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Transgenerational Effect of Fetal Programming on Vascular Phenotype and Reactivity in Nitric Oxide Knockout Mouse Model

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Abstract

Objective—To investigate the transgenerational effect of fetal vascular programming

Methods—Homozygous NOS3 knockout and wild type controls (NOS3+/+WT) were cross-bred to obtain heterozygous offspring that developed in (KO−/−) mothers lacking a functional NOS3 (KOM) versus wild-type control mothers (KOP). The first generation (F1) KOM(+/-) and KOP(+/-) female mice were then bred with WT(+/+) males to obtain a second generation (F2). F2 offspring were genotyped and WT(+/+)-F2 mice were then used for in vivo blood pressure and in-vitro vascular reactivity studies.

Results—WT-F2 mice born to KOM mothers (KOM-F2WT) had significantly higher systolic BP, mean arterial and pulse pressure compared to WT-F2 born to KOP mothers. Male KOM-F2WT offspring had significantly increased response to phenylephrine (PE) compared with male KOP-F2WT. Male offspring had increased contractile responses to PE when compared to female. Acetylcholine responses were decreased in female KOM-F2WT compared with female KOP-F2WT, but the difference was not statistically significant

Conclusion—Our findings support a transgenerational effect of fetal programming on the vascular phenotype, and suggest possible gender specific adaptation

Keywords

Fetal programming; vascular phenotype; transgenerational

INTRODUCTION

In recent years there has been more approval of the concept that intrauterine environment modulates adult diseases. Dr David Barker published a study in 1989 that suggested that the intrauterine environment influences blood pressure during adult life. In addition he concluded that the geographical differences in average blood pressure and mortality from cardiovascular disease in Britain partly reflect past differences in the intrauterine environment.¹ This was followed by multiple studies including one in 2000 that showed that blood pressure levels in adult life are related to the mother's fetal growth and size at birth, with no relation to the father's

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CONDENSATION

The transgenerational effect of intrauterine environment on vascular phenotype and reactivity in Nitric Oxide knockout mouse model

size at birth.² Insults to the fetus during the critical periods of development lead to fetal programming with adaptive changes in the fetal structure, physiology and metabolism as a whole.^{3,4}

The interactions between the fetal genome and the intrauterine environment create the milieu in which the fetus develops. The observed association between an unfavorable fetal environment and diseases in later life may be confounded by the presence of a genetic predisposition for the particular disease. In order to induce an unfavorable uterine milieu, we used in our laboratory an animal model that is deficient in endothelial nitric oxide synthase (e-NOS or NOS-3) the rate limiting enzyme responsible for the generation of nitric oxide (NO) in endothelial cells from its substrate L-arginine.

NO is a potent smooth muscle relaxant and one of the primary modulators of vascular tone.^{5,6,7} NO plays a vital role in maintaining adequate utero-placental perfusion through its vasodilatory properties. By inhibiting its synthesis, maternal hypertension and fetal growth restriction have been produced in previous studies.^{8,9} This model combines the effect of a hostile uterine environment as well as a genetic abnormality affecting vascular function, and thus is ideal to investigate the contribution of the hostile uterine environment on the vascular function in adult life vs. that of parental genetics.

In this study we used the transgenic mouse strain lacking endothelial nitric oxide synthase gene (NOS3^{-/-KO}) and then cross bred it to wild type mice (NOS3^{+/+}) having the NOS3 gene to produce genomically similar heterozygous offspring that developed either in a mother lacking NOS3 or in normal wild type mothers. We have previously shown using this animal model, that maternal heterozygous offspring (KOM) (i.e. those that developed in abnormal maternal uterine environment) have altered fetal and postnatal growth as well as abnormal vascular function later in life compared to the genomically-similar paternal heterozygous offspring (KOP).^{10,11} Most strikingly, the endothelium dependent relaxation to acetylcholine in the former group was completely absent and similar to homozygous knockout animals, while that of the latter group was normal similar to the wild type animals.

Our hypothesis was that this abnormal vascular responsiveness in the first generation offspring may predispose them to abnormal vascular adaptations later during their pregnancy leading to an unfavorable uterine environment and thus abnormal vascular function in the 2nd generation offspring later in life.

Our objective from this study was to study the effect of altered fetal programming in the first generation on the vascular phenotype of the second generation.

MATERIALS AND METHODS

Animals

Mature cycling female and male mice (4–6 weeks old) homozygous for disruption of the NOS3 gene (NOS3-knockout, strain B6.129P2-Nos3^{tm1Unc}, stock no. 002684, NOS3^{-/-KO}) and their age-matched wild-type controls (NOS3-wild-Type, strain C57BL/6J, stock no. 000664, NOS3^{+/+WT}) were purchased from Jackson Laboratory (Barharbour, Maine). They were maintained and bred in the animal care facility at the University of Texas Medical Branch. They were housed separately in temperature- and humidity-controlled quarters with constant 12-hour light: dark cycles and provided with food and water ad libitum. Regular maintenance and care was provided by certified personnel and veterinary staff according to the guidelines of the IACUC. All procedures were performed after approval by the Animal Care and Use Committee of the University of Texas Medical Branch.

Homozygous NOS3 knockout (C57BL/6J-NOS3^{-/-KO}) and wild-type mice (NOS3^{+/WT}) were cross-bred to produce the first generation (F1) maternally-derived (NOS3^{+/mat}) (KOM) and paternally-derived heterozygous NOS3 (NOS3^{+/pat}) (KOP) litters. These offspring are genomically-similar but developed in normal mothers or mothers lacking NOS3 gene respectively. The F1 maternal heterozygous and the F1 paternal heterozygous female mice were subsequently bred with homozygous wild-type (NOS3^{+/WT}) male mice to obtain a second generation (F2) mice. With this breeding scheme, we obtained a second generation of mice that were either heterozygous or wild type. After genotyping by PCR on DNA isolated from their tail, only F2 wild type (WT) mice were used. These offspring were either born to a maternally-derived heterozygous mother and named KOM-F2WT or to a paternally-derived heterozygous mother and named KOP-F2WT.

Blood pressure measurement

In vivo blood pressure was measured by telemetry in conscious unrestrained wild type offspring at 15 weeks of age. The animals were anesthetized with a mixture of ketamine (Ketalar, Parke-Davis, Morris Plains, NJ) and xylazine (Gemini, Rugby, Rockville Center, NY), then the left common carotid artery was located and carefully isolated through a vertical midline skin incision along the neck. A 0.4 mm catheter was then introduced into the carotid artery through a small incision in the vessel wall, and the body of the transducer (PA-C20 model, Data Sciences, St. Paul, MN) was secured. The catheter was then tunneled to a telemetric transducer encased in a miniature capsule. The neck incision was closed using 6-0 silk. Mice were kept warm on a heating pad and monitored closely until fully recovered from anesthesia. Blood pressure was continuously measured for 8 days in the unrestrained conscious animal. Daily mean arterial pressure (MAP), average of systolic (SBP), diastolic (DBP) and pulse pressure (PP) were obtained.

Vascular reactivity experiments

Second generation pups were sacrificed at 14 wks of age by CO₂ inhalation according to the Animal Care and Use Committee guidelines as well as the American Veterinary Medical Association guidelines. Carotid arteries were carefully dissected, isolated, and 2-millimeter segments obtained and mounted in a wire myograph (Model 410A, J.P. Trading I/S, Aarhus, Denmark) over 25 μ m tungsten wires. The preparations were bathed in physiological salt solution maintained at 37°C, pH ~7.4 and a mixture of 95% O₂ and 5% CO₂ was bubbled continuously through the solution. The force was recorded continuously using an isometric force transducer and analyzed using Windaq data acquisition and playback software (DataQ Instruments, Inc., Akron, OH). After stabilization of the tone, the vessels were contracted twice with 60 mmol/L KCl for 30 minutes to enhance reproducibility of responses. The second response to KCl was used as the reference contraction in the final calculations. After 1 hour equilibration in physiologic salt solution, contractile responses to cumulative concentrations of the α_1 -adrenergic agonist phenylephrine (PE; 10⁻¹⁰ to 10⁻⁵ mmol/L), in the presence and absence of the non selective NOS inhibitor L-NAME (10⁻⁵ mmol/L), were assessed. In addition, relaxant responses to cumulative concentrations of the endothelium-dependent vasorelaxant acetylcholine (Ache; 10⁻¹⁰ to 10⁻⁵ mmol/L), the endothelium-independent vasorelaxant sodium-nitroprusside (SNP; 10⁻¹⁰ to 10⁻⁵ mmol/L) and the β -adrenoreceptor agonist isoproterenol (ISO; 10⁻¹⁰ to 10⁻⁵ mmol/L) were evaluated after precontraction of the vessels with phenylephrine (10⁻⁷ to 10⁻⁶ mmol/L) to produce matching contractions in the study groups. After each agent tested, the vessels were washed with Krebs solution and left to recover for $\geq$ 30 minutes until they returned to their basal passive tension.

Drugs and solutions

The drugs that were used in the in vitro experiments were acetylcholine hydrochloride, phenylephrine hydrochloride, and isoproterenol hydrochloride, which were purchased from Sigma Chemical Company (St. Louis, MO). Stock solutions of all of the drugs (10^{-2} mol/L) were prepared in deionized water and stored at -20°C . The composition of physiologic salt solution was NaCl, 119 mmol/L; KCl, 4.7 mmol/L; NaH_2PO_4 , 1.2 mmol/L; NaHCO_3 , 25 mmol/L; MgCl_2 , 1.2 mmol/L; CaCl_2 , 2.5 mmol/L; ethylenediaminetetraacetic acid, 0.026 mmol/L; glucose, 11.5 mmol/L.

Data analysis

For the blood pressure studies, MAP, SBP, DBP and PP were collected and expressed as means $\pm$ SEM over the 8 day period. Data was analyzed using the Student t-test.

For the vascular reactivity studies, data was expressed as mean $\pm$ SEM. Response to KCL was used as a reference to calculate the percent of contraction achieved by the contractile agents studied, while phenylephrine pre-contraction was used to obtain the percent relaxation induced by the vasorelaxant agents used. One-way ANOVA followed by Newman-Keuls multiple comparisons test was used for statistical analysis.

A p-value less than 0.05 was considered significant.

RESULTS

The second generation (F2) mice were weighed weekly from week 4 to week 10 of life. Results are summarized in table 1. There were 4–11 mice per group. The weight of the F2 wild type male mice born to paternally-derived heterozygous mother (KOP) remained significantly higher than the F2 wild type male mice born to maternally-derived heterozygous mothers (KOM), and the difference was statistically significant at every week of life. (Fig. 1)

The WT-F2 male mice born to KOM mothers (KOM-F2WT) had a significantly higher SBP (153 ± 4.4 vs. 120.2 ± 2.6 mmHg, $p=0.001$), MAP (110.5 ± 0.8 , vs. 107.2 ± 0.8 mmHg, $p=0.005$) and PP (59.4 ± 1.6 vs. 21.6 ± 1.8 mmHg, $p=0.001$) compared to the WT-F2 male mice born to KOP mothers (KOP-F2WT) ($n=4-11$ animals per group). A higher DBP was also noted in the KOM-F2WT compared to the KOP-F2WT mice, but the difference was not statistically significant (99.2 ± 8.1 , 78.8 ± 8.7 mmHg, $p>0.05$) (Fig. 2)

For the vascular studies, a total of 6–13 male and female animals were present in each group. The total number of animals used in each experiment and their relative gender are summarized in table 2. Maximal contractile responses to the tested agents are summarized in table 3. Wild type male second generation offspring born to KOM mothers (KOM-F2WT/M) have significantly increased response to PE compared with wild type male second generation offspring born to KOP mothers (KOP-F2WT/M) (137.92 ± 27.47 vs. 78.34 ± 10.81 , $p<0.05$) (Fig. 3) and this difference was abolished after incubation with L-NAME. (220.36 ± 16.02 vs. 214.35 ± 13.54) (Fig. 4) On the other hand, female KOM-F2WT/F and KOP-F2WT/F had similar responses to PE, in the presence (180.17 ± 28.34 vs. 180.93 ± 10.63) or absence (49.09 ± 7.38 vs. 39.71 ± 3.34) of L-NAME. (Figs. 3, 4) Male offspring had an overall increased responses to PE when compared to female offspring, irrespective of their maternal heterozygous status (KOM or KOP), but that difference between male and female offspring was much higher in those born to KOM mothers. (Fig. 3) That difference became non-significant after L-NAME incubation. (Fig 4)

The responses to Ach although not statistically significant, were decreased in KOM-F2WT/F compared to the other groups (Fig. 5). The relaxant responses to sodium nitroprusside and

isoproterenol in the F2WT male and female offspring did not differ when compared inter-gender or intra-gender with those born to KOP or KOM mothers (Figs 6, 7 respectively).

DISCUSSION

Predisposition to diseases has traditionally been attributed to various aspects of genetic transmission. However, only few diseases are monogenic in origin and follow mendelian genetics. Preeclampsia, hypertension and cardiovascular disease in general have been linked to many non-genetic factors such as life style, dietary habits and other socioeconomic factors. One of the major determinants of predisposition to these conditions is what is now called “developmental origin of adult diseases”, a concept started in the 1960’s with Dr Robert McCance and Elsie Widdowson^{12–13} and their original work on the influence of early neonatal nutrition on growth, body size and development. Their work was later followed by Dr David Barker’s epidemiological studies in 1980s which gave rise to the Barker Hypothesis and gave impetus to this entire field of investigation.

Previous work in our lab showed that heterozygous first generation offspring that developed in different maternal intrauterine environment have altered vascular adaptations despite their similar genetic composition.¹⁰ The finding in this study of altered vascular function in adult life in the second generation NOS3^{+/-WT} offspring of NOS3^{+Pat/-Mat} compared to the genomically-similar second generation NOS3^{+/-WT} offspring of NOS3^{-Pat/+Mat} is strong evidence of the transgenerational non-genetic transmission of phenotype, and the critical role of uterine environment in this aspect.

The first part of the experiment on blood pressure carries a special interest. Human data from industrialized nations show that systolic blood pressure in individuals over age 50 continuously rises throughout life, whereas diastolic blood pressure remains more stable.^{14–16} Systolic blood pressure, and pulse pressure in particular, are major determinants of cardiovascular risk^{15,17}, and pulse pressure may be regarded as an index of arterial aging. A study performed in a relatively small cohort of young adults found that after age adjustment, telomere length, which is a molecular marker of biological aging, was inversely related to pulse pressure.¹⁸ Another larger study¹⁹ found the same association but only in men. The inverse association between pulse pressure and longevity was much stronger than the effect of chronological age. Biologic age of individuals with relatively wide pulse pressures is more advanced than their chronological age would indicate and, at least in males, wide pulse pressure denotes a forward resetting of biologic age.¹⁴ The fact that SBP and PP but not DBP were significantly elevated in WT mice born to KOM rather than KOP mothers suggest that they are at higher risk of cardiovascular disease and mortality later in life, and this was suspected by the vascular abnormalities at 14 wks of life especially in male mice.

There is evidence from several studies that intrauterine fetal programming as a consequence of an altered intrauterine environment (whether by diet alteration, hormonal or surgical manipulation) can be passed transgenerationally^{20–24} through either the maternal²³ or paternal cell line.²⁵ We have confirmed this transgenerational altered vascular programming both in vivo as well as in vitro in an animal model of fetal programming induced by genetic predisposition. Moreover, our in vivo data were obtained in the conscious unrestrained offspring, thereby circumventing the known effects of anesthesia or restraint on blood pressure. Despite being genetically wild type, and therefore supposed to have similar vascular phenotype, the F2 offspring born to mothers that developed in an adverse uterine environment (F1-KOM mothers born to P1-KO NOS3^{-/-}) had worse blood pressure profile when compared to wild type F2 offspring born to mother that developed in a normal uterine environment (F1-KOP mothers born to P1-WT NOS3^{+/-}).

The transgenerational transmission of phenotype has been suggested to be mediated in part thru the effects of epigenetic mechanisms on gene regulation.²⁶ For instance, DNA methylation of the cytosine residues, particularly CpG nucleotides in the 5' CpG islands can decrease gene transcription.^{27–28} Histone modification, thru lysine residue methylation or acetylation, affects the chromatin structure and regulates gene expression.^{27,29} Moreover, additional control occurs thru activation or deactivation (mostly by phosphorylation) of many of the enzymes involved in the above two mechanisms. DNA and histone changes have been shown to be induced by diet or other manipulations and can be transmitted to the offspring at the F1 and F2 levels.^{27,30,31} It is still not clear how these epigenetic marks can escape DNA demethylation and histone reorganization after normal fertilization. Interaction between micro-RNA and DNA in the gametes may be one of the mechanisms for maintenance of this epigenetic memory.²⁷ Micro-RNA are a novel class of endogenous, small, non-protein coding RNAs that inhibit gene expression by associating with 3'-untranslated regions of mRNAs, and are thought to regulate a large proportion of protein coding genes.³² They are particularly important in the control of expression of genes involved in early development. Micro-RNA also regulates heterochromatin formation which is thought to be involved in gene regulation and/or silencing.³³

In our experiment, male F2WT mice had increased contractile responses compared to female F2WT. The difference between the 2 genders was more pronounced if they were born to KOM mothers. That gender difference became non-significant after incubation with the non-selective NOS inhibitor (L-NAME). This speaks of a potential protective effect mediated by estrogen via NO. Estrogen has long been debated to have athero-protective effect. That was based on multiple epidemiological^{34,35} and experimental studies. One of the suggested mechanisms was through NO.³⁶ Estrogen been found to decrease myointimal smooth muscle cell proliferation³⁷, inhibit TNF- α induced apoptosis in endothelial cells³⁸ and induce NOS-3 in a variety of tissues³⁹ both by increasing mRNA and protein levels and by increasing NOS3 activity.^{40,41}

We did not include a P1 +/- lineage control in our experiment, rather we compared our F2 KOP and KOM to each other, but not to a completely wild type animals. This was based on our prior observation that heterozygote F1 KOP had similar vascular characteristics as WT F1 mice.¹⁰ Moreover, to obtain the second generation mice we always cross bred F1 heterozygote female mice to WT male mice. By this breeding scheme, we hoped to control for the paternal genotype and phenotype effects in addition to the maternal genotype effect. Thus the results of the experiments will be more indicative of the maternal phenotype effect i.e. the effect of the intrauterine environment that these female heterozygote mice developed in. However, not having a WT female cross bred to heterozygote male might have biased some of our results. Therefore, lineage effect may be one process that could have led to our findings.

This study showed that vascular phenotype was transmitted in a non-genetic fashion across generation. The mechanism is still not clear and needs to be elucidated. Understanding non genetic mechanisms of phenotype transmission can help to elucidate ways to prevent adult disease, impacting public health in the short and long term.

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Figure 1.

Weight in grams of second generation wild type male mice offspring born to paternal (KOP) and maternal (KOM) heterozygous mothers between 4–10 weeks of life (n=4–11 mice/group)

* denotes a probability < 0.05 for SBP in F2WT-KOM vs. F2WT-KOP

† denotes a probability < 0.05 for MAP in F2WT-KOM vs. F2WT-KOP

‡ denotes a probability < 0.05 for PP in F2WT-KOM vs. F2WT-KOP

Figure 2.

Mean blood pressure (mm Hg) measurements in the carotid artery of F2WT male mice born to KOM and KOP mothers. (S: systolic, D: diastolic, BP: blood pressure, MAP: mean arterial pressure, PP: pulse pressure) (n= 4–11 mice/group)

* The *asterisk* denotes a probability value of <0.05 for KOM-F2WT/M vs. KOP-F2WT/M, KOP-F2WT/F and KOM-F2WT/F.

Figure 3.

Concentration-response curves to phenylephrine (PE, 10^{-10} - 10^{-5} M) in the carotid artery of 14-week-old male and female F2 offspring born to KOP and KOM mothers. (n= 6–13 mice per group)

Figure 4. Concentration-response curves to phenylephrine (PE, 10^{-10} – 10^{-5} M) in the presence of L-NAME in the carotid artery of 14-week-old male and female F2 offspring born to KOP and KOM mothers. (n= 6–13 mice per group)

Figure 5. Relaxation-response curves to acetylcholine (Ach, 10^{-10} – 10^{-5} M) in the carotid artery of 14-week-old male and female F2-offspring born to KOP and KOM mothers. (n= 6–13 mice per group)

Figure 6. Relaxation-response curves to sodium nitroprusside (SNP, 10^{-10} – 10^{-5} M) in the carotid artery of 14-week-old male and female F2-offspring born to KOP and KOM mothers. (n= 6–13 mice per group)

Figure 7. Relaxation-response curves to isoproterenol (ISO, 10^{-10} – 10^{-5} M) in the carotid artery of 14-week-old male and female F2-offspring born to KOP and KOM mothers. (n= 6–13 mice per group)

Table 1

Weight in grams of second generation male mice KOP-F2WT and KOM-F2WT (n=4–11 mice/group)

Age (weeks)	KOM-F2WT	SEM	KOP-F2WT	SEM
4	10.74	1.39	19.52	2.98
5	15.21	1.39	23.28	2.67
6	18.01	0.95	26.98	1.79
7	19.92	0.94	26.88	1.97
8	22.16	0.26	25.09	0.17
9	23.15	0.30	26.83	0.11
10	24.87	0.66	27.44	0.22

Table 2

Total number of animals used in each experiment and their relative gender

		Contraction studies n (%)		Relaxation studies n (%)		
		Phenylephrine	Phenylephrine + L-NAME	Acetylcholine	Sodium nitroprusside	Isoproterenol
KOP	Total	26	18	18	18	18
	Male	13 (50)	8 (44)	9 (50)	9 (50)	8 (44)
	Female	13 (50)	10 (56)	9 (50)	9 (50)	10 (56)
KOM	Total	12	14	14	12	14
	Male	6 (50)	7 (50)	7 (50)	6 (50)	7 (50)
	Female	6 (50)	7 (50)	7 (50)	6 (50)	7 (50)

Table 3

Mean and SEM of the maximal contractile response (expressed as percentile to KCl contraction) for phenylephrine (PE) with and without L-NAME

Groups	N	PE	N	PE+L-NAME
KOP-F2WT/M	13	78.34±10.81	8	214.35±13.54
KOP-F2WT/F	13	39.71±3.34	10	180.93±10.63
KOM-F2WT/M	6	137.92±27.47	7	220.36±16.02
MOM-F2WT/F	6	49.09±7.38	7	180.17±28.34

P<0.05 when comparing PE responses in KOP-F2WT/M to KOP-F2WT/F, KOM-F2WT/M to KOM-F2WT/F, KOM-F2WT/M to KOP-F2WT/M.