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NOTE

Naringenin suppresses neutrophil infiltration into adipose tissue in high-fat diet-induced obese mice

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Abstract
Recruitment of immune cells to adipose tissue is altered dramatically in obesity, which results in chronic inflammation of the adipose tissue that leads to metabolic disorders, such as insulin resistance and type 2 diabetes mellitus. The regulation of immune cell infiltration into adipose tissue has prophylactic and therapeutic implications for obesity-related diseases. We previously showed that naringenin, a citrus flavonoid, suppressed macrophage infiltration into adipose tissue by inhibiting monocyte chemoattractant protein-1 (MCP-1) expression in the progression phase to high-fat diet (HFD)-induced obesity. In the current study, we evaluated the effects of naringenin on neutrophil infiltration into adipose tissue, because neutrophils also infiltrate into adipose tissue in the progression phase to obesity. Naringenin suppressed neutrophil infiltration into adipose tissue induced by the short-term (2 weeks) feeding of a HFD to mice. Naringenin tended to inhibit the HFD-induced expression of several chemokines, including MCP-1 and MCP-3, in adipose tissue. Naringenin also inhibited MCP-3 expression in 3T3-L1 adipocytes and a co-culture of 3T3-L1 adipocytes and RAW264 macrophages. However, naringenin did not affect the expression of macrophage inflammatory protein-2 (MIP-2), an important chemokine for neutrophil migration and activation, in macrophages or in a co-culture of adipocytes and macrophages. Our results suggest that naringenin suppresses neutrophil infiltration into adipose tissue via the regulation of MCP-3 expression and macrophage infiltration.

Keywords Naringenin · Obesity · Neutrophil · Macrophage · MCP-3 · MIP-2

Introduction

Obesity-related diseases, such as type 2 diabetes mellitus, are associated with chronic inflammation caused by an increase in the expression of proinflammatory adipokines, including chemokines, in adipose tissue [1, 2]. Chemokines attract various immune cells to adipose tissue, which augments inflammation resulting in the initiation and progression of obesity-related diseases [3]. Among immune cells, macrophages have an important role in the induction of inflammation in adipose tissue. A paracrine loop of inflammatory adipokines, including free fatty acids and tumor necrosis factor-α (TNF-α), between adipocytes and macrophages aggravates inflammation [4–6]. In addition to macrophages, other immune cells such as neutrophils, $CD8^+$ T cells, and B cells also infiltrate adipose tissue during high-fat diet (HFD)-induced obesity, and contribute to the development of adipose tissue inflammation and insulin resistance. Of note, the recruitment of neutrophils to adipose tissue occurs as early as a few days following HFD feeding [3, 7–10].

Naringenin, a flavonoid abundant in citrus fruits, was reported to have various pharmacological effects, including antioxidant, anti-inflammatory, and antidiabetic activity [11]. In addition, naringenin influenced immune cell infiltration and function in several disease models. Naringenin reduced T cell infiltration in skin lesions of an atopic dermatitis model and in the spinal cord of an encephalomyelitis model [12, 13]. Naringenin also reduced neutrophil infiltration in the peritoneal cavity of lipopolysaccharide-induced hyperalgesia and in the bronchoalveolar lavage fluid of lipopolysaccharide-induced lung injury [14, 15]. We previously reported that naringenin exerted anti-inflammatory effects in adipocytes in vitro and adipose tissue from HFD-induced obese mice [16, 17]. Moreover, we showed that naringenin suppressed macrophage infiltration into mouse

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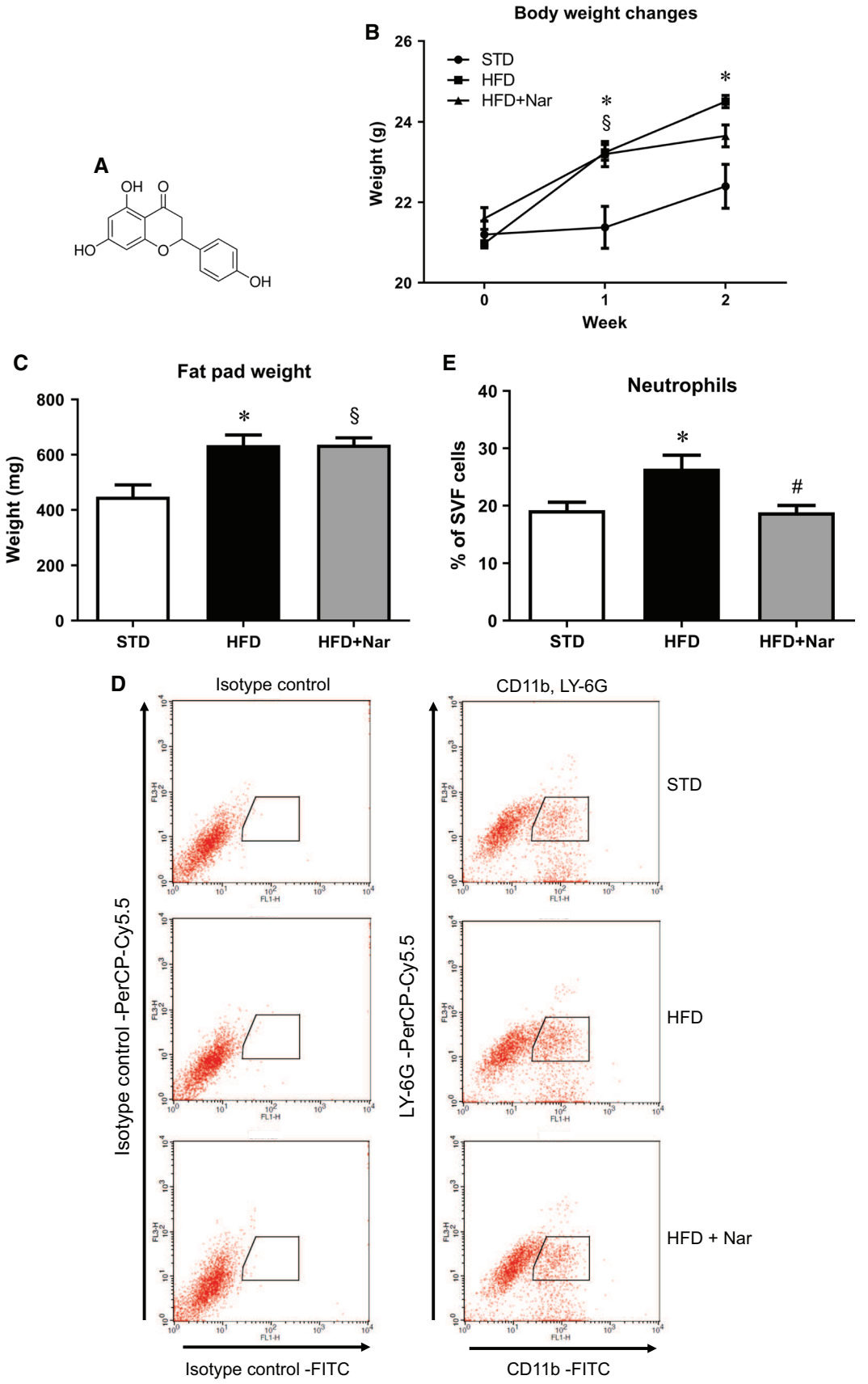
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◂**Fig. 1** Effects of naringenin on neutrophil infiltration into adipose tissue in HFD-fed mice. **a** The chemical structural formula of naringenin. **b** Body weight change. **c** Epididymal fat pad weight. **d** Flow cytometric analysis of neutrophils from the SVF of epididymal fat. **e** Quantified data of flow cytometric analysis. C57BL/6J mice ($n=4$–5) were used for each group. Data are the mean ± SE. $^{*}P<0.05$ for STD vs HFD. $^{\S}P<0.05$ for STD vs HFD + Nar. $^{\#}P<0.05$ for HFD vs HFD + Nar. *STD* standard diet, *HFD* high-fat diet, *Nar* naringenin

adipose tissue in the progression phase (within 2 weeks) to HFD-induced obesity [18]. However, the effects of naringenin on other immune cell infiltration into the adipose tissue of HFD-induced obese mice remains unknown.

In this study, we focused on the effects of naringenin on neutrophil infiltration into mouse adipose tissue in the progression phase to HFD-induced obesity.

Material and methods

Reagents

We purchased the following reagents: NaCl, KH_2PO_4, $MgSO_4$, $CaCl_2$, $NaHCO_3$, HEPES buffer solution, glucose, and dimethyl sulfoxide (DMSO) (Nacalai Tesque, Kyoto, Japan); methyl cellulose (Wako Pure Chemical Industries, Osaka, Japan); naringenin, bovine serum albumin (BSA), isobutylmethylxanthine (IBMX), and dexamethasone (DEX) (Sigma, St. Louis, MO); insulin (Cell Science & Technology Institute, Sendai, Japan); phosphate-buffered saline (PBS), Dulbecco's modified Eagle's medium (DMEM), bovine serum, fetal bovine serum (FBS), and type-1 collagenase (Gibco, Grand Island, NY). The chemical structural formula of naringenin is shown in Fig. 1a.

Animals and animal care

Experiments were approved by the animal care committee of the Kyushu University of Health and Welfare. Male C57BL/6J mice (aged 7 weeks; weight, 20–22 g) were purchased from the Kyudo Animal Laboratory (Kumamoto, Japan). Mice were housed at 5–6 per cage and maintained at 24 ± 2 °C on a 12-h light/dark cycle, and were acclimatized for 1 week prior to experimental use. Mice at 8 weeks of age were randomly divided into the following groups: (1) standard diet (STD)-fed mice treated with 0.2 mL vehicle control (0.5% (w/v) methyl cellulose solution, p.o. once daily) for 14 days; (2) HFD-fed mice treated with vehicle control (methyl cellulose, p.o. once daily) for 14 days; and (3) HFD-fed mice treated with naringenin (100 mg/kg/day, p.o. once daily) for 14 days. Mice were fed STD (10% of calories from fat, D12450B; Research Diets, New Brunswick, NJ) or HFD (60% of calories from fat, D12492; Research Diets) and water ad libitum. Five to six mice were used in each group. Body weight was measured weekly, and epididymal fat pad weight was measured at dissection.

Preparation of the stromal vascular fraction (SVF) from adipose tissue and flow cytometric analysis

The preparation of the SVF from adipose tissue was performed as previously reported [19]. In brief, epididymal fat pads were cut into small pieces and rinsed in a buffer containing 120 mM NaCl, 4 mM KH_2PO_4, 1 mM $MgSO_4$, 750 μM $CaCl_2$, 10 mM $NaHCO_3$, and 30 mM HEPES (pH 7.4). The pieces of fat were incubated at 37 °C for 30 min in 20 mL of the above buffer supplemented with 1% BSA, 280 μM glucose, and 20 mg of type-1 collagenase. The SVF was collected by centrifugation for 15 min at 200×*g*, and resuspended in Stain Buffer (FBS) (BD Biosciences, Franklin Lakes, NJ). After filtration through a 100-μm strainer, the cells were counted and adjusted to 1×10^7 cells/mL. Single cell suspensions (10^6 cells) were incubated with Fc block CD16/32 (clone 2.4G2) (BD Biosciences) at 4 °C for overnight, then stained with FITC- and PerCP-Cy5.5-conjugated antibodies at 4 °C for 30 min. After three washes, the labeled cells were analyzed on a FACSCalibur (BD Biosciences). Antibodies were: FITC rat anti-mouse CD11b (M1/70); PerCP-Cy5.5 rat anti-mouse Ly-6G (1A8); FITC rat IgG2b κ isotype control; and rat IgG2a κ isotype control (all from BD Biosciences).

Real-time PCR analysis

The epididymal fat pads (100 mg) were homogenized and lysed in TRIzol reagent (Invitrogen) to isolate total RNA. 3T3-L1 adipocytes and RAW264 macrophages were cultured in 12-well plates, and then lysed in TRIzol reagent. Reverse transcription was performed (ReverTra Ace qPCR RT Master Mix; Toyobo, Osaka, Japan), in accordance with the manufacturer's instructions. PCR amplification was performed using TaqMan Fast Advanced Master Mix (Applied Biosystems, Carlsbad, CA) in a StepOne Plus Real Time PCR System. Primers and TaqMan probes were: MCP-1 (CCL2), Mm00441242-m1; MCP-3 (CCL7), Mm00443113_m1; MIP-1α (CCL3), Mm00441259_g1; MIP-2 (CXCL2), Mm00436450_m1; TNF-α, Mm00443258_m1; and IL-6, Mm00446190_m1; 18S (Rn18s), Mm03928990-g1 (all from Applied Biosystems). The relative quantity of mRNA was determined using the comparative Ct method and was normalized using 18S ribosomal RNA as an endogenous control.

Cell culture and treatments

3T3-L1 cells (Health Science Research Resources Bank, Osaka, Japan) were maintained in DMEM supplemented

with 10% bovine serum. After pre-adipocytes reached confluence in 12-well plates, they were induced to differentiate into mature adipocytes by replacing medium with 10% FBS-supplemented DMEM containing 0.5 mM IBMX, 0.25 µM DEX, and 5 µg/mL insulin for 2 days. Medium was then replaced with 10% FBS-supplemented DMEM containing 5 µg/mL insulin, and this was changed every 2–3 days for the next 6–7 days. RAW264 cells (RIKEN BioResource Center, Tsukuba, Japan) were cultured in DMEM supplemented with 10% FBS. Adipocytes and macrophages were co-cultured in a contact system, as described previously (5). Briefly, RAW264 cells (1×10^5 cells/mL) were plated onto dishes with differentiated 3T3-L1 adipocytes. For each experiment, we treated cells with naringenin and used 0.5% DMSO as the vehicle control. The total volumes of culture media used for the single cell line and the co-culture system treated with naringenin or vehicle control were 0.6 mL and 1.0 mL, respectively.

ELISA

MCP-3 and MIP-2 levels in cell culture media were measured using specific ELISA kits: Mouse MCP-3 Instant ELISA (BMS6006INST, eBioscience, Santa Clara, CA) and Mouse CXCL2/MIP-2 Quantikine ELISA kit (MM200, R&D Systems, Minneapolis, MN), according to the manufacturer's instructions. The lower limits of detection of these kits were 15.6 pg/mL and 7.8 pg/mL, respectively. The intra- and inter-assay coefficients of variation for ELISA results were less than 10%.

Cytotoxicity assay

The cytotoxicity assay was performed using a Cyto Tox 96 Assay Kit (Promega, Madison, WI), according to the manufacturer's instructions. The cells were treated with naringenin for 6 h, and lactate dehydrogenase (LDH) released upon cell lysis in culture media was measured. Percentage activity was determined by performing a total lysis control in which cells were lysed with the lysis solution included in the kit. The percentage of cytotoxicity was determined using the following formula:

$$\text{Percent}\,(\%)\,\text{cytotoxicity} = 100 \times (\text{experimental LDH release}\,(OD_{490}))/(\text{total lysis LDH release}\,(OD_{490})).$$

Statistical analysis

Data are presented as the mean ± ,SE and analysis was performed using GraphPad Prism 5. Differences between groups were assessed by unpaired *t* test, one-way ANOVA, or two-way ANOVA. Multiple comparisons were made using a post hoc Bonferroni test. Differences between groups were considered significant at $P < 0.05$.

Results

Naringenin suppresses HFD-induced neutrophil infiltration into mouse adipose tissue via the regulation of adipokine expression in the progression phase to obesity

To determine whether naringenin affects neutrophil infiltration into adipose tissue in the progression phase to obesity, we administered naringenin to HFD-fed mice for 2 weeks. We previously found that naringenin (100 mg/kg/day) suppressed the macrophage infiltration into adipose tissues in HFD-induced obese mice [18]. Therefore, we used naringenin (100 mg/kg/day) to evaluate the neutrophil infiltration in this study. Similar to our previous study, HFD, but not naringenin, significantly increased the body weight and epididymal fat pad weight (Fig. 1b, c). We also confirmed that HFD significantly increased neutrophil infiltration into adipose tissue, and revealed that naringenin suppressed this HFD-induced neutrophil infiltration (Fig. 1d, e). We next examined the effects of naringenin on HFD-induced changes in adipokine expression in adipose tissue. We analyzed the expressions of MCP-1, MCP-3, MIP-1α, MIP-2, IL-6, and TNF-α by real-time PCR analysis. These chemokines and cytokines were previously reported to be increased in obese adipose tissues [2, 3]. As shown in Fig. 2, HFD tended to induce the expression of MCP-1, MCP-3, MIP-1α, MIP-2, and IL-6. Naringenin reduced the expressions of these HFD-induced chemokines and/or cytokines especially MCP-3, which was significantly reduced. However, the expression of TNF-α was not affected.

Naringenin suppresses MCP-3 expression in adipocytes

Chemokines such as MCP-1 and MCP-3 derived from adipocytes contribute to obesity-related macrophage infiltration into adipose tissue [20]. In addition, MCP-3 also has a low neutrophil chemotactic potency [21, 22]. Thus, to elucidate the mechanism by which naringenin suppresses HFD-induced neutrophil infiltration into adipose tissue, we next examined the effect of naringenin on MCP-3 expression in vitro. As shown in Fig. 3a, b, naringenin reduced MCP-3 expression at the transcription and secretion level in 3T3-L1 adipocytes. Moreover, to examine the effects of naringenin under conditions of obese adipose tissue, we used a co-culture of 3T3-L1 adipocytes and RAW264 macrophages. Naringenin inhibited the co-culture-induced MCP-3 expression (Fig. 3c, d). Naringenin-induced cytotoxicity was negligible

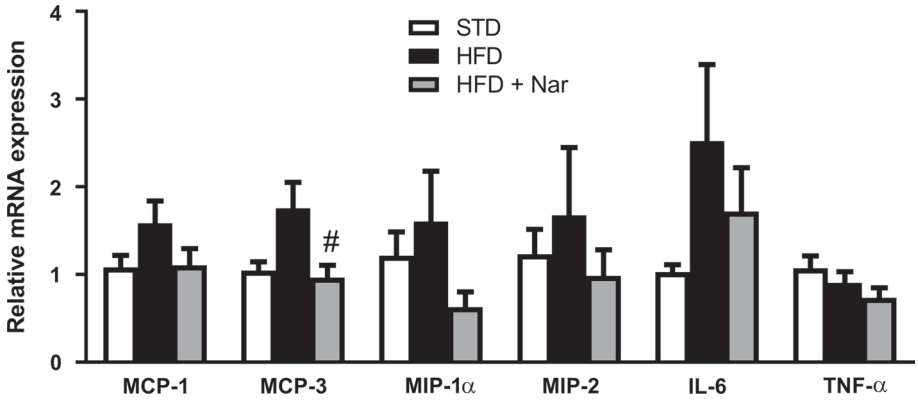

Fig. 2 Effects of naringenin on adipokine expression in adipose tissue of HFD-fed mice. Real-time PCR analysis of MCP-1, MCP-3, MIP-1-α, MIP-2, IL-6, and TNF-α expression in adipose tissue. C57BL/6J mice ($n = 4$–5) were used for each group. Data are the mean ± SE. $^{\#}P < 0.05$ for HFD vs HFD + Nar. *STD* standard diet, *HFD* high-fat diet, *Nar* naringenin

in 3T3-L1 adipocytes and a co-culture of 3T3-L1 adipocytes and RAW264 macrophages (Fig. 3e, f).

Naringenin does not affect MIP-2 expression in macrophages

We analyzed the expressions of several adipokines in adipose tissues (Fig. 2). Similar to MCP-3, MIP-2 is an important chemokine for neutrophil recruitment, and is mainly produced from tissue-resident macrophages [23–25]. Although the results showed no significant difference, HFD and naringenin tended to affect MIP-2 expression. Thus, considering the importance of MIP-2 for neutrophil infiltration, we examined the effects of naringenin on MIP-2 expression using RAW264 macrophages. As shown in Fig. 4a, naringenin did not affect MIP-2 expression at the transcriptional level. In addition, 50 μM naringenin tended to increase MIP-2 expression, but there was no significant difference compared with vehicle control. Moreover, naringenin did not affect the secretion of MIP-2 (Fig. 4b). These results suggest that naringenin did not affect MIP-2 expression in macrophages. In addition, although the co-culture of 3T3-L1 adipocytes and RAW264 macrophages induced the upregulation of MIP-2 expression, naringenin did not affect the co-culture-induced MIP-2 expression (Fig. 4c, d). Naringenin-induced cytotoxicity was negligible in RAW264 macrophages (Fig. 4e).

Discussion

The recruitment of a variety of immune cells is observed in obese adipose tissue, and these events contribute to the initiation and progression of obesity-related diseases, such as insulin resistance and type 2 diabetes mellitus [3]. The regulation of immune cell infiltration into adipose tissue might be a prophylactic and therapeutic target. In the progression phase to obesity, neutrophil infiltration into adipose tissue occurs as early as a few days following HFD feeding [3]. Our previous study showed that HFD increased macrophage infiltration into adipose tissue within 7 days compared with STD-fed mice, and naringenin reduced the HFD-induced macrophage infiltration via the inhibition of MCP-1 expression [18]. In this study, to clarify the effects of naringenin on immune cell infiltration into adipose tissue in the progression phase to obesity, we focused on neutrophil infiltration. Naringenin suppressed neutrophil infiltration into adipose tissue and the expression of several HFD-induced chemokines (Figs. 1, 2). Moreover, naringenin reduced MCP-3 expression in adipocytes, but did not affect MIP-2 expression in macrophages (Figs. 3, 4). Results from our previous and current studies suggest the following mechanism (Fig. 5): (1) naringenin suppresses neutrophil infiltration into adipose tissue via the inhibition of MCP-3 expression in adipocytes; however, the influence of changes in MCP-3 expression on neutrophil infiltration will be low, because the neutrophil attraction potency of MCP-3 is weak; (2) naringenin suppresses MCP-1 and MCP-3 expression in adipocytes, which results in the reduction of macrophage infiltration into adipose tissue. Because macrophages are a major source of MIP-2, which is important for neutrophil infiltration, a reduction in macrophage infiltration by naringenin will result in the reduction of neutrophil infiltration into adipose tissue.

In this study, we analyzed the effects of naringenin on the expressions of several adipokines in adipose tissues from mice fed HFD for 2 weeks (Fig. 2). MCP-1, MCP-3, MIP-1α, and MIP-2 are chemokines involved in the infiltration of macrophages and/or neutrophils. IL-6 and TNF-α are proinflammatory cytokines involved in the induction of insulin resistance [2, 3]. Our results showed that naringenin might reduce the HFD-induced expressions of MCP-1, MCP-3, MIP-1α, MIP-2, and IL-6. These results suggest that naringenin affects the expression of various adipokines, and regulates the infiltration of various immune cells as well as inflammation in adipose tissues during the progression phase to obesity. Of note, HFD tended to induce the expressions of these adipokines except for TNF-α, although there was

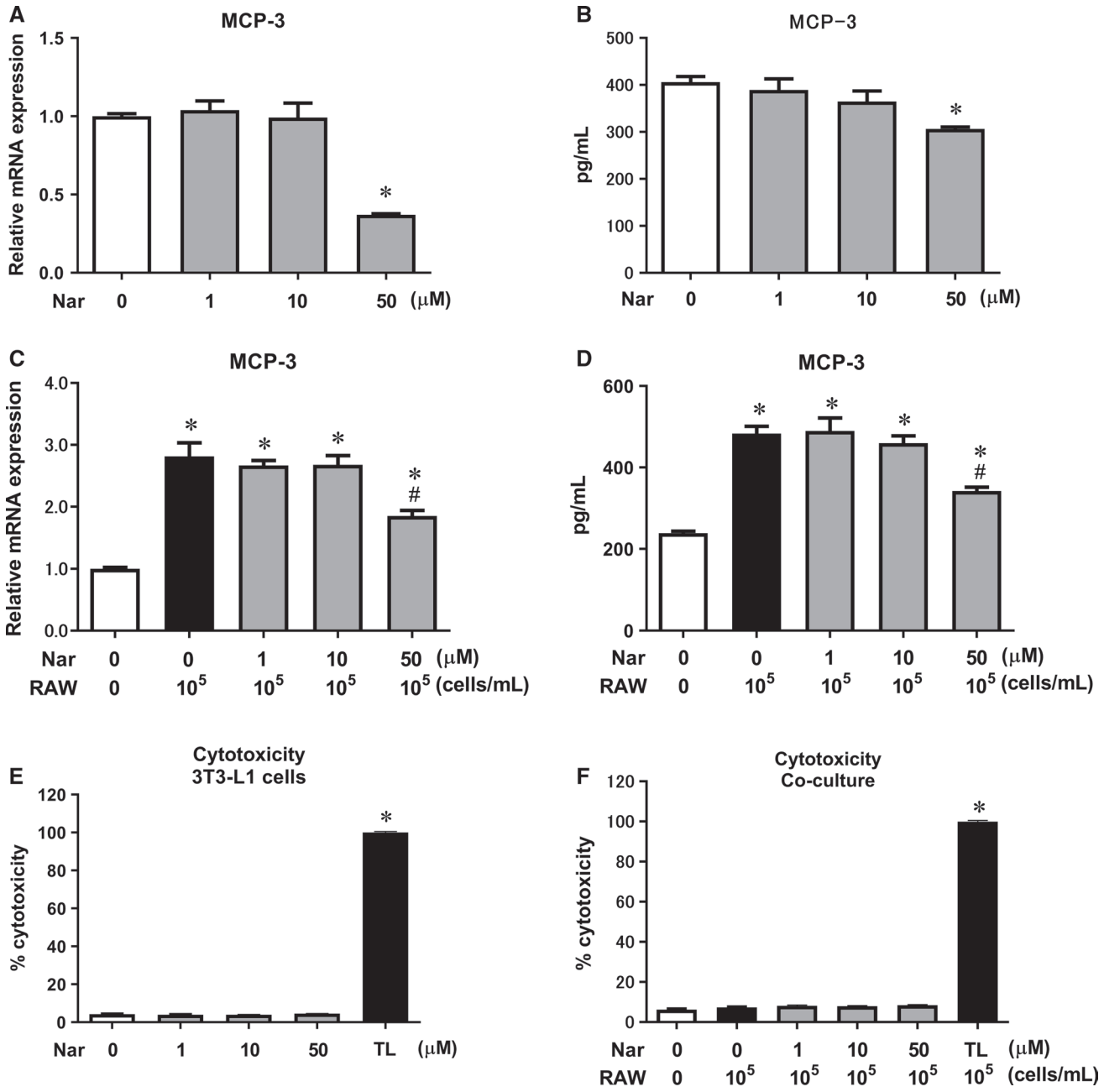

Fig. 3 Effects of naringenin on MCP-3 expression in vitro. **a** Real-time PCR analysis of MCP-3 expression in 3T3-L1 adipocytes treated with naringenin for 3 h. **b** ELISA results of MCP-3 levels in culture media from 3T3-L1 adipocytes treated with naringenin for 6 h. **c** Real-time PCR analysis of MCP-3 expression in a co-culture of 3T3-L1 adipocytes and RAW264 cells. 3T3-L1 adipocytes were pre-treated with naringenin for 30 min and then co-cultured with RAW264 cells for 3 h. **d** ELISA results of MCP-3 levels in culture media from a co-culture of 3T3-L1 adipocytes and RAW264 cells for 6 h. **e** Cytotoxicity analysis of 3T3-L1 adipocytes treated with naringenin for 6 h. **f** Cytotoxicity analysis in a co-culture of 3T3-L1 adipocytes and RAW264 cells treated with naringenin for 6 h. Data are the mean ± SE ($n = 4$–6). $^{*}P < 0.05$ vs vehicle control. $^{\#}P < 0.05$ vs vehicle-treated co-cultures. *Nar* naringenin, *RAW* RAW264 cells, *TL* total lysis control

no significant difference compared with STD. A previous study reported that the long-term feeding of HFD (more than 4 months) significantly increased adipokine expression in adipose tissues [17, 20]. Thus, the short-term feeding of HFD tended to increase adipokine expression, but might be of insufficient duration to show significant differences compared to STD feeding because of individual variation. The expression of TNF-α was not affected in this study; however, we previously reported that 4 months feeding of HFD significantly increased TNF-α expression in adipose

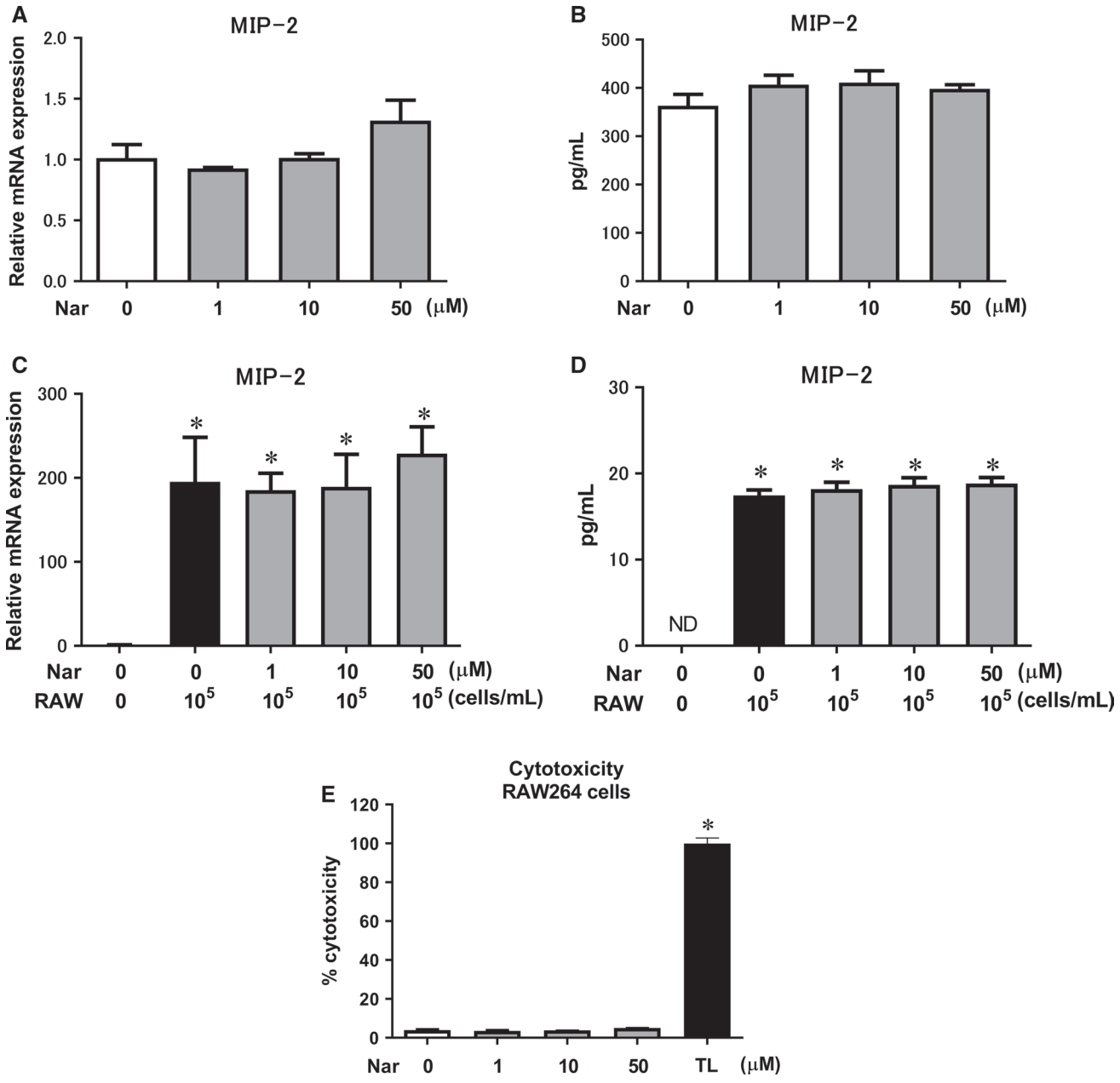

Fig. 4 Effects of naringenin on MIP-2 expression in vitro. **a** Real-time PCR analysis of MIP-2 expression in RAW264 cells treated with naringenin for 3 h. **b** ELISA of MIP-2 levels in culture media from RAW264 cells treated with naringenin for 6 h. **c** Real-time PCR analysis of MIP-2 expression in a co-culture of 3T3-L1 adipocytes and RAW264 cells. 3T3-L1 adipocytes were pre-treated with naringenin for 30 min and then co-cultured with RAW264 cells for 3 h. **d** ELISA of MIP-2 levels in culture media from a co-culture of 3T3-L1 adipocytes and RAW264 cells for 6 h. **e** Cytotoxicity analysis of RAW264 cells treated with naringenin for 6 h. Data are the mean ± SE ($n = 4$–6). $^{*}P < 0.05$ vs vehicle control. *Nar* naringenin, *RAW* RAW264 cells, *ND* not detected, *TL* total lysis control

tissues [17]. A paracrine loop of free fatty acids and TNF-α between matured adipocytes and infiltrated macrophages exists in obese adipose tissues [4–6]. Therefore, insufficient adipocyte hypertrophy and macrophage infiltration related to the short-term feeding of HFD might not affect the expression of TNF-α.

MCP-3 expression is upregulated by the activation of nuclear factor-kappa B (NF-κB) and c-Jun N-terminal kinase (JNK) signaling pathways in adipocytes [20]. We previously showed that naringenin inhibited the activation of NF-κB and JNK signaling pathways in adipocytes [16, 17]. Thus, naringenin likely suppresses MCP-3 expression via the inhibition of these signaling pathways. However, the

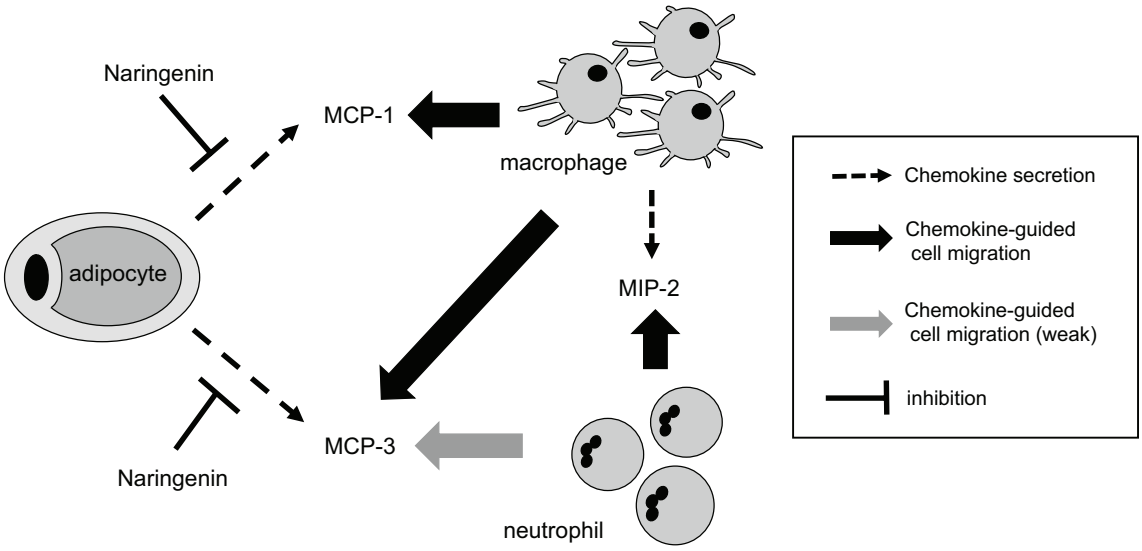

Fig. 5 Schematic representation of the negative regulation of neutrophil infiltration into adipose tissues by naringenin. This figure summarizes the results of our previous and current studies

current study showed that naringenin did not affect MIP-2 expression in macrophages or a co-culture of adipocytes and macrophages. A variety of cytokines and signaling pathways regulate MIP-2 production in macrophages including the IL-6-mediated induction of MIP-2 through the NF-κB/mitogen-activated protein kinase (MAPK) signaling pathway, and the TNF-α-mediated induction of MIP-2 through the NF-κB/MAPK, caspase-3 signaling pathway [26]. Furthermore, naringenin inhibited activation of the NF-κB signaling pathway in macrophages [14, 27], whereas naringenin increased the activation of caspase-3 in THP-1 monocytic cells [28, 29]. These studies suggest that the effect of naringenin on MIP-2 expression may be counteracted by the regulation of diverse signaling pathways exerting an opposite effect on MIP-2 expression.

Neutrophils have a critical role in innate immunity, especially during microbial infection, by producing antimicrobial reagents, including lysozyme, neutrophil elastase, myeloperoxidase, and defensins. Neutrophils also produce various cytokines and chemokines involved in the recruitment of other immune cells and increased tissue inflammation. Studies reported that neutrophils were present in obese adipose tissue. The recruitment of neutrophils to adipose tissue was observed within the first week of HFD feeding [7, 30]. In addition, neutrophil elastase was involved in the development of obesity-induced insulin resistance as demonstrated in neutrophil elastase knockout mice [8]. Although naringenin had a weak inhibitory effect on neutrophil elastase [31, 32], it remains unclear whether naringenin affects the production/secretion of neutrophil elastase. Further studies to verify the effects of naringenin on elastase production in adipose tissue may provide additional evidence for the use of naringenin in obesity-related diseases.

The results of this study indicate that naringenin suppresses neutrophil infiltration into adipose tissue in the progression phase to HFD-induced obesity. Our findings suggest that naringenin exerts its therapeutic effects in obesity-related diseases via the regulation of immune cell functions.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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