Cigarette Smoke Extract Disrupts Transcriptional Activities Mediated by Thyroid Hormones and Its Receptors

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Cigarette smoke contains over 4800 compounds, including at least 200 toxics or endocrine disruptors. Currently, effects of cigarette smoke on thyroid hormones (TH) levels remains to be clarified. Here, we demonstrate that cigarette smoke extract (CSE) possesses thyroid hormone properties and affects synergistically as a partial agonist for thyroid hormone receptors (TRs) in the presence of TH. In transient gene expression experiments, CSE stimulated transcriptional activity with TH in a dose-dependent manner. Stimulatory effects were observed with physiological TH concentrations, although CSE did not activate TRs without TH. CSE (5%) dissolved in phosphate-buffered saline (PBS) supplemented with 1 nM TH was approximately comparable to 3.2±0.1 and 2.3±0.2 nM of TRβ1 and TRβ1, respectively. To illustrate probable mechanisms of the CSE agonistic activity, effects on TR mediated transcriptional functions with cofactors were investigated. With a mammalian two-hybrid assay, CSE recruited the nuclear coactivators glucocorticoid receptor interacting protein 1 (GRIP1) and steroid receptor coactivator 1 (SRC1) to the TR. Unsaturated carbonyl compounds, acrolein, crotonaldehyde, and methyl vinyl ketone, representative constituents of CSE, retained such agonistic properties and possibly contributed to stimulatory effects. The results suggest that CSE recruits a transcriptional activator and may reinforce TH binding to the TR additively, resulting in gene expression. CSE partially agonizes TH action and may disturb the function of various nuclear hormone receptor types and their cofactors to disrupt the physiological processes.

Key words signal transduction; thyroid hormone receptor; endocrine disruptor

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Cigarette smoking may increase the risk of disease development, may reduce the effectiveness of treatment, and eventually induce recurrence.9) Cigarette smoking has a substantial influence on human health. The total number of smokers is increasing globally according to the data provided by WHO.9) Cigarette smoke consists of several chemical substances that are abundant in the gaseous phase of cigarette smoke, are such as acrolein (Acr), crotonaldehyde (CA), and methyl vinyl ketone (MVK), and these compounds are assumed to mediate oxidative stress, which has been implicated in the pathogenesis of smoking-related diseases.2–4) In particular, MVK is abundant in the gaseous phase of cigarette smoke, is present in cigarette smoke extract (CSE), and is a major mediator of cigarette smoke.5–7)

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Especially TRβ1 plays important roles in maintaining the physiological processes. TRs exhibit functionally separable domains, similar to other nuclear receptors. TRs comprise the DNA-binding domain (DBD) and the ligand-binding domain (LBD), which are highly conserved. The LBD has functions for homo- and heterodimerization and transcriptional regulations via exchanging coactivators (CoA) or corepressors (CoR).

We hypothesized that CSE would disrupt TH action. To the best of our knowledge, there is no report that evaluates the current data concerning the influence of cigarette smoking on thyroid gland, including hormonal changes at the molecular level. Further studies are required to explain the effects of smoking on thyroid pathophysiology, with special attention to TH and its receptors. The purpose of the present study was to investigate the effects of CSE on transcriptional activities mediated by TH and TRs and to determine whether CSE affects transcription regulations mediated by TH and TRs in vitro. We showed the possible molecular mechanism of disrupted TH actions induced by CSE.

MATERIALS AND METHODS

Preparation of CSE CSE was prepared according to a previous report. Briefly, mainstream smoke from four cigarettes per trial (Caster Frontier One, cigarette brand name; Japan Tobacco Inc., Tokyo, Japan) was aspirated at a flow rate of 1.050 L/min, passed through a Cambridge glass fiber filter (Heinr. Borgwaldt GmbH, Hamburg, Germany) to remove nicotine and the tar-phase, and bubbled into 20 mL of phosphate-buffered saline (PBS) at 25°C. This procedure was repeated ten times, and finally, 20 mL of PBS containing the smoke of total 20 cigarettes was defined as 100%. In all experiments, we weighed tar-phase CSE and examined the effects of 10% CSE, which exerted maximum cytotoxic activity in TSA 201 cell line, which is derived from HEK293 cells.

Experimental Reagents Acr, CA, and MVK were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). TH was purchased from Nacalai Tesque, Inc. (Kyoto, Japan). Fetal bovine serum (FBS) was purchased from Mediatech, Inc. (Wako Pure Chemical Industries, Ltd., Osaka, Japan). Penicillin/streptomycin solution (penicillin: 100 U/mL, streptomycin: 100 µg/mL) was purchased from Invitrogen Corp. (Wako Pure Chemical Industries, Ltd.). Dulbecco’s modified Eagle’s medium (DMEM) with l-glutamine was purchased from Invitrogen Corp. (Wako Pure Chemical Industries, Ltd.). Dulbecco’s phosphate-buffered saline without calcium and magnesium [PBS (−)] was purchased from Nissui Pharmaceutical Co., Ltd. (Tokyo, Japan).

Cell Culture We employed TSA201, derived from a clone of HEK 293 cells, and human hepatoblastoma cells, HepG2, which is used to examine whether metabolic enzymes of xenobiotics effects on the disrupting actions of CSE. Both were maintained and cultured in DMEM containing penicillin (100 U/mL), streptomycin (100 µg/mL), and l-glutamine (2 mM) supplemented with 10% FBS (HyClone Laboratories Inc., UT, U.S.A.). For treatment with reagents, the medium was replaced to phenol-red-free-DMEM containing 10% charcoal-stripped FBS. The cells were maintained in 5% CO2 and 95% air at 37°C. Cells were subcultured at approximately 80% confluence using 0.02% EDTA and 0.25% trypsin (Wako Pure Chemical Industries, Ltd.). Cells were used for experiments at seven days after seeding. Various nuclear receptors were transiently expressed in the cells cultured in 12-wells dishes (1×10⁴ cells/well) using the calcium phosphate (Invitrogen, MA, U.S.A.) method. After exposure to the calcium phosphate-DNA precipitate for 6 h, phenol red-free DMEM with charcoal-stripped FBS was added, in the absence or presence of CSE (0.1, 1.0, 5.0%), Acr, CA, MVK (1, 10, 50 µM, respectively), and TH for 24 h.

Plasmid Constructions Wild-type of human TRα1 expression vector (pCMX-hTRα1) and human TRβ1 (pCMX-hTRβ1) were gifted from K. Umesono and R. M. Evans (Salk Institute, San Diego, CA, U.S.A.). Other expression plasmids used have been described previously. The reporter plasmids TRE-tk-luc contained thyroid hormone response elements (TRE) (Tα1)’s a malic enzyme (ME) gene promoter connected to upstream of the thymidine kinase (TK) promoter in the pA3-luciferase vector (luc). UAS-EIBTATA-Luc, the Gal4 reporter construct, contains five copies of the Gal4 recognition sequence (UAS) upstream of E1B-TATA-Luc. An internal control, both the pRL-TK vector (Promega, WI, U.S.A.) comprised of the TK promoter and Renilla luciferase cDNA was employed.

Transient Expression Assays Transient expression assays with TSA201 or HepG2 cells were performed by the calcium precipitation method as described previously. Cells were collected to measure the luciferase activities according to the manufacturer’s instructions (Dual Luciferase Reporter Assay System; Promega). Luciferase activities were measured in the lysates using a Promega GLOMAX 20/20 luminometer (Promega). Firefly and Renilla luciferase activities were determined to correct the transfection efficiency and cytotoxicity of the added reagents. Data are presented as the mean ± standard deviation (S.D.).

Mammalian Two-Hybrid and 1.5-Hybrid Assays The mammalian two-hybrid experiment was described in the previous reports. In briefly, the expression plasmid of Gal4-cofactor and VP16-TRβ construct were cotransfected into TSA201 cells with 50 ng of the reporter gene, UAS-EIBTATA-Luc, in the absence or existence of ligand. As cofactors, we employed GRIP1, SRC1, and NCoR in this study. The 1.5-hybrid assay was also performed. In shortly, the ligand binding domain (LBD) of TRα1 or TRβ1 was fused to the DNA binding domain (DBD) of Gal4 inframe in pSG424. Either Gal4-TRα1 or Gal4-TRβ1 and the same reporter gene as described above were cotransfected into the TSA201 cells.

Real-Time Quantitative PCR Total RNAs were isolated using RiboZol®RNA Extraction Reagents (AMRESCO, OH, U.S.A.). For semi-quantitative real time-PCR (RT-PCR) analysis, first strand cDNA was synthesized using the Super-Script™ System (Life Technologies, MA, U.S.A.) with Oligo(dT) as the primer. Primers used for the RT-PCR of the human SPOT14 mRNA have been mentioned previously [forward primer (5’−3’), CGA GAA AGC CCA GGA GGT GA, reverse primer (5’−3’), AGC ATCC GGA AACT G TA GC]. For each sample, 1 ng of template was amplified in PCR performed in triplicate on a LightCycler® 1.5 (Roche Diagnostics, IN, U.S.A.) using LightCycler® FastStart DNA MasterPLUS
SYBR Green I (Roche, CA, U.S.A.). PCR cycles were as follows: 94°C for 5s and 68°C for 4min for 30 cycles. The internal controls glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β-actin were amplified and detected separately.38

**Statistical Analysis**

Statistical analyses were performed using SPSS 17.0 software (SPSS Inc., IL, U.S.A.). All data were expressed as the mean value±S.D. from at least three experiments, each performed in triplicate. Comparisons among the groups were analyzed by one-way ANOVA followed by Dunnnett’s test. With all analyses, an associated probability (p) value) of less than 5% (p<0.05) was considered as significant.

**RESULTS**

In the Presence of Physiological TH, CSE Treatment Leads to a Dose-Dependent Induction of TR-Mediated Transcription

We evaluated the additive effects of CSE and TH on the luciferase signal via TRs/TRE-tk-luc with various concentrations of CSE in TSA201 cells. Figure 1 shows the effects of different CSE concentrations (0.1–5.0%) on the TSA201 cell line, which resulted in a higher signal intensity in cells transfected with TR/TRE-tk-luc than in the controls. Compared to 1 nM TH, CSE conferred mild inductions of TRα1/TRE-tk-luc (Fig. 1a) and TRβ1/TRE-tk-luc (Fig. 1b). The luciferase signal induced by 1 nM TH alone at concentrations of 0.1, 1.0, or 5.0% were 96.1, 141.6, and 226.7%, respectively, when CSE was added to the TRα1/TRE-tk-luc reporter and 100.3, 118.8, and 202.6%, respectively, when CSE was added to the TRβ1/TRE-tk-luc reporter. CSE alone did not activate TR-mediated luciferase transcription as observed in the controls of TRα1 and TRβ1.

In the Presence of Physiological TH Concentration, Representative CSE Compounds Cause Dose-Dependent Induction of TR-Mediated Transcription

Transcription mediated by TRs was mildly induced in the presence of CSE and TH (Fig. 1). Thus, we evaluated the inducing effects of representative CSE constituents on TR-mediated transcriptional activation. CSE constituents with varying concentrations of Acr, CA, and MVK were applied to TSA201 cells where transfected with TR and the luciferase construct. These CSE compounds increased TR-mediated transcription in a dose-dependent manner (Fig. 2). Compared to 1 nM TH, Acr, CA, and MVK, at a concentration of 50 µM, induced luciferase signal to 235, 239, and 211%, respectively, via TRα1/TRE-tk-luc (Fig. 2a). Moreover, 50 µM of CA increased the luciferase signal to 166% via TRβ1/TRE-tk-luc, with the physiological TH concentrations similar to TRα1/TRE-tk-luc (Fig. 2b). In comparison, Acr and MVK did not alter the luciferase signal via TRβ1/TRE-tk-luc in a dose-dependent manner in the presence of physiological TH concentration.

Evaluation of the Inducing Effects of CSE on TR and TH-Activated Transcription

To examine the inducing effects of CSE, we compared CSE and 1 nM TH to varying concentrations of TH alone on the transcriptional activation regulated by TR (Fig. 3). In the presence of physiological TH concentration (1 nM), the effect of 5% CSE on TRα1-mediated transcription was comparable to ≥1.5–5 nM TH (Fig. 3a). The obtained magnitude of 5% CSE and TH is linearly approximated to the concentration with the least square approximation (Fig. 3b). The inducing effects of 5% CSE and 1 nM TH on TRα1/TRE-tk-luc were approximately equal to 3.2±0.1 nM of TH alone, as determined from the calibration graph (Fig. 3b). However, the effect of 5% CSE on TRβ1-mediated transcription in the presence of 1 nM TH was comparable to ≥1.5–5 nM TH (Fig. 3a). The inducing effects of 5% CSE and 1 nM TH on
TRβ1/TRE-tk-luc was approximately equal to 2.3±0.2 nM of TH alone, as determined from the calibration graph (Fig. 3d).

**CSE Induced Gene Transcription via the Activation Function-2 Domain of TRα1 and TRβ1**

To examine the interaction between CSE, LBD, and activation function-2 domain of TRs, we employed a 1.5-hybrid system. Although CSE might bind to TR, CSE did not activate TR-mediated transcription in the absence of TH (Fig. 1). Thus, we examined whether CSE partially aids TH-induced TR mediated transcriptional activation through LBD. In the presence of physiological TH (1 nM), TH-induced stimulation of transcription mediated by Gal4-TRα1/UAS-E1b-TATA-luc (Fig. 4a) and Gal4-TRβ1/UAS-E1b-TATA-luc (Fig. 4b) was observed. In comparison, CSE had no substantial impact on the basal transcription level of Gal4-DBD/UAS-E1b-TATA-luc alone (data not shown). Next, we measured the effects of the representative constituents of CSE on the physiological concentrations of TH. Transcriptional activity was measured in the presence of 50 µM of Acr, CA, and MVK along with TH. As shown in Fig. 4c, CA and MVK upregulated the activity mediated by Gal4-TRα1/UAS-E1b-TATA-luc by approximately 577% and 450%, respectively, compared to that induced by TH alone. Unlike Gal4-TRα1/UAS-E1b-TATA-luc, the representative constituents failed to bind to Gal4-TRβ1 or to reinforce Gal4-TRβ1 binding of TH mediated by any cofactors (Fig. 4d).

**CSE Enhances the Recruitment of CoAs and Not CoRs by TH**

Positively regulated TR genes are mediated by interaction with CoAs. In contrast, CoRs may be related to the negatively regulated genes. We performed a mammalian two-hybrid assay to examine the effect of CSE on TR–cofactor interactions. In this experiment, we employed representative cofactors such as glucocorticoid receptor interacting protein (GRIP, also referred to as SRC2), steroid receptor coactivator (SRC1), and nuclear receptor corepressor (NCoR). With increasing concentrations of TH, the interaction between TR and GRIP1 was enhanced up to 274% in the presence of 5% CSE and 100 nM TH compared to the control without CSE (Fig. 5a). Similarly, the interaction between TR and SRC1 was enhanced up to 356% in the presence of 5% CSE and 100 nM TH compared to the control without CSE (Fig. 5b). On the contrary, CSE did not affect the interactions between TR and NCoR (Fig. 5c).

**CSE Induces the Native Promoter of Malic Enzyme (ME) Gene**

ME gene and SPOT14 gene (also referred to as the thyroid hormone responsive protein spot14, or Thrsp) were originally discovered in the liver.36,37 Consequently, we employed Hep G2, a human hepatoblastoma cell line, because
Fig. 3. CSE as a Partial Agonist Affected TRs-Mediated Transcriptional Activation Only in the Presence of TH

Cmx-Tra (20ng) with 50ng of the TRE-tk-Luc, was co-transfected into TSA-201 cells in the presence of 5% CSE and in the absence of CSE with increasing amounts of TH. Error bar: mean±S.D., *; p<0.05, ***; p<0.001 vs. CSE 5.0%+TH 1.0 nM (a). The obtained results of relative luciferase activities of 5% CSE and TH were linearly approximated to the TH concentration with the least square approximation (b). Similarly, CMX-TRβ1 (20ng) with 50ng of the TRE-tk-Luc was co-transfected into TSA-201 cells in the presence of 5% CSE and in the absence of CSE with increasing amounts of TH. Error bar: mean±S.D., **; p<0.01, ***; p<0.001 vs. CSE 5.0%+TH 1.0 nM (c). To determine the additive effect of CSE in the presence of physiological TH (1 nM), an analytical curve was drawn with the least square approximation (b, d).

Fig. 4. CSE Elevated Transcription Activity Mediated by the TR-LBD

The distal part of TRs, including LBD and the activation function-2 (AF-2) domain, were fused to the Gal4. Gal4-TRα1 (a) or Gal4-TRβ1 (b) was co-transfected into TSA-201 cells with 50ng of the UAS-E1BTATA-Luc, in the presence of 1 nM TH and 5% CSE. ***; p<0.001 (compared to that with stimulation with 1 nM TH). The additive effects of representative CSE constituents on the transcriptional activities via TR-LBD were also examined similarly. Gal4-TRα1 (c) or Gal4-TRβ1 (d) was co-transfected into TSA-201 cells with 50ng of the UAS-E1BTATA-Luc, in the presence of 1 nM TH and 50µM of each compound. Error bar: mean±S.D., ***; p<0.001 vs. TH 1.0 nM. Data are shown as mean±S.D. from at least three transfections carried out in triplicate. *; p<0.05 (compared to that with 1 nM TH stimulation). N.S.: not statistically significant vs. TH 1.0 nM.
ME and SPOT14 genes are specifically expressed in the liver in response to TH/TRs. Since CSE regulates TRE in the presence of liganded-TRs, we performed dual luciferase assay with HepG2 containing negligible endogenous TRs (data not shown). The 5'-flanking region of the ME gene contains a cis-regulatory element that binds TRs and induces transcription of TH via activating the ME promoter. The reporter construct, harboring the ME promoter, ME-tk-Luc, was transfected into HepG2 cells. The TR/β1 expression plasmid with 5% CSE and 100 nm TH when incubated for 24 h upregulated luciferase activity via ME-tk-Luc by 256% compared to that by 100 nm TH alone (Fig. 6).

The Effects of CSE on SPOT14 Expression in HepG2 Cells in Physiological TH Settings Because TH specifically impacts inducible SPOT14 expression in hepatocytes, we examined SPOT14 expression by quantitative real time-PCR of human hepatocytes, HepG2. When RT-PCR was performed using a set of mRNA in the presence of 5% CSE, we found that SPOT14 mRNA expression was higher than that with the control in HepG2 cells (Fig. 7). In the presence of CSE and physiological concentration of TH, the transcriptional level was significantly upregulated by 173% compared to that with vehicle, indicating that CSE specifically acts as a partial agonist to the native gene promoter mediated by endogenous TR, even in the metabolic enzymes of xenobiotics.

**DISCUSSION**

In the current study, we showed that CSE augments TH action by modulating TH binding to the TRs and subsequently upregulates their responsive gene transcription. In the transient gene expression system, an increasing dose of CSE mildly but significantly upregulated gene transcription mediated by liganded-TRs (Fig. 1). Increase in gene transcription is partly due to the recruitment of GRIP1 and/or SRC1 to the TR by CSE (Fig. 5). In particular, some reagents have additive effects on TR mediated transcription in a manner similar to that of CSE. Indeed, Acr, CA, and MVK, the representative constituents of CSE, also retained agonistic action for TRα1 in their maximum concentration, but did not affect TR/β1 (Fig. 2). These results suggested that CA and MVK might have a direct action for TRα1 or might recruit undetermined cofactors to reinforce DNA binding of TRα1. However, both CA and MVK did not directly influence TR/β1 or impact transcriptional regulation mediated by TH/TR/β1 (Fig. 2). Moreover, CSE increases TR-mediated transcription only in the presence of TH, and not by CSE and/or representative compounds alone.

TH contributes to development, growth, and vital metabolic functions through complex biological pathways, affording multiple options to pharmacologically or toxicologically intervene TH signal transduction at multiple points, such as, biosynthesis, cell-specific uptake, export of TH, and nuclear targeting and action. These steps represent typical fragile points for disrupting TH actions, particularly hypothyroidism or hyperthyroidism.

Increasing concerns over health issues and endocrine-disrupting chemicals related to smoking has resulted in a need to evaluate any potential impact on the endocrine system. Cigarette smoking has been related to thyroid disease, although studies on the same have not reported consistent results, with some studies linking smoking to increased TH levels and others to decreased TH levels. Thyroid dysfunction and cigarette smoking are both common in the general population, with a prevalence of approximately 10 and 30%, respectively. These epidemiological studies presumed a direct effect of the constituents of cigarette smoke on the thyroid gland, as some components of tobacco smoke have been reported to interfere with thyroid function. Whether smoking affects the peripheral actions of TH is still unknown.

Till recently, many studies have measured and compared serum TH and TSH concentrations in active, passive, and non-smokers and have determined their association with cigarette smoke exposure. Active and passive exposure to cigarette smoke is associated with a mild inhibitory effect on the TH, which is reflected by higher serum TH levels in nonsmokers compared to smokers. Several studies have described smoke-related changes in free TH levels. Interestingly, Metsios et al. showed that even 1-h passive smoking exposure to levels found in bars/restaurants is accompanied by a statistically significant increase in metabolism and free TH levels in healthy non-smokers. A population-based study has indicated that smoking is negatively associated with hyperthyroidism but positively associated with hypothyroidism. Moreover, a cohort study performed in Sweden showed a significantly increased risk of goiter and nodules among smokers, and the positive association was stronger for toxic than for non-toxic goiter and nodules.

In a report with 4462 individuals, Fisher et al. found that current smokers have higher thyroxine levels and lower TSH levels than non-smokers and former smokers. In the report, they claimed at least two distinct mechanisms for the effect of cigarette smoke on thyroid function: first, related to higher levels of thyroxine-binding globulin and testosterone among smokers compared to non-smokers and second, related to higher levels of tobacco smoke thyrotoxins in heavy smokers compared to light and moderate smokers. Müller et al. reported that in 138 normal women and 135 women with primary hypothyroidism, of whom 84 presented with subclinical hypothyroidism and 51 presented with overt hypothyroidism, smoking increases the metabolic effects of hypothyroidism in a dose-dependent manner. They showed that smoking were associated dose-dependently to serum concentrations of total and low density lipoprotein (LDL) cholesterol in the women with overt hypothyroidism or with subclinical hypothyroidism, suggesting that these effects may be explained by alteration of both thyroid function and hormone action.

The effect of smoking on thyroid function is controversial. This is mainly due to two factors: the presence of 4800 compounds in cigarette smoke and complicated biological steps that regulates the effect of cigarette smoke on thyroid function. As the consequence of cigarette smoke particularly pertaining to transcription has not been reported to date, the underlying physiological mechanisms are not fully understood. Smoke exposure may affect various physiological processes, including hormone biosynthesis and secretion, and interferes with TH release, binding, association with co-factors, transport, intranuclear conformational change, and clearance. These are associated with adverse effects on the TH resulting in changes in circulating hormone levels. Whether these diverse phenomena can be attributed to the same chemical constituents may never be fully resolved, given the complex-
Fig. 5. Effect of CSE on the TR-CoAs or TR-CoR Interaction, Examined Using a Mammalian Two-Hybrid Assay

The TR interaction domain of CoRs or CoAs, which corresponds to the AF-2 was linked to the Gal4-DBD. The LBD of TRβ was linked to the transcriptional activation domain of VP16 to measure the contact between the Gal4-CoR/CoA and VP16-TR. Gal4-GRIP1 (100 ng), or Gal4-SRC1, or NCoR were co-transfected into TSA-201 cells with 100 ng VP16-TR with 100 ng of the UAS-E1b-TATA-Luc, in the absence or presence of 5% CSE. Escalating dose of TH (0.1, 1.0, 10, 100 nM) were added. As shown in the figure, 5% CSE additively enhanced TR-GRIP1 (a) and TR-SRC1 (b) recruitment in a concentration-dependent manner. Five percent of CSE had no substantial effect on the transcription mediated by TR-NCoR (Fig. 5c), Gal4-DBD, and VP16-TR (data not shown). Data are shown as mean±S.D. from at least three transfections carried out in triplicate. ***p<0.001. N.S.: Not statistically significant vs. control.
Employed in metabolic studies. Based on the expression of Hep G2 cells, the Hep G2 immortal cell line is frequently TR positively regulated ME-tk gene promoter in transfected transcription. Furthermore, in Fig. 6, we showed that liganded-additive effects on TH action by increasing TR-mediated thyroid hormone functions and homeostasis in vivo. Suggested that Acr, CA, and MVK might not interfere with via that TH action mainly maintains general transcription factors (Fig. 2b). We mentioned earlier that CA may interact with the complex of TRα and TRE-tk-luc, and general transcription factors (Fig. 2b). We mentioned earlier that TH action mainly maintains via TRβ not TRα. This suggested that Acr, CA, and MVK might not interfere with thyroid hormone functions and homeostasis in vivo.

In this study, we demonstrated that CSE could exert additive effects on TH action by increasing TR-mediated transcription. Furthermore, in Fig. 6, we showed that liganded-TR positively regulated ME-tk gene promoter in transfected Hep G2 cells. The Hep G2 immortal cell line is frequently employed in metabolic studies. Based on the expression of cellular proteins, HepG2 cells are also similar to the human liver cells than other hepatic cell lines, such as Huh7 and NKNT-3. Therefore, we asked whether native gene promoters are directly activated by TRβ in the presence of CSE and TH, even in the metabolic enzymes of xenobiotics (Fig. 7). This result suggested that CSE act as a partial agonist for TRβ, in the presence of physiological concentration of TH and TRβ. Then, we examined whether SPOT14 gene expression in Hep G2 is directly induced by endogenous TRs in the presence of CSE and physiological concentration of TH. Expression of SPOT14 mRNA was also mildly, but significantly, increased in the presence of physiological concentration of TH and CSE when compared to the control. In the HepG2 cells, TRs were scarcely detected by PCR (data not shown). In in vitro experiments, the endogenous gene expressions responses to CSE were milder than that observed in the reporter assays (Figs. 6, 7). TRs barely express in Hep G2 cells. Thus, it was one of the possible reasons why the disrupting effects of CSE were attenuated in HepG2 cells. The increase in transcription might be ascribed partly to the recruitment of CoA, such as, GRIP1 or SRC1, by CSE. These results indicated that the disrupting effects of CSE were not absorbed at any step of transcription in HepG2 cells, even in the metabolic enzymes of xenobiotics (Fig. 7).

To date, many nuclear coregulators have been reported, although most of their specific functions remain to be determined. Every coregulator has various interactive domains consisting of slightly different forms of the LXXLL motif and its combinations and appears to be in contact with nuclear receptors in different conformations. Specific ligands for each NHR also causes conformational changes in its receptor and modifies the coregulator accessibility to the receptor, either as a stimulator or suppressor. Indeed, CSE improved recruitment of GRIP1 or SRC1, but not NCoR, to TRs (Fig. 5). SRC1/GRIP1 preferentially binds to the TR homodimer over the TR-RXR heterodimer. These features might be dependent on the sequence variations of TR, which interacts with corepressor domains. Since, CSE contains numerous constituents, the minute mechanisms and the specific active compounds involved in recruiting such nuclear cofactors remains to be elucidated.

We evaluated the inducing effects of CSE on the transcription level at various settings, such as, luciferase assay with TRE-tk-luc reporter, promoter assay with native gene of malic enzyme, and by examining the mRNA expression level of endogenous gene. In the case of the TRs/TRE-tk-luc assay, stimulatory effects of 5% CSE in PBS supplemented with 1 nM TH was comparable to approximately 3.2 and 2.3 nM of TRα and TRβ, respectively. However, these effects were observed with physiological TH concentrations. Moreover, CSE did not activate TRs without TH. These results suggested that the conformational change of TR induced by CSE might be subtly different from that induced by TH, and it may strengthen the contact to certain parts of CoAs (Fig. 5). SRC, a family member of CoA, has functional specificity for interacting and activation domains, which cooperatively enhance the activation function (AF)-1 and AF-2 domains of steroid receptors. SRC1 preferentially associates with progesterone receptor. On the other hand, GRIP1, also known as SRC-2, is a member of the SRC family and preferentially interacts with glucocorticoid receptor. CSE might exert a diverse effect on gene activation to alter preferences of liganded-TR and SRC family members via undetermined processes and/or cofactors.

**Fig. 6. CSE Activate the ME Promoter by the Liganded-TRs**

ME-tk-luc (200 ng) was introduced into HepG2 cells, in the presence or absence of 100 nM TH (left panel). Expression plasmid of TRβ (20 ng) was co-transfected into HepG2 cells with 200 ng of the reporter gene in the presence or absence of 100 nM TH (right panel). Data are shown as the mean ± S.D. from at least three transfections carried out in triplicate. *p<0.05 compared to stimulation by 100 nM TH alone.

**Fig. 7. Expression Levels of SPOT14 in HepG2 Cells**

Total RNA was prepared using the TRIZOL reagent. Real-time RT-PCR using SYBR Green mix was performed as described in Methods. Relative quantification of the SPOT14 mRNA levels was calculated after normalization of the total amount of cDNA tested to the GAPDH expression level. The ΔΔCt method was used to analyze results. Relative quantification data were expressed as fold change compared with the control (Fig. 7). *p<0.05 compared to vehicle.
more than one activation domain such as AF-1 and AF-2. The AF-2 domain is well-preserved among the NHR superfamily as hormone inducible transcription factors. Thus, we examined the impact of CSE on AF-2-mediated transcription using the 1.5-hybrid system (Fig. 4). CSE had no substantial effect on basal transcription regulated by Gal4-DBD alone (data not shown). However, CSE induced transcription and this was regulated by Gal4-TRα1 or Gal4-TRβ1. These data suggested that CSE might induce conformational changes to modulate ligand binding sites of TRs or it might recruit undetermined cofactors to reinforce DNA binding to TRs in the presence of TH. In comparison, representative constituents of CSE failed to bind to the Gal4-TRβ1, the mainstay of physiological processes, or to reinforce Gal4-TRβ1 binding of TH mediated by any cofactors. CSE also exerts mild but significant effects on NHRs, such as peroxisome proliferator activated receptor (PPARs), estrogen receptor-α and -β, and other nuclear cofactors (manuscript in preparation), in addition to TRs.

When interpreting these findings, it is important to consider that analysis of the relationship between CSE and transcription could not be directly connected to the concentrations of TSH, free TH levels in vivo, and the thyroid structure. The purpose of the present study was to determine in vitro whether CSE affects gene transcription regulated by TH and TRs at the molecular level because many factors such as the iodine status, translocation to the nucleus, and other multiple steps are involved in pathways through which CSE affects TSH concentration and/or TH. Moreover, the influence of smoking on free TH concentrations in vivo has been also been found to be controversial.

In summary, transient gene expression experiments showed that CSE mildly, but significantly, upregulated gene transcription mediated by liganded-TRs with dose-escalation, even in the metabolic enzymes of xenobiotics. In the presence of TH, CSE recruited two nuclear CoAs to the TR to induce gene expression. In this report, we suggested possible molecular mechanisms responsible for TH/TR-mediated transcriptional induction by CSE, although the epidemiological and toxicological significance of our observations remain to be established. It also might be beneficial to determine the therapeutic approaches for treating patients with thyroid dysfunctions and smoking habits. This is the first report to demonstrate that CSE can increase TH action as a partial agonist at the transcriptional level.

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Conflict of Interest The authors declare no conflict of interest.

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