Orally Administered Rutin Inhibits the Gene Expression of Th2 Cytokines in the Gut and Lung in Aged Mice

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ABSTRACT. Rutin is one of the flavonoids derived from plants such as buckwheat and is well known as a powerful antioxidant. To determine whether dietary rutin could modulate mucosal immunity, we examined the gene expression of Th1/Th2 cytokines and the receptors in the gut and lung. Aged (18 months old, 18 M) C3H/HeN female mice were orally administered rutin for 10 days. The small intestine and lung were taken and analyzed by real-time PCR for gene expression. Interleukin (IL)-13 and IL-13R α 2 gene expression was significantly low ($P < 0.05$ respectively) in the small intestine of aged rutin-fed mice. Meanwhile, there was no change in interferon γ gene expression between control and rutin-fed mice. IL-13 gene expression was also downregulated in the lung. To examine the mechanism of the inhibitory effect of rutin on Th2 cytokines in aged mice, intestinal nitric oxide synthase (NOS) expression was evaluated. Rutin inhibited inducible NOS (NOS2) gene expression, but not neuronal NOS and endothelial NOS. Gene analysis of cells collected from the small intestine by laser capture dissection revealed that NOS2 expression was significantly inhibited in crypt regions. Thus, rutin might be effective against a Th2-dominant profile through NOS2 inhibition in aged mice.

KEY WORDS: aging, mouse, rutin, small intestine, Th2 cytokine.

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T helper (Th) cells develop into different subsets with different profiles of cytokine production [1, 21, 27–29, 35]. The balance of these different immune systems controls homeostasis; therefore, a cytokine imbalance may be associated with several diseases. Th2 cytokines, such as IL-4, IL-9 and IL-13, play an important role in the development of allergic diseases, including asthma and atopic dermatitis in particular [32]. A significant increase in the incidence of inflammatory disease and allergy in industrial nations has become a serious problem. Therefore, it is necessary to understand the regulation of the polarization of the host defense.

Nitric oxide (NO) plays an important role in Th cell differentiation [11, 30]. NO production in response to activating signals, such as proinflammatory cytokines or bacterial products, is catalyzed by the enzyme NO synthases (NOSs) and largely controlled by activation of the transcriptional factor nuclear factor (NF)- κ B. It is well known that NF- κ B regulates the expression of a large number of target genes involved in the immune and inflammatory response and is constitutively activated in an animal model of aging [33]. Therefore, the compounds that regulate NO production or NF- κ B activation are of interest for disease control.

Flavonoids are powerful antioxidants that have several well-described biological activities, such as inhibiting platelet aggregation, scavenging superoxide and vasorelaxant effects. Several flavonoids have anti-inflammatory proper-

ties via inhibition of NO production [8, 20] or NF- κ B activation [31]. Furthermore, most flavonoids inhibit histamine release, so they are excellent candidates for clinical studies to treat or prevent allergic diseases. Previous studies have already reported that luteolin, fisetin and apigenin inhibited IL-4 and IL-13 production *in vitro* [10]. These Th2 cytokines are very important for host defense against parasite and allergic disease.

Rutin is one of the flavonoids widely found in various plants and foods, especially in buckwheat. However, the effects of rutin on Th2 cytokine modulation and receptor expression *in vivo* are unclear. Since flavonoids are ingested orally through food or supplements, we are interested to know whether rutin exhibits effects in the mucosa of the gut as well as the lung. In this study, we investigated Th1 and Th2 cytokine gene expression, their receptors as well as NOSs in aged mice to verify whether dietary rutin could modulate immune factors *in vivo*. It is well known that the immune system changes and shows functional depression during aging, but there are few reports demonstrating the effects of flavonoids in geriatric models. A number of studies of age-related changes in the immune system have reported decreased immune function such as a proliferative response of T cells to antigen or mitogen stimuli [15, 36] or antibody production in response to vaccination or infection in an elderly population [6, 16]. Our previous report also showed that the Th2 response against nematode infection was down regulated in aged mice [34]. Oxidative and/or nitrosative stress may be one of the mechanisms responsible for the decline in immune function; therefore, analysis of flavonoids might be more useful in models of aging. In this report, we examined the effects of rutin

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administration in 18-month-old (18 M) mice to identify whether rutin contributes to improvements in age-related immune dysfunction, including cytokine imbalance.

MATERIALS AND METHODS

Mice: Female C3H/HeN mice were purchased from Japan SLC, Inc. (Shizuoka, Japan) and used throughout the experiments. The animals were housed under conventional conditions, given food and water *ad libitum* and were kept on a 12:12-hr light:dark cycle. The animals' environment was maintained at $22 \pm 1.5^\circ\text{C}$ with a relative humidity of $55 \pm 5\%$. The experimental protocol was approved by the institutional ethics commission for animal research. Rutin (Wako Pure Chemical Industries, Osaka, Japan) was dissolved in distilled water, and the suspension (6 mg/25 g body weight) was orally administered to the mice once a day for 10 days using a rounded gavage tube. Control mice were administered saline orally during the same period. On the day following the final oral administration, mice were sacrificed by cervical dislocation, and the small intestine, mesenteric lymph nodes, colon and lungs were collected and frozen in -80°C until use.

Gene expression analysis: RNA extraction, reverse transcription and real-time PCR: Total RNA extraction from whole tissue was performed using TRIzol (Life Technologies, Inc., Frederick, MD, U.S.A.) according to the manufacturer's instructions. RNA concentration was measured with a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE, U.S.A.), and cDNA was synthesized with random primers and SuperScript II (Life Technologies, Inc.). Primer sequences for IL-4, IL-13, STAT6, IL-4R α , IL-13R α 1, IL-13R α 2 and IFN γ have previously been described [25]. IL-6, TNF- α , neuronal NOS (NOS1), inducible NOS (NOS2) and endothelial NOS (NOS3) were designed using Primer Express software (Applied Biosystems, Foster City, CA, U.S.A.) with sequences obtained from GenBank as follows: 5'-TCCATCCAGTTGCTTCTTG-3' (sense) and 5'-TTTCT-CATTTCCACGATTTCCC-3' (antisense) for IL-6, 5'-CCCACGTCGTAGCAAACC-3' (sense), and 5'-GCAGC-CTTGTCCTTGAA-3' (antisense) for TNF- α , 5'-TGTC-CTTAAAGCCATCCAGC-3' (sense) and 5'-CGGTTGT CATCCCTCAGC-3' (antisense) for NOS1, 5'-CGGAGC-CTTAGACCTCAACA-3' (sense) and 5'-CCCTCGAAG-GTGAGCTGAAC-3' (antisense) for NOS2 and 5'-GCTG GTACATGAGTTCAGAGATTG-3' (sense) and 5'-TTC-CACCGCTGCCTTGTC-3' (antisense) for NOS3.

Real-time PCR was performed using the Brilliant SYBR Green QPCR Master Mix (Stratagene, La Jolla, CA, U.S.A.) with an MX3000P system (Stratagene). The amplification conditions were as follows: 95°C for 10 min, 40–50 cycles of 95°C for 30 sec, 60°C for 1 min and 72°C for 1 min. Fluorescence signals measured during amplification were processed after-amplification. Ribosomal RNA primers were utilized, and all data were normalized to constitutive ribosomal RNA values. Quantitative differences between groups

were calculated according to the manufacturer's instructions (Applied Biosystems).

Preparation of frozen blocks and sectioning: Small pieces of the small intestine were slit longitudinally, laid flat with the mucosal surface down, rolled around a wood stick (Swissroll) and embedded in Tissue-Tek O.C.T Compound (Sakura Finetek U.S.A., Inc., Torrance, CA, U.S.A.) in a cryomold. The tissues were frozen on dry ice-acetone, then removed from the cryomold and placed at -80°C in an air-tight container until sectioned. Tissue sections (4 μm) for immunohistochemistry were obtained from frozen blocks using plain coated slides and an HM560 cryostat (Carl Zeiss, Oberkochen, Germany); the slides were immediately placed on dry ice and stored at -80°C until needed.

Immunofluorescent staining: Tissue sections (4 μm) were fixed in cold acetone for 30 min and incubated in 10% normal goat serum in PBS for 1 hr at room temperature. The tissues were then exposed to the anti-NOS2 antibody (Upstate, Lake Placid, NY, U.S.A.), which was diluted in PBS containing 10% normal goat serum, 0.3% Triton X and 0.1% sodium azide, overnight at 4°C . After incubation, the tissues were washed in PBS and incubated with a secondary antibody (Alexa Fluor 488, goat anti-rabbit, Molecular Probes, Eugene, OR, U.S.A.) that was diluted to 1:200 in PBS containing 10% normal goat serum, 0.3% Triton X and 0.1% sodium azide for 2 hr at room temperature. Then, the tissues were washed in PBS, coverslipped with VECTASHIELD (Vector Laboratories, Burlingame, CA, U.S.A.), examined and digitally photographed on an Axio Imager microscope using the Axio Vision 4.6 software (Carl Zeiss).

Laser capture microdissection (LCM): LCM was performed as described previously [23, 24]. Briefly, cryosectioned tissue was stained with HE and dehydrated, and LCM was performed with a PicCell II (Arcturus Engineering, Mountain View, CA, U.S.A.). Cells were captured from the region of crypts and muscles in the small intestine and transferred to CapSure LCM Caps (Arcturus Engineering). Total RNA was extracted using an RNA isolation kit (Stratagene), cDNA was synthesized, and real-time PCR was performed as described above.

Determination of lipoperoxide levels: For lipid peroxidation measurements, the thiobarbituric acid (TBA) assay was performed as described previously [12, 19]. Briefly, a sampling mixture (40 μl of 8.1% SDS, 300 μl of 20% acetic acid, 200 μl of 1.2% TBA and 150 μl of the small intestine homogenate) was incubated for 30 min at 95°C . After cooling on ice, the reactants were supplemented with 300 μl of n-butanol and pyridine (15:1 v/v), shaken vigorously and centrifuged for 5 min at 22°C . The optical density of the supernatant was determined at a wavelength of 532 nm as compared with twofold-diluted standards of malondialdehyde. The lipoperoxide level in 3-month-old (3 M) mice was examined as a control, and data for the 18 M mice were expressed relative to the level of the 3 M mice.

Statistical analysis: All data are expressed as means and SE for each treatment group. Statistical analysis was

performed using the *t*-test for mRNA expression and lipoperoxides levels.

RESULTS

Th1 and Th2 cytokines, the receptors and STAT6 gene expression in the small intestine after rutin administration in 3M mice: After administration of rutin for 10 days, differences in the expression of IL-4, IL-13 and their receptors (IL-4R α , IL-13R α 1 and IL-13R α 2), as well as STAT6, IFN- γ , IL-6 and TNF- α were investigated by excising the small intestines of 3-month-old (3 M) mice and analyzing gene expression by real-time PCR.

Rutin administration resulted in decreased IL-4 and IL-13 mRNA expression (Fig. 1A and 1B). IL-13R α 2 was also decreased after rutin administration (Fig. 1C). IL-4R α , IL-13R α 1, STAT6, IFN- γ , IL-6 and TNF- α showed no change (data not shown). These results suggested that rutin is able to downregulate Th2 cytokines in the small intestine, but is not involved with inflammatory cytokine gene expression.

Effects of rutin in aged mice: To evaluate the effects of rutin in geriatric immune regulation, next, rutin was administered to 18 M mice. We tested both the small intestine and lung, where IL-13 can play an important role in allergic responses.

While IL-4 mRNA expression in 18 M mice showed a decreasing tendency, no significant changes were observed in either the small intestine or lung (Fig. 2A and 2B). However, IL-13 gene expression was inhibited significantly in both the small intestine and lung after rutin administration in the 18 M mice (Fig. 2C and 2D). Unexpectedly, IL-13R α 2 gene expression was downregulated only in the small intestine (Fig. 2E) but not in the lung (Fig. 2F). In this study, the

IL-13 gene expression level in the lung was comparable to that in the small intestine based on the normalized Ct values in the 18 M mice. The expression of IL-4R α , IL-13R α 1, STAT6, IFN- γ , IL-6 and TNF- α mRNA was similar in both groups (data not shown).

Effects of rutin on NOS gene expression and lipid peroxidation: The bioavailability and antioxidative efficiency of rutin has been well studied to date. Rutin inhibits production of ROS and NF- κ B activation *in vitro* [18]. Therefore, the interaction between the antioxidative/antinitrosative effect and inhibitory effect of rutin on Th2 cytokines in aged mice is of interest. To determine the effect of rutin for NO production, the expression of NOS1, NOS2 and NOS3 gene was examined in the small intestine and lung. Figure 3 shows that rutin inhibited NOS2 gene expression in the small intestine, but not in the lung, in the 18 M mice. No effects on NOS1 and NOS3 gene expression were observed in either organ. An effect of rutin on the oxidative status was confirmed by the lipoperoxide levels in the small intestine (Fig. 4). The lipoperoxide level in the 18 M mice was significantly higher compared with that in the 3 M mice. The lipoperoxide level in the small intestine of the 18 M mice was significantly decreased to the level of the 3 M mice control after rutin administration.

Localization of NOS2 expression in the small intestine: NOS1, NOS2 and NOS3 gene expression was also examined in the mesenteric lymph node and colon; however, no changes were observed (data not shown). Interestingly, only NOS2 was inhibited in the small intestine of the 18 M mice as described above. The small intestine is an important organ, not only for digestion and absorption, but also in host defense. Immunofluorescent staining was performed to assess NOS2 expression in the small intestine. The crypt

Fig. 1. IL-4 (A), IL-13 (B) and IL-13R α 2 (C) gene expression in the small intestines of untreated control and rutin-administered 3-month-old mice measured by real-time PCR (n=5). All data are expressed relative to the untreated control. * $P < 0.05$ vs. untreated control.

Fig. 2. IL-4 (A, B), IL-13 (C, D) and IL-13R α 2 (E, F) gene expression in the small intestines (A, C, E) and lungs (B, D, F) of untreated control and rutin-administered 18-month-old mice measured by real-time PCR (n=5). All data are expressed relative to the untreated control. * $P < 0.05$ vs. untreated control.

region and smooth muscle of the small intestine were strongly stained (Fig. 5A). We collected cells from both areas by LCM and compared NOS2 gene expression between control and rutin-administered 18 M mice. Figure 5B shows a significant decrease in NOS2 gene expression in the crypt region; in the muscle region, gene expression also tended to decrease, but not significantly.

DISCUSSION

The prevalence of allergic diseases or chronic inflammatory diseases has increased worldwide over the last quarter century. It is generally accepted that deleterious changes in the environment, eating habits and life-style elicit an inadequate immune system or immune dysfunction in the host. Physiological modulatory effects of flavonoids have been

well-discussed to date, and various foods containing flavonoids with health-promoting benefits have been evaluated *in vivo* and *in vitro*. The advantage of flavonoids is that they can be taken orally from food. They are expected to be candidates for alternative medicine, especially if they have immunomodulatory effects on allergies or inflammation. Rutin has already been reported to ameliorate experimental colitis in rats [4] and mice [17] through attenuation of proinflammatory cytokine gene expression. Some flavonoids, such as quercetin, kaempferol, fisetin and rutin, have been identified to inhibit histamine release from human basophils and rat mast cells [7, 22]. Furthermore, fisetin inhibits Th2 cytokine production from basophils [9]. In this study, we demonstrated that orally administered rutin also inhibited Th2 cytokines, particularly IL-13 in the gut and lung in the 18 M mice; however, no effects on IL-4R α and IL-13R α 1

Fig. 3. NOS2 gene expression in the small intestines (A) and lungs (B) of untreated control and rutin-administered 18-month-old mice measured by real-time PCR (n=5). All data are expressed relative to the untreated control. * $P < 0.05$ vs. untreated control.

Fig. 4. Effect of rutin on lipoperoxide levels in the small intestine. Data are expressed relative to the lipoperoxide levels in the 3-month-old mice. (a) $P < 0.05$ vs. 3 M mice control; (b) $P < 0.05$ vs. 3 M mice control (n≥4).

were observed. IL-13 regulation is still unclear, but previous reports suggested that IL-13 has a prominent role in allergic disease. Additionally, IL-13 has a great effect on smooth muscle contraction, which is important for the elimination of pathogens in the small intestine [39]. Our previous data showed that IL-13Rα1, which is the functional receptor for IL-13, was decreased when IL-13 increased after parasitic worm infection [26]. In addition, IL-4Rα and IL-13Rα1 gene expression increased in IL-13-deficient mice (unpublished data), suggesting that receptor gene expression is downregulated by IL-13 to avoid excessive IL-13 activity. However, our data showed no increase in these receptors when rutin inhibited IL-13 gene expression. Therefore, it is considered that the inhibitory effect of rutin on IL-13 gene expression might not be negated by an increase in the receptors. Thus, rutin might cause the biological activity of IL-13 to decrease *in vivo*. In addition, IL-13Rα2 gene expression was also inhibited by rutin. In contrast to IL-4Rα and IL-13Rα1, IL-13Rα2 is not linked to STAT6 signaling [5] and has been reported as a decoy receptor for IL-13 [2, 38]. Previous data showed that IL-13Rα2 gene expression is dependent on IL-4/IL-13- and STAT6-dependent signaling pathways [2]. Therefore, the

downregulation of IL-13Rα2 confirms the role of rutin in decreasing the biological activity of IL-13. Taken together, intake of rutin might impede the shift towards a Th2-dominant profile in hosts, without any effects on inflammatory cytokine gene expression.

Aging is associated with a decline in immune function, and cytokine imbalance might be an underlying factor. In this study, aged mice were used as a model to investigate whether rutin exhibits controlling effects on immune factors, especially in the gerontic stage. Our results showed that IL-13 gene expression was inhibited in both the small intestine and lung of the 18 M mice administered rutin (Fig. 2), while no change in INFγ gene expression was observed. While IL-13Rα2 gene expression decreased in the small intestine, this effect was not observed in the lung, indicating that different pathways may regulate IL-13Rα2 gene expression.

Previous research has suggested that oxidative stress promotes polarization of T cell differentiation toward a Th2 phenotype [14]. In addition, type-1 cytokine mRNA production is enhanced and type-2 cytokine mRNA levels are reduced in NOS2-deficient mice [11, 37]. Therefore, the regulation of oxidative and nitrosative stress is very important in controlling cytokine imbalance during aging. Another report also demonstrated that several flavonoids inhibited NOS expression through inhibition of the NF-κB pathway [3]. To examine the inhibitory effect of rutin on NO production and lipid peroxide production in aged mice, the expression of individual NOS genes was first examined in the small intestine and lung. Figure 3 shows that rutin inhibited NOS2 gene expression in the small intestine, but not in the lung, of the 18 M mice, which is interestingly consistent with the pattern of IL-13Rα2 gene expression. In addition, rutin did not exhibit any effects on NOS1 and NOS3 gene expression in either organ, suggesting that NOS2 is important for Th2 development in the small intestine. However, IL-13 may be modulated in a different manner in the lung. Next, the lipoperoxide level was measured in the small intestine to confirm the effect of rutin as an antioxidant (Fig. 4). The lipoperoxides level was higher in aged mice, suggesting that oxidative stress

Fig. 5. A: Immunofluorescent staining with anti-NOS2 antibody in the small intestines of 18-month-old mice. Crypt regions and smooth muscle were strongly stained. B: NOS2 gene expression in smooth muscle and crypt regions of the small intestines of untreated control (□) and rutin-administered (■) 18-month-old mice measured by real-time PCR with LCM samples (n=5). All data are expressed relative to untreated control muscle. * $P < 0.05$ vs. untreated control.

increases during aging; however, it was reduced after rutin administration. These results are consistent with previous reports describing the effect of rutin as an antioxidant in diabetic rat tissues [13].

Rutin might be able to regulate immune responses in the small intestine through inhibition of NOS2. Our previous data suggested a regional difference in IL-13 and the receptor gene expression [25]. IL-13 has a prominent role in smooth muscle contractility during nematode infection [39], and IL-13 α 2 is expressed mainly in the smooth muscle of the small intestine [25]. Therefore, whether NOS2 gene expression also shows regional differences is of interest. Immunofluorescent staining revealed that NOS2 is expressed in the crypt region and smooth muscle of the small intestine (Fig. 5A), and NOS2 gene expression decreased significantly in the crypt region (Fig. 5B). Therefore, the inhibitory effect of rutin on Th2 cytokines may

result in NOS2 downregulation, particularly in the crypt region.

In conclusion, a low dose of dietary rutin inhibited Th2 cytokines and NOS2 gene expression in aged mice. Routine intake of rutin from foods may ameliorate the cytokine imbalance and prevent allergic diseases through downregulation of Th2 responses. Further mechanistic and toxicity studies to explore the effectiveness of rutin in treating or preventing allergic diseases in animal models are expected.

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