Carvacrol, a component of thyme oil, activates PPARα and γ and suppresses COX-2 expression[S]

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Abstract Cyclooxygenase-2 (COX-2), the rate-limiting enzyme in prostaglandin biosynthesis, plays a key role in inflammation and circulatory homeostasis. Peroxisome proliferator-activated receptors (PPARs) are ligand-dependent transcription factors belonging to the nuclear receptor superfamily and are involved in the control of COX-2 expression, and vice versa. Here, we show that COX-2 promoter activity was suppressed by essential oils derived from thyme, clove, rose, eucalyptus, fennel, and bergamot in cell-based transfection assays using bovine arterial endothelial cells. Moreover, from thyme oil, we identified carvacrol as a major component of the suppressor of COX-2 expression and an activator of PPARα and γ. PPARγ-dependent suppression of COX-2 promoter activity was observed in response to carvacrol treatment. In human macrophage-like U937 cells, carvacrol suppressed lipopolysaccharide-induced COX-2 mRNA and protein expression, suggesting that carvacrol regulates COX-2 expression through its agonistic effect on PPARγ. These results may be important in understanding the antiinflammatory and antilifestyle-related disease properties of carvacrol.—Hotta, M., R. Nakata, M. Katsukawa, K. Hori, S. Takahashi, and H. Inoue. **Carvacrol, a component of thyme oil, activates PPARα and γ and suppresses COX-2 expression. *J. Lipid Res.* 2010.** 51: **132–139.**

Supplementary key words cyclooxygenase • peroxisome proliferator-activated receptor • thymol • essential oil

Cyclooxygenase (COX), the rate-limiting enzyme in prostaglandin (PG) biosynthesis, has two isoforms, COX-1 and -2. COX-1 is constitutively expressed in most cells, whereas COX-2 is typically absent. However, COX-2 is induced by inflammatory stimuli such as endotoxins and lipopolysaccharide (LPS), suggesting that COX-2 plays a role in inflammation (1–3). Recent studies have shown that COX-2 is involved in not only inflammation but also circulatory homeostasis (4–6).

The peroxisome proliferator-activated receptors (PPARs) are members of a nuclear receptor family of ligand-dependent transcription factors (7). The PPAR subfamily comprises three isotypes, PPARα, β/δ, and γ, which play various roles in lipid and carbohydrate metabolism, cell proliferation and differentiation, and inflammation; they are considered molecular targets against lifestyle-related diseases (8, 9). The PGD_2 metabolite 15-deoxy-$\Delta^{12,14}$ PGJ_2 (15d-PGJ_2) was identified as a potent natural ligand of PPARγ (10, 11). Previously, we reported that 15d-PGJ_2 suppressed LPS-induced expression of COX-2 in differentiated, macrophage-like U937 cells, but not in vascular endothelial cells, and that the expression of COX-2 was regulated by a negative feedback loop mediated through PPARγ, especially in macrophages (12). Likewise, the PPARα agonist fenofibrate inhibited interleukin-1-induced COX-2 expression in smooth muscle cells (13). These findings indicate that PPARs participate in cell type-specific control of COX-2 expression.

Resveratrol, a phytoalexin and antioxidant polyphenolic compound found in red wine and various plant products, has long been suspected to have cardioprotective effects and to be a contributor to the so-called "French paradox" (i.e., the relatively low incidence of coronary heart disease in France compared to other developed countries with comparable diets; 14−16). We demonstrated that resveratrol suppressed COX-2 expression in 184B5/HER-transformed mammary epithelial cells (17), activated PPARα and γ in cell-based reporter assays using bovine arterial endothelial cells (BAEC), and protected the brain against ischemic stroke in mice through a PPARα-dependent mechanism (18). Similarly, polypheno-

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Abbreviations: BAEC, bovine arterial endothelial cell; COX, cyclooxygenase; FID, flame ionization detection; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; LPS, lipopolysaccharide; 15d-PGJ_2, 15-deoxy-$\Delta^{12,14}$ PGJ_2; PG, prostaglandin; PKC, protein kinase C; PPAR, peroxisome proliferator-activated receptor; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; TRP, transient receptor potential.

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[S] The online version of this article (available at http://www.jlr.org) contains supplementary data in the form of two tables and five figures.

lic compounds such as apigenin, chrysin (19, 20), and humulon (21) suppress COX-2 expression, and these compounds activate PPARα and/or γ (22, 23).

The suppression of COX-2 expression and the agonistic activity for PPARs are useful in evaluating the function of food-related components in lifestyle-related diseases. Similar to the case of 15d-PGJ_2, it is possible that COX-2 expression is regulated by PPAR agonism exerted by chemical components in food. In this context, we evaluated commercially available lipids derived from various plants and found that six essential oils have COX-2 suppressive activity. Moreover, we identified carvacrol, from the essential oil of thyme, as a major suppressor of COX-2 expression and activator of PPARα. In addition, PPARγ-dependent suppression of COX-2 promoter activity was observed in response to carvacrol, indicating that carvacrol regulates COX-2 expression through its agonistic effect on PPARγ.

MATERIALS AND METHODS

Materials

Bergamot, castor, clove, croton, eucalyptus, fennel, linseed, olive, orange, sesame, soybean, turpentine oils, and chemical components of thyme oils were from Wako Chemicals (Japan). Cottonseed and safflower oils were from MP Biomedicals. Lavender, lemon, and palm oils were from Spectrum Chemicals. Thyme and rose oils were from ICN Biochemicals and Indofine Chemicals, respectively.

Cell culture

U937 cells (24) and BAEC (25) were grown in RPMI 1640 and DMEM, respectively, supplemented with 10% fetal calf serum and 50 μM 2-mercaptoethanol. For differentiation into monocytes/macrophages, U937 cells were treated with 100 nM 12-*O*-tetradecanoylphorbol-13-acetate (TPA) and allowed to adhere for 48 h, after which they were fed with TPA-free medium and cultured for 24 h before use (26).

RNA purification and quantitative RT-coupled real-time PCR

Total RNA from differentiated U937 cells was isolated using the acid guanidinium thiocyanate procedure and analyzed for gene expression by real-time quantitative RT-PCR (Mx3005, Stratagene). After converting total RNA (5 μg) to cDNA using SuperScript III RT (Invitrogen), quantitative PCR was performed using SYBR green real-time PCR Master Mix-Plus (Toyobo, Japan). The following primer pairs were used for amplification of COX-2 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH): 5′-TGCTGTGGAGCTGTATCCTG-3′ (COX-2 forward), 5′-ACAGCCCTTCACGTTATTGC-3′ (COX-2 reverse), 5′-GGTGAAGGTC GGAGTCAACGGA-3′ (GAPDH forward), and 5′-GAGGGATCTCGCTCCTGGAAGA-3′ (GAPDH reverse). The cycling conditions were 3 min at 95°C, followed by 40 cycles of 95°C for 15 s and 67°C for 30 s. Expression levels of COX-2 mRNA were normalized to those of GAPDH mRNA.

Western blot analysis

Cell lysates were subjected to SDS-PAGE in 10% gels. The separated proteins were electroblotted onto polyvinylidene difluoride membrane (Millipore), probed with human COX-2 antisera (IBL, Gunma, Japan) or anti-actin antisera (Santa Cruz), and visualized using an ECL Western blot analysis system (GE Healthcare) according to the manufacturer's protocol. Chemiluminescence was captured using a cooled CCD Light-Capture camera system (ATTO, Japan) and analyzed using CS Analyzer software version 2.0 (ATTO Densitograph Software, Tokyo, Japan).

Transcription assays

In the COX-2 promoter assay, BAEC were transfected using Trans IT-LT-1 (Mirus) (12). For each well, 0.2 μg of COX-2 reporter vector phPES2 (−327/+59) (24), human PPARγ1 expression vector pCMX-hPPARg1, and 0.02 μg of pSV-β-gal (Promega) were used for transfection. As a control experiment, expression vector pCDNA3.1-GS (Invitrogen) was used instead of pCMX-hPPARg1. Luciferase and β-galactosidase activities were determined, and luciferase activity was normalized to the β-galactosidase standard in BAEC (12). For the PPARα activation assay, BAEC were transfected with 0.15 μg of tk-PPREx3-Luc reporter plasmid (27), 0.15 μg of human PPARα expression vector pGS-hPPARα (Invitrogen, GeneStorm, clone L02932), and 0.02 μg of pSV-β-gal, as described previously (18). For the PPARβ/δ and γ activation assays, their expression vectors, pCMX-hNUCI (PPAR β/δ) and pCMX-hPPARγ, respectively, were used for the transfection instead of the human PPARα expression vector. pCMX-hNUCI was constructed by Dr. Shiho Osada-Ogawa (Kyoto University).

Analysis of thyme oil by GC

The thyme oil was analyzed by GC using a Hewlett Packard 5890 series II plus GC system (now Agilent Technologies, Wilmington, DE). A DB-17 GC column (30 m length × 0.32 mm inner diameter, 0.25 μm film thickness; J and W Scientific, Folsom, CA) was used with He as a carrier gas at a flow of 1 ml/min. Thyme oil (1 ml) was dissolved in ethyl alcohol (10 ml), and 1 μl of this solution was injected. The split flow was adjusted at 50 ml/min. The oven temperature was kept at 50°C for 2 min and increased to 280°C at a rate of 5°C/min. The injector temperature was 240°C. The percentage compositions were obtained from electronic integration measurements, using flame ionization detection (FID; 240°C). The relative FID-area percentages of the characterized components are shown in Fig. 5.

Analysis of thyme oil by GC-MS

Thyme oil was also analyzed by high-resolution MS using a JMS BU-20 high-resolution mass spectrometer (JEOL, Tokyo, Japan) attached to a Hewlett Packard 5890 series II plus GC system. The GC conditions were the same as above. Mass spectra were taken at 70 eV under positive electron impact ionization. The mass range was *m/z* 50–500. The components of thyme oil were identified by mass spectrum library searches conducted using the NIST/EPA/NIH Mass Spectral Search Program, Windows version 1.50, distributed by the Standard Data Reference Program of the US National Institute for Standards and Technology.

RESULTS

Suppression of LPS-induced COX-2 promoter activity in the presence of PPARγ by essential oils

The human COX-2 promoter region (−327/+59) contains nuclear factor-κB, nuclear factor for interleukin 6 expression, and cAMP response element sites (28). Previously, transient transfection assays using the COX-2 reporter vector phPES2 (−327/+59) and expression vector pCMX-hPPARg1 showed that BAEC acquired suppressive regulation of the COX-2 gene by 15d-PGJ_2 (12), indicating the involvement of PPARγ in the regulation of COX-2

expression by 15d-PGJ_2. With this assay, we evaluated commercially available oils derived from 21 kinds of plants (supplementary Table I), each at 0.01% concentration, and found that LPS-induced COX-2 promoter activity was suppressed by thyme (65%), clove (40%), rose (30%), eucalyptus (25%), fennel (22%), and bergamot oils (21%), in descending order of activity (**Fig. 1**). Notably, these oils are essential oils, whereas no suppression of COX-2 promoter activity was found with castor, corn, cottonseed, fusel, lavender, lemon, linseed, olive, orange, palm, safflower, sesame, soybean, or turpentine oils (supplementary Table I). Thyme oil was the strongest suppressor among these essential oils and showed dose-dependent suppression of LPS-induced COX-2 promoter activity (**Fig. 2A**). Previously, we reported that 100 nM TPA induced COX-2 promoter activity in the reporter assay (28). As shown in Fig. 1, croton oil containing TPA induced COX-2 promoter activity (264%), confirming that this assay is useful for screening natural chemicals from various plants. TPA is known to be a potent protein kinase C (PKC) activator (29). Previously, we also reported that combined treatment with TPA and LPS synergistically induced COX-2 promoter activity (28), indicating that activation of PKC is involved in inducing COX-2 promoter activity in a manner independent of LPS treatment. Chelerythrine, a selective inhibitor of PKC (30), did not suppress the LPS-induced promoter activity of COX-2 (Fig. 2B), suggesting that the suppression by essential oils such as thyme oil is not via inhibition of PKC.

Activation of PPARs by essential oils

A natural ligand for PPARγ, 15d-PGJ_2, suppressed the LPS-induced COX-2 mRNA expression in macrophage-like differentiated U937 cells (12). Resveratrol suppressed COX-2 mRNA expression (17) and also activated PPARα and γ in our cell-based assay using BAEC (18). Thus, using this assay system, we evaluated the activation of PPARs by the essential oils that suppressed LPS-induced COX-2 promoter activity (Fig. 1). **Figure 3A** shows that thyme, rose, clove, and bergamot oils had PPARα agonistic activity and that thyme oil also had PPARγ agonistic activity. In the positive control experiment, selective activators of PPARs, such as Wy-14643 (α), GW501516 (β/δ), and pioglitazone (γ), had PPARα, β/δ, and γ agonistic activities, respectively (Fig. 3B). Remarkably, thyme oil activated PPARα and γ with about the same dose dependency (**Fig. 4**), within the range for suppression of the LPS-induced COX-2 promoter activity was observed (Fig. 2A). As a control experiment, pioglitazone suppressed LPS-induced COX-2 promoter activity in BAEC in the presence of the vector encoding PPARγ (supplementary Fig. I). In contrast, we previously found that LPS rapidly downregulates PPARγ expression in U937 cells (12). By this mechanism, the suppressive effect of the PPARγ agonist 15d-PGJ2 on COX-2 expression is less potent than that of dexamethasone, an agonist of the glucocorticoid receptor, which is upregulated by LPS (12). We also examined the effects of LPS on the expression of PPARs and COX-2 in U937 cells and found that PPARγ mRNA alone was rapidly suppressed in response to LPS (supplementary Fig. II).

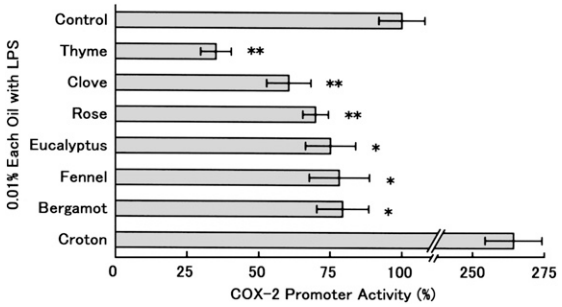

Fig. 1. Effects on COX-2 promoter activity by essential oils in BAEC. BAEC were transiently transfected with phPES2 (−327/+59) together with pCMX-hPPARg1 and pSV-β-gal as an internal control for the transfection. Following transfection, the cells were incubated for 5 h with no stimulant (control) or with each oil (0.01%) and LPS (1 μg/ml). The cells were harvested, lysed, and assayed for both luciferase and β-galactosidase activities. The results are represented as relative luciferase activities, which were normalized against the β-galactosidase standard and presented as the mean ± SD. *, $P < 0.05$, **, $P < 0.01$, compared with cells treated with 1 μg/ml LPS, by an unpaired *t*-test (n = 3).

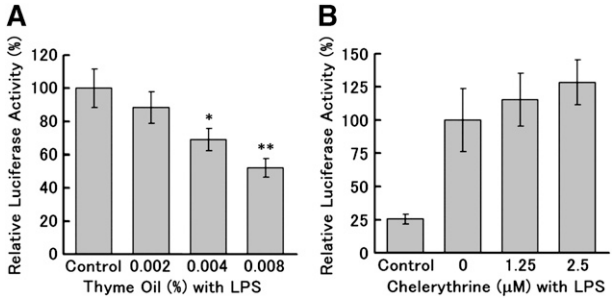

Fig. 2. Dose-dependent suppression of COX-2 promoter activity by thyme oil and the effects of chelerythrine on promoter activity. BAEC were transiently transfected with phPES2 (−327/+59), together with pCMX-hPPARg1, and pSV-β-gal as an internal control for the transfection. Following transfection, the cells were incubated for 5 h with thyme oil (0.002, 0.004, and 0.008%) and LPS (1 μg/ml) (A). In a second experiment, following transfection, the cells were incubated for 5 h with no stimulant (control) or were treated with LPS (1 μg/ml) in the presence or absence with chelerythrine (B). In both cases, the cells were harvested, lysed, and assayed for both luciferase and β-galactosidase activities. The results are represented as relative luciferase activities, which were normalized against the β-galactosidase standard and presented as the mean ± SD. *, $P < 0.05$, **, $P < 0.01$, compared with cells treated with 1 μg/ml LPS, by an unpaired *t*-test (n = 3).

Carvacrol, the major component involved in the activation of PPARα and γ, suppresses LPS-induced COX-2 mRNA and protein expression

We screened for active chemicals in thyme oil that were involved in the suppression of LPS-induced COX-2 promoter activity and activation of PPARα and γ. Thyme oil was analyzed by GC and GC-MS. Eleven major compounds, representing 96.80% (FID-area percentage for GC) of the oil, were identified; the main constituents of the oil were carvacrol (58.29%), *p*-cymene (24.15%), linalyl acetate (4.07%), γ-terpinene (2.39%), β-caryophylene (2.26%), β-myrcene (2.15%), limonene (1.11%), 2-carene (1.05%),

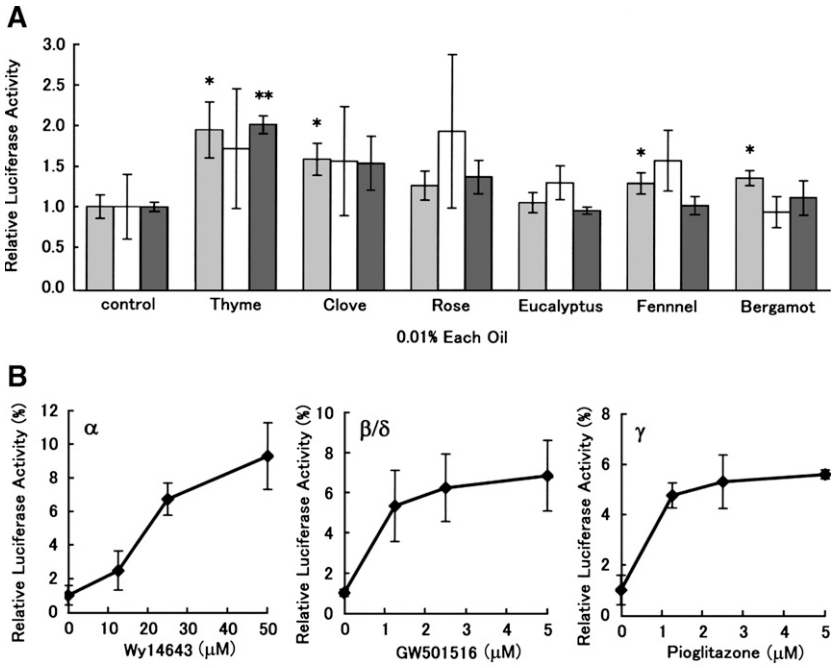

Fig. 3. Effect on activation of PPARs by essential oils. BAEC were transiently transfected with PPRE-luc together with GS-hPPARα (shaded column), pCMX-NUC1 (open column), or pCMX-hPPARg1 (solid column) and pSV-β-gal as an internal control for the transfection. Following transfection, the cells were incubated for 24 h with each essential oil (0.01%) (A). In the positive control experiment, the indicated concentration of selective PPAR activators such as Wy-14643 (α), GW501516 (β/δ), and pioglitazone (γ) were applied to the BAEC transiently transfected with PPRE-luc together with GS-hPPARα, pCMX-NUC1, or pCMX-hPPARg1, respectively (B). In both experiments, the cells were harvested, lysed, and assayed for both luciferase and β-galactosidase activities. The results are represented as relative luciferase activities, which were normalized against the β-galactosidase standard and presented as the mean ± SD. *, $P < 0.05$, **, $P < 0.01$, compared with cells treated with ethanol (control), by an unpaired *t*-test (n = 3).

and borneol (0.60%; **Fig. 5**). Next, commercially available compounds were examined for their effects on LPS-induced COX-2 promoter activity at a concentration of 1 mM; carvacrol and borneol suppressed promoter activity by 80% and 55%, respectively (**Fig. 6**). As shown in Fig. 2A, COX-2 promoter activity was suppressed to 48% by 0.008% thyme oil, which contained about 300 μM carvacrol and 3 μM borneol (Fig. 5). **Figure 7** shows the dose-dependent suppression of COX-2 promoter activity by carvacrol (open columns: 40% and 60% suppression by 200 and 400 μM carvacrol, respectively), indicating that carvacrol is the major component of thyme oil involved in the suppression of the LPS-induced COX-2 promoter activity. On the other hand, COX-2 promoter activity was only marginally suppressed in the absence of PPARγ expression vector (closed column); however, this suppression was statistically significant in the presence of the PPARγ expression vector (open column) in BAEC (Fig. 7), indicating that carvacrol-mediated suppression was partly PPARγ dependent. In the control experiments, carvacrol alone had no effect on COX-2 promoter activity in the transfection assay using BAEC and no suppressive effect on COX-2 mRNA in U937 cells (supplementary Table II). Next, we evaluated carvacrol-mediated PPAR activation. Carvacrol activated PPARα (**Fig. 8A**) and γ (Fig. 8B) at the same concentrations at which the suppression of COX-2 promoter activity was observed (Fig. 7). These carvacrol concentrations are similar to those at which PPARα and γ activities were observed in response to thyme oil (Fig. 4). Moreover, 400 μM carvacrol induced the mRNA expression of the PPARα-dependent carnitine palmitoyltransferase I gene in U937 cells (supplementary

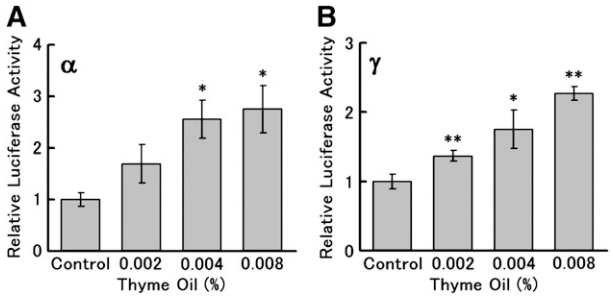

Fig. 4. Dose-dependent activation of PPARα and PPARγ by thyme oil. BAEC were transiently transfected with PPRE-luc together with GS-hPPARα (A) or pCMX-hPPARg1 (B) and pSV-β-gal as an internal control for the transfection. Following transfection, the cells were incubated for 24 h with thyme oil (0.002, 0.004, and 0.008%). The cells were harvested, lysed, and assayed for both luciferase and β-galactosidase activities. The results are represented as relative luciferase activities, which were normalized against the β-galactosidase standard and presented as the mean ± SD. *, $P < 0.05$, **, $P < 0.01$, compared with cells treated with ethanol (control), by an unpaired *t*-test (n = 3).

Component	Rate (%)	Concentration (μM) in 0.008% Thyme Oil
Carvacrol	58.29	303.0
p-Cymene	24.15	123.3
Linalyl Acetate	4.07	14.9
γ-Terpinene	2.39	12.0
β-Caryophyllene	2.26	8.0
β-Myrcene	2.15	10.0
Limonene	1.11	5.5
2-Carene	1.05	5.3
Borneol	0.60	3.2
β-Phellandrene	0.48	2.4
γ-Terpineol	0.25	1.2

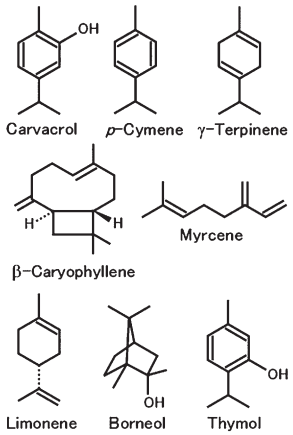

Fig. 5. Major components of thyme oil and their structures. Thyme oil was analyzed by GC and GC-MS. Eleven major compounds, representing 96.80% (FID area percentage for GC) of the oil, were identified (A). The structures of some of the components and thymol are presented (B).

Fig. III). These results indicate that carvacrol is both a suppressor of COX-2 promoter activity and an activator of PPARα and γ.

In BAEC, dehydroxyl-carvacrol, *p*-cymene, did not suppress LPS-induced COX-2 promoter activity, whereas thymol, a structural isomer of carvacrol, did (Fig. 6). Similarly, thymol but not *p*-cymene, activated PPARα and γ (Fig. 8C–F). In addition, 400 μM carvacrol and thymol, but not *p*-cymene, weakly activated PPARβ/δ (supplementary Fig. IV). These results suggest that the hydroxyl group of carvacrol is essential for both the suppression of COX-2 promoter activity and the activation of PPARα and γ.

Next, we examined whether carvacrol suppressed COX-2 mRNA and protein expression in differentiated macrophage-like U937 cells. LPS-induced COX-2 mRNA and protein expression was suppressed by carvacrol to about 14% (1,000 μM in **Fig. 9**) and 40% (400 μM in **Fig. 10**), respectively. Carvacrol-mediated suppression of LPS-induced COX-2 protein expression (Fig. 10) is consistent with the results of the COX-2 reporter assays (Fig. 7). The LPS-induced expression of COX-2 mRNA was suppressed to about 14% by treatment with 1000 μM carvacrol but was not suppressed in response to 400 μM carvacrol (Fig. 9). This discrepancy may be due, at least in part, to the dual regulation of COX-2 mRNA at the transcriptional and posttranscriptional levels (30, 31). The 3′-untranslated region of COX-2 mRNA has 17 copies of the Shaw-Kamen sequence (AUUUA), which is involved in the regulated stabilization of COX-2 mRNA by LPS (31). Regarding this point, we found no evidence that carvacrol is involved in the control of COX-2 mRNA stability, based on reporter assays using the 3′-untranslated region of COX-2 (supplementary Fig. V). From our data, carvacrol suppressed not only promoter activity in BAEC transfected with hPCMX-hPPARg1 but also LPS-induced COX-2 mRNA and protein expression in U937 cells expressing PPARγ.

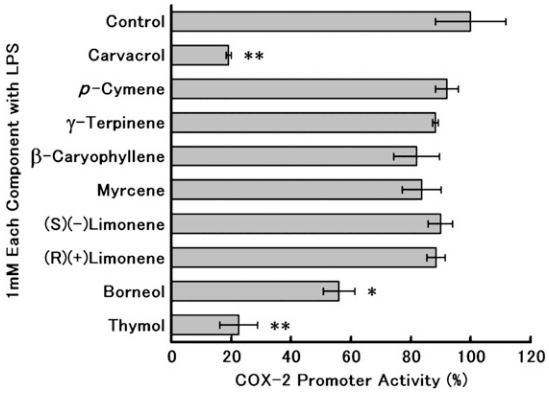

Fig. 6. Effects on COX-2 promoter activity by components of thyme oil and thymol. BAEC were transiently transfected with phPES2 (−327/+59) together with pCMX-hPPARg1 and pSV-β-gal as an internal control for the transfection. Following transfection, the cells were incubated for 5 h with no stimulant (control), with LPS alone (1 μg/ml), or with LPS (1 μg/ml) and each component (1 mM). The cells were harvested, lysed, and assayed for both luciferase and β-galactosidase activities. The results are represented as relative luciferase activities, which were normalized against the β-galactosidase standard. *, $P < 0.05$, **, $P < 0.01$, compared with cells treated with 1 μg/ml LPS alone, by an unpaired *t*-test (n = 3).

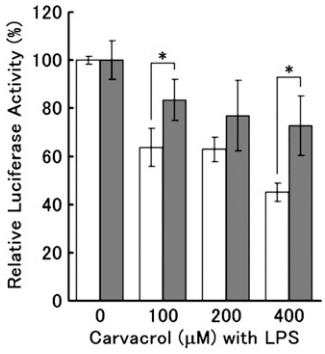

Fig. 7. PPARγ-dependent suppression of COX-2 promoter activity in response to carvacrol. BAEC were transiently transfected with phPES2 (−327/+59), along with either the human PPARγ expression vector, pCMX-hPPARg1 (open column), or the expression vector pCDNA3.1-GS alone (closed column) and pSV-β-gal as an internal control for the transfection. Following transfection, the cells were incubated for 5 h with no stimulant (control) or with carvacrol (100, 200, or 400 μM) and LPS (1 μg/ml). The cells were harvested, lysed, and assayed for both luciferase and β-galactosidase activities. The results are represented as relative luciferase activities, which were normalized against the β-galactosidase standard. *, $P < 0.05$, compared with open and closed columns, by an unpaired *t*-test (n = 3).

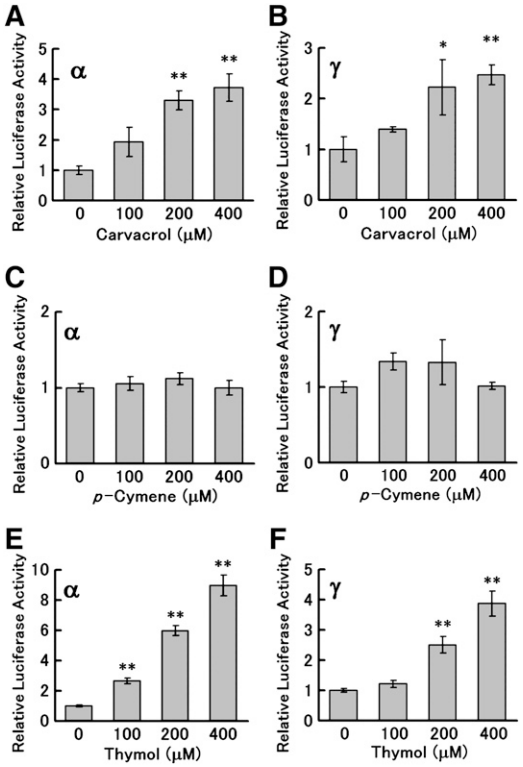

Fig. 8. Activation of PPARα and PPARγ in response to carvacrol and thymol. BAEC were transiently transfected with PPRE-luc together with GS-hPPARα (A, C, E), or pCMX-hPPARg1 (B, D, F), and pSV-β-gal as an internal control for the transfection. Following transfection, the cells were incubated for 24 h with carvacrol, *p*-cymene, or thymol (100, 200, or 400 μM). The cells were harvested, lysed, and assayed for both luciferase and β-galactosidase activities. The results are represented as relative luciferase activities, which were normalized to the β-galactosidase standard. *, $P < 0.05$, **, $P < 0.01$, compared with cells treated with ethanol (control), by an unpaired *t*-test (n = 3).

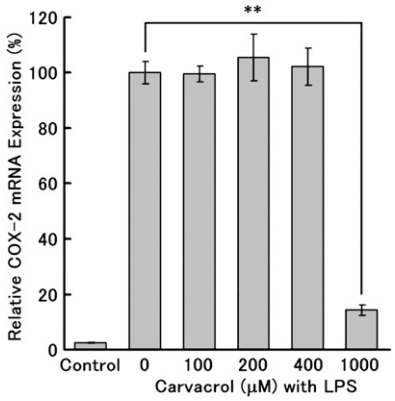

Fig. 9. Suppression of COX-2 mRNA expression by carvacrol in macrophage-like differentiated U937 cells. Macrophage-like differentiated U937 cells were treated for 2 h with 10 μg/ml LPS in the presence or absence of 1 mM carvacrol. Isolated RNA was used for quantitative RT-PCR analysis. The amount of COX-2 mRNA was normalized to that of GAPDH mRNA, and the normalized amount of COX-2 mRNA induced by LPS was taken as 100%. The results represent the means ± SD of three separate dishes. **, $P < 0.01$, compared with cells treated with 10 μg/ml LPS, by an unpaired *t*-test (n = 3).

DISCUSSION

The prevention of lifestyle-related diseases, such as cardiovascular disease, diabetes, and stroke, is of worldwide interest. There have been many studies on the functionality of natural chemicals in food and drink. In this context, resveratrol in red wine is one of the most attractive of the characterized compounds, and experimental information regarding its actions has accumulated (32, 33). We have focused on COX-2 (17) and PPARs (18) as possible molecular targets of resveratrol in preventing lifestyle-related diseases, while others have studied sirtuins and PGC-1α (34, 35). Given that humulon (21, 23) and apigenin (22), as well as resveratrol, suppress COX-2 expression and activate PPARs, we screened commercially available oils, including essential oils, for their effects on COX-2 and PPARs and found that carvacrol, a major component of thyme oil, was active. It is noteworthy that carvacrol activates PPARα and γ (Fig. 8A, B) and suppresses LPS-induced COX-2 protein expression (Fig. 10) in the same concentration range. Moreover, PPARγ-dependent suppression of COX-2 promoter activity was observed in response to carvacrol (Fig. 7), which is consistent with our previous finding that 15d-PGJ_2, a potent natural ligand of PPARγ, suppressed LPS-induced COX-2 expression in differentiated macrophage-like U937 cells and that the expression of COX-2 was regulated by a negative feedback loop mediated through PPARγ (12). Taken together, it is likely that carvacrol regulates COX-2 expression, at least in part, via its agonistic effect on PPARγ.

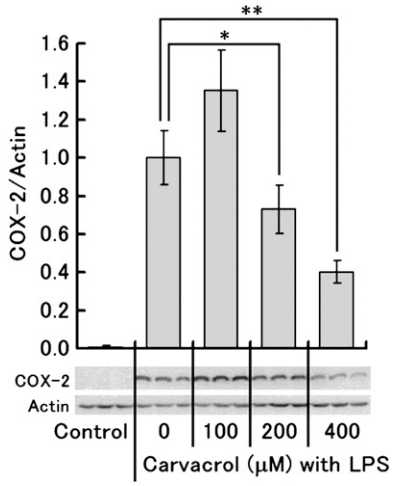

Fig. 10. Suppression of COX-2 protein expression in response to carvacrol in macrophage-like differentiated U937 cells. Macrophage-like differentiated U937 cells were treated for 5 h with 10 μg/ml LPS in the presence or absence of the indicated concentrations of carvacrol. Cellular lysate proteins (5 μg/lane) were separated by 10% SDS-PAGE and transferred onto a polyvinylidene difluoride membrane. Immunoblots were probed with antibodies specific for COX-2 and actin. The expression level of COX-2 was normalized against that of actin, and the normalized amount of COX-2 protein induced by LPS was taken as 1.0. The results represent the mean ± SD of three separate dishes. *, $P < 0.05$, **, $P < 0.01$, compared with cells treated with 10 μg/ml LPS, by an unpaired *t*-test (n = 3).

Because of the high concentration of carvacrol necessary to suppress COX-2 promoter activity and activate PPARs, we must consider the possible existence of a minor contaminant associated with carvacrol. To address this point, we confirmed our results using another source of carvacrol (100% purity) via both HPLC and NMR analyses; these analyses indicated that a minor contaminant was unlikely. Another point to consider, given the high carvacrol concentration, is the possibility of nonspecific effects on the cell. Concerning the binding pocket of PPARs, the molecular size of carvacrol is smaller than that of other synthetic PPAR agonists, which may be one of the reasons for its relatively weak activation of PPARs. We also observed weak activation of PPARβ/δ with 400 μM carvacrol and thymol, both of which elicited an approximate 2-fold increase in activity (supplementary Fig. IV). In contrast, carvacrol and thymol elicited 4- and 8-fold increases in PPARα activity, respectively, and 2.5- and 3.5-fold increases in PPARγ activity, suggesting that carvacrol and thymol activate these factors in a somewhat specific manner. Fatty acids are considered to be intrinsic ligands for PPARs at higher concentrations compared with synthetic ligands. In this case, the presence of fatty acid-binding molecules, which include specific binding proteins and nonspecific interacting lipids, both inside and outside of the cells appears to modulate PPAR activation. A similar mechanism may be at work in the activation of PPARs by carvacrol and other chemical components in essential oils. Recently, carvacrol was reported to be an agonist of transient receptor potential (TRP) V3, a thermosensitive ion channel expressed predominantly in the skin and neural tissues (36, 37). The EC_{50} of carvacrol for TRPV3 is 0.49 mM, similar to the effective concentration for COX-2 and PPARs in our study. Moreover, thymol, but not *p*-cymene, is also an agonist of TRPV3 (EC_{50} = 0.86 mM), suggesting a structural requirement for a hydroxyl group (Fig. 5), which is also similar to our results on the effects on COX-2 and PPARs (Figs. 6 and 8). Members of TRPV activate upon heating: TRPV1 at 42°C, TRPV2 at 52°C, TRPV3 at 39°C, and TRPV4 at 27−42°C. Further study on the relationships among COX-2, PPARs, and TRPV3 may be helpful.

We demonstrated that LPS-induced COX-2 promoter activity was suppressed by not only thyme oil but also clove, rose, eucalyptus, fennel, and bergamot oils (Fig. 1). It is noteworthy that statistically significant activations of PPARs were not found with rose or eucalyptus oils, but there was a tendency for PPARβ/δ activation by thyme, clove, rose, eucalyptus, and fennel oils (Fig. 3A). These results may indicate that some natural chemicals in essential oils act as COX-2 suppressors but not PPAR activators, or that some minor oil components act as PPARβ/δ agonists. Further identification of the chemical compounds in these essential oils is under investigation.

In summary, we identified carvacrol, a chemical component of thyme oil, as a suppressor of COX-2 and activator of PPARα and γ. Although most essential oils such as thyme oil have a wide range of applications in pharmaceutical, medical, and cosmetic fields, their mechanisms of action are not completely understood for many of the described effects, which include antimicrobial, antitumor, antimutagenic, antigenotoxic, analgesic, antiinflammatory, angiogenic, antiparasitic, antiplatelet, antihepatotoxic, and hepatoprotective properties (38). Our results may be important in understanding the antiinflammatory and antilifestyle-related disease properties of carvacrol, although further studies in vivo will be necessary to address the physiological significance of our findings. Our screening system using reporter assays for COX-2 and PPARs may be applicable for studying the functionality of food-related materials.

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