Maternal Exposure to Diesel Exhaust Decreases Expression of Steroidogenic Factor-1 and Müllerian Inhibiting Substance in the Murine Fetus

Miki Yoshida,[a] Seiichi Yoshida,[a, c] Isamu Sugawara,[b, c] and Ken Takeda*[, a, c]

[a]Department of Hygiene Chemistry, Faculty of Pharmaceutical Sciences, Tokyo University of Science, 12 Ichigaya, Funagawara-machi, Shinjuku-ku, Tokyo 162–0826, Japan, [b]Department of Molecular Pathology, The Research Institute of Tuberculosis, 3–1–24 Matsuyama, Kiyose-shi, Tokyo 204–8533, Japan, and [c]CREST, JST (Japan Science and Technology Corporation), 4–1–8 Hon-cho, Kawaguchi-shi, Saitama 332–0012, Japan

(Received March 5, 2002; Accepted March 22, 2002)

We investigated the effect of exposure of pregnant mice to diesel exhaust on male gonad development at the level of mRNA expression. Expression of mRNAs for steroidogenic factor-1 (Ad4BP/SF-1) and Müllerian inhibitory substance (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 mg and 3.0 mg diesel exhaust particles (DEP)/m^3 for 8 hr per day between days 2 and 13 post coitum. Expression levels of mRNAs for steroidogenic cytochrome P450 genes regulated by Ad4BP/SF-1, especially 3β-hydroxysteroid dehydrogenase (3β-HSD) and aromatase, were also decreased by exposure to diesel exhaust. There were no significant differences in levels of estrogen receptor (ER) or androgen receptor (AR) mRNAs between control and exposed mice. The data indicate that exposure of pregnant mice to diesel exhaust affects the expression of genes essential in the early stages of embryonic development.

Key words —— diesel exhaust, steroidogenic factor-1, Müllerian inhibitory substance, fetus, pregnancy, male reproductive system

INTRODUCTION

The number of environmental chemicals known to affect human health by disrupting normal endocrine function through interactions with hormone receptors may continue to increase.[1)] Diesel exhaust is a complex mixture of particulate and vapor-phase compounds. The soluble organic fraction of the particulate materials in diesel exhaust contains thousands of compounds including a variety of polycyclic aromatic hydrocarbons and heavy metals.

It has been reported that diesel exhaust and diesel exhaust particles (DEP) are hazardous to human health and may cause cancer,[2)] allergic rhinitis, and asthma.[3)] It was recently proposed that diesel exhaust may influence reproductive function.[4)] Exposure to diesel exhaust has been shown in developing mice, to induce Leydig cell degeneration, increase the number of damaged seminiferous tubules, and reduce daily sperm production. Furthermore, it has been reported that exposure to diesel exhaust during pregnancy disturbs fetal differentiation of the testis, ovary, and thymus in rats.[5)] Therefore, it has been hypothesized that inhalation of diesel exhaust during gestation impairs reproductive function by disrupting sex organ development. The reproductive system is believed to be particularly susceptible to toxic insult during the gestation period.

Normal development of the testes and male genital tract is a tightly regulated process dependent upon a coordinated cascade of molecular and morphological events, including formation of the testes, regression of the Müllerian ducts, and stabilization of the Wolffian ducts. The sex determining gene located on the Y chromosome (SRY) has been shown to encode a testis-determining factor.[6)] In the presence of an active copy of SRY, the undifferentiated gonad forms a testis within which Sertoli cells synthesize and secrete Müllerian inhibiting substance (MIS), which is required for regression of the Müllerian

*To whom correspondence should be addressed: Department of Hygiene Chemistry, Faculty of Pharmaceutical Sciences, Tokyo University of Science, 12 Ichigaya, Funagawara-machi, Shinjuku-ku, Tokyo 162–0826, Japan. Tel.: +81-3-3260-6725 (Ext. 5076); Fax: +81-3-3260-5856; E-mail: takeda@ps.kagu.sut.ac.jp

ducts.[7] MIS acts downstream of steroidogenic factor-1 (Ad4BP/SF-1)[8] and is required for normal male reproductive tract development. MIS causes regression of the Müllerian duct of the bipotential urogenital ridge, which, if left undisturbed, gives rise to female reproductive tract structures such as the uterus, fallopian tubes, and upper vagina in XY mice.[9] Leydig cells differentiate within the interstitium between the seminiferous cords containing the Sertoli cells, and they express the steroidogenic enzymes required for synthesis of testosterone,[10] which then acts via a specific receptor to virilize the Wolffian ducts and urogenital sinus.[11] In females, absence of testicular MIS and testosterone results in development of Müllerian structures and regression of the Wolffian ducts.

Ad4BP/SF-1, a member of the steroid receptor superfamily of transcription factors, influences reproductive function through regulation of development and differentiation of hormone producing tissues.[12] Ad4BP/SF-1 was identified as a steroidogenic tissue-specific transcription factor regulating the expression of the steroidogenic cytochrome P450 genes.[13] In mice, expression of Ad4BP/SF-1 is limited to endocrine tissues related to reproduction including testicular Leydig and Sertoli cells, ovarian granulosa and theca cells, the three layers of the adrenal cortex, placenta, pituitary gonadotrophs, and hypothalamus.[14–17] A wider role for Ad4BP/SF-1 as a key regulator of gonadal differentiation has been suggested by studies in which the gene encoding Ad4BP/SF-1 was selectively inactivated in transgenic mice.[18] At birth, these mice were found to lack gonads and adrenal glands and to have female genitalia. These reports suggest that Ad4BP/SF-1 is involved in regulation of the genes that are crucial for differentiation of these tissues in the early stages of embryonic development.

In the present study, we examined the effect of exposure to diesel exhaust during critical periods of fetal development on the expression of mRNAs for gonad development-related genes such as Ad4BP/SF-1 and MIS in mouse fetuses.

MATERIALS AND METHODS

Animals —— A total of one hundred pregnant ICR mice were obtained from SLC Co. (Shizuoka, Japan). Twenty of these mice were used in each of the five experiments, 10 for diesel exhaust exposure and 10 for control. We performed two different exposure studies: in the first one three experiments involved low-level exposure to exhaust, and in the second two experiments involved high-level exposure to exhaust. In the exposed groups, the pregnant mice were exposed to diesel exhaust through an airway for 8 hr each day, while the pregnant mice in the control groups were exposed to clean air. Exposure began on day 2 post coitum (p.c., the day the plug was found was taken as day 0 p.c.) and continued until day 13 p.c. No exposure occurred on days 4, 5, 11, or 12 p.c. Mice were given free access to a commercial stock diet CE-2 (Japan Clea Co., Japan) and water.

Exposure to Diesel Exhaust —— A 2300-cc diesel engine (manufactured by Isuzu Motor Co., Japan) was operated at 1050 rpm and 80% load with commercial light oil (Idemitsu Kosan Co., Japan). The engine exhaust was introduced into a dilution tunnel 45 cm in diameter and 625 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 exhaust was delivered directly to the animal exposure chamber.

Animals were exposed for 8 hr/day, and the average concentrations of exhaust constituents were as follows: 0.04 ± 0.01 ppm for NO_2, less than 0.01 ppm for SO_2, 1.25 ± 0.01 ppm for CO, and 0.1 mg/m^3 for particles in the first, second, and third experiments for low-level exposure. In the fourth and fifth experiments, values of 0.7 ± 0.1 ppm for NO_2, less than 0.01 ppm for SO_2, 8.0 ± 0.1 ppm for CO, and 3.0 mg/m^3 for particles were used for high-level exposure.

Harvesting Fetuses —— Blood samples were collected from the abdominal aorta of the pregnant mice under ether anesthesia. After these animals were exsanguinated, the two uterine horns were dissected, and the pups were removed. Each fetus and placenta was weighed. Fetal sex was determined according to the method described by Lambert *et al.*[19]

Quantitative Reverse Transcription (RT)-PCR — After sex determination, we selected 2 to 4 male fetuses from each pregnant mouse. These fetuses were homogenized in 1 ml Isogen (Nippon Gene, Japan) with a homogenizer (HG30, Hitachi Co., Japan), and total RNA was isolated per the manufacturer's protocol. Reverse transcription of total RNA into cDNA was carried out as described elsewhere.[20] Quantitative analysis of specific mRNA expression was using a sequence detection system (ABI PRISM 7700; Perkin-Elmer, Foster City, CA, U.S.A.). Use of this

Table 1. Average Median Weights of Fetuses and Placentas from Control Mice and Pregnant Mice Exposed to Diesel Exhaust (DE) on Day 14 Post Coitum

		Control	DE (0.1 mg DEP/m^3)
Fetal weight (mg)	Total	350.3 ± 48.2	332.6 ± 37.7
	Male	357.1 ± 42.9	331.4 ± 36.5
	Female	349.5 ± 60.4	334.5 ± 46.7
Placental weight (mg)	Total	126.4 ± 15.3	118.8 ± 18.7
	Male	140.8 ± 22.0	126.9 ± 20.1
	Female	120.1 ± 16.1	112.4 ± 21.3

Values are expressed as means ± S.D.

system to determine specific mRNA expression has been described in the manufacturer's user bulletins. Pairs of primers and TaqMan probes were designed by computer (Primer Express Software; Perkin-Elmer) to amplify specific small fragments of the Ad4BP/SF-1, MIS, estrogen receptor (ER), androgen receptor (AR), cholesterol side-chain cleavage (P450scc), 3β-hydroxysteroid dehydrogenase (3β-HSD), 17α-hydroxylase/C17,20-lyase (P450c17), 17β-hydroxysteroid dehydrogenase (17β-HSD), and aromatase genes. The murine glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene, a ubiquitously expressed housekeeping gene, was used as an internal marker of mRNA integrity. PCR amplification was performed in 96-well optical trays with a 50-μl final reaction mixture consisting of 25 μl TaqMan universal PCR master mix (Perkin-Elmer), 200 nM TaqMan fluorescent probe (Perkin-Elmer), 600 nM each primer (Espec Oligo Service Corp., Japan), and 10 μl cDNA sample. Amplification conditions consisted of one cycle of 50°C for 2 min and 95°C for 10 min followed by 45 cycles of 95°C for 15 sec and 60°C for 1 min.

Statistical Analysis —— All values are reported as mean ± standard deviation (S.D.). Differences were analyzed statistically by ANOVA and Student's *t*-test with Thompson's rejection test (JSTAT). *p*-Values of < 0.01 were considered significant.

RESULTS

Fetal Parameters

The average body weight of the pregnant mice did not differ significantly between the control and diesel exhaust exposure groups. In the first experiment, which involved low-level exposure to exhaust, litter size ranged from 4 to 14, and the average litter size in the control group and the diesel exhaust exposure group was 10.0 and 10.2, respectively. The number of fetuses and the sex ratio of the litters in the control and the diesel exhaust exposure group was 80 (male : female, 39 : 41) and 61 (30 : 31), respectively. There was no significant difference in litter size, sex ratio, or implantation rate between the two groups. Data for the median weights of fetuses and placentas from mice in the control and exposure groups in the first experiment are shown in Table 1. The average fetal and placental weights were slightly lower in the exposure group than in the control group, and the difference was greater in male fetuses than in female fetuses. However, these differences were not significant.

In the second and third experiments, which also involved low-level exposure to exhaust, and the fourth and fifth experiments, which involved high-level exposure, we obtained similar results with no significant differences between the two groups in the body weight of pregnant mice, litter size, sex ratio, implantation rate, or fetal and placental weight.

Expression of mRNAs Related to Development of Gonads in Male Fetuses

The relative levels of Ad4BP/SF-1 and MIS mRNA in male fetuses in the five experiments are shown in Fig. 1(A). Levels of Ad4BP/SF-1 and MIS mRNAs in the low-level diesel exhaust exposure groups (0.1 mg DEP/m^3) were approximately 70% of control group levels; this difference was significant ($p < 0.01$). There was a dose–dependent effect of diesel exhaust on expression of Ad4BP/SF-1 and MIS mRNAs. In the high-level diesel exhaust exposure groups (3.0 mg DEP/m^3), expression levels were approximately 50% those of controls. The relative levels of ER and AR mRNAs in male fetuses are shown in Fig. 1(B). There were no significant dif-

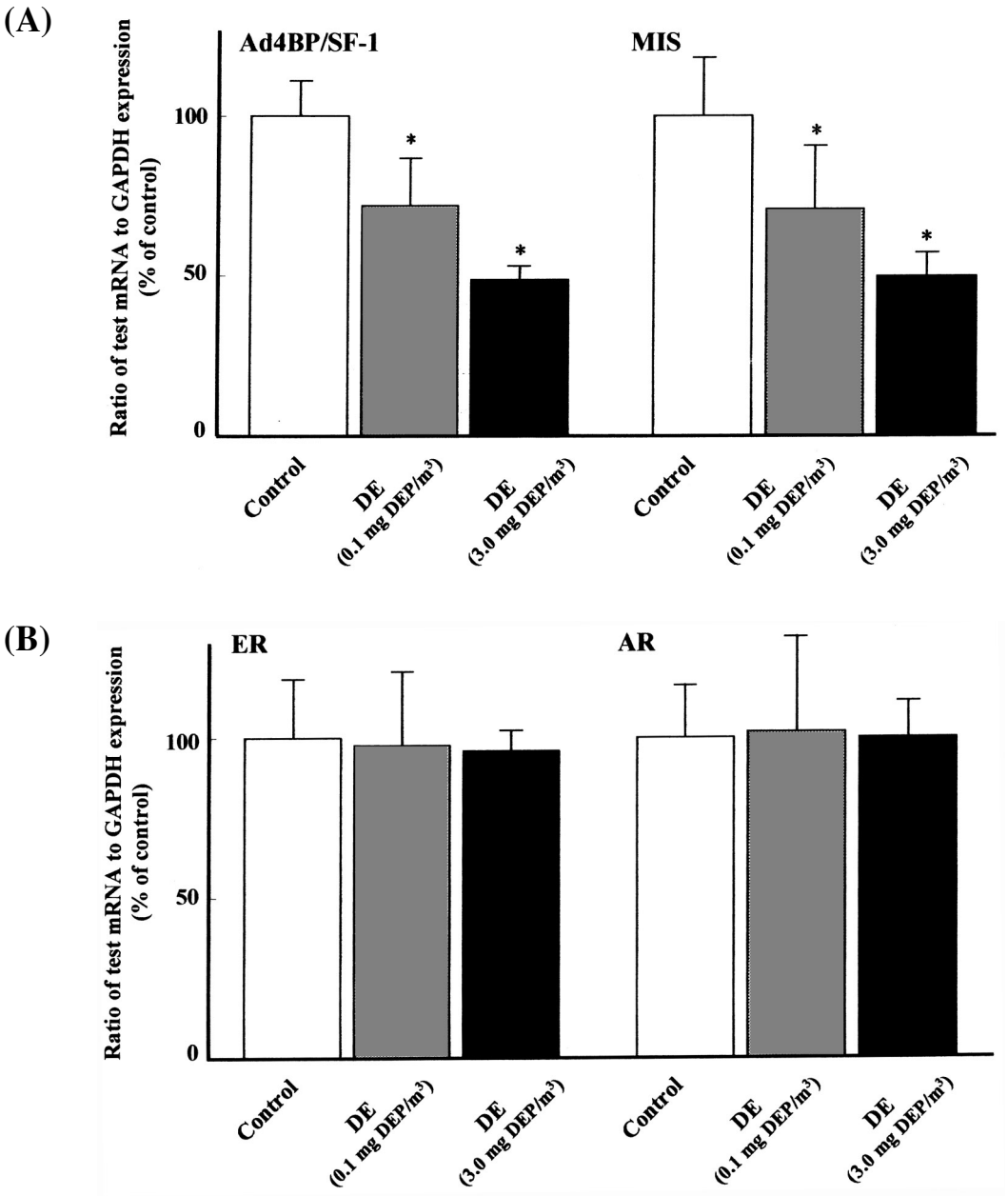

Fig.1. Effect of Maternal Exposure to Diesel Exhaust on Ad4BP/SF-1 and MIS (A) and ER and AR (B) mRNA Levels in Male Fetuses

Total RNA was isolated on day 14 p.c. from the fetuses of control pregnant mice and pregnant mice exposed to diesel exhaust (DE) for quantitative RT-PCR. Levels of Ad4BP/SF-1, MIS, ER, and AR mRNAs were analyzed quantitatively with a sequence detection system (ABI Prism 7700). mRNA expression levels are presented as the ratios of target gene mRNA levels to GAPDH levels to correct for variations in the amounts of RNA. The ratios were then normalized such that the mean ratio of the control is 100%. (A) Control, $n = 25$; DE (0.1 mg DEP/m^3), $n = 23$; DE (3.0 mg DEP/m^3), $n = 15$. (B) Control, $n = 13$; DE (0.1 mg DEP/m^3), $n = 7$; DE (3.0 mg DEP/m^3), $n = 6$. Values are expressed as mean ± S.D. of the expression in fetuses from the same pregnant female; $*p < 0.01$.

ferences in ER or AR mRNA levels between the control and diesel exhaust exposure groups.

Ad4BP/SF-1 has been reported to control transcription of P450 genes related to steroid synthesis. Therefore, we used quantitative RT-PCR to analyze the expression of mRNAs for P450scc, 3β-HSD, P450c17, 17β-HSD, and aromatase. The relative levels of expression of these mRNAs in male fetuses are shown in Table 2. There were no significant differences in the levels of mRNAs between the low-level diesel exhaust exposure groups and the control groups, with the exception of a slight reduction in 3β-HSD and aromatase mRNA levels in the low-level exposure groups. In the high-level diesel exhaust exposure groups, 3β-HSD and aromatase mRNA levels were reduced significantly ($p < 0.01$),

Table 2. Effect of Maternal Exposure to Diesel Exhaust (DE) on Expression of mRNAs for P450 Genes Related to Steroid Synthesis in Male Fetuses

	P450scc	3β-HSD	P450c17	17β-HSD	Aromatase
Control ($n = 13$)	100.0 ± 19.9	100.0 ± 19.8	100.0 ± 23.1	100.0 ± 31.4	100.0 ± 14.9
DE (0.1 mg DEP/m^3, $n = 7$)	104.2 ± 18.1	73.1 ± 10.1	98.0 ± 34.0	101.7 ± 20.7	72.7 ± 8.8
DE (3.0 mg DEP/m^3 , $n = 6$)	76.0 ± 19.8	61.6 ± 34.1*	83.4 ± 12.6	71.7 ± 19.1	46.6 ± 6.2*

mRNA expression levels are presented as the ratios of target transcript levels to GAPDH levels to correct for variations in amounts of RNA. The mean ratio of the control was considered 100%. Values are expressed as means ± S.D. of average expression in fetuses from the same pregnant mouse; *$p < 0.01$.

and levels of other mRNAs were reduced slightly.

DISCUSSION

In the present study, we investigated the influence of diesel exhaust on expression of mRNAs essential for gonad development in male mouse fetuses. The expression of Ad4BP/SF-1 and MIS mRNAs in male fetuses was decreased significantly after pregnant mice were exposed to diesel exhaust.

The steroidogenic tissue-specific transcription factor Ad4BP/SF-1 regulates expression of steroidogenic cytochrome P450 genes. Steroidogenic tissues in mice with targeted disruption of the Ad4BP/SF-1 gene disappear early in development,[16,18,21] suggesting a critical role for Ad4BP/SF-1 in the differentiation of steroidogenic tissues. The findings of the present study suggest that in utero exposure to even low levels (0.1 mg DEP/m^3) of diesel exhaust reduces expression of Ad4BP/SF-1, and that it may affect development of the gonads. Low levels of diesel exhaust also reduced expression of several genes known to play key roles in gonadal development, including one enzyme necessary for testosterone synthesis. The effect of prenatal exposure to estrogenic compounds on Ad4BP/SF-1 mRNA expression has been reported previously.[22] Injection of diethylstilbestrol (DES) or 4-octylphenol during pregnancy reduced expression of Ad4BP/SF-1 mRNA in rat fetal testes.[22] Human male offspring of mothers given DES, a potent synthetic estrogen, to prevent miscarriage are known to have an increased incidence of undescended testes, urogenital abnormalities, and semen abnormalities in comparison to offspring of mothers who did not take DES.[23] A reduction in Ad4BP/SF-1 expression in male fetuses after administration of DES may be associated with these abnormalities. Moreover, levels of P450c17 mRNA were reduced relative to controls in testes from fetuses of DES- and OP-treated mothers.[24] Ad4BP/SF-1 binding sites are present in enzymes including P450c17, P450scc, and aromatase, suggesting that reduced Ad4BP/SF-1 expression after maternal exposure to synthetic estrogens may be associated with expression of P450c17 by Leydig cells.[24] In the present study, we investigated the effect of diesel exhaust exposure during pregnancy on expression of P450 mRNAs in male mouse fetuses. Although there were no apparent differences in expression of P450 mRNAs between the control groups and the low-level exposure groups, a reduction in the levels of these mRNAs, especially aromatase mRNA, was observed in the high-level exposure groups on day 14.5 p.c. These results may be associated with the dose–dependent effect of diesel exhaust on Ad4BP/SF-1 mRNA levels. These findings suggest that low-level maternal exposure to diesel exhaust may not significantly affect expression of several enzymes essential for testosterone synthesis despite reduced Ad4BP/SF-1 mRNA expression; however, high-level exposure may affect fetal steroidogenesis by disrupting expression of these genes.

It is likely that a wide variety of genes in addition to the P450 genes are regulated by Ad4BP/SF-1 in steroidogenic cells. The oxytocin gene was the first gene other than the P450 genes reported to be regulated by Ad4BP/SF-1.[13] Several other genes, including the inhibin α- and β-subunit genes,[25] the luteinizing hormone receptor gene,[26] and the steroidogenic acute regulatory protein (StAR) gene,[27] expressed in steroidogenic cells have Ad4BP/SF-1 sites in their promoter regions, but the effect of such sites on gene expression remains unclear. In the hypothalamus, the ventromedial hypothalamic nucleus (VMH) has the only Ad4BP/SF-1-positive cell population.[15,21] Because the Ad4BP/SF-1-positive cells in the VMH do not express P450 genes, other genes must be targets of Ad4BP/SF-1. According to Barnhart and Mellon,[28] the glycoprotein hormone α-subunit gene is regulated by a gonadotroph-specific element that is identical to the Ad4BP/SF-1

sequence. There is anatomical and physiological evidence that one of the functions of the VMH is regulation of copulatory behavior and that this behavior is influenced by sex steroids secreted by the gonads.[29] The distribution of Ad4BP/SF-1 in the VMH, gonads, and also pituitary suggests that it might be involved in establishment and maintenance of the hypothalamic-pituitary-gonadal axis, which is essential for normal reproductive function.

Although we do not know whether diesel exhaust exposure affects Ad4BP/SF-1 mRNA expression in other tissues during other developmental stages, it is possible that such exposure influences reproductive function widely through its effects on development and differentiation of hypothalamus-pituitary-gonadal tissues.

MIS, a Sertoli cell glycoprotein and a target gene of Ad4BP/SF-1,[8] induces regression of the Müllerian ducts, which give rise to the uterus, the fallopian tubes, and the upper portion of the vagina in females. Development of the male reproductive tract, including descent of the testes into the scrotum, occurs during the fetal period.[30] Because persistence of the Müllerian ducts is usually associated with failure of the testes to descend, it is possible that MIS plays a role in the transabdominal phase of normal testicular descent.[30] Altered MIS production could, therefore, impair normal testicular descent and/or development of the male reproductive tract.[30,31] MIS may also suppress proliferation of germ cells during development.[32] MIS inhibits growth of various types of cells, including certain ovarian tumor cells, and prevents resumption of oocyte meiosis *in vitro*. Because the abnormal germ cells that give rise to most testicular cancers are thought to arise during fetal development,[33] testicular cancers may be related to altered secretion of MIS. We observed a significant reduction in levels of MIS mRNA in male mouse fetuses exposed to diesel exhaust. The reduced expression of MIS mRNA in the exposed male fetuses had a temporal pattern of expression similar to that of Ad4BP/SF-1 mRNA. These data indicate that exposure to diesel exhaust during the fetal period affects gene expression related to gonadal development.

Although we observed a slight decrease in the average fetal and placental weights in the diesel exhaust exposure groups in comparison to those in the control groups, there was no correlation between the decreased weights and the reduced Ad4BP/SF-1 and MIS mRNA expression.

Follicle stimulating hormone (FSH) drives estrogen production by Sertoli cells, increases proliferation of Sertoli cells, and regulates the postnatal decrease in MIS production by Sertoli cells.[34] Estradiol also decreases MIS secretion.[35] Therefore, it is possible that reduced MIS mRNA expression in male fetuses exposed to diesel exhaust in utero is due to changes in serum hormone levels in the pregnant mice. Watanabe and Kurita[5] reported that exposure of pregnant rats to diesel exhaust containing 5.63 mg/m^3 particulate matter caused testosterone levels to increase and estradiol and luteinizing hormone levels to decrease in the mothers. They also observed masculinization of the fetuses. In the present study, we also examined serum levels of estradiol, testosterone, luteinizing hormone, and FSH; however, we found no differences in levels of these hormones between the exposed groups and the control groups (data not shown). There were no significant changes in expression of ER, AR, and luteinizing hormone receptor mRNAs in male fetuses exposed to diesel exhaust in utero. These findings suggest that low-level diesel exhaust exposure decreases expression of Ad4BP/SF-1 and MIS without changing the serum hormone levels in pregnant mice. A preliminary experiment showed that there was no change in Ad4BP/SF-1 mRNA expression in primary cultured mouse Leydig cells exposed to DEP (10 μg/ml) for 6 hr (data not shown), indicating that diesel exhaust exposure during pregnancy affects Ad4BP/SF-1 expression indirectly.

The present data suggest that exposure of pregnant mice to diesel exhaust may influence male genital organogenesis by reducing expression of genes essential to normal gonadal development. However, it is unclear which substances in the diesel exhaust are responsible for the observed changes. Further studies are needed to identify the substances that decrease expression of these genes and are causative of the developmental defects owing to prenatal exposure to diesel exhaust.

Acknowledgements We thank Mr. T. Udagawa and Mr. S. Mizuno for help with the diesel exhaust exposure protocols. We thank the graduate and undergraduate students in the Takeda laboratory, especially Mr. K. Noguchi, for technical help. This work was supported in part by a Grant-in-Aid for Science Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

REFERENCES

1) Colborn, T. (1995) Environmental estrogens: health implications for humans and wildlife. *Environ. Health. Perspect.*, **103**, 135–136.
2) Pope, C. A., III, Bates, D. V. and Raizenne, M. E. (1995) Health effects of particulate air pollution: time for reassessment? *Environ. Health. Perspect.*, **103**, 472–480.
3) Sagai, M., Furuyama, A. and Ichinose, T. (1996) Biological effects of diesel exhaust particles (DEP). III. Pathogenesis of asthma like symptoms in mice. *Free Radic. Biol. Med.*, **21**, 199–209.
4) Yoshida, S., Sagai, M., Oshio, S., Umeda, T., Ihara, T., Sugamata, M., Sugawara, I. and Takeda, K. (1999) Exposure to diesel exhaust affects the male reproductive system of mice. *Int. J. Androl.*, **22**, 307–315.
5) Watanabe, N. and Kurita, M. (2001) The masculinization of the fetus during pregnancy due to inhalation of diesel exhaust. *Environ. Health. Perspect.*, **109**, 111–119.
6) Koopman, P., Munsterberg, A., Capel, B., Vivian, N. and Lovell-Badge, R. (1990) Expression of a candidate sex-determining gene during mouse testis differentiation. *Nature* (London), **29**, 450–452.
7) Lee, M. M. and Donahoe, P. K. (1993) Müllerian inhibiting substance: a gonadal hormone with multiple functions. *Endocr. Rev.*, **14**, 152–164.
8) Shen, W. H., Moore, C. C., Ikeda, Y., Parker, K. L. and Ingraham, H. A. (1994) Nuclear receptor steroidogenic factor 1 regulates the Müllerian inhibiting substance gene: a link to the sex determination cascade. *Cell*, **3**, 651–661.
9) Behringer, R. R., Finegold, M. J. and Cate, R. L. (1994) Müllerian-inhibiting substance function during mammalian sexual development. *Cell*, **4**, 415–425.
10) Saez, J. M. (1994) Leydig cells: endocrine, paracrine, and autocrine regulation. *Endocr. Rev.*, **15**, 574–626.
11) Lala, D. S., Rice, D. A. and Parker, K. L. (1992) Steroidogenic factor I, a key regulator of steroidogenic enzyme expression, is the mouse homolog of fushi tarazu-factor I. *Mol. Endocrinol.*, **6**, 1249–1258.
12) Sadovsky, Y. and Crawford, P. A. (1998) Developmental and physiologic roles of the nuclear receptor steroidogenic factor-1 in the reproductive system. *J. Soc. Gynecol. Investig.*, **5**, 6–12.
13) Morohashi, K., Honda, S., Inomata, Y., Handa, H. and Omura, T. (1992) A common trans-acting factor, Ad4-binding protein, to the promoters of steroidogenic P-450s. *J. Biol. Chem.*, **5**, 17913–17919.
14) Ikeda, Y., Luo, X., Abbud, R., Nilson, J. H. and Parker, K. L. (1995) The nuclear receptor steroidogenic factor 1 is essential for the formation of the ventromedial hypothalamic nucleus. *Mol. Endocrinol.*, **9**, 478–486.
15) Ingraham, H. A., Lala, D. S., Ikeda, Y., Luo, X., Shen, W. H., Nachtigal, M. W., Abbud, R., Nilson, J. H. and Parker, K. L. (1994) The nuclear receptor steroidogenic factor 1 acts at multiple levels of the reproductive axis. *Genes Dev.*, **1**, 2302–2312.
16) Sadovsky, Y., Crawford, P. A., Woodson, K. G., Polish, J. A., Clements, M. A., Tourtellotte, L. M., Simburger, K. and Milbrandt, J. (1995) Mice deficient in the orphan receptor steroidogenic factor 1 lack adrenal glands and gonads but express P450 side-chain-cleavage enzyme in the placenta and have normal embryonic serum levels of corticosteroids. *Proc. Natl. Acad. Sci. U.S.A.*, **21**, 10939–10943.
17) Ikeda, Y., Shen, W. H., Ingraham, H. A. and Parker, K. L. (1994) Developmental expression of mouse steroidogenic factor-1, an essential regulator of the steroid hydroxylases. *Mol. Endocrinol.*, **8**, 654–662.
18) Luo, X., Ikeda, Y. and Parker, K. L. (1994) A cell-specific nuclear receptor is essential for adrenal and gonadal development and sexual differentiation. *Cell*, **20**, 481–490.
19) Lambert, J. F., Benoit, B. O., Colvin, G. A., Carlson, J., Delville, Y. and Quesenberry, P. J. (2000) Quick sex determination of mouse fetuses. *J. Neurosci. Methods*, **15**, 127–132.
20) Yoshida, S., Yamada, H., Sugawara, I. and Takeda, K. (1998) Effect of dibromochloropropane (DBCP) on the hormone receptors of the male rat reproductive system. *Biosci. Biotechnol. Biochem.*, **62**, 479–483.
21) Shinoda, K., Lei, H., Yoshii, H., Nomura, M., Nagano, M., Shiba, H., Sasaki, H., Osawa, Y., Ninomiya, Y. and Niwa, O. (1995) Developmental defects of the ventromedial hypothalamic nucleus and pituitary gonadotroph in the Ftz-F1 disrupted mice. *Dev. Dyn.*, **204**, 22–29.
22) Majdic, G., Sharpe, R. M. and Saunders, P. T. (1997) Maternal oestrogen/xenoestrogen exposure alters expression of steroidogenic factor-1 (SF-1/Ad4BP) in the fetal rat testis. *Mol. Cell. Endocrinol.*, **14**, 91–98.
23) Gill, W. B., Schumacher, F. B., Bibo, M., Straus, F. H. and Schoenberg, H. W. (1979) Association of diethylstilbestrol exposure in utero with cryptorchidism testicular hypoplasia and semen abnormalities. *J. Urol.*, **122**, 36–39.
24) Majdic, G., Sharpe, R. M., O'Shaughnessy, P. J. and Saunders, P. T. (1996) Expression of cytochrome P450 17 alpha-hydroxylase/C17-20 lyase in the fetal rat testis is reduced by maternal exposure to exogenous estrogens. *Endocrinology*, **137**, 1063–1070.
25) Feng, Z. M., Li, Y. P. and Chen, C. L. (1989) Analy-

sis of the 5′-flanking regions of rat inhibin alpha- and beta-B-subunit genes suggests two different regulatory mechanisms. *Mol. Endocrinol.*, **3**, 1914–1925.
26) Tsai-Morris, C. H., Buczko, E., Wang, W., Xie, X. Z. and Dufau, M. L. (1991) Structural organization of the rat luteinizing hormone (LH) receptor gene. *J. Biol. Chem.*, **15**, 11355–11359.
27) Clark, B. J., Soo, S. C., Caron, K. M., Ikeda, Y., Parker, K. L. and Stocco, D. M. (1995) Hormonal and developmental regulation of the steroidogenic acute regulatory protein. *Mol. Endocrinol.*, **9**, 1346–1355.
28) Barnhart, K. M. and Mellon, P. L. (1994) The orphan nuclear receptor, steroidogenic factor-1, regulates the glycoprotein hormone alpha-subunit gene in pituitary gonadotropes. *Mol. Endocrinol.*, **8**, 878–885.
29) Cohen, R. S. and Pfaff, D. W. (1992) Ventromedial hypothalamic neurons in the mediation of long-lasting effects of estrogen on lordosis behavior. *Prog. Neurobiol.*, **38**, 423–453.
30) Hutson, J. M., Williams, M. P. L., Fallat, M. E. and Attah, A. (1990) Testicular descent: new insights into its hormonal control. In *Oxford Reviews of Reproductive Biology* (Milligan, S. R., Ed.), pp. 1–56, Oxford University Press, Oxford, England.
31) Behringer, R. R., Cate, R. L., Froelick, G. J., Palmiter, R. D. and Brinster, R. L. (1990) Abnormal sexual development in transgenic mice chronically expressing mullerian inhibiting substance. *Nature* (London), **10**, 167–170.
32) Hirobe, S., He, W. W., Lee, M. M. and Donahoe, P. K. (1992) Müllerian inhibiting substance messenger ribonucleic acid expression in granulosa and Sertoli cells coincides with their mitotic activity. *Endocrinology*, **131**, 854–862.
33) Skakkebaek, N. E., Berthelsen, J. G., Giwercman, A. and Muller, J. (1987) Carcinoma-in-situ of the testis: possible origin from gonocytes and precursor of all types of germ cell tumours except spermatocytoma. *Int. J. Androl.*, **10**, 19–28.
34) Arambepola, N. K., Bunick, D. and Cooke, P. S. (1998) Thyroid hormone and follicle-stimulating hormone regulate Müllerian-inhibiting substance messenger ribonucleic acid expression in cultured neonatal rat Sertoli cells. *Endocrinology*, **139**, 4489–4495.
35) Stoll, R., Ichas, F., Faucounau, N. and Maraud, R. (1993) Action of estradiol and tamoxifen on the testis-inducing activity of the chick embryonic testis grafted to the female embryo. *Anat. Embryol. (Berl.)*, **188**, 587–592.