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Prenatal Environmental Tobacco Smoke Exposure Promotes Adult Atherogenesis and Mitochondrial Damage in Apolipoprotein E^{-/-} Mice Fed a Chow Diet

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Background—Environmental tobacco smoke (ETS) exposure is recognized as a cardiovascular disease risk factor; however, the impact of prenatal ETS exposure on adult atherogenesis has not been examined. We hypothesized that in utero ETS exposure promotes adult atherosclerotic lesion formation and mitochondrial damage.

Methods and Results—Atherosclerotic lesion formation, mitochondrial DNA damage, antioxidant activity, and oxidant load were determined in cardiovascular tissues from adult apolipoprotein E^{-/-} mice exposed to either filtered air or ETS in utero and fed a standard chow diet (4.5% fat) from weaning until euthanasia. All parameters were significantly altered in male mice exposed in utero to ETS.

Conclusions—These data support the hypothesis that prenatal ETS exposure is sufficient to promote adult cardiovascular disease development. (*Circulation*. 2004;110:3715-3720.)

Key Words: smoking ■ mitochondria ■ pregnancy ■ prenatal exposure delayed effects ■ atherosclerosis

Recent studies suggest that conditions within the fetal environment may promote the development of adult disease.¹ Although this concept has recently begun to be appreciated in the context of cardiovascular disease (CVD),²⁻⁶ the overall effects of the maternal-fetal environment on adult CVD development are largely undefined. Epidemiological studies have suggested that a relationship exists between birth weights and adult CVD development, which implies that the maternal-fetal environment may play an important role in adult CVD development.^{1,7} However, much more is known about the influence of the maternal-fetal environment on childhood vulnerability to reactive airway disease. Neonatal exposure to environmental tobacco smoke (ETS) has been linked to increased incidence of childhood asthma and has been demonstrated in a variety of animal models.^{8,9} Because the pulmonary circulation in utero is not entirely distinct from other organ systems within the developing fetus, it is plausible that ETS exposure could have additional effects on other organ systems, including the cardiovascular system. Importantly, the effects of maternal-fetal ETS exposure on postnatal atherogenesis have not been elucidated.

The American Heart Association has concluded that ETS exposure is a significant risk factor for CVD in both adults and children.¹⁰ Over the past decade, numerous studies have consistently shown that exposure to ETS increases the risk of heart disease death,¹¹⁻¹⁶ and it has been estimated that ETS exposure increases the risk of CVD-related death by 30%.¹⁷ Whereas it is

known that ETS exposure causes a multitude of effects, ranging from endothelial cell injury to altered cardiac cellular metabolism,¹⁸ the mechanisms of ETS-mediated cellular injury and disease development have not been well characterized.

Recently, it has been shown that ETS exposure causes significant mitochondrial damage and altered function in cardiovascular tissues and, when combined with hypercholesterolemia, accelerates both mitochondrial damage and atherogenesis.¹⁹ Mitochondria are involved in a variety of critical cell functions, including oxidative energy production, programmed cell death, growth, and redox signaling. In this report, the effects of prenatal ETS exposure on adult atherogenesis and mitochondrial damage are described. Both atherosclerotic lesion development and mitochondrial damage were significantly higher in 12-week-old male apolipoprotein E^{-/-} (apoE^{-/-}) mice that were exposed to ETS in utero and fed a standard chow diet (4.5% fat) compared with gender-matched controls. These results are consistent with and support the hypothesis that both adult atherogenesis and mitochondrial damage are influenced by the fetal environment.

Methods

Mice

The apoE^{-/-} mouse lacks apolipoprotein E, a high-affinity ligand for lipoprotein receptors. Consequently, these mice exhibit elevated levels of plasma LDL cholesterol and triglycerides and develop atherosclerotic plaques in a fashion similar to that in humans.²⁰ Mice

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were purchased from Jackson Laboratories (Bar Harbor, Me) and shipped directly to an Association for Assessment and Accreditation of Laboratory Animal Care–approved animal facility at the Louisiana State University (LSU) School of Veterinary Medicine. All procedures involving live mice were approved by both the University of Alabama and LSU institutional animal care and use committees. Food and water were available ad libitum except when mice were in the exposure chambers (see below). Pregnant females were fed a breeder chow (11% fat) diet during gestation. Otherwise, all mice were fed diets that contained 4.5% fat by weight (PicoLab Rodent Chow 20).

ETS Exposures

ETS is composed of 85% to 90% sidestream smoke. The remainder is exhaled mainstream smoke. Here, we used sidestream smoke as a surrogate for ETS. Exposures were performed in 1.3-m³ exposure chambers (14.5 volume changes per hour) at the Inhalation Research Facility at the LSU School of Veterinary Medicine in accordance with institutional guidelines. Temperature was maintained at 22.1±0.5°C with a relative humidity of 47%. Breeding was performed, and impregnated females were exposed to either high-efficiency particulate air (HEPA)–filtered air or ETS mixed with HEPA-filtered air, 5 h/d, from gestation days 1 to 19. Hence, offspring were exposed to ETS only in utero. ETS concentrations were monitored by continual online measurements of total suspended particulate (TSP) and carbon monoxide (CO) levels. In addition, TSP levels were measured gravimetrically at 2-hour intervals during the daily 5-hour exposures. A steady state concentration of 10±0.6 mg/m³ TSP was maintained by burning 1R4F standard reference cigarettes (University of Kentucky, Lexington). CO levels in the ETS chamber were 42.2±2.2 ppm.

Tissue Collection

At 12 weeks of age, offspring were euthanized for atherosclerotic lesion and mitochondrial damage assessments. Animals were anesthetized via intraperitoneal injection (ketamine/xylazine 4:1) and exsanguinated by heart puncture. Blood samples were collected directly into syringes containing 200 μL of 50 μmol/L sodium citrate solution. Tissues were harvested and stored immediately as previously described.¹⁹ Because of the limited amounts of tissue available, whole aortas were used for atherosclerotic lesion assessment, and hearts were used for enzyme and mitochondrial DNA (mtDNA) damage analyses.

Atherosclerotic Lesion Quantification

As previously described, atherosclerotic lesion development was quantified by Oil Red O staining of whole aortas (aortic root to the iliac artery).¹⁹ Aortas were photographed en face, with the use of a Nikon 995 digital camera photo system mounted onto a Zeiss Stemi 2000-C dissecting microscope. Digital (TIFF) images were imported into MetaMorph (Universal Imaging Corporation), and atherosclerotic lesion areas were selected by contrast differences for measurement of lesion area. Total atherosclerotic lesion area (mm²) was quantified and normalized as percent positive staining area relative to total aortic area. Sample analysis was performed in a blinded fashion.

Cholesterol Determination

Total blood plasma cholesterol levels were determined as previously described.¹⁹

Aconitase Inactivation as a Measure of Oxidant Load

Oxidant loads related to superoxide (O₂⁻) and peroxynitrite (ONOO⁻) levels were determined indirectly by measuring the activity of aconitase, an enzyme that is specifically inactivated by O₂⁻,²¹ as described previously. Briefly, aconitase activity is measured by monitoring the formation of cis-aconitate from isocitrate at 340 nm. To control for overall reduction in tricarboxylic acid cycle enzymes by oxidative damage, we also assayed for fumarase activity.

Fumarase activity is insensitive to O₂⁻ and is determined by monitoring the increase in absorbance at 240 nm.

Superoxide Dismutase Activity

Total superoxide dismutase (SOD) and mitochondrial SOD (SOD2) activities were determined with the use of the cytochrome *c* reduction assay, as previously described.^{19,22} This assay is based on the ability of SOD to inhibit the reduction of cytochrome *c* by O₂⁻ generated by xanthine/xanthine oxidase. Increased SOD activity results in inhibition of cytochrome *c* reduction, reflected by decreased absorbance at 550 nm. Cyanide and azide (3 mmol/L KCN, 3 mmol/L NaN₃) are used to inhibit SOD1 and SOD3, allowing for direct measurement of SOD2 activity.

Quantitative Polymerase Chain Reaction for Evaluating mtDNA Damage

Quantitative polymerase chain reaction (QPCR) was performed to quantify mtDNA damage, as described previously.¹⁹ The principle of this gene-specific assay is that DNA lesions will block rTth polymerase and therefore will lead to a decrease in amplification. A 16 059-bp QPCR product, which encompasses all but 236 bp of NADH5/6 genes in the mouse mtDNA genome, is amplified with the use of primer set M13597 FOR (13597 to 13620 bp) and 13361 REV (13361 to 13337 bp). Briefly, genomic DNA is quantified, and 15 ng is used for QPCR. Copy number differences are normalized with the use of a short QPCR reaction, which yields products directly related to gene copy numbers with the use of primers 13597F/13713R (5'CCAGCTACTAC-CATCTCAAGT/ GATGGTTTGGGAGATTGGTTGAT GT3') for the mtDNA.

Statistical Analysis

Results are expressed as mean±SEM. Two-way ANOVA tested the null hypothesis that all samples were drawn from a single population. If this test revealed significant differences (*P*<0.05), a Student-Newman-Keuls test was used for group comparisons.

Results

There were no significant differences between ETS-exposed and air control mothers in pregnancy weight and litter sizes, indicating that fertility and viability were not overtly affected by the utilized ETS exposure regimen. Similarly, there were no significant differences in total plasma cholesterol levels between the in utero, ETS-exposed offspring and gender-matched controls at time of euthanasia (12 weeks), although males did have higher total cholesterol than females (Table; *n*=6 per group), a characteristic previously observed.^{23,24} In contrast, in utero ETS exposure was associated with a lower average body weight at 12 weeks of age in males than in controls (*P*<0.05), whereas there were no statistically significant differences observed among the similarly treated female offspring, although ETS-exposed females had lower average weights.

Prenatal ETS exposure increased Oil Red O staining of whole aortas in 12-week-old adult apoE^{-/-} mice fed a standard chow (4.5% fat) diet (Table and Figure 1; *n*=6 per group). Moreover, percent Oil Red O staining area in males was significantly enhanced by in utero ETS exposure compared with controls (121% increase), whereas female offspring were less affected relative to gender-matched controls (39% increase). Regression analysis revealed that cholesterol level was not significantly correlated with the observed increase in Oil Red O–positive staining areas in the ETS-exposed animals (*P*=0.455), whereas there was a significant level of interaction between gender and ETS exposure (*P*<0.05).

Weight, Total Cholesterol, and Percent Oil Red O Staining in Mice Exposed to Filtered Air or ETS In Utero

	Females		Males	
	FA	ETS	FA	ETS
Weight, g	20.0±0.10	18.7±0.70	24.7±0.70	22.3±0.30*
Total cholesterol, mg/dL	563.40±64.68	452.55±46.15	986.07±73.01	878.40±21.33
Oil red O area/total aortic area, %	3.39±0.78	4.68±0.74	3.36±0.81	7.52±0.68*

FA indicates filtered air. Data are mean±SEM.

* $P<0.05$ compared with gender-matched FA group.

Oxidant load assessment was performed by quantifying the activity of aconitase, a citric acid cycle enzyme that is specifically inactivated by $O_2^{\cdot -}$ and $ONOO^-$.²¹ Figure 2A shows that aconitase activity was significantly reduced in adult male apoE^{-/-} mice exposed in utero to ETS. These data are consistent with increased oxidant loads in males (Figure 2A; $P<0.05$; $n=6$ per group). Prenatal exposure of females to ETS resulted in a 22% reduction (not statistically significant) in aconitase activity. Because an aconitase antibody is not commercially available, the activity of fumarase, a citric acid cycle enzyme not affected by increased oxidant stress, also was quantified. There were no significant changes in fumarase activity in either exposure group, suggesting that the observed differences in aconitase activity were not due to a general alteration of protein levels (Figure 2B).

Total SOD was increased in both male and female mice that were exposed to ETS in utero (Figure 3A; $P<0.05$; $n=6$ per group) and positively correlated with percent Oil Red O staining areas ($P<0.05$). In contrast, assessment of SOD activity specific to mitochondria (SOD2 or MnSOD) revealed that although prenatal ETS exposure had no significant impact on SOD2 activity within either gender, female mice that were exposed to ETS in utero had significantly higher SOD2 activities than similarly exposed males (Figure 3B). SOD2 activity was not significantly different between control male and female offspring.

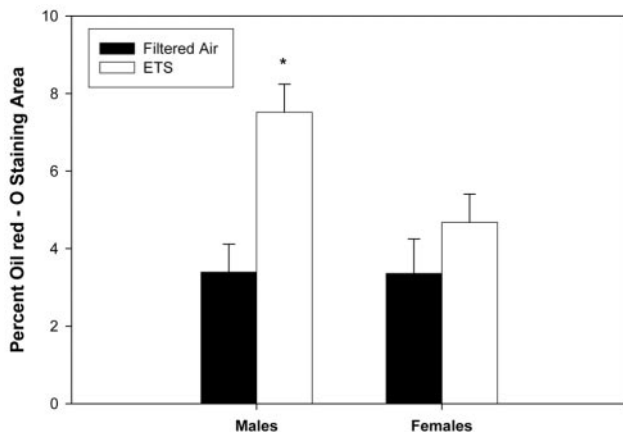


Figure 1. Percent Oil Red O staining of whole aortas collected from 12-week-old apoE^{-/-} mice exposed to ETS or filtered air from gestation days 1 to 19. Positively stained areas are expressed as percent Oil Red O area relative to total aortic area. *Significant difference ($P<0.05$) between in utero ETS-exposed and control animals.

Determination of mtDNA damage by QPCR analysis revealed that cardiovascular mtDNA damage in adult male mice was significantly increased by prenatal ETS exposure (Figure 4; $P<0.05$; $n=6$ per group) compared with gender-matched controls. In addition, males exposed to ETS in utero sustained greater mtDNA damage than their female counterparts (Figure 4; $P<0.05$). Although mtDNA damage in females exposed to ETS in utero was substantially increased compared with gender-matched controls, it was not at statistically significant levels.

Discussion

In the present study, all weaned mice were fed a standard chow (4.5% fat) diet. There were no differences in total cholesterol levels between in utero ETS-exposed and gender-matched control mice at 12 weeks of age. This study did not examine the impact of a high-fat (21%) Western diet. Furthermore, regression analysis revealed that cholesterol level was not significantly correlated with the observed increase in Oil Red O-positive staining areas in the ETS-exposed animals, consistent with the observation that gestational ETS exposure was associated with the increased Oil Red O staining. The results here demonstrate that prenatal exposure to ETS increased adult male susceptibility to CVD development, without a significant change in adult total cholesterol levels, suggesting that in utero ETS exposure was primarily responsible for the observed differences in aortic Oil Red O staining between animals exposed to ETS and filtered air (Figure 1). Examination of oxidant load and mitochondrial damage revealed gender-specific differences in adults after prenatal ETS exposure (Figures 2 and 4), consistent with the results observed with the Oil Red O staining of whole aortas. The mechanisms underlying these gender-specific effects are not clear. Studies in adult female apoE^{-/-} mice have shown that atherogenesis is accelerated with ETS exposure; however, these animals were also fed a high-fat (21%) diet.²⁵ Other studies have shown that males appear to be more sensitive to the effects of certain CVD risk factors (cigarette smoke and hypercholesterolemia) than females,^{5,26} perhaps because of differences in immune response and/or hormonal effects. Studies have also shown that male apoE^{-/-} mice have significantly more lesions than females when fed cholesterol-rich diets.²⁷ Fatty streak lesions are increased in ovariectomized apoE^{-/-} mice, and it has been reported that estrogen hormone therapy reduces atherosclerotic lesion formation in male apoE^{-/-} mice, consistent with the notion that hormonal factors may influence atherogenesis.²⁴ Similarly, CVD risk

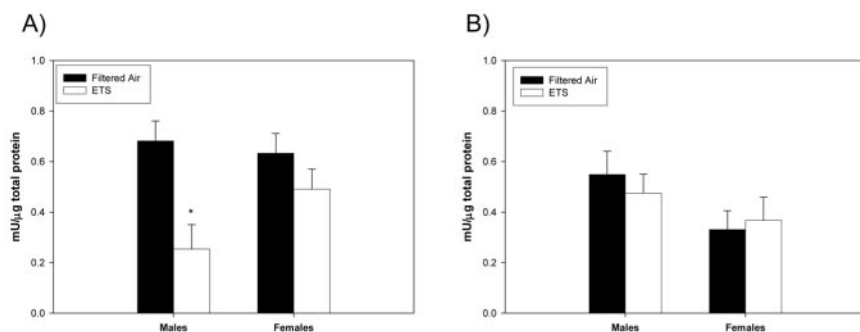


Figure 2. Aconitase and fumarase activities in cardiovascular tissues collected from 12-week-old apoE^{-/-} mice exposed to ETS or filtered air from gestation days 1 to 19. A, Aconitase activity. Decreased activity is consistent with increased oxidant load. B, Fumarase activity. *Significant difference ($P < 0.05$) between in utero ETS-exposed and control animals.

increases dramatically in human females after menopause and is thought to be related to hormonal changes.²⁸

Although a growing body of data suggests that in utero exposure to ETS is a significant factor in pulmonary disease, congenital malformation, learning deficiency, and low birth weights,^{9,26,29–32} the effects of in utero tobacco smoke exposure on adult atherogenesis have been largely overlooked; during the past 3 decades only a few studies have been published concerning fetal ETS exposure and cardiovascular disease, with the general finding that a correlation appears to exist between gestational ETS exposure and heart disease.³³ However, CVD development probably begins decades before the onset of clinical manifestations and may be hastened by prenatal exposure to ETS.

It has been estimated that approximately 13% of pregnant females in the United States continue smoking cigarettes during pregnancy.³⁴ Maternal tobacco smoking has been associated with reduced birth weights, shortened gestation and increased risk of preterm birth, and intrauterine growth restriction.^{35–39} Studies also found that maternal tobacco smoking is associated with overweight or obesity in both childhood and adulthood.^{40–42} Although both the intrauterine growth restriction and obesity are thought of as risk factors for CVD, the impact of gestational ETS exposure on subsequent development of adult CVD is still unknown. In an additional series of studies, total cholesterol levels were quantified in female apoE^{-/-} mice that were the same age as the pregnant dams in this study and exposed for the same length of time (3 weeks). No significant differences in total plasma cholesterol levels between control and ETS-exposed females were observed. These studies, however, do not exclude the possibility that ETS exposure in pregnant female mice may alter total cholesterol levels or cause changes in maternal lipids during pregnancy. It has been reported that no significant differences exist in total cholesterol levels be-

tween smoking versus nonsmoking mothers,⁴³ with a similar finding in their offspring. In contrast, other reports claim decreased total cholesterol levels at 19 weeks of gestation in pregnant women who smoke.⁴⁴ Still others claim that smoking is associated with higher total cholesterol levels in pregnant women^{45,46}; however, both reports that claimed higher total cholesterol levels with smoking also showed that the average birthweight of children was higher in smoking mothers.

Studies have shown that components of tobacco smoke can cross the placenta, resulting in DNA damage to fetal liver, lung, kidney, heart, and brain.⁴⁷ Additional reports have shown that components of tobacco smoke target the mtDNA.^{19,48,49} CO directly inhibits mitochondrial oxidative phosphorylation by reducing the amount of available oxygen (by forming carboxyhemoglobin) to the mitochondria.^{50,51} This effect is exaggerated in the fetus; a maternal carboxyhemoglobin level of 6% corresponds to a fetal carboxyhemoglobin level of 11% and will reduce fetal blood oxygen transport by $\approx 15\%$.⁵² The finding that in utero exposure to 3'-azido-3'-deoxythymidine (AZT) causes increased oxidative damage and dysfunction in mitochondria from multiple tissues, including the heart,^{53,54} suggests that the maternal-fetal environment significantly influences cardiovascular mitochondrial damage and function. These findings are consistent with the hypothesis that fetal ETS exposure compromises mitochondrial function and can promote adult CVD.

ETS-mediated alteration of steady state mitochondrial oxidant and antioxidant concentrations could potentially affect cardiovascular function. For example, ETS-related inducible nitric oxide synthase induction would likely increase mitochondrial $\cdot\text{NO}$ concentrations, inhibiting electron flow and contributing to $\text{O}_2^{\cdot -}$ formation.^{55,56} Mitochondrial $\text{O}_2^{\cdot -}$ is converted to hydrogen peroxide (H_2O_2) by SOD2 and may act as a redox signaling molecule, or H_2O_2 can be reduced to

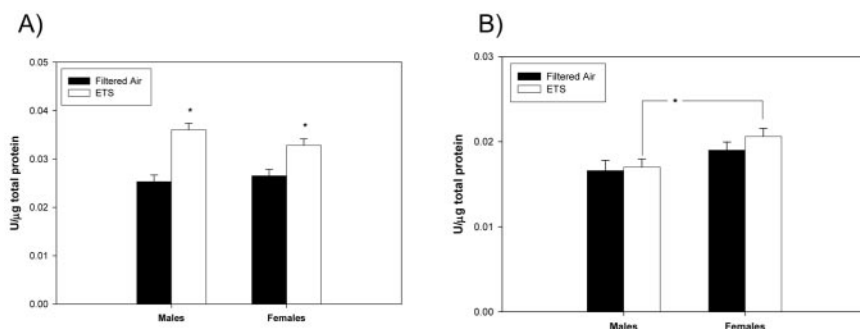


Figure 3. SOD activity in cardiovascular tissues collected from 12-week-old apoE^{-/-} mice exposed to ETS or filtered air from gestation days 1 to 19. A, Total SOD activity. *Significant difference ($P < 0.05$) between in utero ETS-exposed and control animals. B, SOD2 activity. *Significant difference ($P < 0.05$) between male and female mice exposed to ETS in utero.

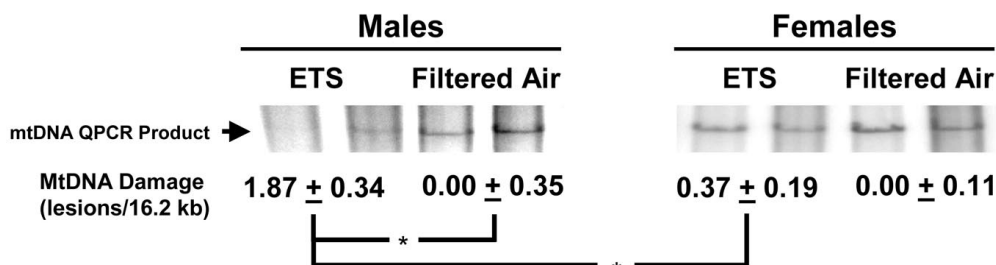


Figure 4. QPCR was used to quantify the relative levels of mtDNA damage between adult (12-week-old) mice exposed to ETS in utero (gestation days 1 to 19) vs controls. Typical amount of QPCR product generated from cardiovascular tissues from 12-week-old mice exposed to either ETS or filtered air in utero is shown. Less QPCR product indicates greater mtDNA damage. Numbers below indicate the relative amount of mtDNA damage (lesions per 16.2 kb) in ETS-exposed animals compared with gender- and age-matched filtered air controls (zero class). *Significant difference ($P < 0.05$) between the indicated groups.

water by mitochondrial glutathione stores. Alternatively, $O_2^{\cdot -}$ undergoes facile reaction with $\cdot NO$ to form $ONOO^-$, which in the presence of CO_2 yields the nitrosoperoxycarbonate anion ($ONOOCO_2^-$), a reactive intermediate that can nitrate proteins,⁵⁷ including SOD2. Inactivation of SOD2 by nitration⁵⁸ may affect mitochondrial redox signaling via altered H_2O_2 production. Adult ETS exposure decreases SOD2-specific activity and increases 3-nitrotyrosine levels in mitochondrial proteins, including SOD2.¹⁹ Because it has been previously shown that both nitrosative and oxidative stress can cause significant mitochondrial damage and dysfunction in vascular cells⁵⁹ and because significantly reduced SOD2 activity results in accelerated atherosclerotic lesion formation in apoE^{-/-} mice,⁶⁰ it is possible that altered oxidative and nitrosative stress within the mitochondrion can contribute to CVD development.⁶⁰ Similar processes may occur as a consequence of in utero ETS exposure. The observed reduction of aconitase activity and increased mtDNA damage in adults exposed to ETS in utero are consistent with the hypothesis that overall oxidative loads within the mitochondrion are increased by prenatal ETS exposure. Furthermore, the association of increased atherosclerotic lesion formation in male apoE^{-/-} mice with in utero ETS exposure and mtDNA damage suggests that mitochondrial damage and function may contribute to CVD development. In this regard, female mice exposed to ETS in utero had far less mtDNA damage and atherosclerotic lesion development than their male counterparts. This is consistent with the suggestion that cardiovascular mitochondrial damage (and thus function) may be related to atherogenesis. Hence, damage sustained in utero could lead to altered mitochondrial function in cardiovascular cells earlier in life, markedly increasing the risk for atherosclerotic lesion development as an adult.

Over the past decade, studies have consistently shown that ETS exposure increases the risk of heart disease death.¹⁴ Nonetheless, the effects of prenatal ETS exposure on adult CVD development have not been defined. The findings of the present study strongly support the hypothesis that prenatal ETS exposure, independent of dietary fat intake, promotes adult CVD development and cardiovascular mitochondrial damage.

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