Introduction

Obesity is closely associated with a low-grade state of inflammation, resulting from enlargement of adipocytes and increased macrophage infiltration into the adipose tissue (1,2). Obese adipose tissue is characterized by abnormal production and secretion of adipokines as well as activation of inflammatory signaling in adipocytes (1,2). As obesity-induced inflammation in adipocytes develops, secretion of pro-inflammatory adipokines, including monocyte chemoattractant protein-1 (MCP-1) and interleukin-6 (IL-6), increases, which in turn leads to a decrease in insulin sensitivity (1,2). Furthermore, it has been suggested that the nuclear factor-κB (NF-κB) pathway plays an important role in facilitating adipocyte inflammation. The NF-κB signal is related to the up-regulation of pro-inflammatory adipokines and the down-regulation of adiponectin which has anti-inflammatory and insulin-sensitizing properties (3-5).

Tumor necrosis factor-α (TNF-α) is one of the most important molecules in obesity. TNF-α released from adipose tissue and macrophages is markedly increased in obese or diabetic subjects (6,7). It induces insulin resistance by modulating the secretion of pro-inflammatory adipokines, such as MCP-1 and IL-6, and directly contributing to the inhibition of adiponectin production (6-9). It has been shown that TNF-α activates the NF-κB pathway and suppresses the expression of peroxisome proliferator-activated receptor-γ (PPARγ), which is a strong transcriptional inducer of adiponectin (10).

Vitamin E exists in nature as eight vitamers that are subdivided into two subgroups called tocopherols and tocotrienols, each including the α-, β-, γ- and δ-forms (11,12). Tocopherols possess a saturated phytol chain, whereas tocotrienols contain an unsaturated side chain. Vitamin E isomers differ from each other by the number of methyl groups in the chroman ring. Although tocopherols and tocotrienols exhibit strong antioxidant activities (11,12), most vitamin E studies have focused on tocopherols, and very little is known about tocotrienols. Tocotrienols are primarily found in oat, wheat germ, rice bran and palm oil (11). However, previous studies have found that
tocotrienols have various physiological activities, including anticancer, cardiovascular-protective, hypcholesterolemic and neuroprotective activities (12,13). Among the tocotrienol isomers, γ-tocotrienol, the most common tocotrienol isomer, has been well documented for its physiological availability. It has been shown that γ-tocotrienol suppresses adipocyte differentiation (14), and oral administration of γ-tocotrienol significantly decreases body fat in rats (15).

Tocotrienols have been shown to possess anti-inflammatory effects in certain cell types, which are mediated by inhibition of the NF-κB activation pathway (16,17). It remains unknown whether γ-tocotrienol exerts such effects in adipocytes. Although administered γ-tocotrienol can be accumulated in adipose tissue (18), there have been no reports on its effects on adipokine regulation. Therefore, in the present study, we examined the effects of γ-tocotrienol on the TNF-α-induced changes in secretion and gene expression of inflammatory-related adipokines, and activation of the NF-κB pathway in 3T3-L1 adipocytes.

Materials and methods

Reagents. Recombinant TNF-α was purchased from R&D Systems (Minneapolis, MN, USA). γ-tocotrienol was from Cayman Chemical (Ann Arbor, MI, USA). Dulbecco’s modified Eagle’s medium (DMEM) and insulin were from Sigma-Aldrich (St. Louis, MO, USA). Isobutylmethylxanthine (IBMX), sodium pyruvate and dexamethasone (DEX) were from Nacalai Tesque (Kyoto, Japan). Fetal bovine serum (FBS) was from Gibco-BRL (Rockville, MD, USA).

Cell culture and treatment. 3T3-L1 cells (Health Science Research Resources Bank, Osaka, Japan), were maintained in DMEM containing 25 mM glucose, 1 mM sodium pyruvate and 10% FBS, in a 5% CO₂ atmosphere at 37°C. Differentiation was induced by replacing the medium with FBS-supplemented DMEM containing 200 nM insulin, 0.5 mM IBMX and 1 µM DEX for 2 days. After another 2 days of incubation in 10% FBS/DMEM medium with 200 nM insulin, the medium was replaced every 2 days with 10% FBS/DMEM medium until >90% of cells were demonstrating the adipocyte phenotype. On days 6-8 of differentiation, 3T3-L1 adipocytes were pre-treated with 0.024-2.4 µM γ-tocotrienol for 6 h and then stimulated for 24 h with 10 ng/ml TNF-α. Following the 24-h incubation, the conditioned medium was collected for measurement of adipokines by ELISA. Total RNA and protein were isolated and the expression levels of the genes and proteins of interest were evaluated by quantitative real-time RT-PCR and Western blotting, respectively.

Real-time RT-PCR analysis. Total RNA was extracted from 3T3-L1 adipocytes using Sepasol-RNA I (Nacalai Tesque, Kyoto, Japan). For complementary DNA synthesis, 1 µg of total RNA was reverse-transcribed using the reverse transcription system (PrimerScript RT reagent kit, Takara Bio, Shiga, Japan). Real-time RT-PCR was performed by the ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA, USA) using SYBR Green fluorescence signals (SYBR Premix Ex Taq II; Takara Bio). The following oligonucleotide primer pairs were used: mouse MCP-1, 5′-CTC ACC TGC TGC TAC TCA T-3′ (forward) and 5′-TGG TGA TCC TCT TGT AGC TCT CC-3′ (reverse); mouse IL-6, 5′-GCT ACC AAA CTG CTG ATT ACC AGG A-3′ (forward) and 5′-CCA GGT AGC TAT GGT ACT CCG AA-3′ (reverse); mouse adiponectin, 5′-GGC CTA CTG CAA CAA TCC GAC-3′ (forward) and 5′-TAC ACC TGG AGC CAG ACT TG-3′ (reverse); mouse PPARγ, 5′-GCC GAT CTT CCT GAG AAA GCC-3′ (forward) and 5′-CCC TTG AAA AAT CAC GTG GG-3′ (reverse); mouse 36B4, 5′-CCT GAG GTG AGG CAG CAG CAG-3′ (forward) and 5′-GCT CCA AGC AGA TGC AGC A-3′ (reverse). Expression levels of RNA, expressed as relative mRNA levels compared to control, were calculated after normalization to 36B4.

Adipokine ELISA. The culture medium from 3T3-L1 adipocytes was collected from each sample 24 h after TNF-α treatment. The concentrations of MCP-1, IL-6 and adiponectin were assayed using a mouse MCP-1 ELISA kit (R&D systems), a mouse IL-6 ELISA kit (R&D systems) and a mouse adiponectin ELISA kit (R&D systems).

Protein isolation and Western blotting. Whole cell lysates were prepared using RIPA buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% Na-deoxycholate and 0.1% SDS) containing a protease inhibitor cocktail (Nacalai Tesque) and complete phosphatase inhibitors (Nacalai Tesque). Lysates were centrifuged at 12,000 g for 10 min at 4°C and the supernatants were boiled in SDS loading buffer. The boiled samples were separated by an SDS-PAGE gradient gel (10-20%) and transferred to PVDF membranes (Bio-Rad Laboratories, CA, USA). Membranes were blocked with blocking reagent (Blocking One-P; Nacalai Tesque) and incubated with anti-IκB-α and anti-phospho-IκB-α (Ser 32) antibodies (Cell Signaling Technology, Beverly, MA, USA), followed by incubation with horseradish peroxidase-conjugated secondary antibodies. Western blot analysis was conducted using an enhanced chemiluminescence detection system (ECL-Plus; Amersham Pharmacia, Arlington, IL, USA).

Quantification of NF-κB activation. To quantify NF-κB activity, nuclear extracts were prepared using Nuclear Extract kit (Active Motif, Carlsbad, CA, USA) and analyzed with a sensitive ELISA-based kit (PathScan Total NF-κB activity, sandwich ELISA kit; Cell Signaling Technology) to quantify NF-κB activity, according to the manufacturer’s instructions.

Statistical analysis. Results are expressed as the means ± SEM. Data were analyzed using one-way analysis of variance (ANOVA) between groups with the Dunnett post-hoc test. Statistical analyses were performed using the SPSS 11.0 software (SPSS Inc., Chicago, IL, USA). Significant differences were considered to be present at P<0.05.

Results

Effects of γ-tocotrienol on adipokine secretion in TNF-α-treated 3T3-L1 adipocytes. To investigate whether γ-tocotrienol affects the TNF-α-induced secretion of adipokines, 3T3-L1 adipocytes were pre-treated with various concentrations of γ-tocotrienol for 6 h and then incubated with 10 ng/ml TNF-α...
for 24 h. Adipokines secreted into the conditioned medium were measured by an ELISA assay. TNF-α-induced increases in MCP-1 and IL-6 secretion were significantly inhibited by γ-tocotrienol treatment (Fig. 1A and B). At the γ-tocotrienol concentration of 2.4 µM, the secretions of MCP-1 and IL-6 were decreased by 27.7 and 36.5%, respectively. By contrast, adiponectin secretion, which was decreased by TNF-α stimulation, was restored by γ-tocotrienol treatment (Fig. 1C). In the presence of 2.4 µM γ-tocotrienol, adiponectin levels were 1.24-fold higher than with TNF-α alone. Thus, treatment with γ-tocotrienol attenuated the effects of TNF-α on the secretions of three adipokines.

**Effects of γ-tocotrienol on adipokine gene expression in TNF-α-treated 3T3-L1 adipocytes.** The gene expression of MCP-1, IL-6 and adiponectin tested by real-time quantitative RT-PCR analysis is shown in Fig. 2. The enhanced expression of MCP-1 and IL-6 mRNA by TNF-α-stimulation was effectively inhibited by γ-tocotrienol treatment (Fig. 2A and B). At 2.4 µM γ-tocotrienol, the gene expression of MCP-1 and IL-6 was suppressed by 55.6 and 62.8%, respectively. γ-tocotrienol also attenuated the inhibiting effect of TNF-α on adiponectin gene expression (Fig. 2C). The expression of adiponectin mRNA was restored to 87.2% of control by γ-tocotrienol treatment. Furthermore, PPARγ mRNA expression, which was suppressed by TNF-α, was restored to the control level by treatment with γ-tocotrienol at all concentrations tested (Fig. 2D). Thus, TNF-α-induced changes in the mRNA transcription levels of adipokines were also effectively suppressed by γ-tocotrienol.
γ tocotrienol inhibits TNF-α-induced activation of NF-κB in 3T3-L1 adipocytes. Activation of transcription factor NF-κB plays an important role in the TNF-α-mediated inflammation progress, the down-regulation of adiponectin and the up-regulation of inflammatory molecules, including MCP-1 and IL-6 (3-5). The release and nuclear translocation of active NF-κB are regulated by phosphorylation of IκB-α (19). To further evaluate whether the anti-inflammatory function of γ-tocotrienol is mediated by NF-κB, the effects of γ-tocotrienol on IκB-α phosphorylation and NF-κB (p65) nuclear translocation were examined by Western blotting and ELISA assay. As shown in Fig. 3, TNF-α increased the phosphorylation level of IκB-α, which was attenuated by treatment with γ-tocotrienol. Furthermore, γ-tocotrienol effectively suppressed the TNF-α-enhanced nuclear translocation of NF-κB (Fig. 4).

Discussion

Obesity is considered to be a state of low-grade inflammation in adipose tissues, which is closely associated with the development of insulin resistance (1,2). This inflammatory condition is partly caused by macrophage infiltration into the adipose tissue and, subsequently, the inflamed adipocyte itself enhances the production of various pro-inflammatory cytokines, including TNF-α, MCP-1 and IL-6 (1,2). It has been reported that TNF-α levels are increased in obese subjects, and that they induce the inflammation of adipocytes through the elevation of inflammatory adipokines (6,7). Furthermore, it is known that TNF-α is one of the negative regulators of adiponectin, attenuating its beneficial effects, such as anti-inflammation and facilitation of insulin sensitivity (8,9). Therefore, anti-inflammatory treatment could be an effective way to prevent or treat insulin resistance and type 2 diabetes. Several reports have suggested that dietary tocotrienols accumulate in adipose tissue and skin (18); however, not much is known about their physiological effects on adipocytes. It has also been reported that treatment with γ-tocotrienol reduced body fat mass in rats (15). A more recent study by Uto-Kondo et al showed that γ-tocotrienol suppressed adipocyte differentiation in 3T3-L1 preadipocytes (14). In the present study, we demonstrated for the first time that γ-tocotrienol effectively attenuated the TNF-α-mediated increase in MCP-1 and IL-6 secretion and decrease in adiponectin secretion in 3T3-L1 adipocytes. Furthermore, the TNF-α-induced changes in the mRNA expression of each adipokine were also inhibited by γ-tocotrienol. These results indicate that γ-tocotrienol affected the TNF-α-mediated changes in the secretion of adipokines at the transcription level.

Activation of the transcription factor NF-κB is considered to play a major role in TNF-α-induced inflammatory responses, including down-regulation of adiponectin and up-regulation of MCP-1 and IL-6 in adipocytes (3-5). NF-κB is activated by TNF-α via phosphorylation and removal of IκB-α, resulting in its translocation to the nucleus and up-regulation of gene expression of pro-inflammatory adipokines, such as MCP-1 and IL-6 (3-5). Adiponectin suppression mediated by TNF-α is also regulated by NF-κB activation. Indeed, Kamon et al showed that TNF-α-induced down-regulation of adiponectin secretion was cancelled by IκB kinase β inhibitor in 3T3-L1 adipocytes (20). Consistent with these studies, we observed that TNF-α enhanced the phosphorylation of IκB-α and the nuclear translocation of NF-κB.

Recent studies have demonstrated in different cell types that the anti-inflammatory effects of tocotrienols are mediated by suppression of the NF-κB pathway (16). A tocotrienol-rich fraction of palm oil showed anti-inflammatory activity by inhibiting NF-κB expression in human monocyctic cells (17). Moreover, treatment of streptozotocin-induced diabetic rats with tocotrienols significantly suppressed the activation of the NF-κB pathway in the kidney and improved the renal function (21). In this study, our results show that γ-tocotrienol inhibits the TNF-α-induced activation of NF-κB in 3T3-L1 adipocytes. These observations indicate that γ-tocotrienol possesses anti-inflammatory properties, such as attenuation of MCP-1 and IL-6 expression, through the suppression of NF-κB activation in adipocytes.
It is well known that adiponectin improves insulin sensitivity, partly through its anti-inflammatory effects (9,22,23). Adiponectin is highly expressed in adipocytes, and is partly transcriptionally activated by PPARγ, which is negatively regulated by TNF-α-induced inflammation (10). Previous studies have found that adiponectin treatment suppressed inflammation-mediated increase in MCP-1 and IL-6 production in 3T3-L1 cells, through attenuation of NF-κB activation and increased PPARγ expression (23). Thus, MCP-1 and IL-6 counteract adiponectin production in adipocytes. In our experiments, γ-tocotrienol reversed the TNF-α-induced decrease in both adiponectin secretion and PPARγ expression. These results suggest that γ-tocotrienol regulates adiponectin production via PPARγ, which may be involved in its anti-inflammatory effects in adipocytes. Moreover, it has been demonstrated that the transcriptional activity of NF-κB is inhibited by PPARγ in other cell types (24). Thus, our results suggest that γ-tocotrienol down-regulates the activation of NF-κB in part by increasing PPARγ expression in adipocytes.

In summary, γ-tocotrienol inhibits the TNF-α-induced inflammatory effects in 3T3-L1 adipocytes, and this action is mediated by suppression of NF-κB activation. These findings provide novel insight into the prevention and treatment of obesity-related pathologies.

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