NADH quinone oxidoreductase 1; CDK, cyclin-dependent kinase.

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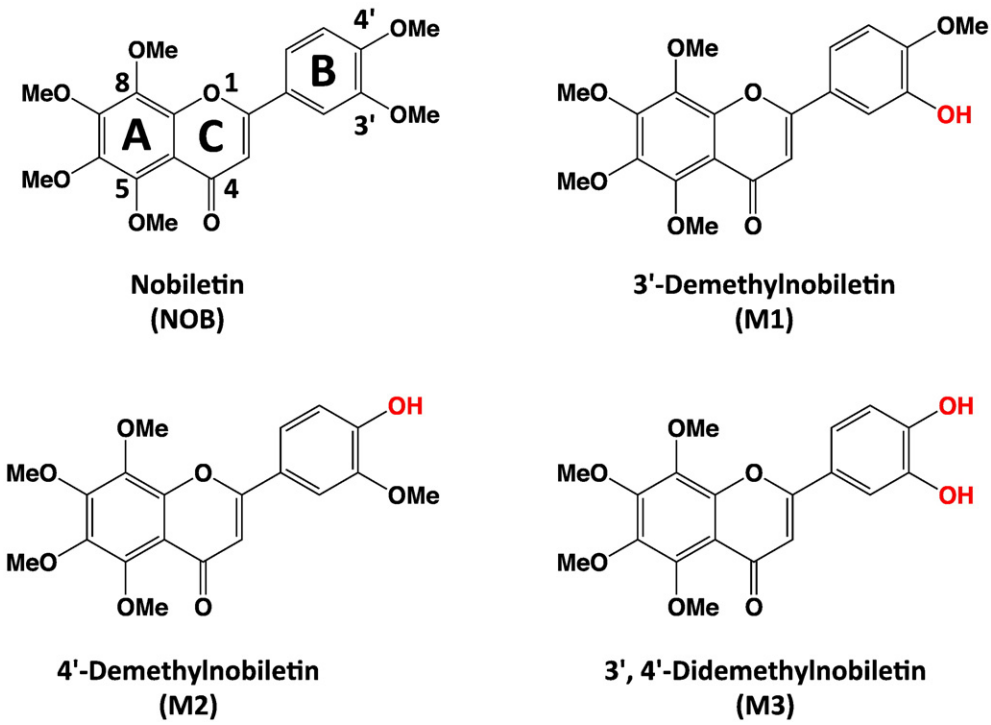

Fig. 1. Chemical structures of nobiletin (NOB), 3′-demethylnobiletin (M1), 4′- demethylnobiletin (M2) and 3′,4′ -didemethylnobiletin (M3).

high abundance in colonic tissues, these metabolites may significantly contribute to the biological effects elicited from oral consumption of NOB.

Accumulating studies have shown the potent bioactivities of aforementioned metabolites of NOB. Cell culture studies revealed that the metabolites of NOB, especially M2 and M3 had much stronger anti-inflammatory and anticancer effects than did NOB [2,13]. M2 and M3 were found to suppress LPS-induced overproduction of nitric oxide (NO) and gene expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) [13]. M2 was also found to inhibit LPS-stimulated nuclear translocation of NF-κB and AP-1, and on the other hand, promote nuclear translocation of nuclear factor erythroid 2-related factor 2 (Nrf2), which in turn triggered the gene expression of Nrf2-dependent enzymes heme oxygenase-1 (HO-1) and NADH quinone oxidoreductase 1 (NQO1) [6]. The stronger inhibitory effects of these metabolites in comparison with NOB have also been demonstrated in human colon cancer cells where the metabolites inhibited cancer cell growth by inducing cell cycle arrest and apoptosis [2]. An *in vivo* study also demonstrated that M2 had more potent inhibitory efficacy than did NOB in suppressing12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced ear inflammation in mice [7]. These findings strongly support the hypothesis that *in vivo* effects of orally administered NOB are closely associated with its metabolites.

Our previous study demonstrated that after oral administration of NOB, mouse colonic tissue was exposed to not only NOB but also considerably high levels of NOB metabolites [2]. NOB and its metabolites have been shown to have different bioactivities, and simultaneous exposure to NOB and its metabolites may result in different biological effects in the colon in comparison with the situation where the colon is exposed to these compounds individually. This is because those different compounds may interact with each other to elicit different biological effects [14,15]. Therefore, studies on the overall effects of the mixture of NOB and its metabolites at the levels found *in vivo* would provide critical information to better understand the biological properties of dietary NOB. The information generated in this type of study would provide a solid scientific basis for using NOB as a nutraceutical to benefit human health. Herein, we determined the inhibitory effects of NOB and its major metabolites as a mixture (NOB-Met) at the concentrations equivalent to those found in the colon of NOB-fed mice in LPS-treated RAW 264.7 macrophages and HCT116 human colon cancer cells. Moreover, we utilized an AOM/DSS-induced colitis-associated colon carcinogenesis model to validate the results from the cell culture studies.

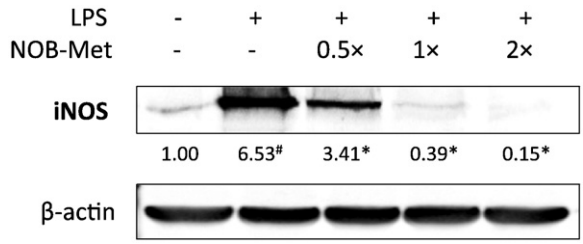

Fig. 2. Inhibitory effects of NOB-Met on LPS-induced protein expression of iNOS in RAW 264.7 macrophages. The cells were seeded in 10-cm dishes for 24h and then treated with LPS or LPS plus NOB and its metabolites M1, M2 and M3 as a mixture (NOB-Met). Three levels of NOB-Met were used at 0.5×, 1× and 2×. NOB-Met at 1× was equivalent to the concentrations (NOB: 2.0 μM; M1: 3.3 μM; M2: 24.1 μM; M3: 12.0 μM) found in the colonic tissues of CD-1 mice fed with 0.05 wt% of NOB in diet for 20 weeks as we reported previously [2]. NOB-Met at 0.5× and 2× were half or twice of the concentrations of NOB-Met at 1×. After 24 h of incubation, cells were harvested for Western blotting as described in the Materials and methods section. The numbers underneath the blots represent band intensity (normalized to β-actin, means of 3 independent experiments) measured by Image J software. The standard deviations (all within±15% of the means) were not shown. β-Actin was served as an equal loading control. # Indicates statistically significant difference between untreated negative control group and LPS-treated positive control group. *Indicates statistically significant difference between NOB-Met-treated groups and LPS-treated positive control group ($P<.05$, $n=3$).