Dietary flavonoids suppress azoxymethane-induced colonic preneoplastic lesions in male C57BL/KsJ-*db/db* mice

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Running title: Dietary flavonoids inhibit the early phase of colon carcinogenesis in *db/db* mice

Key words: flavonoid; ACF; BCAC; colon carcinogenesis; *db/db* mice

Abbreviations: ACF, aberrant crypt foci; AOM, azoxymethane; BCAC, β-catenin accumulated crypt; IGF-1, insulin-like growth factor-1; PCNA, proliferating cell nuclear antigen; C/EBP, CCAAT/enhancer binding protein; PPAR, peroxisome proliferator-activated receptor

Abstract

Obesity is known to be a risk factor for colon carcinogenesis. Although there are several reports on the chemopreventive abilities of dietary flavonoids in chemically induced colon carcinogenesis, those have not been addressed in an obesity-associated carcinogenesis model. In the present study, the effects of 3 flavonoids on modulation of the occurrence of putative preneoplastic lesions, aberrant crypt foci (ACF), and β-catenin accumulated crypts (BCACs) in the development of colon cancer were determined in male *db*/*db* mice with obesity and diabetic phenotypes. Male *db*/*db* mice were given 3 weekly intraperitoneal injections of azoxymethane (AOM) to induce the ACF and BCAC. Each flavonoid (100 ppm), given in the diet throughout the experimental period, significantly reduced the numbers of ACF by 68-91% and BCAC by 64-71%, as well as proliferation activity in the lesions. Clinical chemistry results revealed that the serum levels of leptin and insulin in mice treated with AOM were greater than those in the untreated group. Interestingly, the most pronounced suppression of development of preneoplastic lesions and their proliferation were observed in the quercetin-fed group, in which the serum leptin level was lowered. Furthermore, quercetin-feeding decreased leptin mRNA expression and secretion in differentiated 3T3-L1 mouse adipocytes. These results suggest that the present dietary flavonoids are able to suppress the early phase of colon carcinogenesis in obese mice, partly through inhibition of proliferation activity caused by serum growth factors. Furthermore, they indicate that certain flavonoids may be useful for prevention of colon carcinogenesis in obese humans.

Introduction

Epidemiological studies have shown that obesity is an important cofactor for several types of cancer, including colorectal cancer [1]. Recently, a prospective population based study of about 90000 subjects conducted by the American Cancer Society confirmed that obesity is directly associated with an increased risk of death from colon cancer [2]. In addition, animal studies have also suggested that obesity enhances tumor development [3], while calorie restriction was reported to inhibit a broad range of spontaneous, transplanted, and chemically induced neoplasms [4].

Leptin, a 16-kDa protein encoded by the *ob* gene, was first documented in 1994 as a regulator of body weight gain and energy balance, with its activities displayed in the hypothalamus [5]. It is well known that serum leptin levels are highly elevated in obese individuals [6] and the protein is mainly secreted by white adipocytes [7]. C57BL/KsJ-*db/db* (*db/db*) mice are often used as a genetically altered animal model with the genotypes of obesity and diabetes mellitus [8]. In this mouse strain, a mutation in the cytoplasmic domain of the long form of the leptin receptor (Ob-Rb) results in loss of expression of this isoform [9]. In the absence of Ob-Rb, the mice eat excessively and are already obese at 4 weeks of age. Furthermore, they are also demonstrate hyperleptinemia, hyperinsulinemia, hyperglycemia, and hyperlipidemia, as well as increased levels of cholesterol in plasma [10]. The synthesis of leptin in adipocytes, which may be involved in neoplastic processes, is influenced by insulin, tumor necrosis factor-α, glucocorticoids, reproductive hormones, and prostaglandins [11]. In addition, leptin can act as a growth factor in colonic epithelial cells [12], while it also modulates the proliferation of colonic cryptal cells [13]. Since leptin might be one of the biological

factors involved in the development of colorectal cancer associated with obesity/diabetes, *db/db* mice are quite useful as a model for elucidating the relationship between colon carcinogenesis and obesity/diabetes.

Flavonoids comprise a structurally diverse class of polyphenolic compounds ubiquitously found in plants and produced as a result of plant secondary metabolism [14]. They have several biological effects, such as anti-oxidative and anti-inflammatory activities [15]. We previously reported that chrysin [16], quercetin [17], and nobiletin [18] showed chemopreventive effects toward azoxymethane (AOM)-induced colon carcinogenesis in rats. In addition, administration of green tea polyphenols, including epicatechin, epicatechin gallate, epigallocatechin, and epigallocatechin gallate, resulted in a significant reduction in body weight gain and body fat accumulation in rodents [19, 20]. Furthermore, an *in vitro* study found that certain flavonoids inhibit the growth of 3T3-L1 pre-adipocytes [21]. However, there are known no studies of the effects of flavonoids on obesity-associated carcinogenesis.

In the present study, we first determined the modulatory effects of 6 different flavonoids; flavone, chrysin, apigenin, luteolin, quercetin, and nobiletin (Figure 1A), on leptin secretion from 3T3-L1 cells. Next, we evaluated the effects of dietary chrysin, quercetin, and nobiletin on the occurrence of AOM-induced aberrant crypt foci (ACF) and β-catenin-accumulated crypts (BCACs), putative precursor lesions for colonic adenocarcinoma [22, 23], in *db/db* male mice. We also investigated those 3 flavonoids to determine their effects on clinical chemistry related to the occurrence of colorectal cancer [24]. Since we previously observed high proliferation activities in preneoplastic colonic lesions and non-lesional crypts in *db/db* mice [25], the effects of these flavonoids in regard to proliferation activity in ACF and BCAC were analyzed using an

immunohistochemical methods.

Materials and Methods

Cell culture

3T3-L1 mouse pre-adipocytes were obtained from the American Type Culture Collection (ATCC, Manassas, VA). The cells were maintained in Duibecco's Modified Eagle Medium (DMEM) supplemented with 10% bovine serum (BS), as well as 100 U/ml of penicillin and 100 μg/ml of streptomycin at 37°C in a humidified 5% CO_2 atmosphere.

Intracellular lipid accumulation and leptin secretion

3T3-L1 cells (1 x 10^4/200 μl/well) were seeded into 96-well plates under the growth conditions described above. After reaching confluence, they were incubated for an additional 24 hours (designated as day 0). Then, adipocyte differentiation was induced by treatment with a mixture of methylisobutylxanthine (0.5 mM), dexamethasone (1 μM), and insulin (10 μg/ml), components of an Adipogenesis Assay Kit (Chemicon International, Temecula, CA), in DMEM containing 10% FBS for 48 hours. The medium was then replaced by DMEM containing 10% fetal bovine serum (FBS) and insulin (5 μg/ml), and changed to fresh medium every 2 days, according to a method previously described by Maeda et al. [26], with some modifications. On day 2, each flavonoid (10, 50, and 100 μM) was dissolved in dimethyl sulfoxide (DMSO), then added to DMEM containing FBS and insulin. The final concentration of DMSO was 0.1% (*v*/*v*), which was found to have no effect on cell growth (data not shown). After 12

days, the medium was collected and subjected to ELISA to determine the levels of leptin. The cells were stained with the Oil Red-O component of an Adipogenesis Assay Kit, according to the manufacturer's instructions. Stained oil droplets in 3T3-L1 cells were extracted with dye extraction solution and absorbance of the extracts was measured at 490 nm.

Mice, diet, and carcinogens

Male *db/db* mice were obtained from Jackson Laboratories (Bar Harbor, ME) at the age of 4 weeks and maintained at the Kanazawa Medical University Animal Facility according to the Institutional Animal Care Guidelines. On arrival, all mice were randomized and transferred to plastic cages (2 or 3 mice/cage), and given free access to drinking water and a pelleted basal diet (CRF-1, Oriental Yeast Co., Tokyo, Japan), under controlled conditions of humidity (50 ± 10%), light (12/12 hour light/dark cycle), and temperature (23 ± 2ºC). All mice were quarantined for 1 week before starting the experiment. Nobiletin (>98% purity) was obtained from Nard Chemicals (Hyogo, Japan), while other flavonoids were purchased from WAKO Pure Chemicals (Osaka, Japan). Experimental diets were prepared by mixing each flavonoid (100 ppm) separately with powdered CRF-1 every week during the study. Azoxymethane (AOM), a colonic carcinogen, was purchased from Sigma Chemical Co. (St. Louis, MO).

Experimental procedures

All mice were divided into the following 8 experimental and control groups: AOM alone (group 1, n=9); AOM + chrysin (group 2, n=10); AOM + quercetin (group 3, n=10); AOM + nobiletin (group 4, n=10); chrysin alone (group 5, n=5); quercetin alone

(group 6, n=5); nobiletin alone (group 7, n=5); and untreated (group 8, n=5). The mice in groups 1-4 were given 3 weekly intraperitoneal injections of AOM (15 mg/kg body weight), while those in groups 2 through 7 were fed the experimental diets containing the flavonoids (100 ppm) for the entire 10-week experimental period. Groups 1 and 2 were given the basal diet without flavonoids during the study. At week 10, all mice were euthanized after overnight fasting by an intraperitoneal injection of sodium pentobarbital (1 mg/kg body weight). Blood samples were taken from the portal vein before the mice were killed. A complete necropsy was done, and all organs, including the colon, were removed, with the liver, kidneys, pancreas, and epididymal adipose tissue weighted.

Counting colonic ACF and BCAC

The numbers of ACF and BCAC were determined according to standard procedures described previously [27, 28]. Briefly, the colons were cut, placed on filter paper with the mucosal surface up, and fixed in 10% buffered formalin for at least 24 hours. The fixed colons were stained with methylene blue (0.5% in distilled water) for 20 seconds, dipped in distilled water, and placed on microscope slides to count the number and determine the size of ACF. Rectal mucosa (2.0 cm from the anus) was embedded in paraffin to identify intramucosal lesions, considered to be BCAC. A total of 20 serial sections (4 μm thick each) per rectum were prepared using an *en face* method [28]. For each mouse, 2 serial sections were used to analyze the BCAC. The numbers of BCAC in histological sections stained with β-catenin were counted and are expressed as the number of BCAC per cm^2 of mucosa.

Immunohistochemistry of β-catenin and PCNA

Immunohistochemistry for β-catenin was performed using sections from the distal colon segments with a labeled streptavidin-biotin method (LSAB Kit; Dako, Glostrup, Denmark) and microwave accentuation. Paraffin-embedded sections were heated for 30 minutes at 65ºC, deparaffinized in xylene, and rehydrated through a graded series of alcohol at room temperature. A 0.05-M Tris-HCl buffer (pH 7.6) was used to prepare the solutions and for washing between the steps. The sections were treated for 40 minutes at room temperature with 2% bovine serum albumin and incubated overnight at 4ºC with the primary antibody against β-catenin protein (diluted 1:1000, BD Transduction Laboratories, Lexington, KY). Horseradish peroxidase activity was visualized by treatment with H_2O_2 and diaminobenzidine for 5 minutes. Negative-control sections were immunostained without the primary antibody. Immunoreactivity to determine the presence of BCAC was regarded as positive if apparent staining was detected in the cytoplasm and/or nuclei.

For PCNA immunohistochemistry, formalin-fixed, paraffin-embedded distal colon sections were subjected to deparaffinization and dehydration prior to quenching of endogenous peroxidase activity (1.5% H_2O_2 in methanol for 20 minutes). An antigen-unmasking step was done by placing the slides in a pressure cooker containing 0.01 M sodium citrate (pH 6.0) for 10 minutes. The sections were incubated for 60 minutes with the primary mouse anti-rat PCNA monoclonal antibody (Clone PC-10, DakoCytomation) at a dilution of 1:1500 in 10% goat serum. A secondary antibody, biotinylated goat anti-mouse IgG (Vector Laboratories, Burlingame, CA), was then applied for 30 minutes in a 1:500 dilution. Slides were processed with ABC reagent from a Vectastain Elite kit (Vector Laboratories) using DAB as the substrate. Using

distal colonic mucosa without lesions from 5 mice in each group, 20 fields were randomly selected from each slide and analyzed at 400x magnification. PCNA-positive cell nuclei were determined in 10 ACF and 10 BCAC each from groups 1 through 4. Cells stained positive for PCNA were scored and expressed as a percentage of total cells in each lesion.

Clinical chemistry

The collected blood samples were used for clinical chemistry. Leptin (Quantikine Mouse leptin, ELISA/Assay Kit, R&D Systems Inc.), adiponectin (Mouse/Rat adiponectin ELISA kit, Otsuka Pharmaceutical Co., Ltd., Tokyo, Japan), insulin-like growth factor (IGF)-1 (Quantikine Mouse IGF-1, ELISA/Assay Kit, R&D Systems Inc.), insulin (Insulin measurement kit, Morinaga Institute of Biological Science), triglycerides (Triglyceride E-test, Wako Pure Chemical Industries), cholesterol (Cholesterol E-test, Wako Pure Chemical Industries), and glucose (Glucose CII-test Wako, Wako Pure Chemical Industries) levels were measured. Serum samples without dilution were used for determining insulin, triglycerides, cholesterol, and glucose, while those diluted 100-, 10201-, and 500-fold were used for determining the levels of leptin, adiponectin, and IGF-1, respectively.

Western blotting

3T3-L1 cells (1×10^5/3 ml/dish) were seeded into 35-mm dishes following treatment with quercetin or nobiletin, then washed twice with PBS and lysed in lysis buffer [10-nM Tris (pH 7.4), 1% sodium dodecyl sulfate (SDS), 1-mM sodium metavanadate (V)], and centrifuged at 3200 x g for 5 minutes. Denatured proteins (40

μg) were separated using SDS-PAGE on a 10% polyacrylamide gel and then transferred onto Immobilon-P membranes (Millipore, Billerica, MA). After blocking with Block Ace (Snow Brand Milk Products, Tokyo, Japan) for 1 hour, the membranes were reacted with the appropriate specific primary antibody (1:1000), followed by the corresponding HRP-conjugated secondary antibody (1:1000). The blots were developed using ECL Western blotting detection reagents. Antibodies directed against Pi- mitogen-activated protein kinase/ extracellular signaling-regulated kinase (MEK)1/2 (Ser217/221), Pi-extracellular signaling-regulated kinase (ERK)1/2 (Thr202/Tyr204), Pi-mammalian target of rapamycin (mTOR) (Ser2448), Pi-S6 (Ser240/244), and Pi-eukaryotic initiation factor 4B (eIF4B) (Ser422), as well as horseradish peroxidase (HRP)-conjugated anti-rabbit antibody, were obtained from Cell Signaling Technology (Beverly, MA).

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from the cells under the same conditions used for Western blotting using TRIzol reagent, according to the manufacturer's instructions. cDNA was synthesized using 1 μg of total RNA and an RNA PCR Kit (AMV). PCR amplification was performed using a thermal cycler (PTC-100™, MJ Research, Watertown, MA), and conducted with each sense and antisense primer. The primer sequences and PCR conditions are listed in Table 1. A cyclophilin transcript served as the internal control. PCR products were subjected to electrophoresis through 3% agarose gels and stained with SYBR® Gold.

Statistical analysis

Where applicable, data were analyzed using a Tukey-Kramer multiple comparison

test (GraphPad InStat version 3.05, GraphPad Software, San Diego, CA), Fisher's exact probability test, and Student's *t*-test (two-sided), with $P < 0.05$ as the criterion of significance.

Results

Modulatory effects of flavonoids on Oil Red-O staining and leptin secretion

Adipocyte differentiation was induced by treatment with a mixture of 3-isobutyl-1-methylxanthine, dexamethasone, and insulin in DMEM containing 10% FBS for 48 hours, after which differentiated 3T3-L1 adipocytes were separately treated with the 6 flavonoids (10 μM) or the vehicle for 12 days to determine their effects on intracellular lipid accumulation and leptin secretion. Differentiated 3T3-L1 cells were notably loaded with lipid, as detected by Oil Red-O staining, whereas none of the flavonoids had noticeable effects (Figure 1B). On the other hand, quercetin and nobiletin significantly reduced leptin secretion ($P < 0.01$, Figure 1B), with the reduction by nobiletin remarkable.

General observations of in vivo *experiment*

We selected 3 of the flavonoids, chrysin, quercetin, and nobiletin, for the *in vivo* experiment based on their chemopreventive efficacy previously shown in colon carcinogenesis models [16-18], together with the present data regarding leptin secretion (Figure 1B). To investigate the effects of these flavonoids on the early phase of obesity-related carcinogenesis and serum levels of leptin, we performed short-term *in vivo* assays using histological biomarkers, ACF and BCAC, in the *db/db* mice. During

the study, dietary feeding with the flavonoids did not cause clinical symptoms, including toxicity (data not shown). Food consumption did not significantly differ among the groups. In addition, we did not observe significant changes in regard to body weight gain, epididymal fat weight, or colon length (Table 2). In contrast, the pancreas weight was significantly increased in group 3 (AOM + quercetin, $P < 0.05$) when compared with group 1 (AOM alone).

Frequency of preneoplastic lesions (ACF and BCAC) and PCNA-labeling index

Histological examinations revealed no pathological lesions in any organs except the colon. Table 3 summarizes data for colonic ACF and BCAC formation. All mice in groups 1 through 4, which received AOM with or without a flavonoid, developed ACF. In groups 5 through 8, there were no microscopically observable changes, including ACF and BCAC, in our examinations of colonic morphology. The mean number (± SD) of ACF per colon in group 1 was 25.6 ± 8.9. Dietary administration of chrysin, quercetin, and nobiletin significantly reduced ACF incidence by 91%, 89%, and 68%, respectively ($P < 0.001$ versus group 1), while we also saw a significant reduction (85-100% inhibition, $P < 0.001$) in the numbers of large ACF containing 4 or more aberrant crypts, which are known to be well-correlated with the incidence of colonic adenocarcinoma [29-31], when compared with group 1 (12.5 ± 9.7). Also, large ACF did not develop in the colons of mice in group 3 (AOM + quercetin). As shown in Table 3, the numbers of BCAC per cm^2 in groups 2 (65% inhibition, $P < 0.001$), 3 (71% inhibition, $P < 0.001$), and 4 (64% inhibition, $P < 0.001$) were significantly fewer than that in group 1 (12.5 ± 9.7).

As summarized in Table 3, the mean PCNA-labeling indices of BCAC were greater

than those of ACF in groups 1 through 4. ACF indices in the mice that received dietary flavonoids (28% reduction by chrysin, $P < 0.05$; 30% reduction by quercetin, $P < 0.001$; and 20% reduction by nobiletin, $P < 0.05$) were significantly smaller than that of mice that received AOM alone (group 1, 39.1 ± 5.2). Also, feeding with chrysin (26% reduction, $P < 0.001$), quercetin (41% reduction, $P < 0.001$), and nobiletin (19% reduction, $P < 0.001$) significantly lowered index for BCAC when compared with group 1 (48.8 ± 6.0).

Serum levels of leptin, adiponectin, IGF-1, insulin, triglyceride, cholesterol, and glucose

Serum profile data are listed in Table 4. The serum concentration of leptin in group 1 was significantly greater (67% increase) than that in group 8 (untreated, $P < 0.05$), while dietary administration of quercetin significantly decreased the serum leptin level by 31% ($P < 0.05$) when compared with group 1. Chrysin feeding also decreased the serum leptin level (11% decrease), though it was not significant. Dietary nobiletin did not have an effect on the level of leptin in serum. The serum level of adiponectin in group 1 was significantly higher than that in group 8 ($P < 0.05$). However, dietary administration with the flavonoids (groups 2 through 4) did not have any effects on serum adiponectin levels. The serum level of IGF-1 in group 1 was comparable to that in group 8. Dietary flavonoids (groups 2 through 4) decreased the level, though the differences were not significant. Treatment with the different flavonoids did not have a significant effect on the increase of insulin in serum caused by AOM administration. There were no marked differences in regard to the levels of triglyceride, cholesterol, and glucose among the groups.

Quercetin inhibition of leptin mRNA expression

The Akt/mTOR signaling pathway, including eIF4B, is considered to play a crucial role as a regulator of adipogenesis [32] and leptin secretion [33]. Our previous study indicated that nobiletin decreased the phosphorylation state of eIF4B partly through inactivation of MEK/ERK [34]. Therefore, we investigated the effects of quercetin on the mTOR signaling pathway, because it exhibited a profound suppressive effect on leptin production *in vivo*. Unexpectedly, the phosphorylation state of Akt, ERK, eIF4B, and S6 was increased in quercetin-treated cells, while nobiletin abolished the increase, as previously reported [34] (Figure 2A). The differing effects obtained by treatments with quercetin and nobiletin led us to examine whether quercetin has an effect on the expression of *leptin* mRNA. It is well known that CCAAT/enhancer binding protein (C/EBP)α is an important transcription factor of leptin. Peroxisome proliferator-activated receptor (PPAR)γ plays an important role in adipocyte differentiation, though several PPARγ agonists, including thiazolidinediones, were shown to repress leptin gene expression in adipocytes [35]. In the present study, quercetin and troglitazone significantly reduced leptin mRNA expression, while they did not reduce the level of C/EBPα expression (Figure 2B). Of interest, inverse correlations for mRNA expression between leptin and PPARγ were observed for quercetin and troglitazone (Figure 2B).

Discussion

Our results clearly indicate that dietary administration of the flavonoids chrysin, quercetin, and nobiletin leads to suppression of the development of precursor lesions

(ACF and BCAC) induced by AOM in obese mice, in part by reducing the proliferation activity of the lesions. The order of chemopreventive ability in the present findings was quercetin > chrysin > nobiletin, which is consistent with our previous reports [16, 25]. Interestingly, the tested flavonoids, particularly quercetin, lowered the levels of growth factors in serum, especially leptin.

The high susceptibility of *db/db* mice to colon carcinogenesis might be related to high proliferation activities of normal crypts and pre-neoplasms. Obesity itself along with high levels of serum cholesterol, triglycerides, glucose, insulin, and leptin have been suggested to explain that elevated susceptibility [25]. Recently, leptin was reported to act as a mitogenic factor in cultured human colon cancer cells [36] and mouse colon carcinogenesis [34]. Hyperinsulinemia has also been hypothesized to be an underlying factor linking obesity, type 2 diabetes mellitus, and colon tumorigenesis [37]. As for the mechanism of action, insulin resistance is associated with hyperinsulinemia and increased levels of growth factors including IGF-1, which may promote colon carcinogenesis through their effects on colonic cryptal cell kinetics [38]. In this context, a recent report showing that leptin interacts with IGFs to promote the survival and expansion of *APC* deficient colonic epithelial cells, but not of those expressing wild-type *APC*, is interesting [39].

In the present study, the tested dietary flavonoids did not have effects on body weight gain, epididymal fat pad weight, or food intake. However, quercetin markedly lowered serum leptin and insulin concentrations, which were elevated by injection of AOM. Importantly, treatment with each flavonoid alone (groups 5 through 7) did not have an influence on the level of leptin as compared with the control group (Table 4). However, feeding with the flavonoids decreased the serum levels of IGF-1. Although, it

is not clear how each of the flavonoids in this study reduces the serum concentrations of these growth factors, such reduction may lead to suppression of proliferation activity in preneoplastic lesions. Quercetin modulates several signal transduction pathways involving MEK/ERK, which are associated with proliferation of several types of cancer cells [40], while nobiletin inhibits the proliferation of a variety of human cancer cell lines, partly due to induction of G1 cell cycle arrest [41]. In addition, chrysin induces G2/M cell-cycle arrest in human colon carcinoma SW480 cells [42]. Together with our findings, it is suggested that the reduction of proliferation in preneoplastic lesions (ACF and BCAC) caused by each flavonoid is related to induction of cell-cycle arrest in aberrant cells.

Leptin release is influenced by the amount of *leptin* mRNA expression in adipocytes. That release is regulated by not only the mTOR signaling pathway, but also *leptin* mRNA transcription, which is activated during adipocyte differentiation. C/EBPα, which belongs to the C/EBP family of transcription factors, plays a central role in the control of energy homeostasis and is expressed during the terminal phase of differentiation immediately prior to the expression of many adipose-specific genes [43]. The PPAR family of proteins also plays an important role in adipocyte differentiation [44]. Taken together, logical candidate transactivators of the leptin promoter include C/EBPα and PPARγ. In fact, the proximal promoter of the *leptin* gene contains a functional C/EBP-binding site, which mediates activation of the leptin promoter by co-transfected C/EBPα in 3T3-L1 pre-adipocytes. However, it is surprising that PPARγ agonists, *e.g.*, thiazolidinediones, were found to down-regulate leptin mRNA levels [35]. Furthermore, since the putative PPARγ response element in the leptin promoter is not involved in negative regulation, it has been hypothesized that PPARγ functionally

antagonizes C/EBPα to decrease transcription in response to thiazolidinediones [45].

In the present study, quercetin significantly reduced the mRNA expression of leptin, but not that of C/EBPα, while it up-regulated PPARγ mRNA expression, as did troglitazone (Figure 2B). Consistent with our findings, Fang *et al*. recently reported that quercetin inhibited insulin-stimulated glucose uptake in 3T3-L1 adipocytes by acting as a potential agonist of PPARγ [46]. Furthermore, their competitive ligand-binding assay confirmed that quercetin competes with rosiglitazone in the same binding pocket site as PPARγ. Thus, it is likely that quercetin affects leptin secretion from white adipose tissue in *db/db* mice by acting as a PPARγ agonist. We previously observed that nobiletin suppresses hyperleptinemia in ICR mice given AOM and dextran sulfate sodium [34]. However, no effects of nobiletin were found in the present *db/db* mice that received AOM (Table 4). Since nobiletin suppresses leptin secretion partly by repression of the insulin signaling pathway in 3T3-L1 cells, the differences between the biochemical effects induced by quercetin and nobiletin may explain why the former and not the latter suppresses serum leptin levels in AOM-treated *db/db* mice.

In summary, the present results provide additional evidence that certain daietary flavonoids are potent to inhibit the early phase of colon carcinogenesis in genetically altered obese mice, partly through reduction of proliferation. Those effects were also shown to be related to lowered serum levels of leptin, insulin, and IGF-1. This study investigated the effects of selected flavonoids on colonic pre-malignancy by focusing on lowered levels of serum growth factors, thus additional studies of the exact mechanisms are needed for development of prevention and treatment strategies for obesity-related colonic malignancies.

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Figure legends

Fig. 1. (A) Chemical structures of the studied flavonoids. (B) Modulatory effects of the flavonoids on leptin secretion from 3T3-L1 cells. 3T3-L1 mouse pre-adipocytes were induced to adipocyte differentiation with a mixture of methylisobutylxanthine (0.5 mM), dexamethasone (1 μM), and insulin (10 μg/ml) in DMEM containing 10% FBS for 48 hours. Differentiated 3T3-L1 cells were treated with DMSO alone or various concentrations of nobiletin for 12 days, then the supernatants were removed for measurements of leptin. The cells were washed twice with PBS and stained with Oil Red-O. Stained cells were viewed under an inverted microscope (Leica Microsystems, Tokyo, Japan) (original magnification 1: 200). Leptin secretion was quantified by ELISA. Values are shown as the mean ± SD. Statistical analysis was performed using Student's *t*-test: $P<0.05$.

Fig. 2. (A) Modulatory effects of quercetin, nobiletin, and rapamycin on the Akt/mTOR signaling pathway in differentiated 3T3-L1 cells. (B) Modulatory effects of quercetin and troglitazone on the expression of transcription factors in differentiated 3T3-L1 cells. 3T3-L1 mouse pre-adipocytes (1×10^5 cells in 35 mm dish) were induced to adipocyte differentiation with a mixture of methylisobutylxanthine (0.5 mM), dexamethasone (1 μM), and insulin (10 μg/ml) in DMEM containing 10% FBS for 48 hours. Differentiated 3T3-L1 cells were treated with DMSO alone, quercetin, nobiletin, or rapamycin for 12 days. The cells were washed twice with PBS and analyzed using western blotting and

RT-PCR methods. Rap, rapamycin.

Table 1. List of primer sequences for RT-PCR

Gene	Primer	Sequence (5'-3')	Product size (bp)	Cycles	Denaturation Annealing (°C, sec) Extention
Leptin	**Forward** **Reverse**	**CCA AAA CCC TCA TCA AGA CC** **GTC CAA CTG TTG AAG AAT GTC CC**	**395**	**37**	**95, 45** **57, 45** **72, 45**
C/EBP α	**Forward** **Reverse**	**AGG TGC TGG AGT TGA CCA GT** **CAG CCT AGA GAT CCA GCG AC**	**238**	**25**	**94, 60** **54, 60** **72, 30**
PPAR γ	**Forward** **Reverse**	**GGT GAA ACT CTG GGA GAT TC** **CAA CCA TTG GGT CAG CTC TT**	**268**	**30**	**94, 40** **58, 40** **72, 50**
Cyclophilin	**Forward** **Reverse**	**TTG GGT CGC GTC TCG TTC GA** **GCC AGG ACC TGT ATG CTT CA**	**240**	**20**	**95, 30** **50, 30** **72, 60**

Table 2. General observations in male *db/db* mice

Group No.	Treatment	Body weight (g)	Epididymal fat weight (g)	Pancreatic weights (g/100g body weight)	Length of large bowel (cm)	Food intake (g/day)
1	**AOM**	**48.6 ± 3.3**	**2.42 ± 0.22**	**0.530 ± 0.078**	**11.8 ± 0.4**	**6.30 ± 1.66**
2	**AOM+ 100 ppm CHR**	**47.7 ± 5.3**	**2.31 ± 0.28**	**0.661 ± 0.127**	**11.9 ± 0.4**	**5.70 ± 1.88**
3	**AOM+ 100 ppm QER**	**48.8 ± 2.1**	**2.45 ± 0.31**	**0.666 ± 0.083[a]**	**12.1 ± 0.3**	**6.26 ± 1.77**
4	**AOM+ 100 ppm NOB**	**53.3 ± 3.3**	**2.47 ± 0.40**	**0.555 ± 0.013**	**12.1 ± 0.4**	**5.83 ± 1.92**
5	**100 ppm CHR**	**43.3 ± 9.2**	**2.42 ± 0.35**	**0.802 ± 0.205[a]**	**11.6 ± 0.7**	**6.86 ± 2.03**
6	**100 ppm QER**	**47.9 ± 7.2**	**2.63 ± 0.35**	**0.750 ± 0.209**	**11.6 ± 0.4**	**6.93 ± 2.39**
7	**100 ppm NOB**	**46.9 ± 8.0**	**2.38 ± 0.36**	**0.740 ± 0.057[a]**	**11.9 ± 0.5**	**6.59 ± 1.94**
8	**None**	**48.7 ± 9.5**	**2.35 ± 0.46**	**0.686 ± 0.162**	**11.5 ± 1.0**	**6.48 ± 2.07**

Data are shown as the mean ± SD.

[a] Significantly different in Student's *t*-test, $P < 0.05$ vs group 1.

Table 3. Inhibitory effects of flavonoids on AOM-induced preneoplastic lesion formation and PCNA-index

Group No.	Treatment	Total no. of ACF/colon	Total no. of ACF containing 4 or more ACs/colon	Total no. of BCAC/cm^2	PCNA-labeling index (%)	
					ACF	BCAC
1	AOM	25.6 ± 8.9	4.6 ± 2.4	12.5 ± 9.7	39.1 ± 5.2	48.8 ± 6.0
2	AOM+ 100 ppm CHR	2.3 ± 2.2[a]	0.1 ± 0.3[a]	4.4 ± 3.1[b]	30.5 ± 6.2[b]	35.9 ± 6.3[c]
3	AOM+ 100 ppm QER	2.8 ± 2.0[a]	0	3.6 ± 2.6[c]	27.5 ± 6.3[a]	28.7 ± 9.2[a]
4	AOM+ 100 ppm NOB	8.3 ± 4.9[a]	0.7 ± 1.3[a]	4.5 ± 3.2[b]	31.4 ± 6.2[b]	39.5 ± 6.9[b]
5	100 ppm CHR	0	0	0	0	0
6	100 ppm QER	0	0	0	0	0
7	100 ppm NOB	0	0	0	0	0
8	None	0	0	0	0	0

Data are shown as the mean ± SD.

[a] Significantly different in one-way ANOVA with Bonferroni correction test, $P < 0.001$ vs group 1.

[b] Significantly different in one-way ANOVA with Bonferroni correction test, $P < 0.05$ vs group 1.

[c] Significantly different in one-way ANOVA with Bonferroni correction test, $P < 0.01$ vs group 1.

Table 4. Serum profiles in each groups of male *db/db* mice

Group No.	Treatment	Leptin (ng/ml)	Adiponectin (μg/ml)	IGF-1 (ng/ml)	Insulin (ng/ml)	Triglyceride (mg/dl)	Cholesterol (mg/dl)	Glucose (mg/dl)
1	**AOM**	**181.4 ± 15.6[a]**	**8.0 ± 0.3[a]**	**467.5 ± 93.3**	**4.2 ± 2.6**	**204.0 ± 27.9**	**176.4 ± 13.0**	**791.6 ± 101.4**
2	**AOM+ 100 ppm CHR**	**160.9 ± 39.5**	**8.1 ± 0.2**	**397.3 ± 61.8**	**4.6 ± 1.9**	**183.1 ± 37.2**	**169.5 ± 13.8**	**843.9 ± 78.4**
3	**AOM+ 100 ppm QER**	**125.4 ± 19.3[b]**	**7.8 ± 0.7**	**434.2 ± 53.2**	**2.8 ± 0.8**	**227.4 ± 44.2**	**175.8 ± 21.2**	**882.0 ± 20.6**
4	**AOM+ 100 ppm NOB**	**179.2 ± 44.3**	**8.5 ± 0.8**	**412.2 ± 49.8**	**5.4 ± 2.2**	**248.8 ± 66.7**	**187.0 ± 21.0**	**817.4 ± 59.4**
5	**100 ppm CHR**	**102.3 ± 51.0**	**7.0 ± 0.8**	**538.2 ± 175.8**	**1.5 ± 1.0**	**277.3 ± 94.7**	**151.9 ± 36.2**	**1013.4 ± 79.0**
6	**100 ppm QER**	**100.8 ± 44.9**	**6.5 ± 0.3**	**495.8 ± 95.4**	**2.0 ± 1.3**	**250.4 ± 61.8**	**171.5 ± 28.3**	**966.8 ± 94.0**
7	**100 ppm NOB**	**102.6 ± 35.9**	**6.9 ± 0.7**	**528.1 ± 114.0**	**2.7 ± 2.9**	**243.7 ± 17.5**	**168.0 ± 28.8**	**886.7 ± 102.5**
8	**None**	**108.8 ± 36.8**	**6.4 ± 0.3**	**473.0 ± 35.7**	**2.8 ± 1.7**	**275.0 ± 15.9**	**179.0 ± 24.8**	**1068.2 ± 27.2**

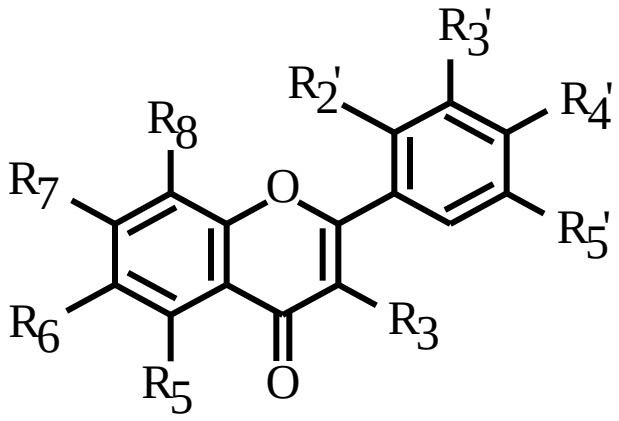
Data are shown as the mean ± SD.

[a] Significantly different in Student's *t*-test, $P < 0.05$ vs group 8.

[b] Significantly different in Student's *t*-test, $P < 0.05$ vs group 1.

Fig. 1 (Miyamoto et al.)

A



	R_5	R_6	R_7	R_8	R_3	R_2'	R_3'	R_4'	R_5'
Flavone	H	H	H	H	H	H	H	H	H
Chrysin	OH	H	OH	H	H	H	H	H	H
Apigenin	OH	H	OH	H	H	H	H	OH	H
Luteolin	OH	H	OH	H	H	H	OH	OH	H
Quercetin	OH	H	OH	H	OH	H	OH	OH	H
Nobiletin	OMe	OMe	OMe	OMe	H	H	OMe	OMe	H

B

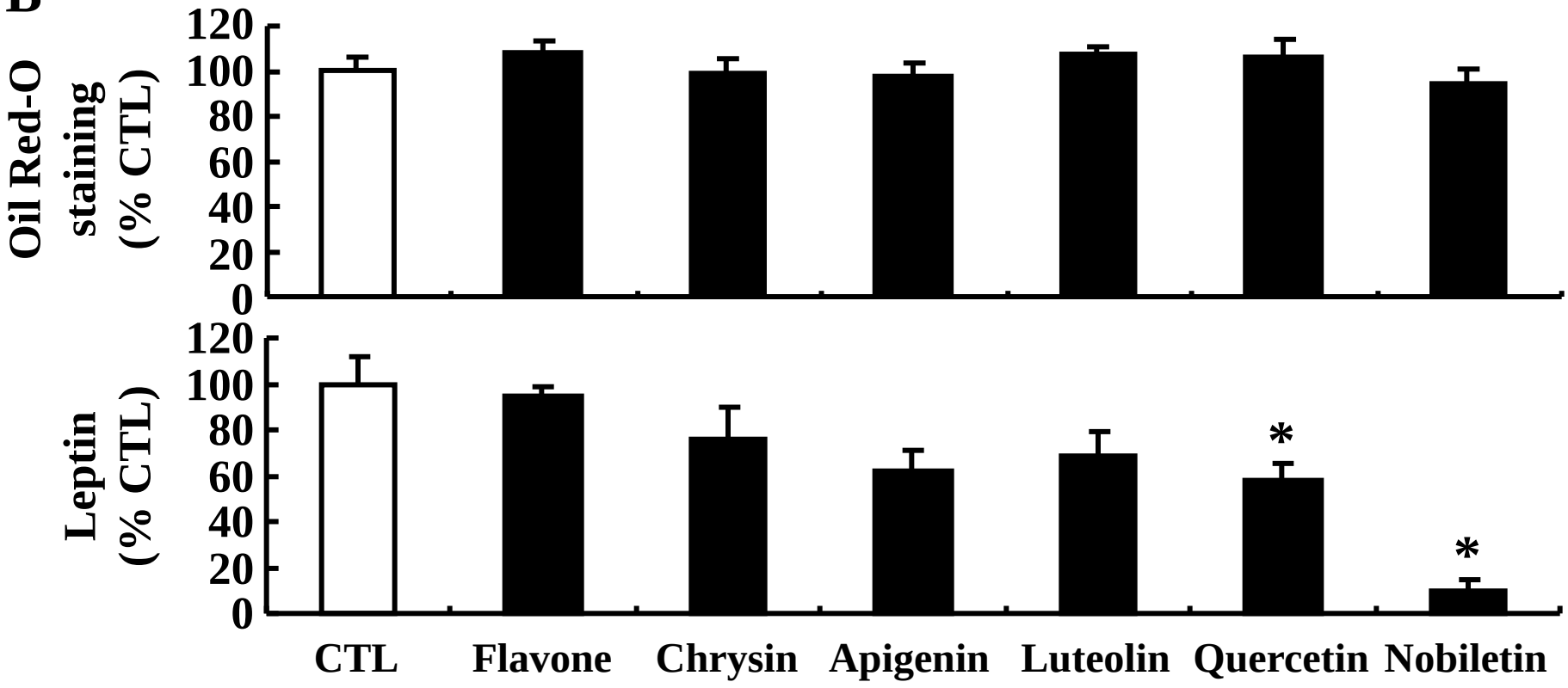


Fig. 2 (Miyamoto et al.)