Nobiletin Induces Oligodendrocyte Lineage Precursor Cells in a Cuprizone-Administered Demyelination Animal Model

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Abstract

We investigated the effect of nobiletin on the oligodendroglial cell lineage in a cuprizone-induced demyelination animal model. Nobiletin, a polymethoxylavone, was administered to the model animal for 3 consecutive weeks (50 mg/kg, intraperitoneally, 2 times/week). Immunohistochemical analysis of the corpus callosum and/or Western blotting analysis of brain extracts revealed that cuprizone administration reduced immunoreactivity for myelin-basic protein (MBP), a marker for mature oligodendrocytes, and increased immunoreactivity for platelet derived-growth factor receptor (PDGFR)-α, a marker for oligodendrocyte precursor cells (OPCs). Nobiletin treatment did not affect these changes in the cuprizone-fed mice. Nobiletin treatment, however, increased the immunoreactivity against proteolipid protein (PLP)/DM-20, a marker for mature oligodendrocytes, OPCs, and oligodendrocyte lineage precursor cells, and also augmented immunoreactivity against oligodendrocyte transcription factor 2 (olig2), a marker for OPCs and their precursor cells. These findings suggest that nobiletin promotes the production of oligodendrocyte lineage precursor cells in a demyelination animal model, and that nobiletin treatment might be suitable for individuals with demyelination diseases such as multiple sclerosis.

Keywords: Nobiletin; Demyelination; Oligodendrocyte precursor cells (OPCs); Olig2; PLP/DM-20

Introduction

Polymethoxyflavones (PMFs) have a broad spectrum of biological activities [1]. Nobiletin is one of the PMFs present in the peel of citrus fruits, and various biological activities have been demonstrated in vitro and in vivo. Nobiletin stimulates the cAMP-dependent protein kinase and extracellular signal-regulated kinase signaling pathways in PC12 cells and cultured hippocampus neurons [2,3]. In in vivo experiments, nobiletin improves memory deficits in an Alzheimer’s disease model animal [4] as well as ischemia-induced memory deficits [5], and improves motor and cognitive deficits in an 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced Parkinson model animal [6].

Multiple sclerosis (MS) is a chronic inflammatory disease of the CNS that is characterized by demyelination and axonal loss [7,8]. The precise cause of MS remains obscure. Some well-characterized experimental model animals allow us to study the mechanisms of MS pathology. Experimental autoimmune encephalomyelitis is the most prevalent animal model of MS [9,10]. This model is used to evaluate the potential efficacy of immunosuppressive agents and to determine the effectiveness of promyelinating agents. Another MS model, induced by cuprizone feeding, exhibits demyelination and astrogliosis. Feeding a diet containing 0.2% cuprizone, a copper chelator, induces these pathological features of MS, which is believed to be due to the vulnerability of mature oligodendrocytes to the toxic agent [11]. Using these models, various agents have been evaluated for their possible beneficial effects on MS pathology, such as FTY720, interferon-β, sildenafil, vitamin D3, testosterone, 17β-estradiol, and benzotropine [12-19]. However, the functional relevance of nobiletin for MS animal models has not been addressed. In the present study, we found an effect of nobiletin on the cuprizone-induced demyelination model.

Materials and Methods

Animals

Mice were maintained under a controlled temperature and photoperiod (23°C, 12-h light and 12-h dark) with food and water ad libitum. All experimental procedures followed the Guideline for Animal Experimentation prepared by the Animal Care and Use Committee of Matsuyama University. C57BL/6j and 129 hybrid mice were used. Demyelination was induced by feeding 4-week old male mice a diet containing 0.2% cuprizone (bis-cyclohexane oxalidihydrzone, Sigma-Aldrich #C9012, St. Louis, MO, USA) mixed into ground standard rodent chow (CRF-1, Oriental Yeast, Tokyo, Japan) for 5 weeks.

PMF treatment

Nobiletin and heptamethoxyflavone (HMF) were generously provided by USHIO Chemix (lot nos. H13166 and H13020, respectively, Omaezaki, Japan). After feeding the mice a cuprizone-containing diet for 2 weeks, nobiletin and HMF treatment were started. These PMFs were suspended in corn oil (Sigma-Aldrich #C8267) as a vehicle and intraperitoneally injected at a dose of 50 mg/kg, twice a week for 3 weeks. The experimental dose of PMFs was selected based on previous reports [6,20,21]. Mice were divided into four experimental groups: a control group (Con+Veh), a cuprizone-control group (CPZ+Veh), a cuprizone-HMF group (CPZ+Hep), and a cuprizone-nobiletin group (CPZ+Nob).

Immunohistochemistry

Mice were anesthetized with pentobarbital (90 mg/kg, Nakalai Tesque, Kyoto, Japan). To prepare cryosections of the brain, the mice were perfused with 4% paraformaldehyde in magnesium-calcium-free phosphate-buffered saline (PBS [−]). Brains were post-fixed with...
the 4% paraformaldehyde solution for 2 days, and rinsed with PBS (−). After serial immersion in 10%, 20%, and 30% sucrose in PBS (−), the brains were embedded in OCT compound (Sakura Finetechical, Tokyo, Japan) and sectioned coronally at a thickness of 30 μm using a cryostat. Sections were stored at −80°C until use. Sections were thawed and re-fixed with 4% paraformaldehyde solution for 30 min. Endogenous peroxidase activity was blocked by incubation with 3% hydrogen peroxide in PBS (−) for 5 min. Sections were incubated overnight with primary antibodies. Immunoreactivity was detected using the EnVision+ system HRP Rabbit (Dako/Japan #K4003, Tokyo, Japan) for rabbit antibodies, HRP-conjugated rabbit anti-rat IgG (1/50000, Jackson ImmunoRes #312-035-003, West Grove, PA, USA) for rat antibodies, or M.O.M. Kit (Vector, Burlingame, CA) for mouse antibodies, and diaminobenzidine. Primary antibodies used in the immunohistochemistry were as follows: rabbit anti-β-amyloid precursor protein (APP; 1/80), Invitrogen #51-2700, Carlsbad, CA, USA), rabbit anti-platelet derived-growth factor receptor-α (PDGFR-α; 1/150, Santa Cruz Biotechnology #sc-338, Dallas, TX, USA), rabbit anti-glial fibrillary acidic protein (GFAP; 1/10, Dako/Japan #N1506, Tokyo, Japan), rabbit anti-ionized calcium-binding adaptor molecule 1 (Iba-1; 1/250, Wako #019-19741, Osaka, Japan), mouse monoclonal anti-oligodendrocyte transcription factor 2 (olig2; 1/300, Merck Millipore #MABN50, Billerica, MA, USA), mouse monoclonal anti-proteolipid protein (PLP; 1/200, Serotec #MCA839G, Toronto, Ontario, Canada) antibodies, and rat monoclonal anti-myelin-basic protein (MBP; 1/20, Merck Millipore #MAB386). The sections for PLP were immunostained by mounting them onto glass slides after blocking the endogenous peroxidase activity, followed by immersion in xylenes, rehydration in PBS (−), and incubation with primary antibodies. The immunohistochemistry procedures were validated using a negative control in which the primary antibodies were omitted. The densities of the anti-MBP, PDGFR-α, PLP, APP, and GFAP antibody stains were measured with Image J, an image processing and analysis program, on photographed sections (Dr. Wayne Randash, NIH, Bethesda, MD). Data obtained are presented as the fold-change of control mean ± SEM. In each experiment, data from the control brains (control group [Con+Veh]) were standardized. When multiple control slides were stained in an experiment, mean values of the control brains were used for standardization of the experimental data. Therefore, control data have error bars in some cases, and in other cases lack error bars. For quantification of olig2 immunoreactivity, immunostaining-positive cells were manually counted (0.11 mm²/section).

Western blotting analysis

The cerebral cortices of mice were homogenized with a glass Dounce homogenizer in RIPA buffer containing 50 mM Tris–HCl (pH 8.0), 150 mM NaCl, 0.1% sodium dodecyl sulfate, 1% NP40, 0.5% sodium deoxycholate, and a complete protease inhibitor cocktail (Boehringer Mannheim GmbH, Mannheim, Germany), and centrifuged for 30 min at 20,000 g. After sodium dodecyl sulfate-polyacrylamide gel electrophoresis, the extracted proteins in the supernatant were transferred to a polyvinylidene difluoride membrane (BioRad Laboratories, Hercules, CA, USA). The blots were probed with the following antibodies, which were detected using the ECL Plus Western Blotting Detection System (GE Healthcare UK Limited, Little Chalfont Buckinghamshire, UK). Blots probed with rabbit anti-GFP antibody (1/100, Dako/Japan #N1506), rabbit anti-Iba-1 antibody (1/4000, Wako #019-19741), or rabbit anti-MBP antibody (1/500, Merck Millipore #MAB386) were reprobed with horseradish peroxidase-conjugated anti-glyceraldehyde 3-phosphate dehydrogenase antibody (1/70000, Sigma #G9295). Data are presented as the fold-change of control mean ± SEM.

Statistical analysis

Student’s t-test was used to analyze the intergroup differences. A p value of less than 0.05 was considered statistically significant. All data are presented as mean ± SEM.

Results

PMFs do not have obvious effects on mature oligodendrocytes or oligodendrocyte precursor cells in cuprizone-fed mice

As shown in our previous study [22], mice treated with cuprizone for 5 weeks exhibit reduced body weight (Figure 1). Treatment with the PMFs (nobiletin and its structurally related compound, HMF) for 3 consecutive weeks (50 mg/kg, intraperitoneally, 2 times/week) partially recovered the reduction in body weight, suggesting that these compounds have some beneficial effects on the cuprizone-induced pathology.

We selected the corpus callosum (CC) for evaluation of demyelination, because cuprizone-induced demyelination was most obvious in the CC within the brain [22]. Immunohistochemical analysis of brain sections using anti-MBP (a marker for mature oligodendrocytes) antibody [13] revealed that the dense staining of the CC in the brain was weakened by cuprizone administration (Figure 2A-2I). As we used comparable areas of brain tissues for the immunohistochemical experiments, reduction in the CC volume by the cuprizone administration was also obvious (Figure 2A, 2C, 2E and 2G, [13]). The cuprizone-induced MBP reduction assessed by immunohistochemistry was confirmed by Western blotting analysis (Figure 2J and 2K). The MBP reduction was not affected by treatment with PMFs such as nobiletin or HMF (Figure 2).

Cuprizone administration induced an increase in the staining of PDGFR-α positive cells in the CC of mice. PDGFR-α is a marker for oligodendrocyte precursor cells (OPCs) [23]. The staining of OPCs was not affected by nobiletin or HMF treatment (Figure 3).

Figure 1: Body weight changes of cuprizone-fed mice under PMF treatment.

Mice were fed a diet containing 0.2% cuprizone from 4 to 9 weeks of age and a PMF (nobiletin or HMF) was intraperitoneally injected at a dose of 50 mg/kg, twice a week for 3 weeks from 6 to 9 weeks of age. Body weight of mice was clearly reduced by cuprizone feeding (Con+Veh) vs. (CPZ+Veh). The inter-group differences in body weight between the cuprizone-control group (CPZ+Veh) and the cuprizone-polymethoxyflavone groups (CPZ+HMF; CPZ+Nob) were significant over the last 10 days. *: p<0.05.
Nobiletin treatment increases the number of oligodendrocyte lineage precursor cells

We next examined, using other antibodies, the possible involvement of PMFs on oligodendrocyte lineage cells. Cuprizone administration reduced the dense staining of the CC by mouse monoclonal anti-PLP antibody (Figure 4) [22]. This antibody is known to respond to PLP expressed in mature oligodendrocytes as well as DM-20, which is a short splice variant protein from the PLP gene expressed in mature oligodendrocytes, OPCs, and oligodendrocyte lineage precursor cells [24]. The antibody (https://www.abdserotec.com/cow-bovine-myelin-proteolipid-protein-antibody-plpc1-mca839g.html) recognizes an epitope of the amino acid sequence shared between these two proteins. The reduced PLP/DM-20 staining in the CC was recovered by nobiletin treatment. HMF also showed a slight recovering effect (Figure 4).

Olig2 is required for oligodendrocyte specification and differentiation, and is a marker for OPCs and oligodendrocyte lineage...
precursor cells [25]. Cuprizone administration induced an increase in the number of olig2-positive cells in the CC. The increase in the olig2-positive cell number was enhanced by nobiletin treatment. HMF also showed a slight olig2-increasing effect (Figure 5).

The PMF-induced augmented immunoreactivity in PLP/DM-20 and olig2 in conjunction with the results of the MBP and PDGFR-α immunoreactivity suggests that PMFs, especially nobiletin, induces oligodendrocyte lineage precursor cells in cuprizone-fed mice.

**Nobiletin treatment slightly reduces astrogial and microglial activation induced by cuprizone administration**

To probe the possible involvement of astrogial cells in the effects of PMFs, we examined astrogial activation by assessing GFAP expression. As reported previously [11,22,26], cuprizone administration severely induced GFAP expression in various brain regions, including the CC. Nobiletin and HMF slightly, but not significantly, reduced the expression levels of GFAP induced by cuprizone administration (Figure 6).

Next, we examined microglia/macrophage activation by assessing the expression of Iba-1. Cuprizone caused strong induction of Iba-1 expression in several brain regions including the CC [27], where numerous mononuclear cells had accumulated (data not shown). Again, nobiletin and HMF, slightly, but not significantly, reduced the expression levels of Iba-1 induced by cuprizone administration (Figure 7).

To assess axonal injury caused by cuprizone administration, we examined the immunoreactivity for APP in the CC of mice. Expression of APP is associated with axonal and neuronal degeneration in MS [28]. APP immunoreactivity in the CC was enhanced by cuprizone treatment. The enhanced immunostaining was not affected by PMFs (Figure 8).

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*Figure 5: Nobiletin increases olig2-immunoreactivity in cuprizone-fed mice. Coronal brain sections were prepared from cuprizone-fed mice with/without PMF treatment and stained immunohistochemically with anti-olig2 antibody. A and B are photos of the CC from the control group (Con+Veh), C and D from the cuprizone-control group (CPZ+Veh), E and F from the cuprizone-HMF group (CPZ+Hep), and G and H from the cuprizone-nobiletin group (CPZ+Nob). Olig2-immunonegative cell number in the CC was increased by cuprizone feeding. The increased olig2-immunoreactive cell number was enhanced by nobiletin treatment. Scale bar=200 um for A, C, E, and G; 50um for B, D, F, and H. *: p<0.05.*

*Figure 6: Effect of nobiletin and HMF on astrogliosis in cuprizone-fed mice. Coronal brain sections were prepared from cuprizone-fed mice with/without PMF treatment and stained immunohistochemically with anti-GFAP antibody. A is photo of the CC from the control group (Con+Veh), B from the cuprizone-control group (CPZ+Veh), C from the cuprizone-HMF group (CPZ+Hep), and D from the cuprizone-nobiletin group (CPZ+Nob). Proteins extracted from the cerebral cortices of mice were applied to Western blotting analysis. GFAP-immunoreactivity was increased by cuprizone feeding. The increased GFAP-immunoreactivity was slightly, but not significantly, reduced by nobiletin and HMF treatment. Scale bar=200 um. *: p<0.05.*

*Figure 7: Effect of nobiletin and HMF on microgliosis in cuprizone-fed mice. Coronal brain sections were prepared from cuprizone-fed mice with/without PMF treatment and stained immunohistochemically with anti-Iba-1 antibody. A is photo of the CC from the control group (Con+Veh), B from the cuprizone-control group (CPZ+Veh), C from the cuprizone-HMF group (CPZ+Hep), and D from the cuprizone-nobiletin group (CPZ+Nob). Proteins extracted from the cerebral cortices of mice were applied to Western blotting analysis. Iba-1-immunoreactivity was increased by cuprizone feeding. The increased Iba-1-immunoreactivity was slightly, but not significantly, reduced by nobiletin and HMF treatment. Scale bar=1 mm. *: p<0.05.*
Nobiletin does not affect APP-immunoreactivity in the CC in cuprizone-fed mice. Coronal brain sections were prepared from cuprizone-fed mice with/without PMF treatment and stained immunohistochemically with anti-APP antibody. A and B are photos of the CC from the control group (Con+Veh), C and D from the cuprizone-control group (CPZ+Veh), E and F from the cuprizone-HMF group (CPZ+Hep), and G and H from the cuprizone-nobiletin group (CPZ+Nob). B, D, F, and H are magnifications of A, C, E, and G, respectively. APP-immunoreactivity in the CC was promoted by cuprizone feeding. The promoted APP-immunoreactivity was not modulated by nobiletin treatment. Scale bar=200 um for A, C, E, and G; 30 um for B, D, F, and H. *: p<0.05.

Discussion

Cuprizone administration decreases mature oligodendrocytes, and increases OPCs

The complexity of oligodendrocyte lineage marker proteins makes it difficult to evaluate compounds that might have some effects on the cell lineage in the brain. In fact, we were confused in the early periods of this study. While cuprizone administration reduced the immunoreactivity against MBP and PLP/DM-20 (Figures 2 and 4), the compound increased the immunoreactivity against PDGFR-α and olig2 (Figures 3 and 5). Anti-MBP antibody reacts with mature oligodendrocytes; anti-PLP antibody reacts with mature oligodendrocytes, OPCs, and oligodendrocyte lineage precursor cells; anti-PDGFR-α antibody reacts with OPCs; and anti-olig2 antibody reacts with OPCs and oligodendrocyte lineage precursor cells [29]. These findings let us to the following interpretation: Cuprizone administration decreases mature oligodendrocytes, and increases OPCs. This interpretation is consistent with the previous report by Kipp et al. that mature oligodendrocytes are vulnerable to cuprizone.

Nobiletin treatment increases the number of oligodendrocyte lineage precursor cells

Nobiletin treatment did not show any obvious effects on mature oligodendrocytes and OPCs in cuprizone-treated mice (Figures 2 and 3). In contrast, nobiletin treatment promoted immunoreactivity against PLP/DM-20 and olig2 (Figures 4 and 5), suggesting that nobiletin increases the number of oligodendrocyte lineage precursor cells.

What are the target cells of nobiletin?

In addition to oligodendroglial lineage cells, there are other constitutive cells such as astroglial and microglial cells as well as neuronal cells in the brain. Nobiletin treatment did not affect neuronal injury induced by cuprizone administration (Figure 8). However, nobiletin slightly reduced astroglial and microglial activation in cuprizone-fed mice (Figures 6 and 7). We could not identify specific cell lineages targeted by nobiletin. Nobiletin might directly promote oligodendrocyte lineage precursor cells, or indirectly affect those precursor cells via inactivation of astroglia and/or microglia in cuprizone-fed mice.

In the present study, we used HMF as a reference for nobiletin because HMF (3,5,6,7,8,3',4'-heptamethoxyflavone) is structurally related to nobiletin (3',4',5,6,7,8-hexamethoxyflavone) and is reported to have several biological functions [21,30,31], HMF did not have a prominent effect on the assays for demyelination and axonal injury as well as glial activation, except for body-weight change (Figure 1). However, HMF showed moderate effects in analyses in which nobiletin showed significant effects, suggesting that HMF has actions similar to nobiletin, although the effect of HMF activity is not as potent. Because both nobiletin and HMF are contained in the peel of citrus fruits [1], administration of the peel of citrus fruits might benefit individuals with demyelination diseases.

Acknowledgements

This work was supported by JSPS KAKENHI Grant number 25461567.

References


