Dual suppression of adipogenesis by cigarette smoke through activation of the aryl hydrocarbon receptor and induction of endoplasmic reticulum stress

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Am J Physiol Endocrinol Metab 296: E721–E730, 2009. First published January 13, 2009; doi:10.1152/ajpendo.90829.2008—Cigarette smoking decreases body weight, whereas molecular mechanisms underlying this phenomenon have not been elucidated. In this report, we investigated regulation of adipogenesis by cigarette smoke and involvement of aryl hydrocarbon receptor (AhR) and endoplasmic reticulum (ER) stress. We found that cigarette smoke extract (CSE) inhibited differentiation of preadipocytes into adipocytes dose dependently. It was associated with a decrease in lipid accumulation, blunted expression of adipocyte markers (adiponectin, PPAR-γ, and C/EBPα), and sustained expression of a preadipocyte marker MCP-1. CSE markedly induced activation of AhR and AhR agonists (2,3,7,8-tetrachlorodibenzo-p-dioxin, benzo[a]pyrene and 3-methylcholanthrene) reproduced the inhibitory effect of CSE on adipocyte differentiation. Furthermore, knockouts of the AhR gene or blockade of AhR by a dominant-negative mutant attenuated the suppressive effects of CSE on adipocyte differentiation. We also found that CSE induced ER stress in preadipocytes and ER stress inducers (thapsigargin, tunicamycin, and A23187) reproduced the suppressive effect of CSE on the differentiation of preadipocytes. Interestingly, AhR agonists did not cause ER stress and ER stress inducers did not activate AhR. These results suggested that cigarette smoke has the potential to inhibit adipocyte differentiation via dual, independent mechanisms, i.e., through activation of the AhR pathway and induction of the unfolded protein response.

Adipocyte; adiponectin; peroxisome proliferator-activated receptor-γ; C/EBPα/enhancer-binding protein-α; monocyte chemoattractant protein-1

PREVIOUS INVESTIGATION suggested a link between cigarette smoking and a reduced gain of body weight. Albanes et al. (2) showed that J cigarette smokers were leaner than nonsmokers, 2 duration of smoking and body weight was inversely correlated, and 3) these phenomena were observed in both sexes and among all ages. Although some reports suggested that smoking may decrease body weight by suppression of appetite, other cross-sectional studies showed that caloric intake was not different between smokers and nonsmokers (39). Animal experiments also showed no acute effects of smoking or nicotine administration on caloric intake (39). Currently, therefore, mechanisms underlying the suppressive effect of smoking on body weight gain are not well-understood. Because substances in cigarette smoke enter from the respiratory tracts into the systemic circulation, there is a possibility that some chemicals in smoke reach and affect the adipose tissue directly, leading to inhibition of adipogenesis and body weight gain (12). Using cultured preadipocytes, we tested this possibility and found that cigarette smoke has the potential to inhibit differentiation of preadipocytes into mature adipocytes in vitro. The aim of the present investigation is to elucidate molecular mechanisms underlying this novel observation.

In the first part of this study, we focus on a role of the aryl hydrocarbon receptor (AhR). Using gas chromatography-mass spectrometric analysis, previous investigation demonstrated that cigarette smoke contains low levels of dioxins, dioxin-like compounds, and other agonists of AhR. Those chemicals were polychlorinated dibenzo-p-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), coplanar polychlorinated biphenyls (Co-PCBs), and polycyclic aromatic hydrocarbons including benzo[a]pyrene (B[a]P) (15, 33, 35). Using a cell-based bioassay to detect and quantify the level of AhR activation (25, 26), we disclosed that cigarette smoke has the high level of dioxin-like potential to activate the AhR pathway in hepatoma cells (24). Using transgenic mouse to monitor AhR activation in vitro, we also provided evidence that exposure of mice to cigarette smoke induced activation of AhR not only in the lung but also in other organs (23). Because AhR may be a putative, negative regulator of adipogenesis (3), AhR agonists in cigarette smoke might inhibit adipocyte differentiation.

In the second part of this investigation, we focus on a role of endoplasmic reticulum (ER) stress. The ER plays an important role in appropriate folding of synthesized proteins. Chemical, physical, and nutritional stress perturbs function of the ER, leading to accumulation of unfolded proteins in the ER (42). Such ER stress triggers several cascades of signal transduction pathways, known as the unfolded protein response (UPR), and affects cellular function. Recently, we reported that J cigarette smoke induced ER stress in bronchial epithelial cells (48) and 2) K-7174, a GATA inhibitor, suppressed adipocyte differentiation via induction of ER stress (44). ER stress and consequent UPR are known to regulate differentiation of some cell types including plasma cells, pancreatic β cells, hepatocytes, osteoblasts, and myocytes (31). Cigarette smoke could inhibit differentiation of preadipocytes via induction of ER stress.
Based on these previous findings, we here investigate whether and how cigarette smoke suppresses adipogenesis, especially focusing on the roles of the AhR pathway and the ER stress response.

MATERIALS AND METHODS

Reagents. Insulin, 3-isobutyl-1-methylxanthine (IBMX), dexamethasone, Oil Red O, tunicamycin, thapsigargin, A23187, and 3-methylcholanthrene (3MC) were purchased from Sigma (Tokyo, Japan). 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) and BaP were obtained from Wako Pure Chemical (Osaka, Japan). The concentrations of these agents used for studies were not toxic to 3T3-L1 cells when examined by microscopic analysis and formazan assay.

Preparation of cigarette smoke extract. To prepare cigarette smoke extract (CSE), mainstream smoke was made from 10-mg-tar cigarettes using constant vacuum flow (10.5 l/min) and collected in a glass bottle, as described previously (23, 48). The smoke from 20 cigarettes was dissolved in 500 ml PBS with vigorous shaking. This undiluted material was regarded as 100% CSE. CSE was stored at -80°C until use.

Induction of adipocyte differentiation. 3T3-L1 preadipocytes purchased from Health Science Research Resources Bank (Osaka, Japan) were maintained in DMEM-F12 (Wako Pure Chemical) supplemented with 10% FBS (basal medium). For the induction of adipocyte differentiation, cells were 1) precultured in basal medium for 2 days, 2) treated with differentiation medium containing 10 μg/ml insulin, 0.25 μM dexamethasone, and 500 μM IBMX (IDI medium) for 2 days, and 3) incubated in basal medium supplemented with insulin alone for 2 days. The cells were further incubated in basal medium for an additional 2 days and subjected to analyses. To examine effects of CSE, AhR agonists, and ER stress inducers, cells were exposed to the individual agents during the initial 24-h incubation in IDI medium.

Establishment of stable transfectants. Using electroporation, 3T3-L1 cells were transfected with pEFBOS-AhR(Arg39) (47) that encodes a dominant-negative mutant of AhR (AhR-DN) under the control of the elongation factor-1α promoter (9 g) together with pcDNA3.1 (3 g; Invitrogen, Carlsbad, CA) that codes for neomycin phosphotransferase. Stable transfectants were selected by G418 (500 μg/ml), and 3T3-L1/AhR-DN cells were established. 3T3-L1/Neo cells were also established as a control by transfection of 3T3-L1 cells with pcDNA3.1 alone.

Primary culture of preadipocytes. Primary cultures of preadipocytes were established as described previously (7). In brief, inguinal fat pads were obtained from wild-type C57BL/6J mice and AhR-null mutant mice (34) and digested in DMEM-F12 containing 2 mg/ml collagenase-1 (Sigma) for 15 min at 37°C with gentle shaking. After gentle pipetting for a few minutes with a P-1000 micropipette, the tissues were passed through a 106-μm mesh, and the resulting cell suspension was centrifuged at 700 g for 10 min to separate the stromal-vascular cells from adipocytes. The pellet was washed and cultured using DMEM-F12 containing 10–20% FBS. Animals were used for the experiments according to regulations and guidelines at...
University of Yamanashi. The experiments were approved by the Yamanashi University Animal Experiment Committee.

**Oil Red O staining.** To quantify lipid accumulation, cells were fixed with 10% formalin in PBS for 10 min, rinsed with 60% isopropanol, and stained by Oil Red O in 60% isopropanol for 20 min. After the staining, cells were rinsed several times with 60% isopropanol and subjected to microscopic analysis. To evaluate the amount of lipid quantitatively, cells were added with isopropanol containing 4% Nonidet P-40 and lysed by agitation for 5 min. Absorbance (520-nm wave length) was measured by a spectrophotometer.

**Northern blot analysis.** Northern blot analysis was performed as described before (30). cDNAs for adiponectin (49), peroxisome proliferator-activated receptor-γ (PPAR-γ; purchased from Addgene, Cambridge, MA) (20), CCAAT/enhancer binding protein (C/EBP)-α (6), monocyte chemoattractant protein-1 (MCP-1) (41), 78-kDa glucose-regulated protein (GRP78) (28), C/EBP-homologous protein (CHOP) (50), AhR (47), and cytochrome P-4501B1 (CYP1B1) (45) were used for preparation of radiolabeled probes. The levels of 28S ribosomal RNA and glyceraldehyde-3-phosphate dehydrogenase mRNA were used as loading controls. Assays were repeated two to three times, and representative results were shown.

**Dioxin-responsive element-based sensing via secreted alkaline phosphatase assay.** Dioxin-responsive element-based sensing via secreted alkaline phosphatase (DRESSA) bioassay was performed using HeXS34 cells to evaluate activity of AhR (25–27). Activity of secreted alkaline phosphatase (SEAP) in culture media was evaluated by a chemiluminescent method using Great Escape SEAP Detection Kit (BD Biosciences, Palo Alto, CA), as described before (26).

**ER stress-responsive alkaline phosphatase assay.** Induction of ER stress was evaluated by ER stress-responsive alkaline phosphatase (ES-TRAP) assay (21). 3T3-L1 cells were transiently transfected with pSEAP2-Control (BD Biosciences) by using GeneJuice Transfection Reagent (Novagen, Madison, WI) and treated with test reagents. Activity of ES-TRAP in culture medium was evaluated using Great Escape SEAP Detection Kit.

**Formazan assay.** The number of viable cells was assessed by formazan assay using Cell Counting Kit-8 (Dojindo Laboratory, Kumamoto, Japan) (21).

**Statistical analysis.** Data were expressed as means ± SE. Statistical analysis was performed using the nonparametric Mann-Whitney U test to compare data in different groups. A P value of <0.05 was considered to be statistically significant difference.

**RESULTS**

**Blockade of adipocyte differentiation by CSE.** To examine whether cigarette smoke has the potential to affect differentiation of preadipocytes into adipocytes, 3T3-L1 preadipocytes were pretreated with serial concentrations of CSE for 6 h and treated with IDI medium (differentiation medium) in the presence of CSE. After 24 h, the cells were further incubated in IDI medium without CSE for an additional 24 h. After the incubation, the cells were cultured in basal medium supplemented with insulin for 2 days, further incubated in basal medium for an additional 2 days, and subjected to microscopic analyses. As shown in Fig. 1A, in the absence of CSE, IDI medium induced differentiation of preadipocytes into adipocytes with substantial lipid accumulation. Treatment with CSE inhibited this process in a concentration-dependent manner. Oil Red O staining revealed that accumulation of lipid was significantly reduced to 67.0 ± 1.1% by 3% CSE and to 42.1 ± 0.9% by 4% CSE vs. 100% in CSE-untreated cells (means ± SE, P < 0.05; Fig. 1B).

It is known that, during differentiation of 3T3-L1 preadipocytes, expression of adiponectin, PPAR-γ, and C/EBPα increases dramatically (9, 11, 22), whereas basal expression of MCP-1 is suppressed (16). To confirm the inhibitory effect of cigarette smoke on adipocyte differentiation, 3T3-L1 cells were treated with IDI containing CSE for 24 h, further incubated for an additional 5 days as described above, and subjected to Northern blot analysis. As shown in Fig. 1C, expression of adipocyte markers adiponectin, PPAR-γ, and C/EBPα was induced during adipocyte differentiation, whereas this induction was attenuated by CSE in a dose-dependent manner. In contrast, expression of the preadipocyte marker MCP-1 was suppressed during adipocyte differentiation, whereas this inhibitory effect was abolished by CSE. These results confirmed that CSE inhibited adipocyte differentiation.

**Fig. 2. Activation of the aryl hydrocarbon receptor (AhR) by CSE. A and B:** reporter cells that produce secreted alkaline phosphatase (SEAP) following activation of AhR were exposed to serial concentrations of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD; A) or CSE (B) for 24 h, and activity of SEAP in culture media was evaluated by chemiluminescent assay. Assays were performed in quadruplicate. Data are expressed as means ± SE. *Statistically significant differences (P < 0.05). RLU, relative light unit. C: 3T3-L1 preadipocytes were treated with 4% CSE for indicated time periods and subjected to Northern blot analysis of cytochrome P-4501B1 (CYP1B1). The level GAPDH mRNA is shown at the bottom as a loading control.
Inhibition of adipocyte differentiation by CSE through activation of AhR. A previous report indicated that AhR may be a putative, negative regulator of adipogenesis (3). Cigarette smoke contains dioxins, dioxin-like compounds, and other agonists of AhR, which might inhibit adipocyte differentiation via activation of AhR. To test this possibility, we first examined the potential of cigarette smoke to activate AhR using the DRESSA assay (25, 26). Reporter cells were exposed to serial concentrations of TCDD or CSE, and activity of the reporter enzyme SEAP was evaluated by chemiluminescent assay. As expected, TCDD caused activation of AhR dose dependently (Fig. 2A). Similarly, CSE induced activation of AhR in a dose-dependent manner (Fig. 2B). This result was further confirmed using an endogenous indicator for AhR activation in adipocytes, CYP1B1 (3). Consistent with the result from the DRESSA assay, Northern blot analysis revealed that CSE substantially induced expression of CYP1B1 in preadipocytes (Fig. 2C), confirming the activation of AhR.

We next examined effects of AhR agonists on the differentiation of preadipocytes. 3T3-L1 cells were treated with IDI in the presence of AhR agonists including TCDD (10 nM), benzo[a]pyrene (BaP; 1 μM), and 3-methylcholanthrene (3MC; 5 μM) and subjected to phase-contrast microscopy (A), quantitative analysis of lipid content (B), and Northern blot analysis of adiponectin, PPAR-γ, and C/EBPα (C). B: assays were performed in quadruplicate. Data are expressed as means ± SE. *Statistically significant differences (P < 0.05).

Inhibition of adipocyte differentiation via ER stress triggered by CSE. Recently, we reported that K-7174 suppressed adipocyte differentiation and that it was associated with induction of ER stress (44). We also showed that, in bronchial
Fig. 4. Attenuation of anti-adipogenic effects of CSE in the absence of AhR. A–C: 3T3-L1 cells were stably transfected with a dominant-negative mutant of AhR (AhR-DN), and 3T3-L1/AhR-DN cells were established. Expression of endogenous AhR and exogenous AhR-DN in 3T3-L1/AhR-DN cells and mock-transfected 3T3-L1/Neo cells was examined by Northern blot analysis (A). The cells were then treated with IDI in the presence of CSE (4%) or TCDD (10 nM) and subjected to microscopic analyses (B) and quantitative analysis of lipid content (C). D and E: preadipocytes were established from adipose tissues in wild-type mice (WT) and AhR-null mutant mice (AhR KO). The cells were then treated with IDI in the presence of CSE or TCDD and subjected to phase-contrast microscopy (D) and quantitative analysis of the number of mature adipocytes (E). C and E: assays were performed in quadruplicate. Data are expressed as means ± SE. *Statistically significant differences (P < 0.05). NS, not statistically significant.
epithelial cells, cigarette smoke can induce ER stress (48). Cigarette smoke, therefore, might inhibit differentiation of preadipocytes via induction of ER stress. To examine this possibility, we first investigated whether cigarette smoke causes ER stress in 3T3-L1 preadipocytes. Expressions of GRP78 and CHOP were well-known endogenous markers for ER stress. Northern blot analysis revealed that treatment with CSE induced expression of GRP78 and CHOP within 3 h and peaked at 6 h (Fig. 5A), indicating that cigarette smoke triggered ER stress in 3T3-L1 preadipocytes. The induction of ER stress by cigarette smoke was further confirmed by the ES-TRAP assay (21). This assay is based on the fact that activity of the reporter enzyme ES-TRAP is markedly reduced in response to ER stress (21). 3T3-L1 cells were transiently transfected with an ES-TRAP gene under the control of a constitutively active viral promoter and treated with CSE for 3–24 h. The culture media and cells were subjected to chemiluminescent assay and formazan assay, respectively. Activity of ES-TRAP was normalized by the number of viable cells estimated by formazan assay. As shown in Fig. 5B, ES-TRAP activity was significantly inhibited by CSE at 9 and 24 h, confirming induction of ER stress by CSE.

To examine whether the induction of ER stress is causative of blockade of adipocyte differentiation by CSE, we tested effects of other ER stress inducers including thapsigargin (inhibitor of sarco/endoplasmic reticulum Ca\(^{2+}\)-ATPase), A23187 (calcium ionophore), and tunicamycin (inhibitor of protein glycosylation). Treatment of 3T3-L1 preadipocytes with these agents substantially induced ER stress evidenced by expression of GRP78 and CHOP (Fig. 6A). The induction of ER stress by tunicamycin was slower than that by thapsigargin and A23187, possibly because accumulation of hypoglycosylated proteins in the ER takes time, or different concentrations were used for individual agents. Using these agents, effects of ER stress on the differentiation of preadipocytes were investigated. 3T3-L1 cells were treated with IDI together with thapsigargin or A23187 for 24 h, or the cells were pretreated with tunicamycin for 6 h and treated with IDI together with tunicamycin for an additional 24 h. The cells were further cultured for an additional 5 days. Microscopic analysis revealed that all ER stress inducers blocked differentiation of 3T3-L1 cells (Fig. 6B, left). Consistent with this result, accumulation of lipids induced by IDI was also abrogated by any of the ER stress inducers (Fig. 6B, right). Furthermore, Northern blot analysis revealed that induction of adipocyte markers by IDI was suppressed by either thapsigargin, A23187, or tunicamycin and that suppression of the adipocyte marker by IDI was reversed by the ER stress inducers (Fig. 6C). These results suggested that ER stress interferes with adipocyte differentiation and that CSE blocks adipogenesis, at least in part, via induction of ER stress.

To examine a possibility that activation of AhR is an event downstream or upstream of ER stress, 3T3-L1 preadipocytes were treated with AhR agonists for 6 or 24 h, and expression of ER stress markers was examined by Northern blot analysis. As shown in Fig. 7A, all of TCDD, B[α]P, and 3MC substantially induced expression of CYP1B1, the marker of AhR activation. However, induction of GRP78 and CHOP was not observed in the cells treated with these AhR agonists. Only B[α]P exclusively induced modest elevation of CHOP mRNA at 24 h, but it was not associated with induction of GRP78. We also examined an effect of ER stress on the activation of AhR. 3T3-L1 cells were treated with inducers of ER stress including thapsigargin, A23187, and tunicamycin for 9 or 24 h and subjected to analysis. The result showed that all ER stress inducers upregulated GRP78 and CHOP, whereas induction of CYP1B1 was not observed (Fig. 7B). These results suggested that activation of the AhR pathway and induction of ER stress by CSE are independent mechanisms underlying the antiadipogenic effect of cigarette smoke.

**DISCUSSION**

Several previous reports suggested that cigarette smoking attenuates body weight gain. Appetite loss by smoking might explain this phenomenon, but direct effects of smoke on adipogenesis have not been reported. In the present investigation, we examined regulatory effects of cigarette smoke on adipogenesis. We found that cigarette smoke has the potential to inhibit differentiation of preadipocytes into adipocytes. Subsequent experiments revealed that cigarette smoke induces activation of AhR and ER stress, both of which play crucial roles in the suppression of adipogenesis by cigarette smoke. Interestingly, AhR agonists did not cause ER stress, and ER stress inducers did not activate AhR. These results disclosed...
that cigarette smoke blocks adipocyte differentiation via dual, independent mechanisms, i.e., through activation of the AhR pathway and induction of the UPR.

Previous reports showed that dioxin inhibited adipocyte differentiation in several cell types, including 3T3-L1 cells, C3H10T1/2 cells, and primary mouse embryonic fibroblasts (MEFs) (3, 8, 40). However, molecular mechanisms underlying the negative regulation of adipogenesis by dioxin have not been fully elucidated. Shimba et al. (46) suggested possible involvement of ERK in the suppression of adipogenesis via AhR, because 1) 3T3-L1 cells overexpressing AhR exhibited higher ERK activity and 2) treatment with ERK inhibitors...
abrogated the anti-adipogenic effect of AhR, possibly through inhibition of PPAR-γ, the crucial factor required for adipogenesis (17). Hanlon et al. (19) also demonstrated that low levels of ERK activation cooperated with AhR-induced factor(s) to generate a suppressor that prevents transcription of PPAR-γ.

The similar mechanisms might be involved in the suppression of adipogenesis by cigarette smoke. However, in our experimental setting, we found that activation of ERK was observed in CSE-exposed 3T3-L1 cells, whereas selective inhibition of ERK by PD98059 did not reverse the anti-adipogenic effect of CSE (our unpublished data), excluding this possibility.

Currently, active entities responsible for the activation of AhR by cigarette smoke in preadipocytes have not been fully determined. Previous assessment using gas chromatography-mass spectrometric analysis showed that the levels of dioxins in cigarette smoke were very low. For example, Aoyama et al. (4) evaluated the amount of dioxins (PCDDs, PCDFs, and Co-PCBs) in mainstream and sidestream smoke. They reported that the total amount of dioxins in cigarette smoke was ranging from 0.4 to 2.4 pg TEQ/cigarette. On the other hand, several reports showed that various polycyclic aromatic hydrocarbons, another major group of AhR agonists, were contained in cigarette smoke (15, 32) and may contribute to intense activation of AhR (24). These data indicate a possibility that main AhR activators in cigarette smoke are not dioxins but an array of polycyclic aromatic hydrocarbons.

We demonstrated in this study that cigarette smoke caused ER stress in preadipocytes currently, it is undetermined what kind of substances in cigarette smoke trigger ER stress. Cigarette smoke contains thousands of chemicals including nicotine, reactive oxygen/nitrogen species, and heavy metals such as cadmium (38). Crowley-Weber et al. (14) previously reported that nicotine had the weak potential to induce activation of the GRP78 and CHOP promoters in human hepatoma cells. Recently, we reported that various heavy metals, especially cadmium, had the potential to induce ER stress in vitro and in vivo (51). We also demonstrated that cadmium induced ER stress via generation of reactive oxygens, especially superoxide anion (52). Similarly, cigarette smoke induced ER stress in pulmonary epithelial cells, which was abrogated by the treatment with antioxidants (48). These findings indicate that nicotine, heavy metals, and reactive oxygen species may be responsible for the induction of ER stress in preadipocytes by cigarette smoke.

In the present report, we showed that ER stress inhibited differentiation of preadipocytes into adipocytes. Several underlying mechanisms may be postulated. First, in eukaryotic cells, secretory proteins enter the subcellular pathway through the ER where immature proteins are folded into native conformation and undergo a multitude of posttranslational modifications. Several autocrine factors, including adiponectin, fibroblast growth factor, and midkine, have been identified as adipogenic proteins in preadipocytes (10, 18, 37). ER stress may affect secretion of these autocrine factors and thereby interfere with adipocyte differentiation. Second, a previous report showed that CHOP suppressed adipocyte differentiation by inhibiting function of C/EBP. That is, CHOP had an ability to dimerize with C/EBP, and CHOP-C/EBP heterodimers could not bind to the classical C/EBP binding site (5). As demonstrated in this report, CHOP was markedly induced by ER stress in preadipocytes. Induction of CHOP by cigarette smoke may be involved in the blockade of adipogenesis.

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3T3-L1 cells were treated with agonists of AhR (TCDD, B[a]P, or 3MC; A) or inducers of ER stress (Thap, A23187, or Tun; B) for 6–24 h and subjected to Northern blot analysis of GRP78, CHOP, and CYP1B1.
that ER stress interfered with insulin signaling via inhibition of IRS-1 (36). As the fourth possibility, interference with the insulin signaling by ER stress may explain the anti-adipogenic effect of cigarette smoke.

The adipose tissue produces an array of adipocytokines essential for the maintenance of normal tissue structure and function. For example, lipoprotein characterized by a paucity of the adipose tissue causes depletion of adipocytokines and consequent insulin resistance, diabetes, and cardiovascular diseases (17). From this point of view, suppression of appropriate adipogenesis by cigarette smoke may represent an additional mechanism whereby smoking can be injurious to human health.

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