Central stiffening in adulthood linked to aberrant aortic remodeling under suboptimal intrauterine conditions

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Thompson JA, Gros R, Richardson BS, Piorkowska K, Regnault TRH. Central stiffening in adulthood linked to aberrant aortic remodeling under suboptimal intrauterine conditions. Am J Physiol Regul Integr Comp Physiol 301: R1731–R1737, 2011. First published September 7, 2011; doi:10.1152/ajpregu.00274.2011.—This study examined perturbed aortic development and subsequent wall stiffening as a link to later cardiovascular disease. Placental insufficiency was induced in pregnant guinea pigs at midgestation by uterine artery ligation. Near term, fetuses were killed and defined as normal birth weight (NBW), low birth weight (LBW), and intrauterine growth restricted (IUGR). Offspring were classified according to birth weight and killed in adulthood. Collagen and elastin content of aortas were analyzed using Sirius red and orcein staining, respectively. Immunofluorescence was used for detection of α-actin and nonmuscle myosin heavy chain (MHC-B), a marker of synthetic-type vascular smooth muscle cells (VSMCs). Ex vivo generation of length-tension curves was performed with aortic rings from adult offspring. Relative elastic fiber content was decreased by 10% in LBW and 14% in IUGR compared with NBW fetuses. In adulthood, relative elastic fiber content was 51% lower in LBW vs. NBW, and the relative elastic laminae adjusted for wall thickness was 25% lower in LBW (P < 0.01). The percent area stained for MHC-B was sixfold higher in LBW vs. NBW fetuses (P < 0.0001) and threefold higher in LBW vs. NBW adult offspring (P < 0.05). The increase in MHC-B in LBW offspring concurred with a 41% increase in total collagen content and a 33 and 56% increase in relative and total α-actin content, respectively (P < 0.05). Thus aortic wall stiffening in adulthood can be traced to altered matrix composition established under suboptimal intrauterine conditions that is amplified postnatally by the activity of synthetic VSMCs.

aorta; elastin; collagen; compliance

SEMINAL EPIDEMIOLOGICAL STUDIES over the past two decades have established a link between the fetal experience and long-term health. Suboptimal conditions in the womb commonly arise from an interference in substrate delivery and availability that prevents the fetus from sustaining its growth trajectory. Such is the case of placental insufficiency, whereby abnormalities in the placental exchange surface impede maternal-fetal blood flow and frequently lead to chronic fetal hypoxemia (24, 30, 38). This antenatal condition accounts for 60% of neonates who are identified as intrauterine growth restricted (IUGR) in the developed world (17). Morphometric indexes of growth impairment are predictive of a number of chronic diseases in adulthood, among them cardiovascular disease (CVD) (3, 13, 25). Despite widespread knowledge of this prebirth risk factor, the developmental disturbance that renders the low birth weight (LBW) fetus vulnerable to later CVD is currently ill-defined.

In pregnancies complicated by placental insufficiency, fetal compromise apparent as slowed and disproportionate growth manifests in the second half of gestation, when rapid growth and maturation of organs are heavily dependent on delivered substrates. This coincides with an important phase of arterial remodeling, during which occurs time- and site-dependent deposition of extracellular matrix (ECM) proteins and transition of vascular smooth muscle cells (VSMC) from synthetic to contractile-type cells. These processes establish the mechanical and functional properties required for hemodynamic homeostasis in extrauterine life (6, 5, 45). Interesting, the accumulation of elastin, which predominates in large arteries (4), is limited to a brief window that spans late gestation and the early neonatal period (6, 5, 28). The resultant high elastin content of the proximal circulation imparts these vessels with a high degree of compliance that is critical for long-term cardiovascular health, since it dampens and synchronizes the pressure waves generated by cardiac ejection. In fact, central arterial stiffening due to altered composition of the ECM is a strong and independent predictor of CVD (1, 7). Given that elastin content is fixed upon conclusion of developmental remodeling (28, 40) and that fetal hypoxemia alters known regulators of ECM deposition (15, 44), permanent central arterial stiffening may arise from aberrant arterial formation in utero in fetuses growing under placental insufficiency.

Perturbed arterial development and subsequent wall stiffening as a mechanism for intrauterine programming of cardiovascular sequelae was explored in the present study. Growth of the fetal guinea pig was impaired by means of an established model of placental insufficiency, whereby placental blood flow is reduced throughout the second half of gestation by uterine artery ligation (UAL) (19, 20). The effects of growth restriction on aortic development in the near-term fetus were assessed by measurement of wall dimensions, ECM protein composition, VSMC content, and phenotype. Adult growth-restricted offspring were studied to determine the permanence of any structural abnormalities of the aorta established in utero and its elastic properties via measurement of the length-tension relationship ex vivo.

MATERIALS AND METHODS

Animal model and surgery. All surgical and experimental protocols were approved by The University of Western Ontario Animal Use Subcommittee. Chronic placental insufficiency was induced in time-
mated guinea pigs by UAL. This technique is commonly used to impair intrauterine growth in rodents (2, 9), since it depletes uterine capacity leading to discordant fetal growth within litters and variable fetal growth restriction (14, 19, 20). Pregnant guinea pigs at 28–30 days of gestation (term ~67 days) were induced in an anesthetic chamber (4–5% isoflurane with 2 l/min O2; followed by 2.5–3% isoflurane with 1 l/min O2 for maintenance). The volume of the anesthetic chamber was 3 liters. Immediately after induction, a subcutaneous injection of Robinulin (glycopyrrolate, 0.01 mg/kg; Sandoz Canada, Montreal, QC) was administered. A midline incision was made below the umbilicus to retrieve the mesometrium associated with one horn of the uterus, and subsequently UAL was performed at the base of the arterial arcade. To maximize fetal survival, the uterine horn with the smallest number of embryos was ligated. The ligature remained in place for the duration of the experiment, and fetuses from the unooperated horn served as control. A subcutaneous injection of Temgesic (buprenorphine, 0.025 mg/kg; Schering-Plough, Kenilworth, NJ) was administered immediately following surgery.

At 63–66 days, UAL pregnant guinea pigs were sedated with an intramuscular injection of Versed (midazolam, 5 mg/kg; Sandoz Canada, Boucherville, QC), and, after 10 min, an intramuscular injection of Vetalar (ketamine, 50 mg/kg; Bioniche Animal Health Canada, Belleville, ON) together with Rompun (xylazine, 3 mg/kg; Bayer, Toronto, ON) was administered for anesthesia. Subsequently, Robinulin was injected subcutaneously (0.025 mg/kg) and 2% Xylocaine (lidocaine; AstraZeneca Canada, Mississauga, ON) was injected along the incision line previously made during surgery. An adjacent incision was then made below the umbilicus, and the fetuses were removed and treated with Vetalar as above. After caesaerian section, the mother was killed with Euthanyl Forte (pentobarbital sodium; Bimeda-MTZ Animal Health, Cambridge, ON) by intracardiac injection. The placement of the ligature was confirmed at autopsy. Body weight and the brain-to-liver ratio of each fetus were measured.

An additional three ligated pregnant guinea pigs were allowed to deliver spontaneously at term, at which time the pups were weighed and then returned to their mothers. At 20 days, guinea pig offspring were weaned, separated by sex, placed on standard chow, and housed in group cages in a temperature (18°C)- and humidity (30%)-controlled environment. From the time of birth, guinea pig offspring were weighed weekly. At 13–15 mo of age, which corresponds to midadulthood (21), offspring were killed by intracardiac injection of Euthanyl Forte (pentobarbital sodium; Bimeda-MTZ).

**Fetal and adult groupings.** To preserve the integrity of fetal tissue by rapid organ collection during caesaerian section, only the most medial and lateral fetus were studied with horns of more than three fetuses. Groupings of late-gestation fetuses were based on morpho-metric indexes of fetal growth. Fetuses were defined as normal birth weight (NBW) if their body weights were within the 25th and 75th percentile of all fetuses, LBW if their body weights were below the 25th percentile and brain-to-liver ratios were below the group median, and IUGR if their body weights were below the 25th percentile and brain-to-liver ratios were above the group median. The group median included all fetuses of each litter. The range of fetal weights used in the present study for classification into appropriate for NBW, LBW, and IUGR are comparable to those used by other studies (9, 26). The group median of the brain-to-liver ratio, including all fetuses, was 0.85; a ratio above this value has been previously defined as IUGR in guinea pigs (10). For groupings of offspring, the smallest pups in the ligated uterine horn were considered growth impaired, and the remaining pups were considered NBW.

**Staining for collagen and elastin in fetal and adult aortas.** Aortas of fetal guinea pigs were perfusion fixed in situ with 4% paraformaldehyde at physiological pressure via the left ventricle, and subsequently a segment of ~1.5 cm was excised where the aorta becomes straight as the descending thoracic aorta, immediately distal to the aortic arch, for histological analyses. Aortas of adult offspring collected from the same anatomical location were placed in 4% paraformaldehyde for fixation. Fixed aortas from fetal and adult guinea pigs were cut into cross sections, embedded in paraffin, and cut in 5-μm cross sections that were baked onto positively charged glass plates by heating in a 50°C oven for 2 days. After deparaffinization, aortic cross sections were rehydrated by passage through a decreasing ethanol series. Collagen content was measured in cross sections stained with 1% Sirius red F3BA (Sigma-Aldrich Canada, Oakville, ON) in a saturated aqueous solution of picric acid for 1 h. Additional aortic sections were stained 30 min in 0.2% orcein (Sigma-Aldrich Canada) for identification of elastic fibers. Stained cross sections were captured on a microscope (Leica DM RB) at ×423 magnification. Duplicates of two to three cross sections per animal and five to six areas per cross section were used for analysis, which was blinded to the operator. Wall thickness was measured as the distance between the internal and external elastic laminae. For collagen quantification, the tunica media was selected, whereas elastin content measurement included both the tunica media and the internal elastic lamina. The area positive for protein (elastin or collagen) was identified by color thresholding using image analysis software (Image Pro 6.0; MediaCybernetics, Bethesda, MD) and expressed relative to the sum of the area nonstained. Total protein content (elastin or collagen) was calculated by multiplying the average wall thickness for each vessel by the percent area stained, as performed by previous studies (23). In orcein-stained cross sections, the individual elastic laminae from internal to external elastic lamina were counted.

**Immunofluorescence staining for α-actin and myosin heavy chain-B in fetal and adult aortas.** Additional deparaffinized and rehydrated aortic cross sections were incubated at room temperature for 10 min in Background Sniper (Biocare Medical, Brampton, ON) for blockage of nonspecific binding, followed by incubation with primary antibodies diluted in Universal Antibody Diluting Solution (Dako Canada, Burlington ON) in a humidified chamber at 4°C overnight: 1:4,000 dilutions of monoclonal mouse immunoglobulin G α-actin antibody (Boehringer Ingelheim, Mannheim, Germany) along with 1:2,000 dilutions of polyclonal mouse immunoglobulin G α-actin antibody (Boehringer Ingelheim, Mannheim, Germany) for identification of elastic fibers. Stained cross sections were captured on a microscope (Leica DM RB) at ×423 magnification using a camera and software for image capture and analysis (Axiovision 4.0; Carl Zeiss Microimaging, Thornwood, NY). For each cross section, 10–12 fields were analyzed. The area positive for staining was identified by color thresholding using image analysis software (Image Pro 6.0; MediaCybernetics). The sum of area stained for α-actin and MHC-B and the number of cells stained with Sytox Green (Invitrogen Life Technologies). Replacement of the primary antibody with PBS or IgG was used as a negative control. Fluorescence VectaShield mounting medium (Vector Laboratories, Burlington, ON) was used for mounting. Slides were stained in duplicate and simultaneously to minimize variation in staining intensity, and all analyses were performed blinded to the operator. All antibodies were tested for specificity by Western blot. Sections were imaged on a microscope (Zeiss) and captured at ×423 magnification using a camera and software for image capture and analysis (Axiovision 4.0; Carl Zeiss Microimaging, Thornwood, NY). For each cross section, 10–12 fields were analyzed. The area positive for staining was identified by color thresholding using image analysis software (Image Pro 6.0; MediaCybernetics). The sum of area stained for α-actin and MHC-B and the number of cells stained with Sytox Green were expressed as a percentage of an area of constant dimension within the media.

**Length-tension relationship in adult aortas.** Immediately after death, an aortic segment excised just distal to the segment used for histology was immediately placed in ice-cold Krebs solution (in mM: 118 NaCl, 25 NaHCO3, 1.11 d-glucose, 4.71 KCl, 2.56 CaCl2·2H2O, 1.13 NaH2PO4 2H2O, 1.12 MgCl2·6H2O, 0.114 ascorbic acid, and 0.0297 mM disodium EDTA) for in vitro measurement of compliance, as previously described for rats (22). Three aortic rings from each animal were mounted isometrically on two parallel stainless steel wires, one connected to a micrometer for fine distance adjustments and the other connected to a force transducer (FT30; Grass Instru-
Twelve guinea pig offspring born from three surgically prepared pregnant sows were studied. The birth weights of guinea pig offspring are shown in Fig. 2. The smallest pups had birth weights ~20% less than the NBW pups and comparable to the late-gestation LBW fetuses and were thus defined as LBW (n = 5). The remaining pups of the litter were NBW (n = 7). During the postnatal period, LBW offspring exhibited catch-up growth that occurred by ~140 days (Fig. 2). In later adulthood, at the time of death (14 mo), the body weights of NBW offspring were not significantly different from that of LBW offspring (1.0 ± 0.1 vs. 1.0 ± 0.1 kg).

Impaired fetal growth in utero leads to deviations in aortic structure that are magnified in adulthood. The relative area positive for elastic fiber staining and the number of elastic lamellae adjusted for media thickness in aortas of late-gestation fetuses and adult offspring are shown in Fig. 3. In late gestation, relative elastic fiber staining in the aorta was reduced by 10 and 14% in LBW and IUGR fetuses, respectively, compared with NBW fetuses (P = 0.14). The total number of elastic laminae in late-gestation fetuses was 14.6 ± 0.8 for the NBW group, 13.4 ± 0.4 for the LBW group, and 13.2 ± 0.5 for the IUGR group. The total number of elastic laminae relative to media thickness was similar between fetal groups. Media thickness was 69.0 ± 0.1 μm in NBW fetuses, 58.5 ± 3.7 μm in LBW fetuses, and 53.9 ± 3.3 μm in IUGR fetuses.

In adulthood, relative elastic fiber staining of the aorta in LBW offspring was 51% lower than that of NBW offspring (P < 0.01). A significant interaction between the effects of NBW and LBW and fetal and adult age groups (P < 0.01) was observed.

RESULTS

UAL leads to fetal growth impairment and postnatal catch-up growth. Twenty six late-gestation fetuses were studied (NBW: n = 12; LBW: n = 8; IUGR: n = 6). Body weights and brain-to-liver ratios of fetuses are shown in Fig. 1. Birth weight was reduced by 25 and 40% in LBW and IUGR fetuses, respectively, compared with NBW animals, and the brain-to-liver ratio was increased by 38% in IUGR vs. NBW fetuses. LBW adult offspring had birth weights 18% lower than their NBW counterparts. All fetuses but one in the IUGR group were derived from the ligated horn.
shown with two-way ANOVA. Relative to NBW, LBW adults had a smaller total number of elastic lamellae (14.5 ± 0.9 in LBW and IUGR fetuses, respectively, although not significantly (P = 0.14) (A). The ratio of elastic laminae to media thickness was 25% lower in LBW compared with NBW adult offspring (P < 0.01), whereas media thickness and the ratio of media thickness to body weight were similar between groups. Permanence in the total number of elastic lamellae between late gestation and adulthood was demonstrated in that the total number of elastic lamellae was similar in normal growth fetuses and adult animals: 14.6 ± 0.8 in NBW late-gestation fetuses and 14.5 ± 0.9 in NBW adult offspring.

In both LBW and IUGR late-gestation fetuses, the relative area positive for collagen staining was increased by 100% relative to that of NBW fetuses (NBW: 0.69 ± 0.14; LBW: 1.46 ± 0.40; IUGR: 1.38 ± 0.29, P = 0.06). Compared with NBW, total collagen content was increased by 29 and 18% in LBW and IUGR fetuses, respectively (NBW: 18.10 ± 2.49; LBW: 23.42 ± 4.65 (P = 0.06); IUGR: 21.28 ± 2.49). In adulthood, no differences were observed for relative collagen content, whereas total collagen content was increased by 41% in LBW relative to NBW offspring (P = 0.1).

There were no differences in the percent area stained for α-actin in the aortas of late-gestation fetuses (NBW: 46.3 ± 4.2; LBW: 46.2 ± 6.9; IUGR: 48.9 ± 4.1). The percent area stained for MHC-B was increased sixfold in the LBW group compared with NBW fetuses, but no significant differences were found between NBW and IUGR fetuses (Fig. 4). The percent area stained for α-actin in cross sections of aortas from adult offspring are shown in Fig. 5. In aortas of adult LBW offspring, the percent area stained for α-actin was 33% higher and the total α-actin content was 56% higher compared with the NBW group (P < 0.05). The number of cells per area within the media was 2.0 ± 0.0 for the NBW adults and 1.6 ± 0.0 for the LBW group. Figure 4 shows a threefold increase in the percent area stained for MHC-B was observed in LBW relative to NBW offspring (P < 0.05). There was no significant interaction between treatment and age group with respect to staining of MHC-B.

Compliance is reduced in aortas of LBW offspring. The length-tension curve was shifted to the left in LBW adult offspring compared with NBW adult offspring (Fig. 6).

**DISCUSSION**

This study is the first to demonstrate, experimentally, a link between aberrant arterial development in the fetal guinea pig under substrate deprivation and central arterial stiffening in adulthood. We showed adult guinea pigs born of LBW to develop increased aortic stiffness that is a consequence of altered media composition likely originating in utero. The marked reduction in relative elastic fiber content observed in LBW adult offspring was present to a lesser degree in LBW and IUGR near-term fetuses. This apparent postnatal magnification of the subtle offset in balance between the elastic and stiff wall components was due to media hypertrophy without concomitant deposition of elastin proteins. The high expression of α-actin in adult LBW offspring suggests that VSMC proliferation or hypertrophy occurred within the aortic media. LBW offspring also displayed increased collagen content along with an abundance of embryonic-type VSMCs that are capable of synthesizing ECM proteins. The striking increase in staining for the marker of these synthetic VSMCs was also present in LBW fetuses, suggesting that a delayed VSMC maturation in utero leads to permanent phenotypic characteristics in the offspring. Our measurement of reduced aortic compliance in LBW offspring by ex vivo generation of length-tension curves substantiates the positive correlations between birth weight and arterial compliance previously reported in human children, adolescents, and adults (8, 12, 32, 41). Furthermore, our data suggest that aortic dysfunction in LBW offspring is linked to structural and cellular defects programmed by intrauterine deficiency that persist and are magnified postnatally. Thus, we provide evidence for a mechanism underlying the high risk of hypertension and CVD repeatedly reported in LBW human adults (3, 13, 27, 29, 33, 34).
Elastin precursors are synthesized during a brief developmental window, predominantly in proximal arteries, and once deposited as insoluble proteins will endure the lifetime of an individual (28, 40). These proteins comprise 90% of elastic fibers, which bear circumferential tension at low distending pressures, affording properties of distension and recoil of the vascular wall (39). Given that transfer of intravascular load from elastin to collagen occurs over the physiological pressure range, the relative reduction in elastic fiber content evident in LBW offspring results in collagen recruitment at lower distending pressure and wall stiffening. The reduced elastic fiber content in adult life may be remnant of changes originating in utero. We observed a subtle decrease in the total number of elastic laminae and relative content of elastin fibers in late-gestation fetuses that were graded in relation to severity of growth impairment, the latter associated with a reduced thickness of the elastic laminae as the number of elastic laminae remained normalized to wall thickness. A disturbance in elastin deposition is permanent, since these proteins are not appreciably synthesized in postnatal life once developmental remodeling is complete (28, 40). The stability of elastic components was demonstrated in the present study, since the total number of elastic laminae was comparable between LBW fetuses and NBW adults. A fixed ECM ratio established before birth that is slightly deficient in elastic components, together with accumulation of other wall constituents during normal postnatal arterial growth, likely accounts for abnormal ECM composition and associated aortic stiffening in later life. The present study did not allow for examination of an adult IUGR group, yet a greater degree of structural changes translating to a further stiffening of the aorta is likely in such offspring. Nevertheless, evident stiffening in the moderately growth-impaired adult offspring speaks to the potency of placental intrauterine insults as a trigger for progression of cardiovascular pathology.

Dynamic elasticity of the aorta and its major branches provided by passive mechanical properties of ECM proteins dampen pressure oscillations generated by ventricular ejection, thereby minimizing energetic demands placed on the heart (1). A compromise in this buffering function of the proximal circulation leads to progressive hemodynamic disturbance and cardiac malfunction. In fact, noninvasive indexes of aortic stiffness are independent and
time in an individual and across generations is a change in the epigenotype. Evidence suggests VSMC phenotype to be regulated at the level of chromatin and thus susceptible to epigenetic modifications (31, 36). With respect to the discrepancy in cellular response in moderately vs. severely growth-restricted fetuses observed in the present study, we have previously shown changes in gene expression and protein deposition in the aorta to be dependent on the severity of intrauterine hypoxia, and this dose-response relationship was varied with the outcome measured (42). Thus, intracellular signals regulating VSMC differentiation may be differentially affected by the level of hypoxia or oxidative stress reached in the IUGR fetuses, and this may account for the maintenance of VSMC maturation despite slowed overall growth in this group. Currently, comparisons between symmetrically and asymmetrically growth-restricted fetuses in terms of developmental outcomes have not been investigated.

In conclusion, placental insufficiency in the pregnant guinea pig results in a reduction in relative elastic fiber content of the fetal aorta that is related to the severity of growth restriction, a finding that agrees with recent studies in the hypoxic sheep fetus (43). The current study is novel in that it examines both the immediate and long-term effects of fetal growth impairment on aortic structure and function. In so doing, the subtle reduction in elastic fiber content exhibited by the growth-restricted fetus was shown to be amplified later in adulthood, and this was associated with a decreased compliance of the aorta. Aortas of growth-impaired offspring also deviated from those of NBW offspring with respect to the content of collagen and VSMCs as well as VSMC phenotype. Our data suggest that altered VSMC phenotype in the LBW offspring derives directly from an interference in VSMC maturation in utero.

**Perspectives and Significance**

This study provides evidence that fetal growth impairment is associated with changes in media composition and cellular properties of the aorta that persist postnatally and lead to reduced compliance in adulthood. Thus, we have identified a developmental disturbance that may be key in the programming of cardiovascular pathology that is known to occur in LBW human offspring. Clinical inferences drawn from this model with respect to phenotypic outcomes are validated in that the timing of developmental processes are likely similar between the guinea pig and human, since both are precocious developers. Identification of phenotypes consequent to particular prenatal insults is the first step in testing prenatal and postnatal therapeutic targets for the LBW and IUGR infant. The potential of early and aggressive intervention in offspring of complicated pregnancy has not been realized, since this population has essentially been omitted in current CVD risk estimation, treatment, and preventative strategies. Hence, further investigation with use of animal models in the characterization of cardiovascular profiles is required to address these individuals who are born susceptible.

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