Effect of Diesel Exhaust on Development of Fetal Reproductive Function in ICR Female Mice

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Diesel exhaust (DE) is a serious air pollution problem in big cities. Most suspended particulate matter (SPM) less than 2.5 μm in diameter consists of diesel exhaust particles (DEPs), which are reported to cause pulmonary carcinogenesis, allergic rhinitis, and bronchial asthma-like diseases. It has been recently reported that DE also affects the circulatory and reproductive systems. Yoshida *et al.* reported that mRNA expression of steroidogenic factor-1 (Ad4BP/SF-1) and of Müllerian inhibitory substance (MIS), which are essential for male gonadal differentiation, decreased significantly in male fetuses when maternal mice were exposed to DE at levels of 0.1 or 3.0 mg DEP/m^3 for 8 hr per day between 2 and 13 days postcoitum (dpc). In this study, maternal mice were exposed to DE 0.1 mg DEP /m^3 for 8 hr per day between 2 and 13 dpc. Expression levels of Ad4BP/SF-1 and MIS mRNA in female fetuses were not decreased. However, expression levels of bone morphogenetic protein-15, reported to be related to development of the oocyte, were significantly decreased in comparison with that in the control group. Our data suggest that female fetuses of pregnant mice exposed to DE *in utero* are less sensitive to the expression levels of mRNAs for Ad4BP/SF-1 and MIS compared with males and that DE may affect development of the oocyte in the female fetus.

Key words —— diesel exhaust, fetus, female reproductive system, mRNA expression, pregnancy

INTRODUCTION

Air pollution has become a very serious problem in the world's big cities. Diesel exhaust (DE), which contains diesel exhaust particles (DEPs) and diesel exhaust gases such as nitrogen dioxides, carbon oxides, and sulfur dioxide, is an important air pollutant. Lung cancer,[1,2] allergic rhinitis,[3–5] and bronchial asthma-like diseases[6–12] can be caused by exposure to DE or DEP. The effect of DE on the reproductive system has been examined recently along with endocrine-disrupting chemicals. Yoshida *et al*. reported ultrastructural changes in Leydig cells of mice exposed to DE (0.3 mg DEP/m^3 through the airway, 12 hr daily, up to 6 months) and reduced in luteinizing hormone (LH) receptor mRNA expression in Leydig cells of mice exposed to DE 1.0 mg DEP/m^3.[13] Tsukue *et al*. reported that the weight of accessory reproductive organs in male F344 rats was significantly decreased with exposure to 0.3 mg DEP/m^3 and increased with exposure to 3.0 mg DEP/m^3, and that serum LH and testosterone levels increased significantly at both 0.3 and 1.0 mg DEP/m^3.[14]

During fetal life, the reproductive system of male and female mammalian embryos consists of an indifferent gonad that is indistinguishable morphologically between the sexes. Adjacent to the gonad are simple ducts, the Müllerian and Wolffian ducts, and the anlagen of the female (oviduct, uterus, and upper part of the vagina) and male (epididymis, vas deferens, and seminal vesicles) reproductive tract, respectively. Thus the embryo is initially sexually undifferentiated.

In the female, the absence of Müllerian inhibiting substance (MIS) permits continued development of the Müllerian duct, and the absence of testosterone leads to degeneration of the Wolffian duct. Thus female development has traditionally been considered as a default state resulting primarily from the absence of the testis-determining factor and, as a consequence, the failure of MIS and testosterone synthesis.

Wnt-4 is initially required in both sexes for formation of the Müllerian duct, and Wnt-4 in the developing ovary appears to suppress the development of Leydig cells. When sex-specific differentiation

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of the gonads commences at around 11.5 days postcoitum (dpc) in mice, Wnt-4 expression is downregulated in the male gonad but is maintained in the female gonad.[15]

Wnt-7a is expressed along the Müllerian duct epithelium in mice of both sexes from 12.5 to 14.5 dpc, but it is then lost from the male epithelium following Müllerian duct regression. After Wnt-4 initiates duct formation,[15] Wnt-7a functions as an epithelial-to-mesenchyme signal, rendering the mesenchyme competent to respond to MIS signaling through the MIS receptor.[16]

Two nuclear receptors, dosage-sensitive sex reversal-adrenal hypoplasia congenita, a critical region on the X chromosome gene-1 (Dax-1) and steroidogenic factor-1 (Ad4BP/SF-1), are required for adrenal development and function. *In vitro* assays suggest that Dax-1 represses Ad4BP/SF-1 mediated transcription. There has been a recent report that Dax-1 inhibits Ad4BP/SF-1-mediated steroidogenesis not only *in vitro* but also *in vivo*.[17] Dax-1 as well as Wnt-4 plays a concerted role in both the control of female development and the prevention of testes.[18] Yoshida *et al*. reported that expression of mRNAs for Ad4BP/SF-1 and MIS, which are essential for male gonadal differentiation, decreased significantly in male fetuses when maternal mice were exposed to diesel exhaust at levels of 0.1 and 3.0 mg DEP/m^3 for 8 hr per day between 2 and 13 dpc.[19]

The expression pattern of estrogen receptor (ER) between the time of implantation and development of the reproductive tract may be the same in male and female mice.[20] Cytochrome P450 1A1 (CYP1A1) is a substrate-inducible microsomal enzyme that oxygenates polycyclic aromatic hydrocarbons such as the carcinogen benzo[*a*]pyrene as the initial step in their metabolic processing to water-soluble derivatives.[21] Mechanistic studies of CYP1A1 induction have provided insight into P450 induction, polycyclic aromatic hydrocarbon carcinogenesis, dioxin action, aryl hydrocarbon receptor (AhR) function, and receptor-mediated mammalian gene expression.[22]

Members of the transforming growth factor (TGF)-β superfamily are potent regulators of cell proliferation and differentiation in a number of organ systems, and three members, growth differentiation factor (GDF)-9, bone morphogenetic protein (BMP)-15, and BMP-6, are expressed by the oocyte and may mediate effects attributed to the oocyte.[23] BMP-15 and GDF-9 may interact directly (*i.e*., form heterodimers) or functionally (*i.e*., play redundant or antagonistic roles). Important roles for BMP-15 and GDF-9 have been found in oocyte-somatic cell interactions during folliculogenesis and the periovulatory period.[24]

Yoshida *et al*. reported that even low-level (0.1 mg DEP/m^3) DE reduced expression of genes essential to normal male gonadal development, such as MIS and Ad4BP/SF-1, and may affect the development of the male gonads.[19] Alterations in gene expression in the female fetus, however, are largely unknown. To clarify the effects of DE on the development of fetal reproductive function, we investigated body weight and expression of specific genes in female ICR fetuses on 14 dpc (the day the plug was found was taken as 0 dpc), the time of sexual development.

MATERIALS AND METHODS

Animals —— Mice used in the experiment were derived from 44 pregnant Slc: ICR mice on the first day of pregnancy. The 44 pregnant mice were divided into two groups and placed in separate inhalation chambers. Group 1 ($n = 22$) was exposed to diesel engine exhaust at 0.1 mg DEP/m^3; group 2 ($n = 22$) was exposed to clean air (control) in a clean room. Exposure was started at 2 dpc and continued until 13 dpc. No exposure occurred on 4, 5, 11, or 12 dpc. Mice were given free access to a commercial stock diet (CE-2: Japan Clea Co., Tokyo, Japan) and water. The pregnant females were killed on 14 dpc under deep ether anesthesia using the intracardiac method. The uterine horns were dissected, and fetuses and placentas were removed. Each fetus and placenta was weighed, and the fetuses were frozen in about five volumes of RNA *later* (Ambion, The RNA Company, TX, U.S.A.) and stored at –20°C. The paws of the fetuses were removed and stored at –80°C for later sex determination.
Generation of DE —— A 2369-cc diesel engine (manufactured by Isuzu Motor Co., Tokyo, Japan) was operated at 1050 rpm with an 80% load with commercial light oil (Idemitsu Kosan Co., Tokyo, Japan) in the Research Institute of Tuberculosis, Japan Anti-Tuberculosis Association. The engine exhaust was introduced into a dilution tunnel 218 mm in diameter and 555 cm in length. There the exhaust was mixed at a ratio of 1 : 8 with temperature- and humidity-controlled clean air that was first passed through a high-efficiency particulate air filter and a

charcoal filter. The diluted DE was delivered directly to the animal exposure chamber (0.6 m^3 in size). The concentration of fine particles was measured with a mass monitoring system (TEOM; Rupprecht & Patashnick Co., NY, U.S.A.). Exposure was carried out for 8 hr per day (9:30–17:30), and the average concentration of each constitute of the exhaust was 0.1 mg DEP/m^3. The densities of gaseous materials, including NO (2.2 ± 0.34 ppm), NOx (2.5 ± 0.34 ppm), NO_2 (0.0 ppm), CO (9.8 ± 0.69 ppm), and SO_2 (< 0.1 ppm, not detectable) were evaluated using an infrared gad analyzer (ULTRAMAT-S; Fuji Electric Co., Tokyo, Japan), the chemiluminescent detection method (ECL-30 Type; Yanagimoto Mfg. Co., Japan), and a flame photometric detector (Exhaust Gas Analyzer Bex-70 HD; Best Co., Japan).

Sex Determination of Fetuses —— The sex of the fetuses was determined using the method of Lambert *et al.*[25] The fetal paw was transferred to a 1.5-ml Eppendorf tube with 20 μl of TKM buffer (10 mM Tris, pH 7.6, 10 mM KCl, 2 mM EDTA, and 4 mM $MgCl_2$). The cells were separated and homogenized with a 200-μl pipette (Eppendorf, Brinkmann Instruments, Inc., Westbury, NY, U.S.A.) using a filter tip; 380 μl of TKM buffer and 37.5 μl of 10% sodium dodecyl sulfate (SDS) were then added and vortexed. The tube was incubated at 55°C for 5 min to denature the proteins. Proteins were precipitated by adding 200 μl of saturated NaCl, vortexing, and centrifugation for 5 min at 12000 × *g*. An aliquot of the supernatant (500 μl) was transferred into a fresh microfuge tube containing 900 μl of 100% ethanol to precipitate the DNA. The DNA was pelleted by centrifugation and washed once in 70% ethanol. Ethanol was removed by pipetting, and the DNA pellet was dried for 3 min in room air and then dissolved in 100 μl of 10 mM Tris HCl, pH 8, 0.1 mM EDTA (TE) for 10 min at 55°C.

Two microliters of dissolved DNA was used for multiplex PCR in a 50-μl reaction overlaid with mineral oil. Taq polymerase diluted in buffer provide by the manufacturer was used with 0.2 mM of each dNTP (PCR Nucleotide Mix, Roche Diagnostics Corp., NJ, U.S.A.), 0.2 μM of male-specific Sry primers, and 0.12 μM of IL3-specific primers. A thermal cycler (TaKaRa PCR Thermal Cycler MP, TaKaRa Biomedicals, Shiga, Japan) was used under the following conditions: 95°C for 4.5 min followed by 45 cycles at 95°C for 35 sec, at 50°C for 1 min, and at 72°C for 1 min, with a final extension at 72°C for 5 min. The PCR products were subjected to 2% agarose gel electrophoresis.

RNA Preparation —— The total number of pregnant mice was 14 in each group on the basis of the sex determination; we randomly chose 1–3 female fetuses from each pregnant mouse. After we homogenized these fetuses in 1 ml of ISOGEN (Nippon Gene, Japan, a commercially available solution of phenol and guanidine isothiocyanate) with an HG 30 (HITACHI Co., Japan), total RNA was isolated from 400 ml of homogenized fetus with 600 ml of fresh ISOGEN added according to the protocol recommended by the manufacturer. The concentration and purity of the RNA were determined by measurement of the optical density at 260 and 280 nm. The RNA solutions were diluted to a working concentration in double-distilled water (DDW).

Quantitative Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) —— DNase treatment was applied to 2000 ng of total RNA dissolved in 16 μg of DDW, 2 μl of RQ1 DNase 10× reaction buffer (Promega), 0.5 μl of Rnasin RNase Inhibitor (40 U/μl, Promega), and 1 μl of RQ1 Rnase-Free DNase (1 U/μl, Promega) for 60 min at 37°C. Inactivation by RQ1 DNase Stop Solution (Promega) and 22 μl of DDW, and denaturing of total RNA were carried out at 65°C for 10 min, and then on ice for more than 5 min. Reverse transcription of total RNA into cDNA was performed with 2 μl of M-MLV reverse transcriptase (Invitrogen), 20 μl of total RNA, 10 μl of random primers (40 ng/ml, TaKaRa), 20 μl 5 × First Standard Buffer (Invitrogen), and 25 μl of PCR Nucleotide Mix (each dNTP 2 mM; Roche) for 60 min at 37°C. Inactivation of the enzyme was carried out for 5 min at 95°C.

Quantitative analysis of specific mRNA expression was performed using a sequence detection system (ABI PRISM® 7700; Perkin-Elmer, Foster City, CA, U.S.A.). Use of this system to determine specific mRNA expression has been described in the manufacturer's user bulletins. Pairs of primers and TaqMan probes were designed on a computer (Primer Express software; Perkin-Elmer) to amplify specific small fragments from Ad4BP/SF-1, Dax-1, ER, Wnt-4, Wnt-7a, MIS, cytochrome P450 aromatase (aromatase), CYP1A1, BMP-6, or BMP-15. The gene expression of the each primer was verified on 2% agarose gel electrophoresis using PCR products. The murine glyceraldehydes-3-phosphate de-hydrogenase (GAPDH) gene, a ubiquitously expressed housekeeping gene, was used to provide an internal marker of mRNA integrity. The probes were labeled with 6-carboxy-fluorescein (FAM) and the fluorescence was quenched with 6-carboxy-

Table 1. Parameters of Fetuses from ICR Pregnant Mice Exposed to DE or Clean Air

	Control	DE (DEP 100 μg/m^3)
Pregnant mice: a	22	22
(presence of a vaginal plug)		
Number of pregnant mice: b	15	16
Implantation rate: b/a × 100 (%)	68.2	72.7
Number of fetuses: c	193	202
Sex rati ♂/♀	77/115	95/105
Average of conception: c/b	12.9 ± 5.3	12.6 ± 3.3
Dams' body weight (g)	52.2 ± 6.9	49.0 ± 4.0
Fetal weight (mg)		
Total	306.9 ± 44.0	301.2 ± 45.3
Male	320.2 ± 54.7	306.6 ± 48.2
Female	292.9 ± 42.3	297.6 ± 43.1
Placental weights (mg)		
Total	123.4 ± 20.7	124.5 ± 21.7
Male	127.6 ± 22.4	127.5 ± 22.9
Female	116.9 ± 13.7	118.6 ± 13.2

The average ± S.D. of the median weight of fetuses and placentas from the same pregnant mice in the control and exposed groups at 14 pcd.

tetramethyl-rhodamine (TAMRA). PCR amplification was performed in a 96-well optical tray with caps and a 25-μl final reaction mixture consisting of 12.5 μl of TaqMan Universal PCR Master Mix (Applied Biosystems), 2 μM of TaqMan fluorescent probe (Applied Biosystems), 3 μM of each primer (Espec Oligo Service Corp.), and the cDNA sample. The program conditions were at 95°C for 10 min, followed by 40 cycles at 95°C for 15 sec and at 60°C for 1 min.

Statistics —— All values are expressed as mean ± S.D. Differences between the exposed group and control group were evaluated using analysis of variance (ANOVA). The statistical significance of differences in implantation rates and sex ratio was assessed using the chi-square test (for nonparametric tests). Differences in litter size, maternal body weight, and fetal and placental weights were analyzed statistically using Student's *t*-test. A *p*-value of 0.05 was considered significant.

RESULTS

Implantation Rate and Maternal and Fetal Weights

The pregnancy rate was 72.7% (16 to 22) in the exposure group and 68.2% (15 to 22) in the control group (Table 1). Litter size was 12.63 ± 3.32 in the exposure group and 12.87 ± 5.26 in the control group. The fetal male : female sex ratio was 95 : 105 in the exposure group and 77 : 115 in the control group; sex determination was inconclusive in some fetuses.

The body weight of dams was 49.00 ± 4.02 g in the exposure group and 52.18 ± 6.90 g in the control group. Fetal weights (total, males, and females, respectively) were 301.20 ± 45.31 mg, 306.59 ± 48.17 mg, and 297.56 ± 43.05 mg in the exposure group and 306.86 ± 44.02 mg, 320.25 ± 54.74 mg, and 292.92 ± 42.27 mg in the control group. Placental weights (total, males, and females, respectively) were 124.46 ± 21.66 mg, 127.51 ± 22.86 mg, and 118.64 ± 13.19 mg in the exposure group, and 123.37 ± 20.66 mg, 127.56 ± 22.36 mg and 116.88 ± 13.67 mg in the control group. Weights in the exposure group did not differ significantly from those in the control group.

mRNA Expression in Female Fetuses

To clarify the effects of DE on the sex development of female fetuses, we examined mRNA expression levels in the whole female fetal body at 14 dpc. Expression levels of aromatase, CYP1A1, MIS, DAX-1, and Ad4BP/SF-1 mRNAs in female fetuses did not differ significantly from those in the control group (Table 2). Levels of ER, Wnt-4, and Wnt-7a, which occur in female fetuses, also did not differ statistically in the exposure group in comparison to that in the control group. On the other hand, the expression level of BMP-15 mRNA in female fetuses

Table 2. Effect of Maternal Exposure to DE on Expression of mRNA in Female Fetuses

	Control	DE (DEP 100 μg/m^3)	(% of control)
Aromatase	14.0 ± 9.2	14.0 ± 3.7	(99.5)
CYP 1A1	21.5 ± 14.5	19.6 ± 11.4	(90.9)
MIS	28.5 ± 16.9	31.7 ± 29.9	(111.2)
DAX-1	61.6 ± 33.1	47.5 ± 12.5	(77.1)
Ad4BP/SF-1	284.8 ± 76.5	314.2 ± 101.5	(110.3)
ER	1223.7 ± 581.4	1216.8 ± 449.6	(99.4)
Wnt-4	2115.3 ± 487.6	2402.5 ± 638.4	(113.6)
Wnt-7a	1372.3 ± 566.9	1215.8 ± 554.0	(88.6)
GDF-9	20.3 ± 10.1	17.9 ± 6.8	(88.0)
BMP-6	517.0 ± 179.6	445.0 ± 212.0	(86.1)
BMP-15	32.0 ± 10.0	21.5 ± 9.8*	(67.3)

Total RNA was isolated at 14 pcd from the fetuses of control pregnant mice and pregnant mice exposed to DE for quantitative RT-PCR. Levels of aromatase, CYP1A1, MIS, DAX-1, Ad4BP/SF-1, ER, Wnt-4, Wnt-7a, GDF-9, and BMP-6 and -15 mRNA were analyzed quantitatively with a sequence detection system (ABI PRISM® 7700). The mRNA expression levels are shown as ratios between each gene and the GAPDH gene to correct for variations in the amounts of mRNA. The ratios have been normalized so that the mean control ratio is 1. The values ($\times 10^{-6}$) are shown as mean ± S.D. of mRNA expression of one to three fetuses from each pregnant mouse (control group: $n = 22$, DE-exposure group: $n = 22$). *$p < 0.01$ *vs* control by Student's *t*-test.

was significantly decreased in comparison to that in the control group ($p < 0.01$), although expression levels of BMP-6 and GDF-9 mRNAs fetuses did not differ significantly between exposed female fetuses and control female fetuses.

DISCUSSION

To examine the effect of gravid-period exposure to DE on the sex development of female fetuses at the mRNA level, we quantitatively analyzed fetal expression levels of certain mRNA on day 14 of pregnancy, which is the time of sex differentiation. In a previous study, expression of mRNA for Ad4BP/SF-1 and MIS, which are essential for male gonadal differentiation, decreased significantly in male fetuses when maternal mice were exposed to DE levels of 0.1 and 3.0 mg DEP/m^3 for 8 hr per day between 2 and 13 dpc. Therefore, we performed a parallel experiment in female fetuses.

We obtained ICR mice at 1 dpc and exposed them to 100 μg DEP/m^3 from 2 dpc to 13 dpc. SPM is made up mainly of DEP, and the concentration we used is the mean. Dams were dissected at 14 dpc, the time of gonad differentiation in male fetuses,[19] and fetuses and placentas were extracted. We found no statistical effect on pregnancy rate, litter size, or fetal and placental weight in the exposure group. Our findings indicate DE does not influence the pregnancy at 100 μg DEP/m^3 from 2 dpc to 13 dpc.

mRNA expression of MIS and Ad4BP/SF-1 did not differ significantly between exposed and nonexposed female fetuses. This seems to differ from past observations of male fetuses, in which mRNA levels decreased.[19] It may be possible that these two types of mRNA possess different sensitivities between males and females.

mRNA expression of BMP-15 in the exposed group was significantly decreased compared with that in the control group ($p < 0.01$), but we did not examine BMP-15 mRNA expression in male fetuses. BMP-15, BMP-6, and GDF-9 are TGF-β superfamily members reportedly expressed in the mammalian oocyte from the type 3a follicle (one-layer) stage through ovulation.[26–28] Members of the TGF-β family such as GDF-9, MIS, and BMP-6 and -15 have all been shown to exert specific effects on ovarian cell function.[29] In this study, it was not determined whether BMP-15 mRNA expression is specific to the reproductive glands because we used the whole fetal body. There is no report of BMP-15 mRNA expression in fetuses; the presence of BMP-15 has not been confirmed except in the ovary. Expression of mRNAs for BMP-6 and GDF-9, which are related to BMP-15, did tend to decrease, but not significantly, in the exposure group. The absolute value of the mRNA expression of BMP-15 was very low, and thus we understood that these changes were subtle.

Wnt-4 in addition to Ad4BP/SF-1 regulates expression of Dax-1,[18] a putative repressor of Ad4BP/

SF-1 in the gonad. Wnt-4 plays a key role in female sex development by regulating duct formation, controlling steroidogenesis in the gonad, and possibly supporting oocyte development.[15] Wnt-7a functions as an epithelial-to-mesenchymal signal and renders the mesenchyme competent to respond to MIS signaling through the MIS receptor.[16] The expression of mRNA for ER and Wnt-4 and -7a did not differ significantly in comparison to that in the control group. Differences in the expression of mRNA for Dax-1 and aromatase were also not significant. Most types of mRNA expression did not change, and thus DE may not have an effect at the time of sex differentiation in female mouse fetuses.

In conclusion, female fetuses born from pregnant mice exposed *in utero* to DE are less sensitive to it than males in terms of expression levels of mRNAs for Ad4BP/SF-1 and MIS. However, DE might affect development of oocytes in female fetuses.

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