Green Tea Polyphenol Epigallocatechin-3-gallate Suppresses Toll-like Receptor 4 Expression via Up-regulation of E3 Ubiquitin-protein Ligase RNF216* 

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Toll-like receptor 4 (TLR4) plays an essential role in innate immunity through inflammatory cytokine induction. Recent studies demonstrated that the abnormal activation of TLR4 has a pivotal role in obesity-induced inflammation, which is associated with several diseases, including hyperinsulinemia, hypertriglyceridemia, and cardiovascular disease. Here we demonstrate that the highly absorbent 67-kDa laminin receptor (67LR), suppressed TLR4 expression through E3 ubiquitin-protein ring finger protein 216 (RNF216) up-regulation. Moreover, our data indicate cyclic GMP mediates 67LR agonist-dependent RNF216 up-regulation. Moreover, we show that the highly absorbent 67LR agonist (-)-epigallocatechin-3-O-gallate (EGCG3°Me) significantly attenuated TLR4 expression in the adipose tissue. EGCG3°Me completely inhibited the high-fat/high-sucrose (HF/HS)-induced up-regulation of tumor necrosis factor alpha (TNFα) in adipose tissue and serum monocyte chemoattractant protein-1 increase. Furthermore, this agonist intake prevented HF/HS-induced hyperinsulinemia and hypertriglyceridemia. Taken together, 67LR presents an attractive target for the relief of obesity-induced inflammation.

Dyslipidemia is a critical risk factor for the development of atherosclerosis and cardiovascular disease, the major causes of death of patients with obesity (2, 4). Recent reports indicate that obesity induces a chronic inflammatory state, which has been shown to play a critical role in the development of dyslipidemia and atherosclerosis (4). Macrophage-derived tumor necrosis factor alpha (TNFα) induces dyslipidemia and increases lipolysis in adipose tissue, and plasma TNFα is associated with very low-density lipoprotein-cholesterol (LDL-C)/triglyceride (TG) concentrations (5, 6). Moreover, macrophage infiltration into adipose tissue is negatively correlated with plasma high-density lipoprotein cholesterol (HDL-C) concentrations and positively correlated with circulating TG levels in obese patients (7). Hepatic steatosis, adipose tissue inflammation induced by a HF diet, is reported to be extensively reduced in monocyte chemoattractant protein-1 (MCP-1) homozygous knock-out mice compared with that in wild-type mice (8).

Emerging evidence suggests that the abnormal activation of Toll-like receptor 4 (TLR4) acts as the first trigger for the obesity-elicited production of inflammatory cytokines, such as MCP-1 and TNFα. Despite increased body weight and fat content, inflammation was not evident in TLR4−/− mice (9, 10). Moreover, mice lacking TLR4 are protected against HF diet-induced insulin resistance, and they do not show the expression of pro-inflammatory genes, including TNFα and MCP-1 (9, 10).

Cyclic GMP (cGMP) is a well known secondary messenger that plays a central role in vascular homeostasis and penile erection. The regulation of cGMP is a well established strategy for cardioprotection, vasodilatation, and increased blood flow. However, little is known about the role of cGMP in the regulation of inflammation.

Here we show that a natural 67LR agonist, (-)-epigallocatechin-3-O-gallate (EGCG), significantly suppressed TLR4 glutaryl coenzyme A synthase; IRS-2, insulin receptor substrate-2; LDL-C, low-density lipoprotein-cholesterol; MCP-1, monocyte chemoattractant protein-1; RNF216, ring finger protein 216; SCD-1, stearoyl-CoA desaturase-1; SGC, soluble guanylyl cyclase; SREBP-1, sterol regulatory element-binding protein-1; TG, triglyceride; TLR4, Toll-like receptor 4; UCP-2, uncoupling protein-2; 67LR, 67-kDa laminin receptor; ANP, atrial natriuretic peptide.

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expression in macrophages through cGMP production. However, EGCG treatment did not affect TLR4 expression at the mRNA level, whereas EGCG induced the ubiquitination of TLR4. In this pathway, cGMP induction up-regulated E3 ubiquitin-protein ligase ring finger protein 216 (RNF216). Moreover, the highly absorbent 67LR agonist (−)-epigallocatechin-3-O-(3-O-methyl)-gallate (EGCG3O3), the characteristic compound in green tea (11), significantly attenuated TLR4 expression in adipose tissue and was accompanied with the up-regulation of RNF216. This agonist significantly suppressed the HF/HS diet-induced increase in the production of inflammatory cytokines and HF/HS diet-induced dyslipidemia, including the increase in serum TG levels and low-density lipoprotein-cholesterol (LDL-C) to high-density lipoprotein cholesterol (HDL-C) ratio. Taken together, 67LR and cGMP induction presents a possible novel target for the relief of obesity-induced inflammation.

Results

67LR Agonist Strongly Suppressed TLR4 Expression through RNF216 Up-regulation—Emerging evidence suggests that TLR4 acts as a trigger of obesity-induced inflammation. Furthermore, TLR4 deficiency is reported to prevent lipid-induced NFκB DNA binding to the IL-6 and MCP-1 promoters. Consistent with NFκB activation, lipid infusion stimulated TNF-α, IL-6, and MCP-1 mRNA expression in adipose tissue of wild-type mice, but not in TLR4−/− mice (9–13).

Several studies have shown that the natural 67LR agonist EGCG has an inhibitory effect on TLR4-dependent inflammation (14, 15). Therefore, to determine the effect of EGCG on TLR4 expression in macrophages, peritoneal macrophages were treated with the indicated concentrations of EGCG. As shown in Fig. 1A, EGCG suppressed TLR4 expression in a dose-dependent manner. The same result was obtained from the flow cytometry analysis of EGCG-treated peritoneal macrophages (Fig. 1B). To determine the effect of EGCG on TLR4 expression in a mouse model, mice received intraperitoneal injections of EGCG (10 mg/kg/day) for 3 days, which resulted in a significantly reduced TLR4 expression in splenocytes derived from EGCG-treated mice, as compared with that of vehicle-treated mice (Fig. 1C). To determine the effect of EGCG on the sensitivity of macrophages to the TLR4 ligand, macrophages obtained from EGCG-injected mice were treated with lipopolysaccharide (LPS). As shown in Fig. 1D, macrophages derived from EGCG-treated mice showed a limited response to the TLR4 ligand. Our data also showed EGCG pretreatment directly suppressed LPS susceptibility in isolated macrophages Fig. 1E. Taken together, EGCG suppressed both TLR4 expression and LPS sensitivity in isolated macrophages.

Because EGCG suppressed TLR4 expression without affecting TLR4 mRNA levels (Fig. 2A), we hypothesized that EGCG enhanced the degradation of TLR4. Ubiquitination is a crucial step in protein degradation brought about by the ubiquitin/ proteasome pathway. To test this hypothesis, macrophages from EGCG-treated mice were immunoprecipitated with anti-TLR4 antibody and evaluated by immunoblot analysis using an anti-ubiquitin antibody. As shown in Fig. 2B, the 67LR agonist induced TLR4 ubiquitination.

RNF216, also known as Triad3A, is an E3 ubiquitin-protein ring finger protein that regulates TLR4 degradation (16). As shown in Fig. 2C, EGCG treatment up-regulated RNF216 expression in a time-dependent manner. Our data showed that EGCG increased RNF216 at the protein level consistent with the effect of EGCG on RNF216 at the mRNA level (Fig. 2D). To determine whether RNF216 is involved in EGCG-induced TLR4 down-regulation, we evaluated the effect of RNF216 knockdown on the EGCG-elicited decrease in TLR4 expression. As shown in Fig. 2, E and F, RNF216 knockdown signifi-
cantly attenuated the reducing effect of EGCG on TLR4 expression.

Next, to determine whether EGCG increases RNF216 expression in vivo, mice received intraperitoneal injections of EGCG, which resulted in the up-regulation of RNF216 expression in splenocytes (Fig. 2G). The same result was obtained from macrophages derived from EGCG-treated mice (Fig. 2H). Taken together, these results indicate that EGCG decreased TLR4 expression through RNF216 up-regulation.

**EGCG Induces RNF216 Up-regulation through the cGMP Pathway**—cGMP is a secondary messenger involved in the regulation of vascular homeostasis and its induction is a well established strategy for the treatment of vasodilatation by increasing blood flow (17). Because 67LR, the target molecule of EGCG (18, 19), induces cGMP up-regulation when bound to EGCG (20–21), we hypothesized that EGCG would increase cGMP production in macrophages. As shown in Fig. 3A, EGCG up-regulated intracellular cGMP levels in a dose-dependent manner. Guanylyl cyclases belong to a family of enzymes that catalyze cGMP synthesis from GTP. To determine the role of cGMP in the EGCG-induced down-regulation of TLR4, the effect of a soluble guanylyl cyclase (sGC) inhibitor on EGCG-elicited down-regulation of TLR4 was evaluated. As shown in Fig. 3B, the sGC inhibitor NS-2028 significantly attenuated EGCG-induced TLR4 down-regulation.

To confirm the involvement of cGMP in EGCG-elicited TLR4 down-regulation, we examined the influence of the NO-independent sGC activator BAY 41-2272 on TLR4 expression in macrophages. The data presented in Fig. 3C suggest that cGMP induction is sufficient to reduce TLR4 expression levels in macrophages. To evaluate whether cGMP up-regulates RNF216 expression, the effects of the cGMP inducer on RNF216 expression were assessed. As shown in Fig. 3D, the cGMP inducer BAY 41-2272 increased RNF216 expression in a dose-dependent manner. Guanylyl cyclases belong to a family of enzymes that catalyze cGMP synthesis from GTP. To determine the role of cGMP in the EGCG-induced down-regulation of TLR4, the effect of a soluble guanylyl cyclase (sGC) inhibitor on EGCG-elicited down-regulation of TLR4 was evaluated. As shown in Fig. 3B, the sGC inhibitor NS-2028 significantly attenuated EGCG-induced TLR4 down-regulation.

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time-dependent manner. We also confirmed that BAY 41-2272 treatment significantly increased RNF216 mRNA expression in a dose-dependent manner (Fig. 3E). Our data demonstrated that sGC (NO-dependent cGMP synthase) inhibitor pretreatment completely canceled EGCG-induced RNF216 up-regulation at the protein level, indicating that EGCG increases RNF216 in a sGC-dependent manner (Fig. 3G). Importantly, the cGMP inducer-elicited TLR4 down-regulation was completely canceled by RNF216 knockdown (Fig. 3, H and I). Taken
together, these findings indicate that the well known secondary messenger cGMP, which is involved in vascular homeostasis, plays a crucial role in EGCG-induced TLR4 down-regulation through RNF216 up-regulation.

The Highly Absorbent 67LR Agonist EGCG3’Me Inhibits HF/HS Diet-induced Inflammation—Macrophages are mostly responsible for local inflammation through TNFα production in adipose tissue. Also, macrophage-derived TNFα is known to induce hyperinsulinemia and induction of macrophage infiltration followed by MCP-1 expression, which is involved in a feedback loop resulting in additional TNFα production (8). TNFα also causes hyperinsulinemia (22). A recent clinical study demonstrated a significant correlation between serum TNFα levels and states of hyperinsulinemia (23). To confirm 67LR involvement in a high absorbent EGCG analogue, EGCG3’Me-induced TNFα suppression, we transfected mouse primary macrophages with a lentivirus encoding scrambled control shRNA or shRNA against 67LR. Previous study showed that saturated fatty acids including palmitate (C16:0) triggered inflammatory signaling through TLR4 and suggested plays the crucial role in obesity-related chronic inflammation (13).

Remarkably, the silencing of 67LR abrogated the inhibitory effect of the 67LR agonist EGCG3’Me on palmitate-induced TNFα expression (Fig. 4, A and B). To determine the effect of this highly absorbent 67LR agonist on HF/HS diet-induced inflammation, 12-week-old male C57BL/6J mice were fed the HF/HS diet supplemented with EGCG3’Me (0.9 mg/mouse) for 8 weeks. As shown in Fig. 4C, the EGCG3’Me diet significantly suppressed TLR4 expression in the adipose tissue. Our data also showed that EGCG3’Me intake up-regulated RNF216 expression in adipose tissue (Fig. 4D). Interestingly, our data also suggested that LPS treatment significantly suppressed RNF216 expression at the protein level in macrophages (Fig. 4E). Food intake was slightly increased in EGCG3’Me-containing diet groups compared with that in the HF/HS diet group (data not shown).

HF/HS diet intake significantly up-regulated TNFα expression in adipose tissue. In contrast, the 67LR agonist completely canceled HF/HS diet-induced TNFα expression (Fig. 4F). We also found that MCP-1 expression in adipose tissue was up-regulated in the HF/HS group compared with that in the control group (Fig. 4G). However, EGCG3’Me suppressed the increase in HF/HS diet-induced MCP-1 expression (Fig. 4G). Our results also suggested that EGCG3’Me treatment strongly suppressed the IL-6 level, which is a major player in inflammation in adipose tissue (Fig. 4H). On the other hand, EGCG3’Me did not suppress but rather increased leptin, which is another regulator involved in obesity-induced inflammation in adipose tissue (Fig. 4I). EGCG3’Me treatment strongly suppressed serum MCP-1 levels (Fig. 4J). Collectively, these results demonstrate that the highly absorbent 67LR agonist strongly attenuated HF/HS diet-induced inflammation in vivo.

EGCG3’Me Suppresses HF/HS Diet-induced Dyslipidemia and Obesity—Adipose tissue inflammation is associated with hyperinsulinemia. TNFα is the main inflammatory cytokine implicated in hyperinsulinemia. Clinical studies suggest a significant correlation between serum TNFα levels and states of hyperinsulinemia (23, 24). Therefore, we hypothesized that EGCG3’Me suppresses lipogenesis by improving HF/HS-induced hyperinsulinemia. To examine the potential anti-hyperinsulinemia effects of EGCG3’Me, fasting serum insulin levels were evaluated. As shown in Fig. 5A, EGCG3’Me attenuated the HF/HS diet-induced increase in serum insulin. The down-regulation of hepatic insulin receptor substrate 2 (IRS-2) expression is observed in chronic hyperinsulinemia (25). Likewise, in this study, HF/HS diet intake reduced IRS-2 expression. However, there was no difference in IRS-2 expression levels between mice fed the HF/HS diet containing EGCG3’Me and those fed the control diet (Fig. 5B).

Adipose tissue inflammation is associated with obesity-induced hyperinsulinemia. As shown in Fig. 5C, TNFα expression significantly correlated with fasting insulin levels ($R_s = 0.719$, $p < 0.05$, Spearman rank test, $n = 18$).

Hyperinsulinemia is reported to induce dyslipidemia through the up-regulation of fatty acid synthesis (26). Therefore, we hypothesized that EGCG3’Me suppresses lipogenesis by improving HF/HS-induced hyperinsulinemia. Epidemiological and clinical studies have found the LDL-C/HDL-C ratio to be an excellent predictor of cardiovascular disease (27). The HF/HS diet significantly increased the LDL-C/HDL-C ratio. Conversely, the highly absorbent 67LR agonist suppressed the HF/HS-induced increase in the serum LDL-C/HDL-C ratio (Fig. 5D). EGCG3’Me also abrogated the HF/HS-induced increase in serum TG levels (Fig. 5E).

The liver plays a central role in fatty acid metabolism, including oxidation, synthesis, and storage. As shown in Fig. 5F, EGCG3’Me canceled the HF/HS diet-induced TG accumulation in the liver.

Therefore, these results suggest a correlation between fasting insulin levels and the LDL/HDL ratio ($R_s = 0.713$, $p < 0.05$, Spearman rank test, $n = 18$; Fig. 5G). Furthermore, there was a significant correlation between fasting insulin and liver TG levels (Fig. 5H). Taken together, EGCG3’Me intake ameliorated HF/HS diet-induced dyslipidemia accompanied with hyperinsulinemia.

Sterol regulatory element-binding protein-1 (SREBP-1) activates the transcription of genes encoding enzymes active in fatty acid biosynthesis in the liver and is involved in the development of fatty livers (28). Hyperinsulinemia triggers dyslipidemia through SREBP-1 (26). As shown in Fig. 5I, there was a 3.3-fold increase in SREBP-1 expression in the HF/HS diet group compared with that in the control diet group. However, the HF/HS diet containing EGCG3’Me produced no significant increase in SREBP-1 expression compared with the control diet.

SREBP-1 is involved in the expression regulation of acetyl-CoA carboxylase 1 (ACC1), fatty acid synthase (FAS), and sterol-CoA desaturase (SCD-1). Expression levels of SREBP-1-regulated downstream genes were increased in mice fed the HF/HS diet compared with those fed the control diet. However, there was no significant increase in the expression levels of these genes in mice fed the HF/HS diet containing EGCG3’Me compared with those fed the control diet (Fig. 5, J–L).

The HF/HS diet was also associated with up-regulation of genes coding for cholesterol synthesis enzymes, including 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMGCAR) and 3-hy-
droxy-3-methyl-glutaryl-CoA synthase (HMGCS). However, EGCG3'Me intake significantly diminished HMGCR (Fig. 5M) and HMGCS (Fig. 5N) expressions. Importantly, EGCG3'Me did not increase plasma levels of AST or ALT (data not shown).

Uncoupling protein-2 (UCP-2) is a recently identified member of the mitochondrial transporter superfamily that uncouples proton entry in the mitochondrial matrix from ATP synthesis and has been associated with the decreased risk of obesity (29). TNFα reportedly inhibits UCP-2 expression in adipose tissue (30). As shown in Fig. 5O, UCP-2 expression levels were reduced in the adipose tissue of mice fed the HF/HS diet compared with those fed the normal diet. In the group fed the
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HF/HS diet supplemented with EGCG3'Me, UCP-2 expression was not suppressed when compared with the control diet group. Hyperinsulinemia is reported to trigger SREBP-1 expression and induce dyslipidemia (26). Our data showed a significant correlation between the decrease in fasting serum insulin levels and suppression of SREBP-1 (Fig. 5P). Moreover, the highly absorbent 67LR agonist EGCG3'Me attenuated HF/HS diet-induced obesity (Fig. 5, Q and R). Taken together, these findings indicate that 67LR presents a potential novel target for the therapy of obesity-induced inflammation.

Discussion

Recent studies suggest that chronic low-grade inflammation is a characteristic of obesity (31, 32). Moreover, macrophage activation in the adipose tissue is a crucial step that drives the development of obesity and related diseases (32). The role of TNFα is crucial in obesity-induced inflammation, which promotes the development of hyperinsulinemia, dyslipidemia, and cardiovascular disease (6, 7). Studies show that TNF receptor knock-out (TNFR1 KO) mice are protected from diet-induced obesity and hyperinsulinemia (33). Macrophage-derived TNFα is involved in a feedback loop to increase TNFα production through MCP-1 secretion by adipocytes (8). Furthermore, the suppression of UCP-2 expression is induced by macrophage-derived TNFα (30). In this context, TLR4 plays a crucial role in obesity-induced chronic low-grade inflammation. Indeed, TLR4 knock-out mouse are insensitive to obesity-induced inflammation and hyperinsulinemia (9, 10). These findings suggest that TLR4 inhibition is sufficient to repress obesity-induced chronic inflammation. However, there is currently no clinically applicable approach to suppress TLR4 signaling. Here we showed that the 67LR agonist EGCG suppressed TLR4 expression both in vitro and in vivo and found that cGMP, a well-known secondary messenger involved in vascular homeostasis and sexual arousal-elicited penile erection, plays a crucial role in 67LR agonist-induced TLR4 suppression.

Studies have shown that TLR4 signaling is negatively regulated by Toll interacting protein through the inhibition of downstream signaling. However, little is known about the repression of these mediators of TLR4 at the protein level. We previously reported on the inhibitory effect of EGCG on TLR4 signaling by the EGCG receptor in a 67LR-dependent manner (34). However, little is known on the upstream mechanism. In the present study, (i) we revealed that EGCG induced ubiquitination of TLR4, (ii) we also confirmed that EGCG decreased TLR4 expression through E3 ubiquitin-protein ligase RNF216 through a knockdown experiment based on two different clones, (iii) EGCG increased cGMP levels in macrophages and the EGCG-induced TLR4 down-regulation was completely canceled by the soluble guanylate cyclase (cGMP synthesis enzyme) inhibitor, (iv) cGMP induction is sufficient to suppress TLR4 expression, and (v) cGMP-induced TLR4 down-regulation was canceled by silencing RNF216 through a knockdown experiment based on two different clones. Collectively, we showed the novel mechanisms of EGCG/sGC/cGMP/RNF216 in down-regulation of TLR4 expression without affecting mRNA levels. However, the precise mechanisms involved in the RNF216-dependent decrease in TLR4 expression have not been fully elucidated. This mechanism could be particularly valuable to clarify the role of cGMP in down-regulation of TLR4.

The highly absorbent 67LR agonist EGCG3'Me inhibited HF/HS diet-induced TNFα up-regulation in the adipose tissue accompanied with canceling the HF/HS diet-elicited MCP-1 increase in the adipose tissue and serum. Furthermore, EGCG3'Me intake strongly abrogated the inhibitory effect of the HF/HS diet on UCP2 expression. Taken together, the inhibition of TNFα expression could play a major role in the pharmacological effect of this 67LR agonist.

HF/HS diet highly induces SREBP-1, a master regulator of hepatic lipogenesis, which is crucial in liver TG accumulation, and a positive regulator of ACC, FAS, and SCD-1 (28). EGCG3'Me suppresses HF/HS-induced liver TG accumulation and SREBP-1 up-regulation. ACC and FAS are known to be involved in lipogenesis and the up-regulation of ACC and FAS expression levels has been linked to TG accumulation in the liver. SCD-1 catalyzes the de novo synthesis of oleate and palmitoleate, the major fatty acids in TGs (35). Targeting SCD-1 by antisense oligonucleotides results in the up-regulation of the metabolism rate and prevention of diet-induced obesity and steatosis (35). EGCG3'Me normalized the HF/HS-induced increase of ACC, FAS, and SCD-1 in the liver. These results suggest that EGCG3'Me improved HF/HS diet-induced hypertriglyceridemia by the suppression of SREBP-1. Hyperinsulinemia triggers SREBP-1 expression and induces dyslipidemia (26). Our data revealed a significant correlation between the decrease in fasting serum insulin levels and suppression of dyslipidemia, suggesting that the 67LR agonist may inhibit dyslipidemia accompanied with hyperinsulinemia.

Several studies have stated the LDL-C/HDL-C ratio to be a good predictor of cardiovascular disease risk and a monitor for the evaluation of lipid-lowering therapies (27). EGCG3'Me strongly abrogated the increase in the HF/HS-induced LDL-C/HDL-C ratio. Moreover, EGCG3'Me canceled the HF/HS diet-induced up-regulation of HMGCR and HMGCS. Because HMGCR is the rate-controlling enzyme of the mevalonate
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pathway that produces cholesterol, EGCG3”Me may reduce the LDL-C/HDL-C ratio by the inhibition of HMGCR expression.

IRS-2 is a cytoplasmic protein that acts as an adaptor between the insulin receptor and downstream effectors to mediate insulin signaling (36). Previous experiments suggest IRS-2 to be a major player in the activities of hepatic insulin (25), and the disruption of insulin IRS-2 causes type 2 diabetes (25, 36). Taken together, the anti-insulin resistance effect of EGCG3”Me may be in part due to suppression of the down-regulation of HF/HS diet-induced IRS-2 expression.

UCP-2 presents a potent candidate target for obesity because it may contribute to the uncoupling of oxidative phosphorylation and consumption of chemical energy as heat (29). Recent studies have clarified the role of UCP-2 in the regulation of whole body energy homeostasis. In humans, UCP-2 mRNA expression in adipose tissue is reportedly lower in obese subjects than in healthy individuals (29, 38); thus, UCP-2 may be associated with the development and progression of obesity.

Benifuuki extract, which contains EGCG3”Me, showed no side effects on hematological examination, general biochemical examination, or analysis of serum iron content (39). We previously reported that benifuuki conveyed a significant anti-obesity effect in an animal model compared with the effect of major green tea cultivar yabukita (11). Benifuuki contains more methylated catechins including EGCG3”Me compared with that from yabukita, the Japanese green tea cultivar. However, our comprehensive analysis of compounds in both green tea cultivars showed very different metabolite profiles between yabukita and benifuuki (40). In this study, for the first time, we successfully isolated the sufficient highly absorbent 67LR agonist (−)-epigallocatechin-3-O-(3-O-methyl)-gallate for long term oral-administrated study and proved its effect on a HF/HS diet in vivo. Our data revealed that oral intake of this polyphenol strongly suppressed HF/HS diet-induced chronic inflammation and hyperinsulinemia accompanied by TLR4 down-regulation. Our data also suggest that EGCG3”Me is an important component in the anti-obesity effect of benifuuki.

Recent studies showed EGCG markedly suppressed non-LPS (PMA/adenosine diphosphate)-induced phosphorylation levels of p38 MAP kinase (41, 42). Considering the mechanisms of PMA/adenosine diphosphate-induced MAP kinase activation, those data suggested the existence of different inhibitory mechanisms from TLR4 signal inhibition. However, considering the mechanisms of (PMA/adenosine diphosphate)-induced phosphorylation levels of IRS-2 mRNA expression in adipose tissue strongly suggested the TLR4 signaling plays a crucial role in development of atherogenesis (47). Our data suggested that the anti-inflammatory effect of cGMP on TLR4 may contribute to the protective effect of NO/cGMP in blood vessels. Moreover, recent studies showed that diabetes states and oxidative stress deteriorate the eNOS/cGMP axis (48, 49). Because inflammation is the trigger in endothelial dysfunction, down-regulation of cGMP may contribute to the progression of atherogenesis through disruption of the immunosuppressive effect.

Unwilling TLR4 signaling-induced low grade inflammation are widely involved in several types of disease including obesity-induced inflammation (13), atherogenesis (47), Alzheimer’s disease (50), tumor metastasis (45), and the adverse effect of opioids (37). However, the effective countermeasures for suppressing TLR4 signaling is not established. Because direct/indirect up-regulation of cGMP is a widely used applicator clinically, cGMP induction could be the novel approach to suppress TLR4 signaling.

In conclusion, the present work indicates that the highly absorbent 67LR agonist EGCG3”Me significantly reduced TLR4 expression and ameliorated HF/HS diet-induced inflammation and accompanying hypolipidemic effects. Together, these finding suggest that 67LR may be an ideal therapeutic target for the treatment of obesity-induced chronic inflammation.
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Experimental Procedures

Study Approval—All animal studies were performed in accordance with the regulations (protocol number 105) and notification procedures (protocol number 6) required by the Japanese government regarding the welfare of experimental animals. The study protocol was approved by the Animal Care and Use Committee of Kyushu University, Fukuoka, Japan (approval number A22-146).

Antibodies and Reagents—BAY 41-2272 was from Enzo Life Sciences (ALX-420-030-M005). We purchased the cGMP assay kit from Cayman Chemical (581021). EGCG (E4143) were obtained from Sigma. The AST/ALT activity assay kit was purchased from Wako. Anti-β-actin antibody (Sigma, 061M48080), anti-Ub antibody (sc-8017), anti-RNF216 antibody (ab25961), and anti-TLR4 antibody (ab22048) were purchased as indicated. Antibody validation is available on the manufacturer’s websites. Phycoerythrin (PE)-labeled anti-TLR4 antibody was purchased from ebioscience (12-9924-82). Cells were transfected with Lipofectamine™ RNAiMAX Transfection Reagent (Life Technology) and siRNA (Sigma) as per the manufacturer’s protocol. Biochemical analyses of plasma components, including TG, HDL-C, LDL-C, and MCP-1 were measured using the TG E-test, the transaminiase CII-test (each from Wako Pure Chemical Industries, Ltd., Osaka, Japan), HDL-C, LDL-C, and LDL-C Quantification Kit (BioVision, Inc., Milpitas, CA), and MCP-1 ELISA kit (Wako Pure Chemical Industries, Ltd.), respectively. All kits were used in accordance with the manufacturer’s instructions. Flow cytometry analysis was performed using Verso (BD Bioscience).

Mice and Diet and Sample Preparation—Male C57/BL6 mice (12 weeks old) were purchased from Charles River Laboratories Japan, Inc. (Kanagawa, Japan), and maintained in a humidity- and temperature-controlled facility under a 12-h light-dark cycle (light from 8 a.m. to 8 p.m.). All mice were acclimated and fed an AIN-93G diet for 1 week. AIN-93G and HF/HS diets were obtained from KBT Oriental Co., Ltd. (Tokyo, Japan). Mice were assigned to the following three groups: 1) control group, fed an AIN-93G diet; 2) HF/HS group, fed a HF/HS diet; or 3) EGCG3°Me group, fed the HF/HS diet containing EGCG3°Me (0.9 mg/day). In the control group, mice were fed a control diet. In the HF/HS group, mice were fed by a HF/HS diet. In EGCG3°Me group, mice were fed EGCG3°Me containing the HF/HS diet. To determine the effect of EGCG3°Me on food intake, we weighted the food intake. At the end of 8 weeks, plasma was collected after overnight food deprivation and stored at −80 °C. After blood collection, mice were anesthetized by isoflurane overdose. Adipose tissues and organs were harvested. The central lobe of the liver was excised from each mouse and placed in a plastic tube containing RNAlater™ solution (Ambion, Inc., Austin, TX), which was maintained at 4 °C for 24 h and then stored at −80 °C. cDNA was synthesized from total RNA (1 μg) using the PrimeScript RT reagent kit (Takara Bio, Inc., Tokyo, Japan). Gene expression was analyzed by real-time quantitative PCR using the SYBR Green procedure and a Thermal Cycler Dice® Real Time System (Takara Bio, Inc.). Because the effect of the 67LR agonist on HF/HS diet-induced inflammation in vivo was not predicted, we did not use a power calculation to reduce an appropriate group size; instead we used a small group size of treated and control mice, which was demonstrated as sufficient for statistical significance tests. The investigators were not blinded to allocation during experiments and outcome assessment.

TNFα Production by Palmitate-stimulated Peritoneal Macrophages—Thioglycollate-elicited peritoneal macrophages were obtained from specific pathogen-free, 8-week-old, male mice by injection of 1 ml of sterile 3% thioglycollate solution (Difco Laboratories, Inc., Detroit, MI), stored for 4 days in 10 ml of phosphate-buffered saline, and then washed once with Roswell Park Memorial Institute (RPMI) 1640 medium (Wako Pure Chemical Industries, Ltd.) supplemented with fetal bovine serum (FBS) (Biological Industries, Kibbutz Beit Kama, Israel), 100 units/ml of penicillin, and 100 units/ml of streptomycin. The cells were resuspended in RPMI 1640 medium supplemented with 10% FBS at a cell viability of 95%, as determined by trypan blue dye exclusion, then plated and incubated for 4 h at 37 °C in a humidified incubator containing 5% CO2 to allow macrophage adherence. The inhibitory effect of intraperitoneal (i.p.) EGCG injection (10 mg/kg/day i.p.) on LPS sensitivity in macrophages was performed based on isolated macrophages (5 × 105 cells) from each mouse treated with LPS (50 ng/ml) for 24 h. The effect of EGCG injection (10 mg/kg/day i.p.) on LPS sensitivity was determined by ELISA (R&D Systems) based on TNFα production. Isolated macrophage cells (1 × 105 cell/ml in a 24-well plate) were treated with 200 μM palmitate for 3 h. pLKO.1 knockdown vectors, encoding scrambled control shRNA or shRNAs targeting the 67-kDa laminin receptor (67LR), were purchased from Sigma. For lentivirus production, a lentiviral expression vector was cotransfected in accordance with the manufacturer’s protocol.

Western Blot Analysis—Cells (5 × 105 cells/sample) were seeded onto a 12-well plate and treated with various agents as indicated in the figure legends. Cells were lysed in the above mentioned lysis buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 50 mM NaF, 30 mM Na3P2O7, 1 mM phenylmethanesulfonyl fluoride, 2 mg/ml of aprotinin, and 1 mM pervanadate. Western blotting was performed as previously described. Anti-67LR serum used for Western blot analysis was obtained from a rabbit that was immunized with synthesized peptide corresponding to residues 161–170 of mouse 67LR. Macrophages were seeded at the density of 1 × 105 cells/ml in 5-ml dishes and treated with EGCG (5 μM) and cultured for 12 h. Beads used in immunoprecipitation were purchased from Novex by Life Technologies.

Flow Cytometer—Macrophages were seeded at the density of 1 × 105 cells/ml in 5-ml dishes and treated with the indicated concentrations of EGCG and cultured for 24 h. Cells were incubated with Accumax (Innovative Cell Technologies). Cells were incubated with the following primary antibody: anti-mouse TLR4 (Bioscience catalog number 12-9924-82) (1:30 dilution). Staining procedures were performed according to the manufacturer’s protocols. Flow cytometry analysis was performed using Verso (BD Bioscience).

Quantitative Real-time Analysis—Cells (1 × 105 cells/sample) were seeded onto a 12-well plate and treated with various agents as indicated in the figure legends. Total RNA was
extracted by using TRizol reagent (Invitrogen) based on the manufacturer’s protocol. The RNA was assessed by using a Nanodrop® and stored at −80 °C. Quantitative PCR analysis was carried out based on the relative standard curve method to assess the target gene expression. 1 µl of cDNA was assessed as the template for the experiment, which was performed on the Thermal Cycler Dice Real-time System (Takara Bio). Primers were designed as described (11).

Statistics—All statistical analyses were performed using one-way analysis of variance and the Tukey–Kramer test with KYP software (Kyens Lab, Inc., Tokyo, Japan). In cases where the KS normality test indicated non-parametric distribution, Dunn’s multiple comparisons test was performed using GraphPad Prism® version 6.0. None of the investigators were blinded to group allocations. The Spearman’s rank test was used to identify correlations with SPSS software (IBM-SPSS, Inc., Chicago, IL). Data are presented as the mean ± S.E. A probability (p) value of <0.05 was considered statistically significant.

Author Contributions—M. K., Y. N., M. Y., T. S., K. T., and H. T. designed the experiments; M. K., Y. N., M. Y., T. S., K. T., Y. H., J. B., S. Y. M., S. Y., S. W., Y. W., and Y. T. performed the experiments; M. K., Y. N., and H. T. wrote the paper. All authors analyzed the results and approved the final version of the manuscript.

References
EGCG Attenuates Inflammation through Up-regulation of cGMP