Hypertensive disorders in pre-term pregnancy: management and long-term consequences
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In utero programming of adult vascular function in transgenic mice lacking low-density lipoprotein receptor

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Abstract

Objective: The objective of this study was to examine the role of maternal hypercholesterolemia in fetal programming of adult vascular function using transgenic mice lacking the low-density lipoprotein receptor (LDLR).

Study Design: Homozygous LDLR knockout mice (B6.129S7-Ldlrtm1Her/J, LDLR⁻/⁻KO) and their wild-type controls (C57BL/6J, LDLR⁺/⁺WT) were cross-bred to produce 4 litter groups: LDLR⁻/⁻KO, maternally derived heterozygous (LDLR±Mat), paternally derived heterozygous (LDLR±Pat) and LDLR⁺/⁺WT. Female and male offspring were killed at 10-12 weeks of age, and carotid arteries were used for in vitro experiments.

Results: The dose responses to phenylephrine were significantly higher in LDLR⁺/⁻KO and LDLR±Mat male offspring. The contractile responses to phenylephrine in female mice were significantly increased only in the LDLR⁻/⁻KO offspring. Maximal Ca²⁺ contraction was higher in LDLR⁻/⁻KO male and female offspring.

Conclusion: Despite being genomically similar, heterozygous offspring that developed in a hypercholesterolemic maternal environment had abnormal vascular responses later in life compared with those that developed in a normal environment.


**Introduction**

Epidemiologic studies have shown that the atherogenic process already begins during fetal development.\(^1\)\(^2\) Fatty streaks, the earliest lesions of arteriosclerosis, are already present in fetal arteries. The process of formation is enhanced greatly in fetuses who are born to mothers with hypercholesterolemia.\(^3\)\(^4\) This suggests that a high maternal level of cholesterol during fetal development may accelerate the atherogenesis process that occurs later in adult life.\(^5\) However, the effect of high maternal cholesterol level on the intrauterine environment and fetal vascular development is still not clear.\(^6\)\(^-\)\(^8\) Arteriosclerosis is characterized by a long lag time between onset and clinical manifestations.\(^9\) The endothelial dysfunction is considered an early marker of arteriosclerosis, preceding angiographic and ultrasonic evidence of arteriosclerotic plaques.\(^10\)\(^11\) Damage to the endothelium alters the balance between vasoconstricting and vasodilating agents and initiates a number of events that promote or exacerbate arteriosclerosis. In the process of arteriosclerosis, lipoprotein particles, especially low-density lipoprotein (LDL), enter the arterial wall and undergo various modifications, such as oxidation. Modified LDL is chemotactic for monocytes and macrophage. This sets off a cascade of cellular responses within the artery and induces the production of cytokines such as tumor necrosis factor \(\alpha\), interleukin-1, interleukin-6 and interleukin-8. These cytokines increase the binding of LDL to the endothelium.\(^12\)\(^-\)\(^14\) Oxidation of LDL has been proposed as an important mechanism in the pathogenesis of the arteriosclerotic process.\(^15\)\(^16\) Although the placenta is impermeable to the LDL particles themselves, the maternal oxidative end-product that results from the maternal hypercholesterolemic status may increase lipid peroxidation products in the fetal plasma, either directly or indirectly. Furthermore, it has been shown in animal models that fetal genes can be up- or downregulated, depending on the hypercholesterolemic status of the mother.\(^17\)\(^-\)\(^20\) Napoli et al\(^19\) showed an upregulation of 57 genes and a downregulation of 82 genes (such as the fibroblast growth factor binding protein gene that is involved in neovascularization and cancer growth) in the aorta of pups that were born to hypercholesterolemic mothers. The maternal cholesterolemic status can impact fetal vascular development and beyond, either through the transmission of a genetic predisposition for hypercholesterolemia or through altered fetal programming of vascular function. Epidemiologic studies may not differentiate between these 2 potential influences on vascular function in adult life.

Epidemiologic studies have their limitations. The genetic heterogeneity of the human population and the variability in diets and other confounders make it almost impossible to measure the exact interaction between the maternal environment and the genetic contribution to the fetus. Knockout animals are genetically altered
animals, with ≥1 specific genes disrupted. With these exact gene disruptions, a knockout animal model not only give us the opportunity to identify specific interaction between environmental and genetical contribution to the fetus but also to find out the mechanism behind this specific interaction, which is a great advantage compared to epidemiologic studies. We sought to study the effect of maternal hypercholesterolemia on fetal programming using an animal knockout model.\textsuperscript{21-23} We used an LDL receptor knockout mouse model (LDLR\textsuperscript{−/−}KO) lacking a functional LDL receptor.\textsuperscript{10,24,25} The LDL-receptor (LDLR) is involved in the clearing of lipoproteins from the circulation, and its lack leads to hypercholesterolemia and arteriosclerosis. LDLR\textsuperscript{−/−}KO mice fed a normal diet develop moderate fatty streak lesions and intimal thickening with foam cells and smooth muscle cell infiltration.\textsuperscript{26} By crossbreeding LDLR\textsuperscript{−/−}KO mice and their wild-type control mouse (LDLR\textsuperscript{+/+}WT), heterozygous litters that have developed in normal vs hypercholesterolemic environments, but are genomically similar, can be produced and used to determine the genetic vs environmental contributions to fetal programming. The objective of this study was to examine the effect of maternal hypercholesterolemia on fetal vascular programming with the use of transgenic mice lacking LDLR. To assess vascular function in the offspring, we used the carotid artery, a resistance artery, and an artery with a predilection for atherosclerotic plaques and evaluated the vascular reactivity, which is 1 of the first mechanisms affected by the arteriosclerotic process.

**Material and methods**

**Animals**
Female and male homozygous knockout mice disrupted for LDLR gene (LDLR\textsuperscript{−/−}KO, LDLR\textsuperscript{−/−} knockout, strain: B6.129S7-Ldlr\textsuperscript{mut1Her}/J,) and their wild-type age-matched controls (LDLR\textsuperscript{+/+}WT, LDLR\textsuperscript{+/+} wild-type strain: C57BL/6J, LDLR\textsuperscript{+/+}WT) were obtained from Jackson Laboratory (Bar Harbor, ME). All of these animals were from the same bench and thus with the same genetic and environment background. The animals were housed separately in temperature- and humidity-controlled quarters with constant 12-hour light:dark cycles and were provided with food and water ad libitum. Female and male LDLR\textsuperscript{−/−}KO mice and their LDLR\textsuperscript{+/+}WT were crossbred to produce heterozygous pups that developed in a hypercholesterolemic knockout mother (LDLR\textsuperscript{±} maternally derived pups; LDLR\textsuperscript{±Mat}) or normal wild-type mother (LDLR\textsuperscript{±} paternally derived pups; LDLR\textsuperscript{±Pat}). Except for the parental source of the single defective allele, both groups of heterozygous pups were genetically similar and had 1 functional LDLR gene. In addition, female and male LDLR\textsuperscript{−/−}KO
mice and their LDLR^{+/-WT} controls were bred to obtain LDLR^{-/-KO} and LDLR^{+/-WT} homozygous litters. Adult 3-month-old male and female offspring from these 4 groups of litters were used for the in vitro experiments. Each experimental group consisted of 6-10 offspring. The animals were killed by Co_{2} inhalation in a closed chamber. The carotid arteries were dissected and used for the in vitro vascular reactivity experiments. The Animal Care and Use Committee of the University of Texas Medical Branch approved all procedures.

**Diet**

All mice were fed the same regular diet (Teklad 7012; Harlan Teklad LM-485 Mouse-Rat Sterilizable diet; Harlan Teklad, TX) that consisted of 19.92% protein, 5.67% fat, 4.37% fibers, 6.48% ash, and 53.66% nitrogen-free extract.

**Drugs and solutions**

The drugs that were used in the experiments were acetylcholine hydrochloride, phenylephrine hydrochloride, serotonin hydrochloride, and calcium from Sigma (St. Louis, MO). Stock solutions of all drugs (10^{-2} mol/L) were prepared in deionized water and stored at -20°C. The Krebs solution that was used was prepared in the following manner: glucose, 11.5 mmol/L; NaCl, 119.0 mmol/L; KCl, 4.7 mmol/L; MgSO_{4}, 1.2 mmol/L; KH_{2}PO_{4}, 1.2 mmol/L; NaHCO_{3}, 25.0 mmol/L; ethylenediaminetetraacetic acid, 0.026 mmol/L; and CaCl_{2}, 2.5 mmol/L. In the experiments that used high K^{+} Ca^{2+}-free solution (80 mmol/L K^{+}), Ca^{2+} was omitted from the physiologic salt solution, and K^{+} replaced NaCl to maintain osmolality of the solution.

**In vitro experiments**

Two-millimeter segments of the carotid artery from the 4 groups of offspring were isolated and mounted in a wire myograph (Multi Myograph System-610M; J.P. Trading J/S, Aarhus, Denmark) over 25-μm tungsten wires. The preparations were bathed in Krebs solution and maintained at 37°C (pH 7.4), and a mixture of 95% O_{2} and 5% CO_{2} was bubbled continuously through the solution. The force was recorded continuously by an isometric force transducer and analyzed with Power Lab data acquisition and playback software (ADI Instruments, Colorado Springs, CO).

The experiments were performed at a ring diameter of the vessel equal to 0.9 of the optimal diameter. The optimal diameter is an estimate of the vascular diameter in–situ at a specific transmural pressure. The optimal diameter and the passive tension applied to the vessels (3.5 mN) were determined in previous experiments that were done in our laboratory. After stabilization of the vascular tone, the rings were contracted twice with 60 mmol/L KCl to enhance the vascular
responsiveness. The second response was used as reference contraction for data analysis. After equilibration in Krebs solution, contractile responses to cumulative concentrations of the $\alpha$-adrenergic agonist phenylephrine ($10^{-10}$ to $10^{-5}$ mol/L) and serotonin ($10^{-10}$ to $10^{-5}$ mol/L) were performed and followed by a single dose of acetylcholine ($10^{-6}$ mmol/L) to evaluate endothelial function. Only experiments with intact endothelium were used in our final analysis. We also evaluated the relaxant response to the endothelium-dependent acetylcholine ($10^{-10}$ to $10^{-5}$ mmol/L) after precontraction with phenylephrine ($10^{-7}$ to $10^{-6}$ mmol/L). After equilibration of the vessel in high-K$^+$ Ca$^{2+}$-free solution, contractile responses to cumulative concentrations of Ca$^{2+}$ (0.05-5 mmol/L) were studied to evaluate the responsiveness of the vascular smooth muscle.

**Data analysis**

Data are expressed as mean ± SEM, with “n” representing the number of animals used in each experiment. For the vascular reactivity studies, the concentration-response curves to the agents that were tested were constructed. Responses at each concentration and the maximal effect were compared among the different groups. The 4 groups of the female animals were compared, and a comparison was made among the 4 groups of male animals. One-way ANOVA followed by Newman-Keuls post-hoc test were used for statistical analysis. A probability value of < .05 was considered significant.

**Results**

**In vitro vascular reactivity**

Concerning the reaction of phenylephrine among the 4 male groups, the dose-responses to phenylephrine in the carotid artery were significantly higher in the LDLR$^{-/-}$KO and LDLR$^{+/-}$Mat male offspring, compared with LDLR$^{+/+}$WT and LDLR$^{\pm}$Pat mice. Maximal effect in %: LDLR$^{-/-}$KO (N = 6) 102.9 ± 12.17 mN, LDLR$^{+/-}$Mat (N = 7) 80.2 ± 6 mN vs LDLR$^{+/+}$WT (N = 8) 40.70 ± 4 mN and LDLR$^{\pm}$Pat (N = 6) 56.7 ± 8.1 mN. ($P < .05$; Figure 1, A). The contractile responses to phenylephrine among the 4 groups of female mice were significantly increased only in the LDLR$^{-/-}$KO offspring, compared with the other groups. Maximal effect in %: LDLR$^{-/-}$KO (N = 7) 102.7 ± 11.69 mN, LDLR$^{+/-}$Mat (N = 10) 74.4 ± 9.2 mN vs LDLR$^{+/+}$WT (N = 8) 61.5 ± 3.8 mN and LDLR$^{\pm}$Pat (N = 6) 64.8 ± 8.0 mN. ($P < .05$; Figure 1, B).
Concerning the vascular reactivity to calcium among the male groups, the dose response curve to cumulative concentrations of calcium was significantly higher in the LDLR⁻/⁻KO adult male offspring (n = 6) at 1 mmol/L, 1.5 mmol/L, and 2 mmol/L concentration, compared with the other groups of homozygous control male mice (n = 8), heterozygous paternal male mice (n = 6; Figure 2, A).

The maximal effect among the 4 groups of female adult offspring mice to Ca⁺² was higher in the LDLR⁻/⁻KO mice, compared with the other groups: LDLR⁻/⁻KO (N = 6), 2.16 ± 0.27 mN; LDLR⁺/⁻Mat (n = 10), 1.32 ± 0.09 mN; LDLR⁺/⁺WT (n = 8), 1.46 ± 0.21 mN; and LDLR⁺/⁺Pat (n = 6), 1.37 ± 0.3 mN; P < .05; Figure 2, B).

The responses to cumulative concentrations of serotonin did not differ among the 4 groups of offspring, in either the male or the female groups. The same numbers of pups were used in each group as in the experiments with phenylephrine and calcium. Similarly, there were no differences in the responses to cumulative concentrations of acetylcholine in either male or female offspring (Figure 3, A and B).

**Comment**

In this study, we used a knockout mouse lacking the LDLR gene as a model to study the role of maternal hypercholesterolemia in fetal programming of vascular...
With our cross-breeding scheme, we were able to compare the contractile properties of the carotid arteries between mice completely lacking a functional LDLR allele, wild-type mice, mice heterozygous for the LDLR gene disruption that developed in a normal maternal environment, and heterozygous mice developing in a maternal environment completely lacking a functional LDLR gene. In both

![Figure 2](image1)

**FIGURE 2.** Calcium dose-response and concentration-response curves

A. Calcium dose-response curves in the carotid arteries from male LDLR^{-/-}KO, maternally derived heterozygous LDLR^{+/-} (LDLR^{+/-}Mat), paternally derived heterozygous LDLR^{+/-} (LDLR^{+/-}Pat), and LDLR^{+/+}WT offspring. The asterisk denotes a probability value of < .05, compared with the corresponding responses in the other groups. B. Calcium concentration-response curves in the carotid arteries from female LDLR^{-/-}KO, maternally derived heterozygous LDLR^{+/-} (LDLR^{+/-}Mat), paternally derived heterozygous LDLR^{+/-} (LDLR^{+/-}Pat), and LDLR^{+/+}WT offspring. The asterisk denotes a probability value of < .05, compared with the corresponding responses in the other groups.

![Figure 3](image2)

**FIGURE 3.** Acetylcholine concentration-response curves

A. Acetylcholine concentration-response curves in the carotid arteries of male mice LDLR^{-/-}KO, maternally derived heterozygous LDLR^{+/-} (LDLR^{+/-}Mat), paternally derived heterozygous LDLR^{+/-} (LDLR^{+/-}Pat), and LDLR^{+/+}WT. The responses are presented as percent relaxation of the phenylephrine contraction. B. Acetylcholine concentration-response curves in the carotid arteries of female mice LDLR^{-/-}KO, maternally derived heterozygous LDLR^{+/-} (LDLR^{+/-}Mat), paternally derived heterozygous LDLR^{+/-} (LDLR^{+/-}Pat), and LDLR^{+/+}WT. The responses are presented as percent relaxation of the phenylephrine contraction.

function. With our cross-breeding scheme, we were able to compare the contractile properties of the carotid arteries between mice completely lacking a functional LDLR allele, wild-type mice, mice heterozygous for the LDLR gene disruption that developed in a normal maternal environment, and heterozygous mice developing in a maternal environment completely lacking a functional LDLR gene. In both
male and female homozygous LDLR\(^{-/-}\) KO offspring, a lack of expression of a functional LDLR allele was associated with increased contractile responses to phenylephrine and calcium in the isolated carotid artery in vitro. Interestingly, the contractile response to phenylephrine in the heterozygous maternally derived male offspring was similar to the knockout mice; the heterozygous paternally derived male offspring had a contractile response that was similar to the wild-type mice, despite the genomically similar background of the heterozygous offspring. This difference between the genomically similar heterozygous offspring can be due to altered fetal programming of adult vascular function by a maternal environment lacking a functional LDLR allele. Interestingly this could not be confirmed in the female mice. Although there was a trend toward a difference in vascular reactivity between the heterozygous maternally derived female mice compared with the knockout mice, this difference was not statistically significant.

These findings support a role for maternal hypercholesterolemia during fetal vascular programming in the early onset of altered vascular responses in later life.\(^2,3,19\) The process of arteriosclerosis seems to be a chronic inflammatory condition that is converted to an acute clinical event by the induction of plaque rupture, which in turn leads to thrombosis. The process may take years to become clinically evident. However, our results show that the process may start very early in life. The earliest lesions, the fatty streaks, which by themselves may not be of major clinical significance, develop at various sites predicated by hemodynamic and mechanical factors. The LDL enters the arterial wall and undergoes modification including oxidation, which leads to the production of inflammatory mediators like the cytokines.\(^12,16\) The monocytes in the vessel wall take up the oxidized lipoproteins and induce their differentiation into lipid-laden foam cells. Damage to the endothelium will result in altered vascular reactivity, which is an early marker of this process.\(^10,11,16\) The vascular responses to phenylephrine in the LDLR\(^{-/-}\) KO and the LDLR\(^{\pm\text{Mat}}\) offspring confirm an early vascular dysfunction as a consequence of a high maternal cholesterol level.

The higher than normal vascular response to calcium in the male and female LDLR\(^{-/-}\) KO mice may be explained by the process of proliferation and migration of vascular smooth muscle cells. The vascular smooth muscle cells respond to the cascade of events that are associated with the process of arteriosclerosis, and as discussed earlier, with proliferation and migration from the media and into the intima. The increased migration and proliferation increases the vessel’s contractile potential.\(^27,28\) The vascular contractility in the LDLR\(^{\pm\text{Mat}}\) and the LDLR\(^{\pm\text{Pat}}\) offspring to calcium was not significantly different from that of the wild-type offspring. We would have expected a higher contractile reaction of the heterozygous LDLR\(^{\pm\text{Mat}}\) offspring to calcium, as was seen in the LDLR\(^{-/-}\) KO offspring. This can be explained by the fact that vascular smooth muscle cell
proliferation and migration is a process that takes a long time and the use of older offspring may have shown a difference. The offspring were also fed a normal diet. On this diet, these transgenic mice produce moderate fatty streaks, although a western diet with higher cholesterol content may have accelerated the process of arteriosclerosis. In the cascade of events that leads to arteriosclerosis, the process of vascular smooth muscle cells proliferation and migration occurs at a later stage than foam cell formation. This may explain the reason that we did see similar exaggerated responses to phenylephrine in the LDLR⁻/⁻KO and the LDLR⁺/Mat but not yet in the reaction to calcium. Further research may shed more light on the vascular responses of LDLR⁺/Mat offspring later in life, particularly when the offspring are fed a high cholesterol diet (eg, western-type diet).

We also found gender difference in the response to phenylephrine between heterozygous LDLR⁺/Mat offspring, with increased contractile responses in males but not females. This could be due to the protective role of estrogen. Further research in ovariectomized animals may further our understanding.

In conclusion we have shown that maternal hypercholesterolemia seems to accelerate the process of arteriosclerosis in their offspring and in consequence alters their vascular reactivity. This animal model of maternal hypercholesterolemia supports the role of an adverse intrauterine environment on fetal vascular programming, which leads to an abnormal vascular function in later life. Hypercholesterolemia during pregnancy, such as seen in LDLR knockout mice, leads to change in vascular reactivity of the offspring early in life, even in those offspring that receive a normal diet.
References


