

Hypertension

JOURNAL OF THE AMERICAN HEART ASSOCIATION



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Hypertension. 2012;60:378-386; originally published online June 25, 2012;

doi: 10.1161/HYPERTENSIONAHA.112.190876

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

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Print ISSN: 0194-911X. Online ISSN: 1524-4563

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Uteroplacental Insufficiency and Lactational Environment Separately Influence Arterial Stiffness and Vascular Function in Adult Male Rats

Marianne Tare, Helena C. Parkington, Kristen J. Bubb, Mary E. Wlodek

Abstract—Early life environmental influences can have lifelong consequences for health, including the risk of cardiovascular disease. Uteroplacental insufficiency causes fetal undernutrition and impairs fetal growth. Previously we have shown that uteroplacental insufficiency is associated with impaired maternal mammary development, compromising postnatal growth leading to hypertension in male rat offspring. In this study we investigated the roles of prenatal and postnatal nutritional environments on endothelial and smooth muscle reactivity and passive wall stiffness of resistance arteries of male rat offspring. Fetal growth restriction was induced by maternal bilateral uterine vessel ligation (restricted) on day 18 of pregnancy. Control offspring were from mothers that had sham surgery (control) and another group from mothers with their litter size reduced (reduced; litter size reduced to 5 at birth, equivalent to the restricted group). On postnatal day 1, offspring (control, restricted, and reduced) were cross-fostered onto control or restricted mothers. At 6 months, mesenteric and femoral arteries were studied using wire and pressure myography. In restricted-on-restricted rats, wall stiffness was increased, and sensitivity to phenylephrine and relaxation evoked by endothelium-derived hyperpolarizing factor and sodium nitroprusside were impaired in mesenteric arteries. In femoral arteries, relaxation to sodium nitroprusside was reduced, whereas wall stiffness was unaltered. Cross-fostering restricted offspring onto control mothers alleviated deficits in vascular stiffness and reactivity. Control or reduced offspring who suckled a restricted mother had marked vascular stiffening. In conclusion, prenatal and early postnatal environments separately influence vascular function and stiffness. Furthermore, the early postnatal lactational environment is a determinant of later cardiovascular function. (*Hypertension*. 2012;60:378-386.) • [Online Data Supplement](#)

Key Words: uteroplacental insufficiency ■ lactational environment ■ artery ■ arterial stiffness ■ endothelium ■ smooth muscle

Adverse environmental conditions during early life increase the risk for the development of chronic diseases in adulthood, including cardiovascular disease (CVD).¹ Fetal growth restriction is associated with increased risk of ischemic heart disease, stroke, and hypertension.^{1–4} Abnormalities in vascular function often precede the development of CVD, although the mechanisms have yet to be resolved. Vascular mechanisms that may contribute to adverse cardiovascular sequelae include alterations in endothelial and smooth muscle reactivity and arterial wall stiffness. In humans of low birth weight, endothelium-dependent vasodilation is generally impaired.^{5–7} Arterial stiffness has been identified as an important independent risk factor for CVD.⁸ An inverse relationship exists between birth weight and arterial stiffness.⁹ Prepubertal children born small have increased stiffening of carotid arteries.⁶

Intrauterine growth restriction occurs in ≈10% of pregnancies and is commonly attributed to placental dysfunction in

Western societies.¹⁰ Experimental models of uteroplacental insufficiency where uterine perfusion is reduced during the last third of pregnancy in rats yield growth restricted offspring (≈10%) that develop hypertension, and there is increasing evidence for altered vascular function.^{11,12} The early postnatal environment has also been identified as a critical period, such that rapid childhood growth in infants born small or thin is associated with endothelial dysfunction¹³ and heightened cardiovascular risk.^{14,15} The early lactational environment may also influence cardiovascular risk.¹⁶ Breastfeeding, particularly in the early postnatal period, is associated with reduced adulthood cardiovascular risk,¹⁴ although paradoxical, extended durations of breastfeeding (>4 months) may be associated with increased vessel stiffening.¹⁷

Uteroplacental insufficiency in rats impairs mammary development in the mother, resulting in lower milk production with altered composition.¹⁸ Growth-restricted male off-

Received January 9, 2012; first decision February 1, 2012; revision accepted June 5, 2012.

From the Department of Physiology (M.T., H.C.P., K.J.B.), Monash University, Clayton, Victoria, Australia; Department of Physiology (M.E.W.), University of Melbourne, Melbourne, Victoria, Australia.

The online-only Data Supplement is available with this article at <http://hyper.ahajournals.org/lookup/suppl/doi:10.1161/HYPERTENSIONAHA.112.190876/-/DC1>.

Correspondence to Marianne Tare, Department of Physiology, Monash University, Clayton Campus, Victoria 3800, Australia. E-mail Marianne.Tare@monash.edu

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Hypertension is available at <http://hyper.ahajournals.org>

DOI: 10.1161/HYPERTENSIONAHA.112.190876

spring born to uteroplacentally restricted mothers developed high blood pressure, and this was prevented after cross-fostering onto control mothers with normal lactation and improved postnatal nutrition and offspring growth.¹⁹ Although prenatal and early postnatal environments are determinants of adulthood cardiovascular risk, their separate influences on vascular function and underlying mechanisms have yet to be elucidated. Here we used cross-fostering to investigate the roles of growth restriction and lactational environment in male rats on artery stiffness and endothelial and smooth muscle function.

Materials and Methods

An expanded Materials and Methods section is available in the online-only Data Supplement.

Animals and Cross-Fostering Protocol

Surgery was performed on day 18 of pregnancy in Wistar-Kyoto rats.^{19,20} Pregnant rats were allocated to control (sham surgery) or restricted groups. The restricted group underwent bilateral uterine vessel (artery and vein) ligation to induce uteroplacental insufficiency.²⁰ All of the litters delivered at term on day 22 of pregnancy. At birth, half of the litters from the control group had their litter size reduced to 5 to match the restricted group (reduced litter; control reduced from 10–14 to 5 pups; see Figure S1).^{18,21} A cross-fostering at birth approach was used because the key aim of the study was to determine the relative contributions of the prenatal and postnatal environments in programming vascular dysfunction. Pups from each of the 3 groups, control, reduced, and restricted, were cross-fostered 1 day after birth onto a different control or restricted mother.^{19,22} Descriptions of cross-foster groups indicate pup type—cross-fostered on-mother type (ie, restricted-on-control refers to a restricted pup cross-fostered onto a control mother; refer to Figure S1). Six experimental groups were studied, including control-on-control ($n=12$ mothers), control-on-restricted ($n=11$ mothers), reduced-on-restricted ($n=7$ mothers), reduced-on-control ($n=11$ mothers), restricted-on-control ($n=9$ mothers), and restricted-on-restricted ($n=12$ mothers). One male offspring per litter per measure was studied. Systolic blood pressure was measured at 5 months by the tail-cuff method.¹⁹

Tissue Collection and Vascular Testing

When the rats were 6 months of age, small mesenteric and femoral arteries were isolated. Arteries were mounted onto a wire myograph for testing of smooth muscle and endothelial reactivity and onto a pressure myograph for assessment of passive mechanical wall properties, as described previously.^{23–25} Endothelial function was tested using acetylcholine (ACh; 10^{-10} to 10^{-6} M) in submaximally constricted (60% to 70% of maximum, with the level of preconstriction not different across all of the groups) arteries.²⁵ The contributions of NO and endothelium-derived hyperpolarizing factor (EDHF) were assessed (see the online-only Data Supplement).^{23,26}

Statistical Analysis

Please see the online-only Data Supplement. Data are presented as mean \pm SEM with “n” representing the number of animals. Data were analyzed using ANOVA with Bonferroni correction for multiple comparisons or unpaired Student *t* testing, and differences were accepted as significant when *P* value was <0.05 .

Results

Offspring Growth and Blood Pressure

Uteroplacental restriction resulted in a halving of litter size (from 10 to 5) compared with controls, as published previously.¹⁹ On postnatal day 1, body weights of restricted male

Table. Offspring Body Weight and Blood Pressure

Pup-on-Mother	Day 1 Weight, g	Body Weight 6 mo, g	5 mo Systolic Blood Pressure, mm Hg
Control-on-control	5.83 \pm 0.18*	372 \pm 8*	129 \pm 2*
Control-on-restricted	5.56 \pm 0.16*	366 \pm 6†	128 \pm 4†
Reduced-on-restricted	5.51 \pm 0.21*†	371 \pm 4*†	138 \pm 4†‡
Reduced-on-control	5.28 \pm 0.25§	361 \pm 8*†‡	133 \pm 4†‡
Restricted-on-control	4.92 \pm 0.20†‡§	345 \pm 11†‡	136 \pm 4†‡
Restricted-on-restricted	4.41 \pm 0.22‡	347 \pm 6‡	147 \pm 3‡

Data are mean \pm SEM (n=6–12).

Significant differences (*P*<0.05) across groups for each parameter are indicated by different symbols, for example, “*” is different from “†” but not different from “*†.”

offspring were lower than controls (Table). Toward the end of lactation, body weights of males from all of the groups were not different except for the restricted-on-restricted group, which were \approx 26% lighter. Restricted-on-control males experienced accelerated growth during early lactation, and reduced-on-restricted males had slowed growth during lactation. All of other groups were not different from the control-on-control group during lactation.^{19,22} At 6 months of age, restricted-on-restricted males were lighter (\approx 7%) than control-on-control offspring (Table).

By 5 months of age, restricted-on-restricted and reduced-on-restricted offspring had developed increased blood pressure (by 18 and 9 mmHg, respectively) compared with control-on-control offspring. Cross-fostering restricted males onto control mothers resulted in blood pressures that were not significantly different from those of control-on-control males (Table), similar to our previous report for this model.¹⁹

Passive Mechanical Wall Properties

Mesenteric Artery

The stress-strain relationship for arteries from the male restricted-on-restricted group was shifted to the left, indicating increased wall stiffness (*P*=0.0002; Figure 1A). Wall stiffness was restored in arteries from restricted offspring cross-fostered onto control mothers (Figure 1B). Mesenteric arteries from all of the offspring cross-fostered onto restricted mothers (ie, control-on-restricted, reduced-on-restricted, or restricted-on-restricted) were stiffer. Strikingly, wall stiffness was particularly exacerbated in offspring born of normal weight but cross-fostered onto a restricted mother (ie, control-on-restricted or reduced-on-restricted; Figure 1C and 1D). Arteries from reduced-on-control offspring were stiffer than those from the control-on-control group (*P*=0.01; Figure 1E).

Femoral Artery

In contrast to the mesenteric artery, the stress-strain relationship for femoral arteries from restricted-on-restricted offspring was not different versus control-on-control offspring (Figure 1F). As observed with mesenteric artery, wall stiffness was significantly increased in arteries from offspring born of normal weight but cross-fostered onto a restricted mother, that is, control-on-restricted (Figure 1H; *P*=0.02) and reduced-on-restricted (Figure 1I; *P*=0.002). Stress-strain

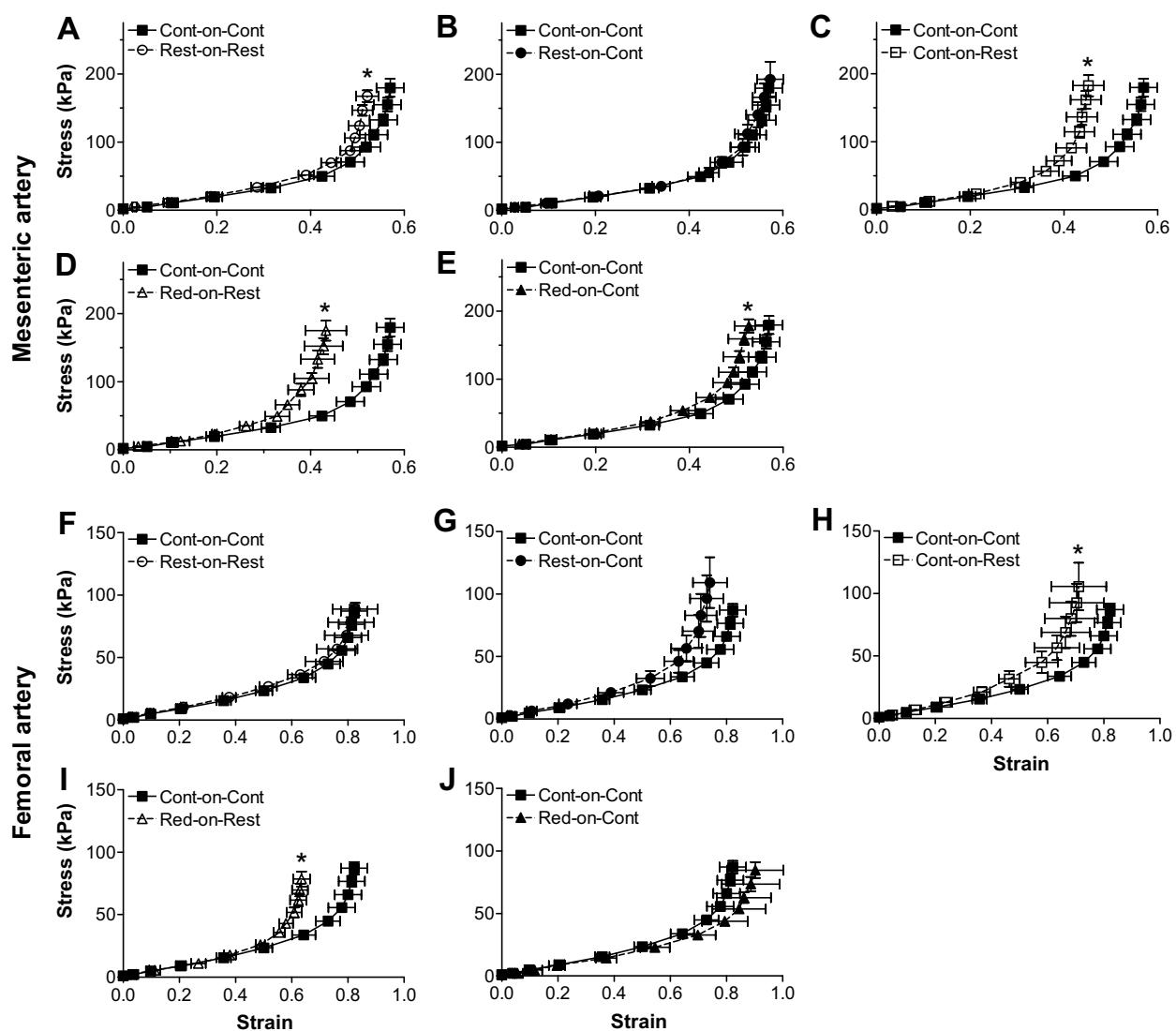


Figure 1. Passive stress-strain relationships for mesenteric (**A** through **E**) and femoral (**F** through **J**) arteries from the 6 groups of rats. For clarity, data are shown over 5 graphs, with control-on-control data appearing in every graph. Data are mean \pm SEM (n=5–12 rats per group). *Difference ($P<0.05$) between groups, ANOVA. Cont indicates control; Rest, restricted; Red, reduced.

relationships of femoral arteries from reduced or restricted offspring cross-fostered onto control mothers were not different ($P=0.06$ and $P=0.09$, respectively) from those from control-on-control offspring (Figure 1J and 1G).

Mesenteric and Femoral Artery Reactivity

Contraction

For mesenteric arteries, sensitivity to the α_1 -adrenoceptor agonist phenylephrine (pD_2) was reduced in arteries from restricted-on-restricted compared with control-on-control offspring (pD_2 , 5.54 ± 0.04 , n=10, versus 5.72 ± 0.06 , n=9; $P=0.02$; Figure 2A). Sensitivity to phenylephrine was restored in mesenteric arteries from restricted offspring cross-fostered onto control mothers (pD_2 , 5.69 ± 0.04 , n=8; Figure 2A). The sensitivity to phenylephrine in mesenteric arteries from control-on-restricted males was intermediate (pD_2 , 5.63 ± 0.03 , n=10) and not different from control-on-control or restricted-on-restricted groups (Figure 2A).

For both reduced litter groups, sensitivity to phenylephrine was intermediate, but not different, from either control-on-control or restricted-on-restricted groups (pD_2 reduced-on-control, 5.62 ± 0.06 , n=9; reduced-on-restricted, 5.62 ± 0.05 , n=5; Figure 2B). In contrast, sensitivity to phenylephrine was not altered in the femoral artery for any of the cross-foster groups (Figure 2C and 2D). Contraction evoked by high K^+ physiological saline solution was not different across any of the cross-foster groups for either the mesenteric or femoral artery (data not shown).

Relaxation

For the mesenteric artery, the maximal relaxation evoked by the NO donor sodium nitroprusside (SNP) was reduced in the male restricted-on-restricted group compared with the control-on-control group (Figure 3A; please see online-only Data Supplement). The impairment in maximal relaxation was partially restored in arteries from restricted off-

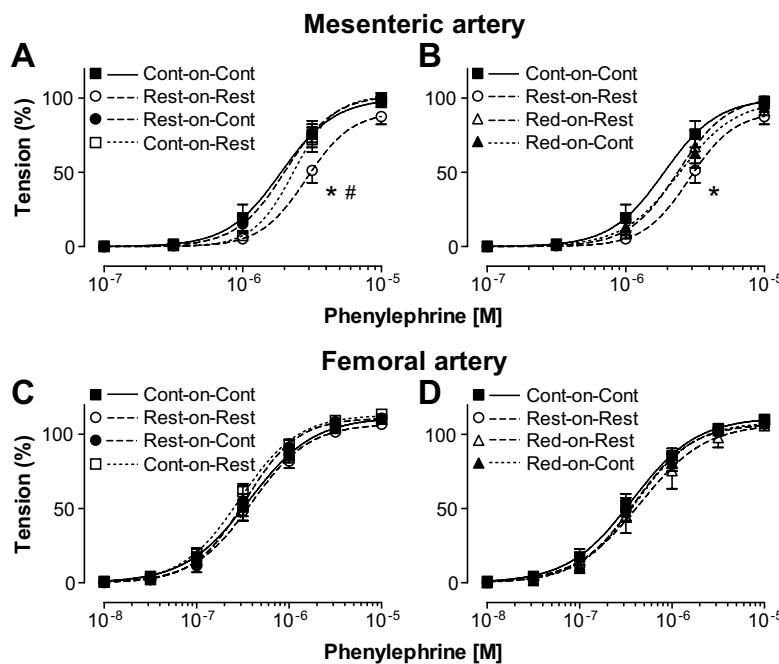


Figure 2. Contraction evoked by phenylephrine in mesenteric (**A** and **B**) and femoral (**C** and **D**) arteries from the 6 groups of rats. Data are mean \pm SEM ($n=5$ to 10 rats per group). For clarity, data for the mesenteric artery for the 6 groups of rats are divided over graphs **A** and **B** with control-on-control and restricted-on-restricted groups appearing on both graphs. For the femoral artery, data for the 6 groups of rats are divided over graphs **C** and **D**, with data for control-on-control and restricted-on-restricted groups appearing on both graphs. Difference in pD_2 ($P<0.05$) between: *control-on-control and restricted-on-restricted groups and between #restricted-on-control and restricted-on-restricted groups. Cont indicates control; Rest, restricted; Red, reduced.

spring cross-fostered onto control mothers (Figure 3B). Mesenteric arteries from control and reduced offspring cross-fostered onto restricted mothers did not have impaired maximal relaxation, but sensitivity to SNP was increased so that it was different compared with the restricted-on-restricted

group (Figure 3C and 3D). Reduced-on-control offspring had enhanced sensitivity to SNP compared with the restricted-on-restricted group; however, maximal relaxation was intermediate between control-on-control and restricted-on-restricted offspring (Figure 3D).

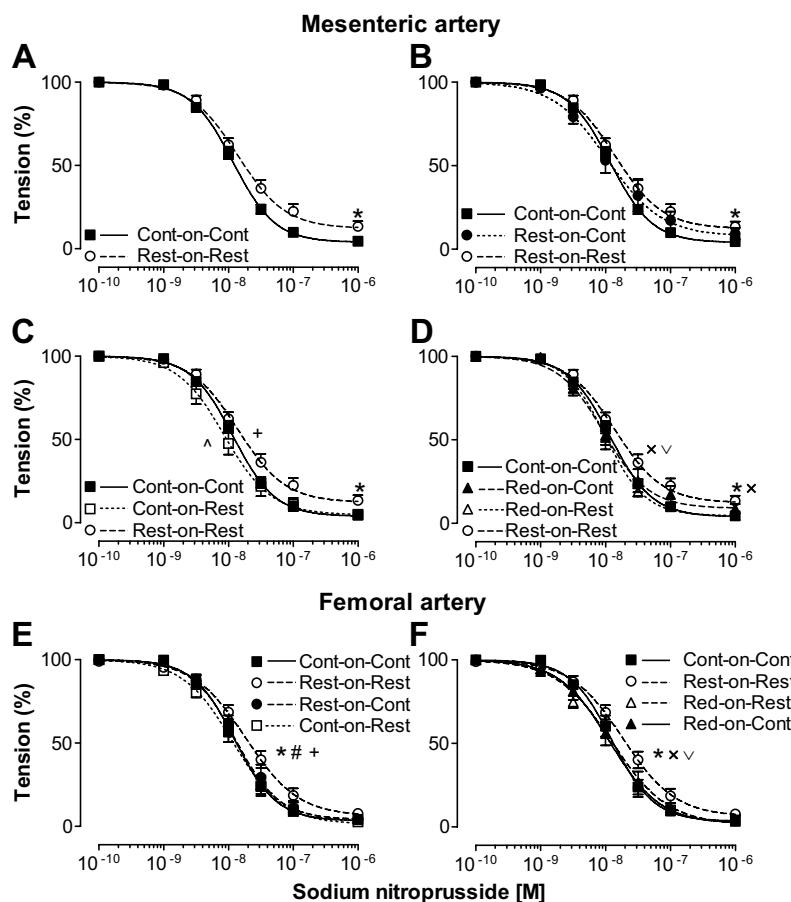


Figure 3. Smooth muscle relaxation evoked by sodium nitroprusside in mesenteric and femoral arteries from the 6 groups of rats (**A** through **F**). For clarity, the data for control-on-control and restricted-on-restricted groups appear in all of the graphs. Data are mean \pm SEM ($n=5$ –10 rats per group). Difference ($P<0.05$) in pD_2 and/or maximal relaxation between: *Cont-on-Cont and Rest-on-Rest; ^Cont-on-Cont and Cont-on-Rest; +Cont-on-Rest and Rest-on-Rest; xRed-on-Rest and Rest-on-Rest; vRed-on-Cont and Rest-on-Rest; #Rest-on-Cont and Rest-on-Rest. Cont indicates control; Rest, restricted; Red, reduced.

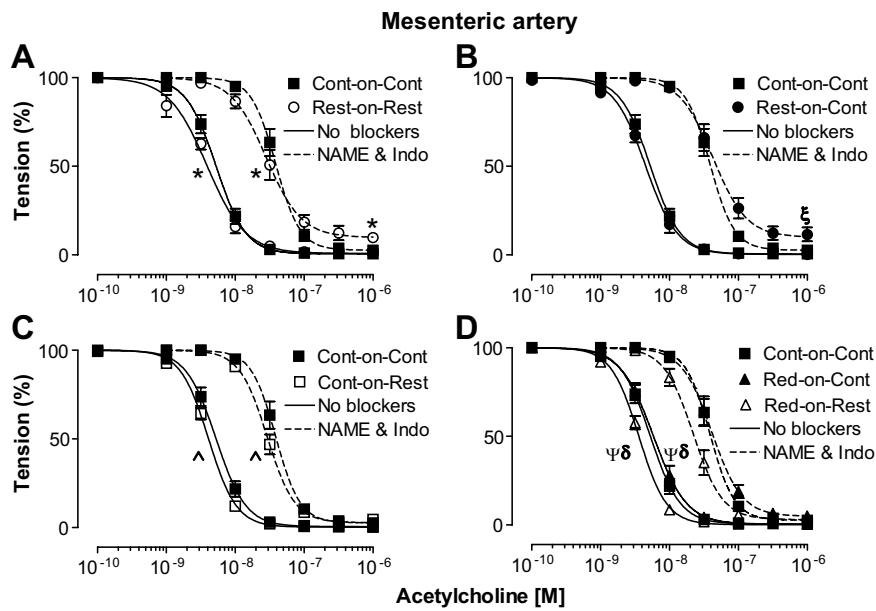


Figure 4. Endothelium-dependent relaxation evoked by acetylcholine (ACh) in mesenteric arteries from the 6 groups of rats (A through D). For clarity, data are shown over 4 graphs, with control-on-control data appearing in each graph. Responses in the absence of blockers are indicated by the solid lines. Responses in the presence of N^G -nitro-L-arginine methyl ester (NAME) and indomethacin (Indo) and attributed to endothelium-derived hyperpolarizing factor (EDHF) are indicated by the dashed lines. Data are mean \pm SEM ($n=6$ –11 rats per group). Difference ($P<0.05$) in pD_2 and/or maximal relaxation between: *Cont-on-Cont and Rest-on-Rest; ξ Cont-on-Cont and Rest-on-Cont; δ Cont-on-Cont and Red-on-Rest; \wedge Cont-on-Cont and Cont-on-Rest; ψ Red-on-Cont and Red-on-Rest. Cont indicates control; Rest, restricted; Red, reduced.

Sensitivity to SNP was reduced in femoral arteries of restricted-on-restricted compared with control-on-control offspring (Figure 3E) but was restored in arteries from restricted offspring cross-fostered onto control mothers (Figure 3E). Cross-fostering control or reduced offspring onto restricted mothers did not impair SNP-mediated relaxation in femoral arteries (Figure 3E and 3F; please see online-only Data Supplement).

Endothelium-Dependent Relaxation

Mesenteric Artery

Sensitivity to ACh was enhanced in arteries from restricted-on-restricted compared with those of control-on-control offspring (Figure 4A), and this was restored in mesenteric arteries from restricted offspring cross-fostered onto control

mothers (Figure 4B). Only arteries from groups of offspring cross-fostered onto restricted mothers exhibited enhanced sensitivity to ACh (Figure 4A, 4C, and 4D; please see online-only Data Supplement).

EDHF is an important vasodilator in mesenteric arteries, and its contribution is revealed in the presence of N^G -nitro-L-arginine methyl ester and indomethacin. Maximal EDHF-mediated relaxation was reduced in arteries from restricted-on-restricted offspring compared with control-on-control offspring (Figure 4A). Sensitivity to ACh was still enhanced in the restricted-on-restricted group. Cross-fostering restricted offspring onto control mothers did not prevent the reduction in maximal EDHF-mediated relaxation but normalized the sensitivity to ACh (Figure 5B). Sensitivity to ACh in the presence of N^G -nitro-L-arginine methyl ester and indo-

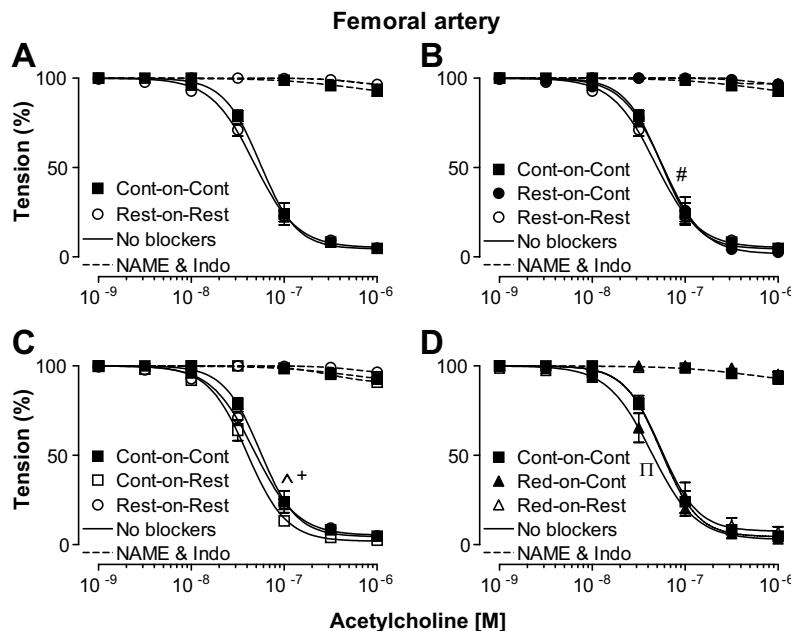


Figure 5. Endothelium-dependent relaxation evoked by acetylcholine (ACh) in femoral arteries from the 6 groups of rats (A through D). Responses in the absence of blockers are indicated by the solid lines and those in the presence of N^G -nitro-L-arginine methyl ester (NAME) and indomethacin (Indo) are indicated by the dashed lines. For clarity, data are shown over 4 graphs, with control-on-control data appearing in every graph and restricted-on-restricted data appearing in graphs A through C. Data are mean \pm SEM ($n=5$ –11 rats per group). Difference ($P<0.05$) in pD_2 between: #Rest-on-Cont and Rest-on-Rest; \wedge Cont-on-Cont and Cont-on-Rest; +Cont-on-Rest and Rest-on-Rest; IICont-on-Cont and Red-on-Cont. Cont indicates control; Rest, restricted; Red, reduced.

methacin was enhanced in arteries from all of the offspring groups cross-fostered onto restricted mothers regardless of the prenatal environment (Figure 4C and 4D). However, maximal EDHF relaxation was impaired in arteries from all of the restricted offspring groups regardless of lactational environment (Figure 4A and 4B). Analysis of the area under the curve for EDHF-dependent and EDHF-independent relaxation revealed no differences between groups.

Femoral Artery

Total endothelium-dependent relaxation in the femoral was not altered between restricted-on-restricted and control-on-control groups, although there was a tendency toward increased sensitivity in the restricted-on-restricted group ($P=0.055$; Figure 5A) that was restored in arteries from offspring cross-fostered onto control mothers (Figure 5B; please see the online-only Data Supplement). Sensitivity to ACh was increased in arteries from control offspring cross-fostered onto restricted mothers (Figure 5C) and from reduced offspring cross-fostered on control mothers (Figure 5D). In femoral arteries, endothelium-dependent relaxation was abolished in N^G -nitro-L-arginine methyl ester and indomethacin (Figure 5), indicating the lack of involvement of EDHF, as described previously.^{23,26} The contribution of NO-dependent mechanisms to total endothelium-dependent relaxation (area under the curve) was not different between groups.

Discussion

This study demonstrates that vascular stiffness and function in adulthood can be separately programmed by prenatal and early postnatal environments. Growth restriction in late pregnancy gives rise to changes in resistance artery wall stiffness and endothelial and smooth muscle function in adulthood that are not the same across all arterial beds (please see Table S4). When this prenatal insult is coupled with a poor postnatal lactational environment, alterations in vascular function are exacerbated, and these offspring develop higher blood pressures¹⁹ (please see Table S4). Importantly, improvement in the lactational environment in growth-restricted offspring restores vascular function and, as reported previously, prevents the development of high blood pressure and nephron deficit.¹⁹ This study reveals that vascular stiffness is a relatively sensitive indicator of perturbations that have occurred in the prenatal and/or the postnatal environment.

Vascular stiffness is a key independent and predictive marker of cardiovascular risk. Humans of low birth weight have increased arterial stiffness.^{6,9} The present study demonstrates that uteroplacental insufficiency is associated with regional differences in arterial wall stiffness in male offspring. Interestingly, we have reported previously that female restricted-on-restricted offspring do not have increased arterial wall stiffness in mesenteric and femoral arteries and are normotensive.²⁵ The passive mechanical wall properties of the arterial wall are mainly determined by the deposition and organization of the major extracellular proteins collagen and elastin^{27,28} and are likely to be vulnerable to early life insults.^{29,30} In the present study we demonstrate that both prenatal and postnatal nutritional environments separately

influence vascular stiffness. A poor prenatal environment is associated with increased arterial wall stiffening. Lactational environment also had a critical role in determining vascular stiffness. A poor lactational environment, underpinned by poor quality and quantity of milk and slowed postnatal growth,¹⁸ resulted in enhanced vascular stiffening. Furthermore, offspring born of normal weight but raised in reduced litters had increased vascular stiffening. The reduced suckling stimulus on the mammary gland of otherwise control mothers reduces milk production and slows postnatal growth of the offspring.¹⁸ This has implications for developmental programming studies that routinely use reduced litter groups as controls.

A striking finding to emerge from this study was that stiffness in both mesenteric and femoral arteries was increased when there was a change in prenatal versus postnatal nutritional environments but was exacerbated when the early postnatal environment was poor. Improvement of postnatal environment generally attenuated vascular stiffness in both mesenteric and femoral arteries. Alteration in postnatal nutritional factors may directly impact on vascular stiffness.^{29,31} In children born small for gestational age, exposure to overnutrition during the lactation period is associated with increased cardiovascular risk.¹⁶ Mismatch between prenatal and postnatal nutrition results in altered cardiovascular function in the offspring.^{19,32} The developmental origins of health and disease hypothesis proposes that the adaptive responses made by the fetus to adverse environments to ensure survival in utero may be detrimental for long-term postnatal survival. Our results here suggest that the “predictive-adaptive response” appears to be strong for arterial wall stiffness, particularly for the femoral artery in male offspring. Indeed, we have shown that a poor prenatal environment coupled with a poor postnatal environment or a good prenatal and postnatal environment is not associated with increased stiffness in the femoral artery. However, exposure to a poor lactational environment in normally grown offspring results in marked stiffening of the femoral artery, and this was also the case for mesenteric arteries. However, mesenteric arteries did respond slightly differently compared with the femorals in that any insult, whether it be prenatal or postnatal, did result in stiffening. Improvement of the lactational environment alleviated increased arterial wall stiffness in mesenteric arteries. This study has demonstrated that, apart from the prenatal environment, the lactational period is a critical time in the determination of vascular wall stiffness, a risk factor for CVD. This is consistent with human studies indicating that lactational nutrition and duration can influence cardiovascular health of the offspring.^{13,16,17,33}

Vascular smooth muscle and endothelial dysfunction are important factors in the etiology of CVD. As with undernutrition models of growth restriction,³⁴ the type and timing of the insult leading to placental insufficiency determine the extent and severity of vascular dysfunction and hypertension in the offspring. Growth restriction induced by clipping the maternal abdominal aorta and ovarian arteries at the beginning of the last third of pregnancy produces offspring that already have significantly increased blood pressure by 4 weeks of age.^{11,35} With maturity, sex differences emerged in

blood pressure, where growth-restricted males remained hypertensive but females did not. Aortas of growth-restricted offspring have enhanced α -adrenoceptor-mediated contraction and impaired NO-mediated endothelium-dependent relaxation, with unaltered smooth muscle guanylate cyclase function.¹¹ Using a similar model, hypertension was maintained in both sexes with maturity, and mesenteric artery contraction was enhanced, with maximal endothelium-dependent relaxation unaltered in males but sensitivity increased in females.¹² Guanylyl cyclase function was unaltered in arteries from male but was enhanced in female growth-restricted offspring.¹² This model is associated with the development of maternal hypertension during pregnancy, which is known to adversely affect fetal development and may further influence the extent of vascular dysfunction in the offspring. The model of growth restriction used in the present study was induced later in pregnancy²¹ and is not associated with maternal hypertension (unpublished observations).

In this study there was no enhancement of vascular contraction to α -adrenoceptor stimulation, but the reduction in sensitivity may reflect downregulation of receptor expression. Contraction to high potassium was unaltered, revealing that overall contractile capacity was unchanged. In our model of growth restriction, mesenteric and femoral arteries of female offspring had unaltered contraction to α -adrenoceptor stimulation or to high potassium.²⁵ For some models of maternal nutrient restriction, impaired contraction to α -adrenoceptor stimulation has also been reported in femoral arteries of young male and female offspring³⁶ and carotid arteries of fetuses.³⁷ Evidence from growth restriction and maternal undernutrition models indicates that the effect on offspring vascular contraction is variable, region specific, sex dependent, and sometimes agonist specific.^{34,36-38}

The endothelium plays a central role in the regulation of vascular tone. Endothelial dysfunction is evident in humans born small and in experimental models of growth restriction and maternal undernutrition.^{6,34} There are also some reports in humans that endothelial function is normal in young individuals of low birth weight.^{39,40} Endothelium-dependent relaxation of rat aorta is impaired in growth restriction, and this is underpinned by reduced NO production.¹¹ Conversely, total endothelial function was not impaired in mesenteric resistance arteries, although the role of the important vasodilator in this vascular bed, EDHF, was not assessed.¹² In the present study, total endothelium-dependent relaxation was not impaired in either mesenteric or femoral arteries, although there was a small increase in sensitivity to ACh in mesenteric arteries of growth-restricted offspring. Although maximal EDHF-mediated relaxation was reduced in mesenteric arteries of restricted-on-restricted offspring, sensitivity to ACh was increased, and this may contribute to the enhanced sensitivity to ACh in total endothelium-dependent relaxation. There was no indication that NO-mediated relaxation was altered in the mesenteric artery. Endothelium-dependent relaxation in the femoral artery is mediated by NO in adult rats,^{23,26} and this response was not diminished in restricted offspring. Some dysfunction was evident in the smooth muscle guanylyl cyclase pathway, as revealed by reduced

maximal relaxation and sensitivity in the mesenteric and femoral arteries, respectively, in response to the NO donor SNP. In contrast, responses to SNP in the aorta and mesenteric arteries of male growth-restricted offspring resulting from the aortic clip model were unaltered.^{11,12} Reductions in fetal nutrient and/or oxygen supply are factors that contribute to growth restriction in complicated pregnancies. Interestingly, these insults have differential effects on SNP relaxation in fetal rat aorta, with undernutrition associated with enhanced maximal relaxation and hypoxia having no effect.⁴¹ Furthermore, total endothelium-dependent relaxation was unaltered with either insult.⁴¹ That total endothelium-dependent relaxation was not impaired in either artery in the present study may also reflect an increased role of endothelial NO to maintain tissue perfusion in the presence of reduced smooth muscle responsiveness. Adult offspring exposed to 40% maternal nutrient restriction during prenatal life showed unaltered mesenteric artery endothelium-dependent relaxation or smooth muscle response to SNP,⁴² whereas those exposed to 50% to 70% maternal nutrient restriction showed impaired endothelium-dependent relaxation attributable to reduced NO contribution,³⁸ and this may reflect the increase in vascular oxidative stress and, hence, reduced NO bioavailability in such models.⁴³

Perspectives

Improving the early postnatal lactational environment and postnatal growth may, in general, be of benefit in reducing the extent of vascular dysfunction and wall stiffening. Interestingly, in normally grown offspring, compromise of the lactational environment and postnatal growth results in marked stiffening of the vessel wall. Thus, lactational environment plays an important role in shaping the vascular phenotype of the offspring, whether they are born small or normally grown. This could have implications for the antenatal nutrition of preterm neonates in intensive neonatal care units. Even in the absence of overt hypertension, the adverse changes in wall stiffness and vascular function brought about by poor prenatal or lactational environments may render the offspring vulnerable to increased CVD risk when challenged with a "second hit," including such lifestyle factors as high-salt or high-fat diets, metabolic syndrome, obesity, or even with inevitable ageing. In the absence of safe and effective interventions during prenatal life that can reduce the extent of growth restriction, a focus on the early postnatal and lactational environments may be beneficial in reducing adulthood CVD risk.

Acknowledgments

We thank Kerryn Westcott and Amy Mibus for their contributions.

Sources of Funding

We thank the National Health and Medical Research Council of Australia, the National Heart Foundation of Australia, Channel 7,

and the University of Melbourne for grant support. K.J.B. was supported by an Australian Postgraduate Award.

Disclosures

None.

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Novelty and Significance

What Is New?

- The lactational environment plays an important role in influencing the vascular phenotype, whether offspring are born small or normally grown.

What Is Relevant?

- Even in the absence of hypertension, the adverse changes in wall stiffness and vascular function programmed by poor prenatal or

lactational environments may render offspring vulnerable to increased CVD risk when challenged with a “second hit.”

- Early postnatal and lactational environments interventions may reduce CVD risk.

Summary

Prenatal and early postnatal environments separately influence vascular function and stiffness. Early postnatal lactational environment determines later cardiovascular function.

ONLINE SUPPLEMENT

UTEROPLACENTAL INSUFFICIENCY AND LACTATIONAL ENVIRONMENT SEPARATELY INFLUENCE ARTERIAL STIFFNESS AND VASCULAR FUNCTION IN ADULT MALE RATS

Marianne Tare¹, Helena C. Parkington¹, Kristen J. Bubb¹ and Mary E. Wlodek²

¹ Department of Physiology, Monash University, Clayton Campus, Victoria 3800, Australia;² Department of Physiology, The University of Melbourne, Victoria 3010, Australia.

Author for correspondence:

Marianne Tare,
Department of Physiology, Monash University, Clayton Campus, Victoria 3800, Australia.
Telephone: +61 3 9905 2557
E-mail: Marianne.Tare@monash.edu

Materials and Methods

Animals and cross-fostering protocol

Experiments were approved by The University of Melbourne Pharmacology, Physiology, Biochemistry & Molecular Biology and Bio21 Institute Animal Ethics Committee (AEC no. 05120). Wistar Kyoto rats (9-13 weeks of age) were mated and surgery was performed on day 18.^{1,2} On day 18 of gestation, pregnant rats were randomly allocated to Control (sham surgery) or Restricted groups (Fig. S1). The Restricted group underwent bilateral uterine vessel (artery and vein) ligation to induce uteroplacental insufficiency.¹ All pregnant rats delivered naturally at term (22 days). At birth, half of the litters from the Control group had their litter size reduced to five to match the Restricted group (Reduced litter, Control reduced from 10-14 to 5 pups) as we have previously demonstrated that reducing litter size at birth programs nephron deficits and hypertension.^{3,4} Pups from each of the three groups – Control, Reduced and Restricted were cross-fostered 1 day after birth onto a different Control or Restricted mother.^{2,5} Descriptions of cross-foster groups indicate pup type – cross-fostered on – mother type (*i.e.* Restricted-on-Control refers to a Restricted pup cross-fostered onto a Control mother) (Fig. S1). Six experimental groups were studied: Control-on-Control ($n=12$ mothers), Control-on-Restricted ($n=11$ mothers), Reduced-on-Restricted ($n=7$ mothers), Reduced-on-Control ($n=11$ mothers), Restricted-on-Control ($n=9$ mothers) and Restricted-on-Restricted ($n=12$ mothers) (Fig. S1). Pups were allowed to wean naturally when 35 days of age.²

Body weight and blood pressure measurements

Body weight was measured on postnatal day 1 and at 6 months. Growth rates of the male offspring during lactation have been previously published.² Systolic blood pressure was measured at 5 months by an indirect, tail-cuff method.² One male offspring per litter per measure was studied.

Tissue collection

At 6 months of age, rats were anaesthetized with an intraperitoneal injection of a solution containing Ketamine (Parnell Laboratories, Pty. Ltd., Alexandria, NSW, Australia, 50 mg/kg body weight) and Ilium Xylazil – 20 (Troy Laboratories, Pty. Ltd., Smithfield, NSW, Australia, 10 mg/kg body weight). Second and third order mesenteric arteries and distal branches of the femoral artery were isolated. Ring segments were mounted onto a Mulvany-style wire myograph for measurement of isometric tension.^{6,7} Arteries were superfused with physiological saline solution (PSS, mM: 120 NaCl, 5 KCl, 25 NaHCO₃, 1 KH₂PO₄, 11 glucose, 1.2 MgSO₄, 2.5 CaCl₂), bubbled with 95% O₂ – 5% CO₂ at 35°C. Endothelial function was tested using acetylcholine (ACh, 10⁻¹⁰-10⁻⁶M, Sigma) in submaximally constricted (60-70% of maximum, with the level of pre-constriction not different across all groups) arteries. The contributions of different factors to endothelium-dependent relaxation was evaluated by repeating the protocol in the presence of the nitric oxide (NO) synthase inhibitor N^ω-nitro-L-arginine methyl ester (L-NAME, 2×10⁻⁴M, Sigma) and cyclooxygenase inhibitor indomethacin (10⁻⁶M, Sigma). Prostanoids make only a minimal contribution to endothelium-dependent relaxation in these arteries,²³ thus NO synthase and cyclooxygenase were blocked simultaneously. The remaining relaxation was attributed to endothelium-derived hyperpolarizing factor (EDHF).

Passive mechanical wall properties

Segments of mesenteric and femoral arteries were mounted onto a servo controlled pressure myograph (Living Systems, Burlington, VT, USA) and superfused with Ca²⁺-free PSS, containing 1mM EGTA to prevent active changes in smooth muscle tone.⁷ Each artery was pressurized in 10mmHg increments from 0-200mmHg and vessel length, wall thickness and outside diameter measured at each pressure in order to calculate wall stress and strain as described previously.⁷

Smooth muscle and endothelial function

To assess contraction, phenylephrine (10^{-9} - 10^{-5} M, Sigma Aldrich, Castle Hill, NSW, Australia) was added cumulatively. Contractions were expressed as a percentage of the contraction evoked by high K⁺ PSS (isotonic replacement of Na⁺ with 100mM K⁺) at the end of the experiment. Endothelium-independent smooth muscle relaxation was tested using sodium nitroprusside (SNP, 10^{-10} - 10^{-6} M, Ajax Chemicals, Auburn, SA, Australia) applied cumulatively to arteries submaximally constricted with phenylephrine.

Endothelial function was tested in arteries submaximally constricted with phenylephrine.⁸ Increasing concentrations of acetylcholine (ACh, 10^{-10} - 10^{-6} M, Sigma) were applied cumulatively. To determine the contribution of different factors to endothelium-dependent relaxation, the endothelium was subsequently stimulated in the presence of the nitric oxide (NO) synthase inhibitor N^ω-nitro-L-arginine methyl ester (L-NAME, 2×10^{-4} M, Sigma) and cyclooxygenase inhibitor indomethacin (10^{-6} M, Sigma). Prostanoids make only a minimal contribution to endothelium-dependent relaxation in these arteries,⁶ thus NO synthase and cyclooxygenase activities were blocked simultaneously. Relaxation remaining in the presence of L-NAME and indomethacin was attributed to endothelium-derived hyperpolarizing factor (EDHF).^{6,9}

Statistical analysis

Data are presented as mean ± standard error of the mean (S.E.M.) with *n* representing the number of animals. Concentration-response curves were generated for the constrictors and vasodilators using Prism (GraphPad Software, San Diego CA, USA) and sigmoidal curves fitted to the data using the least squares method.⁷ The concentration of agonist that evoked half maximal response (EC₅₀) and the pD₂ (-logEC₅₀) and maximal response (E_{max}) were determined and compared between groups. Endothelium-dependent relaxation was also compared by calculating the area under the curve using Prism, and as described.¹⁰ Stress-strain relationships were determined as described previously.¹¹ Data were analysed using ANOVA with Bonferroni correction for multiple comparisons or unpaired Student's t-testing and differences were considered significant when *P* < 0.05.

Results

Stimulation of the endothelium evoked concentration-dependent relaxation in mesenteric and femoral arteries. Values for pD₂ and maximal relaxation (E_{max}) for total endothelium-dependent relaxation and for relaxation in the presence of L-NAME and indomethacin are shown in Tables S1&2. The responses evoked by the NO donor sodium nitroprusside are summarised in Table S3.

The effects of late gestation uteroplacental insufficiency and the lactational environment on blood pressure and smooth muscle reactivity, endothelial function and passive wall stiffness in mesenteric and femoral arteries are summarised in Table S4. Additional information pertaining to the cardiovascular phenotype of the offspring based on results reported previously² is also included in Table S4.

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Table S1. Endothelial function in mesenteric arteries

Group	Total		L-NAME & Indo		n
	pD ₂	E _{max} (%)	pD ₂	E _{max} (%)	
Control-on-Control	8.28±0.02	0.7±0.3	7.41±0.03	2.7±0.7	9
Control-on-Restricted	8.38±0.01 [†]	0.2±0.2	7.54±0.02 [†]	4.6±2.5	9
Reduced-on-Restricted	8.45±0.02 [§]	0.4±0.3	7.66±0.03 [§]	3.4±1.6	5
Reduced-on-Control	8.24±0.03	0.6±0.3	7.39±0.04	5.0±1.7	10
Restricted-on-Control	8.35±0.02	0.3±0.2	7.37±0.05	11.6±4.0 [‡]	9
Restricted-on-Restricted	8.41±0.03 [*]	1.4±0.8	7.55±0.05 [*]	9.8±2.6 [*]	10

*P<0.05 Control-on-Control vs Restricted-on-Restricted. [†]P<0.05 Control-on-Control vs Control-on-Restricted. [‡]P<0.05 Control-on-Control vs Restricted-on-Control. [§]P<0.05 Control-on-Control vs Reduced-on-Restricted. ^{||}P<0.05 Reduced-on-Control vs Reduced-on-Restricted.

Table S2. Endothelial function in femoral arteries

Group	Total		L-NAME & Indo		n
	pD ₂	E _{max} (%)	pD ₂	E _{max} (%)	
Control-on-Control	7.26±0.03	4.8±1.7	-	93.2±3.2	10
Control-on-Restricted	7.40±0.02 [†]	2.4±0.6	-	90.9±2.4	9
Reduced-on-Restricted	7.27±0.04	7.0±3.1	-	94.2±2.7	5
Reduced-on-Control	7.36±0.04 [§]	3.2±1.1	-	95.3±2.3	10
Restricted-on-Control	7.24±0.03	2.5±0.5	-	96.6±1.5	8
Restricted-on-Restricted	7.33±0.02 ^{*,‡}	5.0±0.6	-	96.6±1.6	11

*P<0.05 Restricted-on-Control vs Restricted-on-Restricted. [†]P<0.05 Control-on-Control vs Control-on-Restricted. [‡]P<0.05 Control-on-Restricted vs Restricted-on-Restricted. [§]P<0.05 Control-on-Control vs Reduced-on-Control. – indicates that a sigmoid curve could not be fitted to the data.

Table S3. Sodium nitroprusside induced relaxation in mesenteric and femoral arteries

Group	Mesenteric Artery			Femoral artery		
	pD ₂	E _{max} (%)	n	pD ₂	E _{max} (%)	n
Control-on-Control	7.93±0.02	4.5±1.1	9	7.90±0.04	4.1±1.1	10
Control-on-Restricted	8.06±0.05 [†]	5.1±2.5	8	7.93±0.04	2.7±0.5	9
Reduced-on-Restricted	8.00±0.03	4.7±1.6	5	7.90±0.04	3.5±1.1	5
Reduced-on-Control	8.03±0.06	8.7±2.9	10	7.94±0.06	3.3±1.4	9
Restricted-on-Control	7.97±0.07	9.2±3.4	9	7.89±0.05	4.3±1.5	8
Restricted-on-Restricted	7.86±0.05 ^{‡§}	13.4±3.1 ^{*§}	10	7.73±0.05 ^{*¶‡§}	7.7±2.6	10

^{*}P<0.05 Control-on-Control vs Restricted-on-Restricted. [†]P<0.05 Control-on-Control vs Control-on-Restricted. [‡]P<0.05 Control-on-Restricted vs Restricted-on-Restricted. [§]P<0.05 Reduced-on-Restricted vs Restricted-on-Restricted. ^{||}P<0.05 Reduced-on-Control vs Restricted-on-Restricted. [¶]P<0.05 Restricted-on-Control vs Restricted-on-Restricted.

Table S4. Summary of the effects of late gestation uteroplacental insufficiency and the lactational environment on cardiovascular function*

Early environment		Cardiovascular function in adulthood							
Prenatal	Postnatal (lactation)	Blood pressure	Nephron endowment	Smooth muscle function		Endothelial vasodilator dysfunction		Arterial wall stiffness	
(Pup-on-Mother)				Mes	Fem	Mes	Fem	Mes	Fem
Good <i>(Control-on-Control)</i>	Good	↔	↔	↔	↔	↔	↔	↔	↔
Good	Reduced litter on Good <i>(Reduced-on-Control)</i>	↔	↔	↔	↔	↔	↔	↑↑	↔
Good	Reduced litter on Poor <i>(Reduced-on-Restricted)</i>	↑	↔	↔	↔	↔	↔	↑↑↑↑	↑↑↑↑
Good	Poor <i>(Control-on-Restricted)</i>	↔	↔	↔	↔	↔	↔	↑↑↑↑	↑↑↑↑
Poor	Good <i>(Restricted-on-Control)</i>	↔	↔	↔	↔	↓EDHF	↔	↑	↔
Poor	Poor <i>(Restricted-on-Restricted)</i>	↑	↓	↓P, ↓S	↓S	↓EDHF	↔	↑↑	↔

* Cardiovascular phenotype based on results of present study and those reported previously.² EDHF, endothelium-derived hyperpolarizing factor; Mes, mesenteric artery; Fem, femoral artery; P, phenylephrine; S, sodium nitroprusside; ↑ response increased, ↓ response reduced, ↔ response not statistically different, compared with control. More arrows indicate greater relative severity.

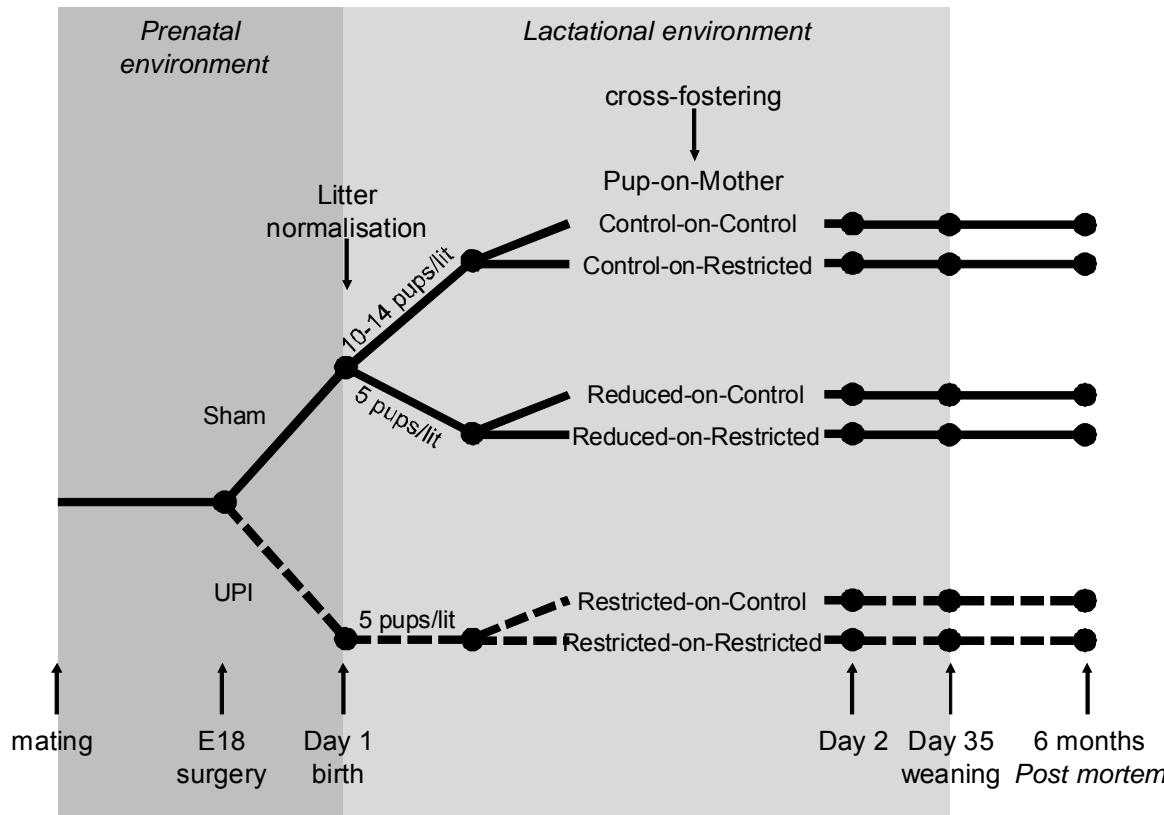


Figure S1.

Schematic representation of the experimental model. On embryonic day 18 (E18) rats designated to the Restricted group underwent bilateral uterine vessel (artery and vein) ligation to induce uteroplacental insufficiency (UPI) and rats in the Control group underwent sham surgery. At birth, half of the litters from the Control group (10-14 pups/litter) were randomly reduced to 5 pups/litter. Pups from each of the three groups – Control, Reduced and Restricted were cross-fostered 1 day after birth onto a Control or Restricted mother. Pups were allowed to wean naturally at postnatal day 35 and vascular studies were performed when the offspring were 6 months of age. Lit = litter.