Cigarette Smoke Extract Modulates Functions of Peroxisome Proliferator-Activated Receptors

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INTRODUCTION

Both active as well as passive cigarette smoking is an established vascular risk factor.¹ Cigarettes have proven biological toxicities for atherogenesis and well-established causal links to cardiovascular events.¹ Apart from oxidized and small dense particles, elevated low-density lipoprotein-cholesterol (LDL-C) is a hallmark of atherosclerotic change of arteries and is risk factor of systemic vascular events. Cigarette smoking is well known to increase triglycerides, very low-density lipoprotein-cholesterol (VLDL-C), and glucose; and to decrease the level of high-density lipoprotein-cholesterol (HDL-C) unarguably. On the contrary, the influence of cigarette smoking on LDL-C levels has been controversial.²–⁶ It is still uncertain whether smoking elevates LDL-C significantly. On the other hand, there have been numerous evidences showing strong association between smoking and triglycerides, VLDL-C, glucose, and HDL-C. In our previous study, we performed sub-analysis of cross-sectional data from 3574 middle aged Japanese people consisted of 2947 men and 627 women, whose lipid profile was paying special attention to the LDL-C levels.⁷ As a result, average LDL-C levels were 131.5, 130.6, and 125.7 (mg/dL) in non-smokers (n = 1190), ex-smokers (n = 1060), and smokers (n = 956), respectively. In our cohort study groups, LDL-C levels of smokers were lower than those of others with statistically significant (p < 0.001), though the other metabolic parameters deteriorated and inversely worse than non-smokers and ex-smokers (data not shown).³ Other studies reported that unlike triglyceride, HDL-C, VLDL-C, total cholesterol, and glucose, there is no close and significant relationship between smoking and LDL-C. Therefore, we hypothesized that cigarette smoke extract (CSE) may modulate LDL-C metabolism. In our detailed literature search, we failed to find any report that correlates the influence of cigarette smoking on LDL-C metabolism at molecular level. The aim of our current study was to examine the effects of CSE on transcriptional regulation mediated peroxisome proliferator-activated receptors (PPARs) and its interaction with cofactors to elucidate differences in the molecular mechanism between CSE and other agonists of PPARs. We constructed several mutant PPARs (mPPARs) with amino acid substitution in the ligand-binding domain, which according to the molecular modeling, may affect the binding of agonists. In transient expression assays, each wild-type peroxisome proliferator-activated receptor (PPAR) mediated transcription stimulated by CSE was faintly yet significantly elevated compared to the control. The CSE-induced transcriptional activation was abolished in the H323A, H323Y, S342A, and H449A mPPARs, although the activation elevated by pioglitazone was reserved. In the mPPAR; with Y473A and mPPARβ/δs with H286Y and Y436A, the pioglitazone-induced or L165041-activated transcriptional elevations were decreased and were lower than that of CSE-induced stimulation. These results suggested that CSE activated both mutant PPARs to be selectively different from those ligands. Mammalian two-hybrid assay illustrated that CSE could mildly recruit SRC1 or GRIP1 to the wild-type PPARγ. Representative ingredients, such as acrolein and crotonaldehyde present in CSE, could stimulate some proliferator-activated receptor (PPAR) mediated transcription stimulated by CSE to decreased and were lower than that of CSE-induced stimulation. These results suggested that CSE activated both mutant PPARs to be selectively different from those ligands. CSE mildly regulates the cholesterol metabolism-related genes, such as low density lipoprotein (LDL) receptor and Liver X receptor (LXR)β. In conclusion, these CSE effects the nuclear hormone receptors and their cofactors thereby disturbing metabolic phenomena. Therefore, CSE might be involved in cholesterol metabolism.

Key words transcription; low density lipoprotein (LDL)-cholesterol; signal transduction; co-factor

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MATERIALS AND METHODS

CSE Preparation  We arranged CSE according to our previous report.\textsuperscript{9} Briefly, mainstream smoke from four cigarettes at a time (Caster Frontier One, cigarette brand name; Japan Tobacco Inc., Tokyo, Japan) was passed through a Cambridge glass fiber filter (Heiner. Bergwaldt GmbH, Hamburg, Germany) to eliminate nicotine and the tar-phase and bubbled into 20mL of phosphate buffered saline (PBS) at 25°C, at a flow rate of 1.050 L/min. We repeated this procedure ten times. Finally, we obtained 20mL of PBS containing the smoke of total 20 cigarettes and defined it as 100%. Since 10% CSE exerts maximum cytotoxic activity in TSA201 cell line, we weighed tar-phase CSE and examined the effects of 10% CSE.

Reagents Acrolein, crotonaldehyde, and methylvinlyketone were obtained from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Fetal bovine serum (FBS) was obtained from Bio West Co. (Nussel, France). GW590735 (Cayman Chemicals, #10099880, MI, U.S.A.), -I65041 (Sigma, #076K4605, St. Louis, MO, U.S.A.), and pioglitazone (Enzo Life Sciences, #ALX-270-367, NY, U.S.A.) were purchased and dissolved in dimethyl sulfoxide (DMSO) as 5 mM, 10 mM, and 50 mM stock solutions, respectively. Final concentration of DMSO was 0.1% v/v in medium. Ethylenediaminetetraacetic acid (EDTA) trypsin solution (EDTA: 2.2 mM, trypsin: 0.25%) was used to suspend the cells. Penicillin/streptomycin solution (penicillin: 50000 U/mL, streptomycin: 50 mg/mL) was supplied by Mediatech, Inc. (Wako Pure Chemical Industries, Osaka, Japan). Penicillin/streptomycin solution (penicillin: 50000 U/mL, streptomycin: 50 mg/mL) was supplied by Invitrogen Corp. (Wako Pure Chemical Industries, Ltd.). Dulbecco’s Modified Eagle’s Medium (DMEM) without phenol red (Wako Pure Chemical Industries, Ltd., Osaka, Japan) was passed through a Cambridge glass fiber filter (Heiner. Bergwaldt GmbH, Hamburg, Germany) to eliminate nicotine and the tar-phase and bubbled into 20mL of PBS at 25°C, at a flow rate of 1.050 L/min. We repeated this procedure ten times. Finally, we obtained 20mL of PBS containing the smoke of total 20 cigarettes and defined it as 100%. Since 10% CSE exerts maximum cytotoxic activity in TSA201 cell line, we weighed tar-phase CSE and examined the effects of 10% CSE in the current experiments derived from HEK293 cells, according to the previous report.\textsuperscript{10}

Cell Culture TSA201 cells derived from HEK293 human embryonic kidney-293 cells and HepG2 cells (human liver cancer cells) were purchased from JCRB Cell Bank (National Institute of Biomedical Innovation, Osaka, Japan). TSA201 cells and HepG2 cells were sub-cultured at 37°C in a 5% CO\textsubscript{2} atmosphere in DMEM containing 2mM l-glutamine (Nissui Pharmaceutical), 10% FBS (HyClone laboratories Inc., UT, U.S.A.), 100 U/mL penicillin G, and 100 µg/mL streptomycin sulfate.

Plasmid Constructions The pCMX-mouse peroxisome proliferator-activated receptor (PPAR) α, pCMX-mouse PPARβ/δ, pCMX-mouse PPARγ and GAL4-PPAR expression vectors were generously gifted by Professor R. M. Evans (Salk Institute, La Jolla, CA, U.S.A.). UAS-E1B-TATA-Luc, the GAL4 reporter construct, containing five copies of GAL4 recognition sequence (UAS) upstream of E1B-TATA-Luc, was employed as depicted in the previous report.\textsuperscript{11–13} An internal control, the pRL-TK vector (Promega, WI, U.S.A.) comprised of the TK promoter and Renilla luciferase cDNA was employed. The cDNA mutants for both PPARβ/δ and PPARγ constructs were prepared by oligonucleotide-directed mutagenesis method and verified by DNA sequencing as depicted previously.\textsuperscript{11–13} Both the expression vectors, VP16-SRC1 and VP16-GRIP1 have been constructed as described earlier with some modifications as follows.\textsuperscript{14,15} Briefly, the VP16-E2F-5 (2-301) construct was made by subcloning an EcolRI fragment from Gal4-E2F-5 (2-301) at EcolRI sites in AASV-VP16, which expresses the trans-activation domain (aa 413-490) of the herpesvirus VP16 trans-activator protein. The thyroid hormone receptor interaction domain of F-SRC1 (residues 213-1061) and that of GRIP1 (residues 480–1462) were fused downstream of the VP16 activation domain in frame to create VP16-SRC1 and VP16-GRIP1, respectively. The reporter plasmid, PPRE-tk-Luc, was gifted by Professor J. Szwaya (Dana Farber Cancer Institute, Boston, MA, U.S.A.).

Transient Expression Assays We carried out transient expression assays with TSA201 cells based on the calcium precipitation method as described previously.\textsuperscript{9} Briefly, to analyze the effects of CSE on PPAR isoforms, PPRE-tk-Luciferase (PPRE-tk-Luc) as a reporter with a PPAR expression construct or pCMX (mock) was co-introduced into TSA201 cells by using the calcium phosphate transfection method. We also introduced pGL4.70 as the internal control in the same cells. After 6h, the culture medium was substituted with phenol red-free DMEM (Wako Pure Chemical Industries) containing an activated charcoal treated 10% FBS. After additional 20h, the cells were harvested to measure the luciferase activities according to the manufacturer’s instructions (Dual Luciferase Reporter Assay System; Promega) using a Promega GLOMAX 20/20 luminometer (Promega). Both firefly and renilla luciferase activities were evaluated to analyze the transfection efficiency and cytotoxicity of the added reagents.

Mammalian Two-Hybrid Assays We performed the mammalian two-hybrid experiment as described previously.\textsuperscript{9–12} In short, both the expression plasmids Gal4-PPAR and VP16-cofactor constructs were co-introduced into TSA201 cells with 100ng of the reporter gene, UAS-E1B-TATA-Luc, in the absence or presence of CSE. We showed SRC1 and GRIP1 as cofactors in this report.

Quantitative (q) RT-PCR CSE was added to HepG2 cells and 24h later, the cells were harvested and total RNA was extracted using RNAiso Plus (TaKaRa Bio, Shiga, Japan). Then, cDNA was prepared by subjecting 1µg of the total RNA to a reverse transcription reaction, using iScriptTM RT Supermix (BioRad, Hercules, CA, U.S.A.). Using this cDNA as a template, qPCR was carried out in a CFX ConnectTM Real-Time

| Gene       | Sense primer (5′→3′) | Antisense primer (5′→3′) |
|------------|----------------------|-------------------------|
| Human LDLR | GCTTGTCTGCACCTGCAAA  | AACTGCGGAGAGAGGACCTTT  |
| Human LXRα | GCAGAACATCTGGCAAGCT  | TCAGGCGGATCTGTTCTCCT   |
| Human LXRδ | CAGATGCGAACCTTTCTGCG| CTGCGTTTCCGAATCTTCTTCT |
| Human FXR  | TACATCGGAAAGAGTGTGCAA| ACTGCTTCATTACGGTGCTGAT |
| Human GAPDH| AGCCACATCGCTCAGACAC | GCCCAATACGACCAATCC   |

Table 1. Primer Sequences Used in qPCR and PCR
PCRs Detection System (BioRad) using iTaqTM Universal SYBR® Green Supermix (BioRad). For each sample, 1ng of template was amplified in PCR performed in triplicate. PCR conditions consisted of an initial denaturation at 98°C for 30s, followed by 40 cycles of the following: 98°C for 10s, 60°C for 20s. Table 1 shows the primers used in the PCR.

Statistical Analysis We performed statistical analyses with SPSS 17.0 software (SPSS Inc., IL, U.S.A.). All data are expressed as mean value ± standard deviation (S.D.) from at least three experiments, each performed in triplicate. Analysis was done using one-way ANOVA followed by Dunnett’s test for comparison among the groups. We have considered all analyses with the associated probability (p-value) of less than 5% (p < 0.05) as significant.

RESULTS

CSE Weakly Stimulates Wild Type PPAR Isoforms We initially screened CSE to the wild type PPAR isoforms (Fig. 1). CSE were able to stimulate each PPARα, PPARβ/δ, and PPARγ, by 202%, 440%, and 451%, respectively, to the vehicle. Though we also examined suppressive effects of CSE in the presence of each conventional ligand, we observed that CSE did not repress transcriptional activity by orthogonal combination, such as PPARα and GW507435, PPARβ/δ and L165041, and PPARγ and pioglitazone, at all. Above all, CSE stimulated PPARβ/δ more than other PPARα isoform.

CSE Binds to the PPAR Isoforms in a Fashion Different from Each Respective Ligand Since 5% CSE weakly but significantly activated PPAR isoforms (Fig. 1), we analyzed its action on PPAR isoforms harboring a mutation in the LBD, with respect to a PPRE-regulated reporter.15) We used site directed mutagenesis to change amino acid residues at H323A/Y, S342A, H449A, and Y473A sites in the PPARγ (Fig. 2B), and at H286Y and Y436A sites in the PPARβ/δ (Figs. 2A, 2E).12–17) We examined the transcriptional activities of these mutants with a reporter construct regulated PPAR, PPRE-tk-Luc, and pioglitazone, or L165041. As the positive control, 5µM of pioglitazone significantly increased luciferase activity in H323A, H323Y, S342A, and H449A, but not in Y473A (Fig. 2B). Then, we studied dose-dependent simulation of each mutant PPARγ (mPPARγ) (Figs. 2C, 2D). Results showed that the CSE-induced activation was significantly impaired in the S342A mPPARγ. However, the Y473A showed escaped impairment compared to that by pioglitazone. Indeed, in mPPARβ/δ, CSE stimulated both H286Y and Y436A mPPARβ/δ significantly higher than that of the L165041 (Figs. 2E, 2F). CSE is able to bind to PPARβ/δγ by different manners from the positive controls, respectively, so that CSE could activate mPPARβ/δγ significantly and characteristically.

Representative Chemicals Weakly Activate PPARβ/δγ-Mediated Transcription in a Dose-Dependent Manner We showed that transcriptions mediated by PPARβ/δγ were mildly elevated in the presence of CSE (Figs. 1, 2). Hence, we estimated the effects of representative compounds containing CSE on PPARβ/δγ mediated transcriptional stimulation. CSE ingredients with varying concentrations of acrolein, crotonaldehyde, and methylvinylketone were respectively added to TSA201 cells co-transfected with PPARγ (Fig. 3A) or PPARβ/δ (Fig. 3B), the reporter construct, and internal control. These CSE compounds increased PPAR-mediated transcription in a dose-dependent manner, except methylvinylketone (Fig. 4). Compared to 5µM of pioglitazone, unsaturated carboxyl compounds, acrolein and crotonaldehyde, at the highest concentration of 100µM, induced luciferase signal to 76%, and 112%, respectively, via PPARβ/δ/PPRE-tk-luc (Fig. 3). In comparison, methylvinylketone did not alter the luciferase signal due to its cytotoxic effect on TSA201. In the PPARβ/δ, similar dose-dependent results were observed to PPARγ (Fig. 3B). Here, compared to 10µM of L165041, acrolein and crotonaldehyde, at the highest concentration of 100µM, induced luciferase signal to 71%, and 51%, respectively, via PPARβ/δ/PPRE-tk-luc.

CSE and Pioglitazone Activate PPARγ Synergistically Since pioglitazone is the unique medicine as a PPARβ/δγ stimulant, which is currently available for clinical usage, we examined the inducing effects of CSE in the presence of pioglitazone to see whether it has synergistic or additive effects on transcriptional activation. We compared CSE to PPARγ with 0.1µM pioglitazone to varying concentrations of CSE on the transcriptional activation regulated by pioglitazone (Fig. 4A). In the presence of pharmacological concentration of pioglitazone, the effect of 5% CSE on PPARγ-mediated transcription was 532% higher than that of pioglitazone alone (Fig. 4A). On the contrary, CSE failed to exert synergistic or additive effects without exogenous PPARγ, even in the presence of the same dose of pioglitazone (Fig. 4B). Additionally, we performed to compare CSE to PPARβ/δ with 1.0µM L165041 to varying concentrations of CSE on the same setting as PPARγ (Fig. 4C). In the presence of L165041, the effect of 5% CSE on PPARβ/δ-mediated transcription was 327% higher than that of L165041 alone (Fig. 4C). Without the expression plasmid of PPARβ/δ, CSE did not show synergistic effects even in the existence of positive ligand (Fig. 4D).

CSE Enhances the Recruitment of Co-activators and Not Co-repressors To verify probable mechanisms of the CSE agonistic activity, we examined the effects of PPARγ-mediated transcriptional functions with cofactors in mammalian two-hybrid system. Results showed that CSE faintly recruited the nuclear coactivators SRC1 and/or GRIP1 to the PPARγ (Fig. 5) but has no effect on co-repressors, such as NCoR and/or SMRT (data not shown).
CSE Mildly Regulates Genes Involved in Cholesterol Metabolism

We examined the effects of CSE on the expression of genes associated with LDL-cholesterol metabolism in HepG2 cells. Results showed that 5% CSE stimulated the expressions of LDL-receptor (LDL-R), liver X receptors LXRα and LXRβ, and farnesoid X receptor (FXR). Through quantitative RT-PCR using a set of mRNAs, we found that 5% CSE mildly upregulated both LDL-R and LXRβ mRNA expressions by 122% and 119%, respectively, compared to that by the control, in HepG2 cells (Fig. 6).

Fig. 2. CSE Binds to the PPAR Isoforms in a Different Fashion from Each Respective Ligand

To examine the molecular mechanism of the modulating effects of CSE on PPAR isoforms, we employed mutant PPARβ/δγ to compare CSE interactions with known ligands. The numbering of the amino acid residues of PPARβ/δ is based on the mouse PPARs sequence (A). Schematic representation of protein structures of each isoform and truncated PPARγ (A). The numbering of the amino acid residues of PPARγ and PPARβ/δ is based on the human PPARγ1 and PPARβ/δ sequence, respectively. The PPARβ/δγ harboring mutation inside LBD expression plasmids for the indicated mutants and reporter construct, PPRE-tk-Luc (50 ng) were co-transfected into TSA-201 cells in the absence or presence of 5 µM of ligand for PPARγ (B), 10 mM of ligand for PPARβ/δ (E) or 5% of CSE. We also examined each mPPARs’ function in a dose-dependent manner by adding increasing amounts (0.1, 1.0, and 5%) of CSE to the culture media. Results are shown as mean ± S.D. from at least three transfections performed in triplicate (C, D, F). *p < 0.05, **p < 0.01, ***p < 0.001, vs. corresponding ligand or vehicle.

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DISCUSSION

Since the effects of cigarette smoking on LDL-C levels has not reached to the crucial conclusions, in the current study, as mentioned above, we tested the hypothesis that whether CSE affects the target genes associated with metabolic pathway of LDL-C. Even though our epidemiological observation made us quite clear, there has been several reports depicting that smoking habit elevated LDL-C in epidemiology investigations. Thus, by using quantitative RT-PCR, we examined whether CSE affects the expression of genes (such as PPARα, PPARβ/δ, PPARγ, ATP-binding cassette, sub-family A transporter 1(ABCA1), PGC1a, PGC1b, SREBP1a, SREBP1c, SREBP2, Fatty acid synthase (FASN), acetyl-CoA carboxylase (ACC), sirtuin (SIRT), CD36, FOXO1, STAT5B, Liver X receptor (LXR) α, LXRβ, Farnesoid X receptor (FXR), vitamin D receptor, hydroxymethylglutaryl-CoA (HMG-CoA) reductase, and uncoupling protein (UCPs)), associated with LDL-C metabolism in HepG2 cells (data not shown except Fig. 6). We observed that CSE mildly upregulated only LDL-R and LXRβ, which are known to be related to cholesterol metabolism. Representative compounds, acrolein, crotonaldehyde, and methylvinylketone, containing CSE, did not induce those genes at all, even at cytotoxic concentrations (data not shown).

LDL-C level is regulated by many factors including hormones, such as thyroid hormone and/or estrogen. For example, LDL-C in patients with thyrotoxicosis, such as Graves’ disease, tends to decrease in levels due to excess catabolism because thyrotoxicosis causes transcriptional stimulation of LDL receptors in the liver. In the previous study, we investigated whether CSE had effects on the effects of triiodothyronine (T3). We reported that CSE recruits transcriptional co-activators and may strengthen T3 binding to its receptor, resulting in upregulated gene expressions. The results suggested that both LDL-R and LXRβ might be elevated, possibly due to CSE, via T3, and its receptors and/or direct action of CSE-mediated PPARβ/δ/γ activation pathways in vivo.

PPARs consist of three isoforms, alpha (α), beta/delta (β/δ), and gamma (γ). Orthodox and/or synthetic ligands for each
isoform have the potential for usage as anti-hyperlipidemia and/or anti-insulin resistant agents in diabetes. We hypothesized that CSE would modulate LDL-C metabolism by modulating PPARγ. Expression plasmids of wild type PPARγ (50 ng) was co-transfected into TSA-201 cells with 50 ng of the reporter construct, PPRE-tk-Luc, in the presence of 0.1 μM pioglitazone and escalating dose of CSE. In the presence of pharmacological concentration of the ligand (0.1 μM), CSE synergistically stimulated PPARγ in a dose-dependent manner (A). However, CSE did not show synergistic or additive effects in the absence of the expression plasmids CMX-PPARγ (B). We also performed to compare CSE to PPARβδ with 1.0 μM L165041 to varying concentrations of CSE on the same setting as PPARγ (Fig. 4C). In the presence of L165041, CSE synergistically elevated PPARβδ-mediated transcription in a dose-dependent manner (C). Without the expression plasmids of CMX-PPARγ, CSE did not present synergistic effects even in the presence of ligand (D). pCMX is introduced as a mock. Data are shown as mean ± S.D. from at least three transfections performed in triplicate. * p < 0.05, *** p < 0.001 vs. comparable control.

isoform have the potential for usage as anti-hyperlipidemia and/or anti-insulin resistant agents in diabetes. We hypothesized that CSE would modulate LDL-C metabolism by modulating PPARγ. Although CSE has faint stimulatory effects on PPARβδγ, CSE exerts synergistic effect on activation of PPARγ via pioglitazone, the synthetic thiazolidine compound that is used in anti-insulin resistant medicine, or L165041 (Fig. 4). We also demonstrated that CSE contacts PPARβδγ in a different manner from pioglitazone or L165041 (Fig. 2). Mammalian two-hybrid assay illustrated that CSE effect transcriptional activation via PPARγ, partly due to enhanced recruitment of co-activators (Fig. 5). We also studied the effects of CSE on other co-factors, such as co-activators CBP, p300, and PGC1αβ, and co-repressors NCoR and SMRT. Results showed that 5% CSE did not have any substantial effect on the transcription mediated by these other co-factors (data not shown).

In the previous study, we described that in the presence of physiological concentration of T3, CSE synergistically increases transcriptional activities via T3 receptors. In the patients with clinical hyperthyroidism or subclinical hyperthyroidism, in spite of increased activity of the HMG-CoA reductase, we observed decrease in the level of total plasma cholesterol, LDL-C, apoprotein B, and lipoprotein-(a) (Lp(a)). These manifestations are mainly due to upregulated LDL-R expression, as the result, LDL-R-mediated catabolism of LDL particles promptly proceeded. On the contrary, simultaneous decreases in the HDL-C levels have been observed in the hyperthyroidism. These phenomena are predominantly due to increased cholesteryl ester transfer protein (CETP)-mediated transfer of cholesteryl esters from HDL to the VLDL and increased hepatic lipase-mediated catabolism of HDL. Triglyceride levels remains unchanged. The lipid profiles described in the patients with hyperthyroidism elsewhere, were inconsistent with our results. Therefore, we could not illustrate the reason behind the lower levels of LDL-C in smoker than that of ex-smoker and/or non-smoker. This indeed is still enigma to us. To figure this out and replicate it in the experimental animals, we performed animal experiments with the mouse line, C57/B6.129P2-apoetm1unc/j acquired from The Jackson Laboratory, and fed them with high-fat diet (D12451, 45 kcal% Fat, Research Diets., Inc.). Since LDL-C metabolism in mice...
vehicle.

5B) recruitment in a dose-dependent manner. Data are shown as mean ± S.D. from at least three transfections carried out in triplicate. *p < 0.05, ***p < 0.001 vs. vehicle.

**Fig. 5. CSE Enhances the Recruitment of Co-activators and Not Co-repressors**

The PPARγ interaction domain of DNA-binding, which corresponds to the AF-1, was replaced to the Gal4-DBD (Fig. 2A). The co-activator was linked to the transcriptional activation domain of VP16 to measure the contact between the Gal4–PPARγ (50 ng) and VP16–SRC1 (50 ng), or VP16–GRIP1 (50 ng), were co-transfected into TSA-201 cells with 100 ng of the UAS-E1BTATA-Luc, in the absence or presence of escalated dosing up to 5% of CSE. As shown in the Fig. 5, 5% CSE significantly enhanced PPARγ-SRC1 (Fig. 5A) and PPARγ-GRIP1 (Fig. 5B) recruitment in a dose-dependent manner. Data are shown as mean ± S.D. from at least three transfections carried out in triplicate. *p < 0.05, ***p < 0.001 vs. vehicle.

**Fig. 6. CSE Mildly Regulates Genes Related to the Cholesterol Metabolism**

We prepared total RNA from the HepG2 cells, which had been exposed by 5% CSE for 24h, using RNAiso Plus. We carried out semi-quantitative real-time RT-PCR using SYBR Green mix according to the conventional methods. Relative quantification data were displayed as fold change compared with the control (Fig. 6). *p < 0.05, **p < 0.01 compared to vehicle.

is completely different from human, we used apoprotein-E-knockout mice and fed them high-fat diet. In brief, each group consists of five mice which were administered with CSE, with saline as a control group, where 15µL/g is a low dose group and 30µL/g is a high dose group (dose/body-weight (g)/d, injected intraperitoneally) for two weeks. High-dose group showed that the level of LDL-C decreased non-significantly at 165.7 ± 35.7 mg/dL that is lower than that of control mice (169.0 ± 10.8). On the other hand, CSE-administered mice displayed significant slight elevation in the levels of total cholesterol, free cholesterol, non-esterified fatty acid, triglyceride, and fasting blood sugar compared to those of control mice (data not shown). Even with such specific experimental conditions, we could not clearly replicate the significant effects of CSE on LDL-C metabolism in vivo so far.

Many epidemiological studies had previously reported that PPARβ/δ/γ play important roles in the regulation of lipid metabolism, especially LDL-C. Ooi et al. reported that PPARδ agonist, GW501516, decreases plasma triglycerides and increases HDL-cholesterol in humans, demonstrated by a randomized, double-blind, crossover trial of 6-week intervention periods with placebo or the agonist. These results suggest that PPARβ/δ/γ plays a role in cholesterol metabolism in humans as well. On the other hand, there are many reports describing the association between LDL-C and smoking, that smoking habit abrogates lipid profile, e.g., increase in level of LDL-C, plasma triglycerides, and VLDL triglycerides, and decrease in the plasma HDL-cholesterol levels in smokers. However, there are still controversies on the direct effect of smoking habit on LDL-C level. Though cigarette smoke contains high concentration of prooxidants, such as, acrolein, crotonaldehyde, methylvinylketone, free oxygen radicals, and peroxynitrites, the minute mechanisms by which smoking deteriorates the plasma lipoprotein metabolism, especially LDL-C, remains unclear. This is the reason behind our main concern with the interrelationships between CSE and lipids and lipoproteins. However, little is known about molecular mechanisms regulating LDL-C metabolism by CSE, granting that CSE disturb the plasma level lipids. Therefore, miRNA profile studies and/or appropriate animal model selection is required for further examinations.

In summary, CSE mildly but significantly upregulated nuclear receptors associated lipid profiles (LDL-R and LXLβ) at the transcription level. CSE also recruited two nuclear co-activators to the PPARγ to induce transcriptional activations. In the current study, we proposed possible molecular mechanisms associated with cholesterol metabolisms perturbed by CSE, although the epidemiological and toxicological implications of our notes remain to be extrapolated. Most importantly, LDL-C oxidized and/or small dense LDL-C, seems to be a sign of developing atherosclerotic change in arteries and seems to be one of the major risks of cardiovascular diseases apart from plasma levels of LDL-C.

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REFERENCES

1. Katsiki N, Papadopoulou SK, Fachantidou AI, Mikhailidis DP. Smoking and vascular risk: are all forms of smoking harmful to all types of vascular disease? Public Health, 127, 435–441 (2013).

2. Sinha AK, Misra GC, Patel DK. Effect of cigarette smoking on lipid profile in the young. J. Assoc. Physicians India, 43, 185–188 (1995).

3. Freeman DJ, Griffin BA, Murray E, Lindsay GM, Gaffney D, Packard CJ, Shepherd J. Smoking and plasma lipoproteins in men: effects on low-density lipoprotein cholesterol levels and high-density lipoprotein subfraction distribution. Eur. J. Clin. Invest, 23, 630–640 (1993).

4. Pollini G, Maugeri U, Tringali S, Jedrychowski W. Serum lipids and lipoproteins in relation to smoking among the apparently healthy workers: a preliminary survey. The Pavia Study. G. Ital. Med. Lav., 7, 119–125 (1985).

5. Halfon ST, Green MS, Heiss G. Smoking status and lipid levels in adults of different ethnic origins: the Jerusalem Lipid Research Clinic Program. Int. J. Epidemiol., 13, 177–183 (1984).

6. Li S, Yun M, Fernandez C, Xu J, Srinivasan SR, Chen W, Berenson GS. Cigarette smoking exaggerates the adverse effects of age and metabolic syndrome on subclinical atherosclerosis: the Bogalusa Heart Study. PLOS ONE, 9, e96368 (2014).

7. Miyawaki T, Hirata M, Moriyama K, Sasaki Y, Aono H, Saito N, Nakao K. Metabolic syndrome in Japanese diagnosed with visceral fat measurement by computed tomography. Proc. Jpn. Acad., 81, 471–479 (2005).

8. Moriyama K, Futawaka K, Tagami T. Cigarette smoking extracts as endocrine disrupting chemicals. Smoking Research Foundation Annual Research Report, 2013, 334–338 (2013).

9. Hayashi M, Futawaka K, Matsuhita M, Hatai M, Yoshikawa N, Nakamuk K, Tagami T, Moriyama K. Cigarette smoke extract disrupts transcriptional activities mediated by thyroid hormones and its receptors. Biol. Pharm. Bull., 41, 383–393 (2018).

10. Hayashi M, Futawaka K, Matsuhita M, Koyama R, Fun Y, Fukuda Y, Nushiha A, Nezu S, Tagami T, Moriyama K. GH directly stimulates UCP3 expression. Growth Horm. IGF Res., 40, 44–54 (2018).

11. Tagami T, Park Y, Jameson JL. Mechanisms that mediate negative regulation of the thyroid-stimulating hormone alpha gene by the thyroid hormone receptor. J. Biol. Chem., 274, 22345–22355 (1999).

12. Tagami T, Yamamoto H, Moriyama K, Sawai K, Usui T, Shimatsu A, Naruse M. A selective peroxisome proliferator-activated receptor-gamma modulator, telmisartan, binds to the receptor in a different fashion from thiazolidinediones. Endocrinology, 150, 862–870 (2009).

13. Benson SC, Pershadisingh HA, Ho CI, Chittoiboyina A, Desai P, Pravenec M, Qi N, Wang J, Avery MA, Kurtz TW. Identification of telmisartan as a unique angiotensin II receptor antagonist with selective PPARγ-modulating activity. Hypertension, 43, 993–1002 (2004).

14. Tagami T, Jameson JL. Nuclear corepressors enhance the dominant negative activity of mutant receptors that cause resistance to thyroid hormone. Endocrinology, 139, 640–650 (1998).

15. Vasavada HA, Ganguly S, Chorney M, Mathur R, Shukla H, Swaroopa A, Weissman SM. SSH4, a mammalian cDNA expression vector. Nucleic Acids Res., 18, 3680 (1990).

16. Uppenberg J, Svensson C, Jaki M, Bertilsson G, Jendeborg L, Berkenstam A. Crystal structure of the ligand binding domain of the human nuclear receptor PPAR. J. Biol. Chem., 273, 31108–31112 (1998).

17. Nolte RT, Wisely GB, Westin S, Cobb JE, Lambert MH, Kurokawa R, Rosenfeld MG, Willson TM, Glass CK, Milburn MV. Ligand binding and co-activator assembly of the peroxisome proliferator-activated receptor. Nature, 395, 137–143 (1998).

18. Batic-Mujanovic O, Beganlic A, Salihefendic N, Pranic N, Kus-

ljuzic Z. Influence of smoking on serum lipid and lipoprotein levels among family medicine patients. Med. Arch., 62, 264–267 (2008).

19. Nakashishi N, Nakamura K, Ichikawa S, Suzuki K, Tataru K. Relationship between lifestyle and serum lipids and lipoprotein levels in middle-aged Japanese men. Eur. J. Epidemiol., 15, 341–348 (1999).

20. Whitehead TP, Robinson D, Allaway SL. The effects of cigarette smoking and alcohol consumption on blood lipids: a dose-related study on men. Ann. Clin. Biochem., 33, 99–106 (1996).

21. Sinha AK, Misra GC, Patel DK. Effect of cigarette smoking on lipid profile in the young. J. Assoc. Physicians India, 43, 185–188 (1995).

22. Nie Y, Luo F, Wang L, Yang T, Shi L, Li X, Shen J, Xu W, Guo T, Lin Q. Anti-hyperlipidemic effect of rice bran polysaccharide and its potential mechanism in high-fat diet mice. Food Funct., 8, 4028–4041 (2017).

23. Samsoe G. Cardioprotection by estrogens: mechanisms of action—the lipids. Int. J. Fertil. Menopausal Stud., 39 (Suppl 1), 43–49 (1994).

24. Raziel A, Rosenzweig B, Botvinic V, Beigel I, Landau B, Blum I. The influence of thyroid function on serum lipid profile. Atherosclerosis, 41, 321–326 (1982).

25. Bonde J, Breuer O, Lütjohann D, Sjöberg S, Angelin B, Rudling M. Thyroid hormone reduces PCSK9 and stimulates bile acid synthesis in humans. J. Lipid Res., 55, 2408–2415 (2014).

26. Shende VR, Singh AB, Liu J. A novel peroxisome proliferator response element modulates hepatic low-density lipoprotein receptor gene transcription in response to PPARα activation. Biochem. J., 472, 275–286 (2015).

27. Duan Y, Chen Y, Hu W, Li X, Yang X, Zhou Z, Kong D, Yao Z, Hajjar DP, Liu L, Liu Q, Han J. Peroxisome Proliferator-activated receptor α activation by ligands and dephosphorylation induces proprotein convertase subtilisin kexin type 9 and low density lipoprotein receptor expression. J. Biol. Chem., 237, 23667–23677 (2012).

28. Ness GC. Thyroid hormone. Basis for its hypcholesterolemic effect. J. Fla. Med. Assoc., 78, 383–385 (1991).

29. Kung AW, Pang RW, Lauder I, Lam KS, Janus ED. Changes in serum lipoprotein(a) and lipids during treatment of hyperthyroidism. Clin. Chem., 41, 226–231 (1995).

30. Aviram M, Luboshitzky R, Brook JG. Lipid and lipoprotein pattern in thyroid dysfunction and the effect of therapy. Clin. Biochem., 15, 62–66 (1982).

31. Rizos CV, Efthas MS, Liberopoulos EN. Effects of thyroid dysfunction on lipid profile. Open Cardiovasc. Med. J., 5, 76–84 (2011).

32. Fan W, Shen C, Wu M, Zhou ZY, Guo ZK. Association and interaction of PPARα and γ gene polymorphisms with low-density lipoprotein-cholesterol in a Chinese Han population. Genet. Test. Mol. Biomarkers, 19, 379–386 (2015).

33. Ooi EM, Watts GF, Sprecher DL, Chui DC, Barrett PH. Mechanism of action of a peroxisome proliferator-activated receptor (PPAR)-delta agonist on lipoprotein metabolism in dyslipidemic subjects with central obesity. J. Clin. Endocrinol. Metab., 96, E1508–E1516 (2011).

34. Skogsberg J, Kannisto K, Cassel TN, Hamsten A, Eriksson P, Ehrenborg E. Evidence that peroxisome proliferator-activated receptor delta influences cholesterol metabolism in men. Arterioscler. Thromb. Vasc. Biol., 23, 637–643 (2003).

35. Freeman DJ, Griffin BA, Murray E, Lindsay GM, Gaffney D, Packard CJ, Shepherd J. Smoking and plasma lipoproteins in man: effects on low density lipoprotein cholesterol levels and high density lipoprotein subfraction distribution. Eur. J. Clin. Invest., 23, 630–640 (1993).

36. Chui PC, Guan HP, Lehrke M, Lazar MA. PPAR gamma regulates adipocyte cholesterol metabolism via oxidized LDL receptor 1. J. Clin. Invest., 115, 2244–2256 (2005).

37. Halfon ST, Green MS, Heiss G. Smoking status and lipid levels
in adults of different ethnic origins: the Jerusalem Lipid Research Clinic Program. *Int. J. Epidemiol.*, 13, 177–183 (1984).

38) Seidel D, Walli A. Influence of smoking on cellular lipid metabolism. *Klin. Wochenschr.*, 62 (Suppl. 2), 28–31 (1984).

39) Baldán Á, de Aguiar Vallim TQ. miRNAs and high-density lipoprotein metabolism. *Biochim. Biophys. Acta*, 1861 (12 Pt B), 2053–2061 (2016).

40) Guan Y, Breyer MD. Peroxisome proliferator-activated receptors (PPARs): novel therapeutic targets in renal disease. *Kidney Int.*, 60, 14–30 (2001).