Coronary endothelial function and vascular smooth muscle proliferation are programmed by early-gestation dexamethasone exposure in sheep

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Volk KA, Roghair RD, Jung F, Scholz TD, Lamb FS, Segar JL. Coronary endothelial function and vascular smooth muscle proliferation are programmed by early-gestation dexamethasone exposure in sheep. Am J Physiol Regul Integr Comp Physiol 298: R1607–R1614, 2010. First published March 24, 2010; doi:10.1152/ajpregu.00824.2009.—Exposure of the early-gestation ovine fetus to exogenous glucocorticoids induces changes in postnatal cardiovascular physiology. We sought to characterize coronary artery vascular function in this model by elucidating the contribution of nitric oxide and reactive oxygen species to altered coronary vascular reactivity and examining the proliferative potential of coronary artery vascular smooth muscle cells. Dexamethasone (dex, 0.28 mg·kg⁻¹·day⁻¹ for 48 h) was administered to pregnant ewes at 27–28-day gestation (term 145 days). Coronary arteries were isolated from 1- to 2-wk-old dex-exposed offspring and aged-matched controls. Compared with controls, coronary arteries from dex-exposed lambs demonstrated enhanced vasoconstriction to endothelin-1 and ACh that was abolished by endothelial removal or preincubation with the nitric oxide synthase inhibitor L-NNA, membrane-permeable superoxide dismutase + catalase, or apamin + charybdotoxin, but not indomethacin. The rate of coronary vascular smooth muscle cell (VSMC) proliferation was also significantly greater in dex-exposed lambs. Protein levels of the proliferating cell nuclear antigen were increased and α-smooth muscle actin decreased in dex-exposed coronary VSMC, consistent with a proliferative state. Finally, expression of the NADPH oxidase Nox 4, but not Nox 1, mRNA was also decreased in coronary VSMC from dex-exposed lambs. These findings suggest an important interaction exists between early-gestation glucocorticoid exposure and reactive oxygen species that is associated with alterations in endothelial function and coronary VSMC proliferation. These changes in coronary physiology are consistent with those associated with the development of atherosclerosis and may provide an important link between an adverse intrauterine environment and increased risk for coronary artery disease.

vascular reactivity; reactive oxygen species; nitric oxide

BEGINNING WITH EPIDEMIOLOGIC examination of birth weight data (2, 3), and progressing through numerous animal studies (16), research regarding developmental programming of adult-onset diseases has expanded greatly in the past 25 years. There are substantial published data indicating that an adverse intrauterine environment predisposes to the onset of adult diseases that include, but are not limited to, hypertension, atherosclerosis, diabetes, and metabolic syndrome (16). Two models commonly employed in this area of research are altered maternal nutrition and augmented steroid hormone exposure. The phenotypic consequences of either of these challenges are surprisingly similar, leading to the hypothesis that they may share key common mechanisms (6, 10, 29).

Understanding the mechanisms that link maternal conditions and disease in the offspring is confounded by the complexity and interrelations of diseases like hypertension and atherosclerosis. One difficulty lies in isolating primary, underlying defects and separating them for secondary effects of the resultant phenotype. For example, hypertension may result directly from endothelial dysfunction or another cause that then contributes to endothelial dysfunction. It is, therefore, advantageous to study offspring at a young age, prior to the development of overt phenotypes, to identify primary defects that have been programmed in utero.

We have examined an ovine model of developmental programming that utilizes brief exposure of ewes to exogenous steroids early in gestation and results in significantly elevated blood pressure in the offspring at 4 mo (6, 22, 25). When studied during the first 2 wk following birth, prior to the development of hypertension, these lambs display marked alterations in vascular reactivity and autonomic function (25, 30). An intriguing observation is the disparity in vasoreactive responses among various vascular beds. Compared with control animals, coronary artery segments from dex-exposed lambs display an increased vasoconstrictive response to endothelin-1 (25), whereas mesenteric and femoral artery responses are decreased (30). These divergent responses may be related, in part, to vessel-specific differences in the nitric oxide-cGMP pathway. Our findings, and those from other laboratories [reviewed by McMillen and Robinson (16)], have suggested that nitric oxide availability and endothelial function are early effectors leading toward hypertension and coronary artery dysfunction (30). Studies from other investigators have also suggested an important role for altered nitric oxide and reactive oxygen species (ROS) signaling in systemic vascular dysfunction in programmed animals, although examination of pathways in the coronary vasculature from these animals is limited (8, 9, 21).

In the present study, we sought to further characterize coronary artery vascular function in early-gestation dexamethasone-exposed lambs. Specifically, the contributions of nitric oxide and ROS to altered vascular reactivity were examined. Because of its role in atherosclerosis, the proliferative potential of coronary artery vascular smooth muscle cells (VSMC) was also explored.

MATERIALS AND METHODS

Sheep model. All procedures were performed within the regulations of the Animal Welfare Act and the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the University of Iowa Animal Care and Use Committee. Ewes were bred and maintained at the Iowa State University Agricultural Station, as described previously (30). Exogenous steroid exposure of the pregnant ewe was accomplished by infusion of dexamethasone (0.28 mg·kg⁻¹·day⁻¹; Gensia Sicor Pharmaceuticals, Irvine, CA) on days

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27 and 28 of gestation (term being 145-day gestation) via a jugular venous catheter. The ewes were allowed to deliver naturally. Ewes and offspring were transferred to the University of Iowa Animal Care Unit prior to experiments being performed.

Coronary artery ring myography. Between 8 and 15 days after birth, the lambs were euthanized with intravenous pentobarbital sodium (50 mg/kg; Abbott Laboratories, Abbott Park, IL). The circumflex coronary artery was quickly harvested, and the loose adventitia and connective tissue were removed before sectioning into 3-mm rings. In some artery segments, the endothelium was removed by rubbing with a rubber policeman. The rings were mounted in individual 18-ml isolated water-jacketed chambers, and contractile forces were measured using 32-gauge wires connected to an isometric force transducer. Contractile responses were recorded with Powerlab software (ADInstruments, Colorado Springs, CO) and stored on an Apple computer. The circulating bathing solution, a bicarbonate-buffered physiological salt solution (PSS) was kept at 37°C and bubbled with 95% O2-5% CO2 to maintain a pH of 7.35. The composition of PSS was (in mM) 130 NaCl, 4.7 KCl, 1.18 KH2PO4, 1.17 MgSO4, 14.9 NaHCO3, 1.6 CaCl2, 5.5 dextrose, and 0.03 Na2-EDTA. The artery rings were allowed to equilibrate for 1 h at a passive tension of 0.7 g before the start of the experiments as previously reported (25). Contractions were first elicited with 120 mM KCl to provide a normalization value for subsequent contractile responses. After the KCl response was recorded, the artery rings were washed extensively with PSS and reequilibrated to baseline for 1 h. Dose-response curve to cumulative additions of endothelin-1 (ET-1, 10−10 M to 10−7 M) and ACh (10−4 M to 10−5 M) were constructed for endothelial intact and endothelial denuded (rubbed) vessels, as well as in the presence of the nitric oxide synthase blocker, Nω-nitro-l-arginine (l-NNA, 10−4 M), peegylated superoxide dismutase (PEG-SOD, 58 units/ml) and peegylated catalase (PEG-Cat, 250 units/ml), apamin (0.1 mM) and charybdotoxin (0.01 mM), and indomethacin (10 μM). Individual contractility curves were best fit with the standard log[agonist] vs. response nonlinear regression equation using the graphing and statistical software package Prism (GraphPad Software, La Jolla, CA).

VSMC cell culture. The left anterior descending coronary artery and second- and third-order mesenteric arteries were harvested, loose connective tissue removed, and the VSMC enzymatically dispersed with a mixture of collagenase and elastase, as described previously (25). Cells were plated in 100-mm dishes, grown to near confluence (75–90%). Freezing and subsequent thawing of cells were performed to allow all samples for proliferations studies (see below) to be performed at the same time, under as similar conditions as possible, including use of same reagents and sera. To synchronize each culture for subsequent seeding, serum was removed for 48 h before lifting with trypsin and seeding for study of the passage 2 (P2) cells.

Characterization of coronary VSMC by immunofluorescence. Because enzymatic dispersion of an arterial segment would initially release endothelial cells, fibroblasts, and VSMCs, it was important to characterize our P2 cultures using immunofluorescent cell type-specific markers. Cells grown to near confluence in 35-mm dishes were washed three times and fixed with a 5-min exposure to 100% methanol. Dual-labeling cocktails of primary and secondary antibodies were designed for detection of α-smooth muscle actin and von Willebrand factor (vWF). The primary antibody for vWF was raised in rabbit and the secondary, which was conjugated to rhodamine, was directed at a rabbit primary and species cross absorbed so that it did not cross react with any mouse primary. The primary antibody for α-smooth muscle actin was raised in mouse and the secondary, which was conjugated to fluorescein, was directed at a mouse primary and species cross absorbed, so that it did not cross react with any rabbit primary. All four antibodies were obtained from Millipore (Billerica, MA). Fluorescence images were acquired using SPOT software and camera (Diagnostic Instruments, Sterling Heights, MI) attached to a Nikon Eclipse E400 fluorescence-capable microscope.

Cell proliferation. After serum starvation (48 h) of a near-confluent T25 flask of second passage VSMC, cells were lifted with trypsin and seeded into 12-well plates at 5,000 cells per well. VSMC media were changed every other day. On the days between media changes, one well of cells was lifted with trypsin and counted in a Beckman Coulter Z2 series dual-threshold particle counter (Beckman Coulter, Fullerton, CA). VSMC were counted between thresholds set at 12–30 μm in diameter based on the size-frequency distribution showing a distinct single population of particles with boundaries that were clearly distinct from debris.

Immunoblotting. Near confluent (~80%) second-passage VSMCs were harvested into PBS using a cell scraper. Because of differing rates of cell proliferation, cells were therefore, harvested on dissimilar days in culture. Cells were pelleted by centrifugation and sonicated in a buffer containing 50 mM Tris, 150 mM NaCl, 0.2 mg/ml sodium azide, 0.1% SDS, 1% NP-40, 0.5% sodium deoxycholate, 0.4 mM DTT, 100 μg/ml PMSF, 2 μg/ml aprotinin, and 20 μg/ml leupeptin. Protein samples (5 μg/lane) were separated by SDS-PAGE and transferred to nitrocellulose membranes (Whatman, Florham Park, NJ). The Odyssey infrared two-color imaging system and reagents (LI-COR, Lincoln, NE) were used for immunoblotting and subsequent quantitation of specific proteins, as previously described (19). Briefly, membranes were blocked with Odyssey blocking buffer 1 h at room temperature followed by incubation in a 1:5,000 dilution of an antibody for proliferating cell nuclear antigen (PCNA; PC10 mouse mAb no. 2586; Cell Signaling Technology, Danvers, MA) or 1:1,000 for Cyclin E1 (no. 4129, Cell Signaling Technology) overnight at 4°C. The anti-mouse secondary antibody (Odyssey, 700 nm) was used at a dilution of 1:1,000 for 1 h at room temperature. Following quantification of the band of interest, the membrane was washed and reprobed overnight (4°C) with a 1:5,000 dilution of an α-actin antibody (rabbit polyclonal; Abcam, Cambridge, MA) designed as a loading control. The anti-rabbit secondary antibody (Odyssey, 800 nm) was applied at a dilution of 1:1,000 for 1 h at room temperature. As an independent evaluation of equal loading, the membranes were stained with Ponceau S.

Quantitative RT-PCR. Total RNA was purified from second-passage VSMC using the QIAzol and RNeasy procedures following the manufacturer’s instructions (Qiagen, Valencia, CA). RNA was quantitated using a NanoDrop ND-1000 spectrophotometer (Labtech International, East Sussex, UK). Reverse-transcription reactions were performed on 1 μg total RNA within the addition of oligo dT, dNTPs, DTT, RNasin, and Superscript III reverse transcriptase (Invitrogen). Quantitative real-time RT-PCRs (qRT-PCRs) utilized the TaqMan reagent and instrumentation systems (Applied Biosystems, Foster City, CA). Primer/probe sets were designed for ovine NOX1, NOX4, and GAPDH by first PCR cloning a large region of each via either published sequence or relying on highly conserved regions for degenerate primer design. Once a large section was PCR amplified, it was sequenced and intron-spanning primer/probe sets were designed for qRT-PCR using the Primer3 (26) following guidelines established by Applied Biosystems. The following oligonucleotides were used: sheep GAPDH, forward: GGCATCGTGAGGAGAGAGCCTTAT, reverse: AACAGGGGATGATTTTGGG, probe: CATCCTGGCAACCCAGAAAAGCTTG; sheep NOX1, forward: CCTTTGAGAAGGCCCAACAAAG, reverse: TAAATATCCAAGGCGAGACCA, probe: TACTACACACAGACAAATACCTGCGGTACGG; sheep NOX4, forward: ttgctctcatagctgtgatg, reverse: ttgctgtaagttgcttt, and probe: atgtacgacggggtgcctagataaca. Relative standard curves were...
generated for each run using pooled RNA harvested from tissues where the target molecules were abundantly expressed (NOX4-kidney, NOX1-kidney, and GAPDH-VSMC). All individual samples were run in triplicate, and each final average was calculated from three separate reverse transcription reactions that were generated from three separate RNA harvests. When compared across equally loaded (by quantitated RNA amounts) control and programmed cells, there was no significant change in GAPDH RNA quantity, supporting its use as an endogenous control for the NOX1 and NOX4 quantification.

**Chemicals.** FBS was from Atlanta Biologicals (Lawrenceville, GA). The cell culture supplements HEPES, penicillin/streptomycin, and nonessential amino acids were from Gibco/Invitrogen. All other chemicals were from Sigma-Aldrich (St. Louis, MO).

**Data analysis.** All values are presented as means ± SE. Statistical comparisons were performed by Student’s unpaired, two-tailed t-test or ANOVA with a post hoc Tukey test. Dose-response curves were analyzed by area under the curve. A value of P < 0.05 was considered significant.

### RESULTS

Control and dexamethasone-exposed lambs did not differ significantly in age (11.5 ± 0.5 days vs. 10.9 ± 2.7 days, respectively), or weight (9.1 ± 1.7 kg vs. 8.2 ± 2.8 kg, respectively) on the day of death. The control group (n = 8, 4 males) comprised 4 twin deliveries, while the dexamethasone group (n = 7, 4 males) comprised 1 triplet and 2 twin deliveries.

**Vascular reactivity.** Responses of the coronary artery segments to KCl (120mM KCl) were not significantly altered by antenatal dexamethasone exposure (data not shown), allowing for normalization of subsequent responses to be expressed as %KCl response. The contractile responses to ET-1 and ACh were significantly enhanced in intact coronary arteries from dex-exposed compared with control animals (Fig. 1, A and G). However, this difference in responsiveness was not observed in endothelial denuded vessels (Fig. 1, B and H) or in the presence of t-NAME (Fig. 1, C and I), PEG-SOD + PEG-Cat (Fig. 1, D and J) or apamin + charybdotoxin (Fig. 1, E and K). Contractile responses in the presence of indomethacin were significantly increased in both groups (P < 0.05), although the differences between control and dex-exposed vessels remained (Fig. 1, F and L).

**VSMC proliferation.** Dual-labeling immunofluorescence was used to characterize the cells isolated for the proliferation assay. Nearly all cells expressed α-smooth muscle actin, while no cells expressed the endothelial marker vWF, consistent with cells being of vascular smooth muscle lineage (Fig. 2A). The effectiveness of the vWF antibody was verified on human umbilical vein endothelial cell cultures (Fig. 2B).

After synchronization by 48 h of serum starvation, cells were counted every other day for 12 days while in 10% serum-containing medium. The average cell proliferation curves for control and dex-exposed VSMC lines are depicted in Fig. 3. Cells derived from dex-exposed lamb coronary arteries proliferated significantly faster than those from control animals (Fig. 3A, P < 0.05). The number of cells per well was significantly different at days 10 and 12, at a time when cells still appeared to be growing exponentially. In contrast, proliferation of VSMC, isolated from second- and third-order mesentery arteries from these same animals and performed in parallel with the coronary studies, was not different between groups (Fig. 3B).

**Immunoblotting.** Expression of proliferating cell nuclear antigen (PCNA) was determined in coronary VSMC lysates derived from serum-exposed control and dex-exposed cell lines (Fig. 4, top). The single band at 37 kDa is consistent with the predicted size of PCNA. PCNA protein was significantly more abundant in the dex-exposed cells compared with controls. Protein levels of β-actin (band near 50 kDa), used as a loading control, were similar between groups. When expressed as PCNA/α-actin, the ratio is almost 6-fold greater in dex-exposed compared with control VSMC (Fig. 4, bottom). Expression of smooth muscle α-actin, a marker of differentiation state, was also significantly decreased in dex-exposed coronary VSMC compared with control (15.5 ± 0.6 vs. 18.3 ± 1.6 AU, respectively, P < 0.05). Expression of another proliferation marker, Cyclin E1, a protein necessary for the transition from G1 to S-phase of the cell cycle, tended to increase in cultured VSMC from dex-exposed compared with control animals, although it failed to reach statistical significance (5.62 ± 1.02 vs. 3.40 ± 0.47 AU, respectively, P = 0.07).

**mRNA expression.** Because the SOD + catalase results suggested a role for ROS production in regulating the programmed differences in coronary artery reactivity, we examined the expression of the NADPH oxidases Nox1 and Nox4 in coronary VSMC. Of note, Nox1 and Nox4, enzymes that normally produce ROS in VSMC, have also been shown to be associated with proliferation and differentiation in VSMC (4). Using qRT-PCR, we found the ratio of Nox4/GAPDH was significantly lower in dex-exposed VSMC compared with controls (80 ± 15 AU vs. 440 ± 125 AU, P < 0.05), while no difference was detected in Nox1 expression. Attempts at quantitating Nox1 and Nox4 protein by immunoblot using commercially available antibodies were unsuccessful.

### DISCUSSION

Following Barker’s initial epidemiological studies, a large number of additional studies, performed in a multitude of populations, have substantiated the link between an adverse intrauterine environment and morbidity from coronary artery disease (2, 5, 20). Despite this evidence, few studies have directly attempted to examine mechanisms underlying developmental programming of coronary dysfunction. In the same animal model used in the present studies, we previously demonstrated that coronary arteries from “programmed” postnatal sheep display increased basal superoxide production, alterations in contractile function, and differences in vascular smooth muscle calcium regulation in the absence of gross histological changes (23–25). We now observe additional alterations in coronary vascular function consistent with those associated with risk for atherosclerosis, including endothelial dysfunction and enhanced VSMC proliferation.

We previously reported enhanced ET-1 and ACh contractile responses of coronary artery rings isolated from dex-exposed lambs (25). In those studies, evidence suggestive of both endothelial and smooth muscle dysfunction was present, although endothelial effects were not evaluated directly. We have now extended the studies to include the effects of endothelial removal, nitric oxide synthase blockade with l-NAME, scavenging of reactive oxygen species with PEG-SOD and PEG-catalase, inhibition of calcium-dependent K+ channels [endothelial-derived hyperpolarizing factor (EDHF)-mediated
vasorelaxation] with apamin + charybdotoxin and inhibition of prostaglandin synthesis with indomethacin. In intact vessels, ET-1 elicits constriction of VSMC by binding to two G protein-coupled receptors (ET\textsubscript{A} and ET\textsubscript{B}), as well as endothelium-dependent relaxation via ET\textsubscript{B} receptor induction of NO release (13). The net ET-1-mediated reactivity occurs through a balance of these effects. Similarly, ACh acts via muscarinic receptors on endothelium and vascular smooth muscle, which, in turn, signal both vasorelaxation and vasoconstriction. The observation that ET-1 and ACh-mediated constriction was greater in intact dex-exposed coronaries compared with controls but that the difference was completely abrogated by
denuding the endothelium suggests a primary role for endothelial dysfunction in contributing to the enhanced contractility in dex-exposed vessels. Endothelial dysfunction is typically thought to be related to impairment in the generation and function of NO as a vasodilator (27). Along these lines, we found that preincubation of coronary arteries with the NOS inhibitor, l-NNA, abolished differences in contraction to ET-1 and ACh between groups. Of note, we previously demonstrated a significant decrease in endothelial eNOS protein expression in dex-exposed newborn coronary arteries, which, in turn, may result in decreased NO production (25). It is also possible that NO bioavailability may be related to enhanced inactivation, resulting from interaction with superoxide. Increased ROS production may decrease NO bioavailability by three distinct mechanisms (32). First, superoxide may react directly with NO to form peroxynitrite anions. Second, ROS may cause oxidative degradation of tetrahydrobiopterin, an essential cofactor for eNOS, thus causing an “uncoupling” of the enzyme. Finally, ROS may cause a redox-dependent inhibition of the enzyme dimethylarginine dimethylaminohydrolase, which is followed by an increase in concentration of the endogenous eNOS inhibitor asymmetric dimethylarginine. Our previous observation of enhanced endothelial superoxide production in dex-exposed lambs is consistent with these mechanisms contributing to our findings.

The failure of indomethacin to abrogate any differences in responsiveness between control and dex-exposed coronary arteries suggests little role for programmed changes in prostaglandin production contributing to vascular dysfunction in the dex-exposed group. However, differences in contractile responses between groups were eliminated by preincubation with apamin + charybdotoxin and with membrane-permeable superoxide dismutase (PEG-SOD) and catalase (PEG-catalase), indicating an important role of EDHF, including H2O2. Specifically, with both apamin + charybdotoxin and PEG-SOD + PEG-catalase, the differences in contractility between control and dex-exposed vessels were obviated not by reducing the response of the dex group but by enhancing the response of control vessels, suggesting a fundamental deficiency in H2O2-mediated EDHF activity in dex-exposed coronary arteries. A potential role for EDHF in regulating the observed differences in vascular function is intriguing, and to our knowledge, it has not been explored in animal models of developmental programming. The molecular nature of EDHF is controversial and likely encompasses a variety of soluble factors, including arachidonic acid metabolites, potassium, and H2O2, as well as...
conducted hyperpolarization moving from endothelial cells to VSMC via gap junctions (7). H2O2 has been identified as a particularly important EDHF in the coronary vasculature (17, 18). While it has been suggested that H2O2 may act to maintain tissue perfusion in states of oxidative stress (when NO-mediated vasodilation is impaired), our findings suggest that both of these complementary pathways are impaired in the programming of coronary dysfunction.

In addition to endothelial dysfunction, smooth muscle cell migration and proliferation are integral to atherogenesis (11). We found that cultured coronary VSMC from dex-exposed lambs proliferated at a greater rate than those from control animals. The finding of increased levels of PCNA, which is expressed mainly in the S-phase of the cell cycle and serves as a proliferative marker, is consistent with the proliferation results (1). We attempted to further define the VSMC phenotype by examining expression of smooth muscle α-actin in the presence and absence of serum. Smooth muscle α-actin represents a marker of differentiation, increasing in expression with elevation of myofilament density (12, 31). The finding that the level of this protein was decreased in dex-exposed coronary VSMC from dex-exposed lambs proliferated at a greater rate than those from control animals. The finding of increased levels of PCNA, which is expressed mainly in the S-phase of the cell cycle and serves as a proliferative marker, is consistent with the proliferation results (1). We attempted to further define the VSMC phenotype by examining expression of smooth muscle α-actin in the presence and absence of serum. Smooth muscle α-actin represents a marker of differentiation, increasing in expression with elevation of myofilament density (12, 31). The finding that the level of this protein was decreased in dex-exposed coronary VSMC in the presence of serum is suggestive of a tendency for these cells to remain in a dedifferentiated, proliferative state. The mechanism(s) regulating the proliferative response in dex-exposed coronary VSMC is(are) not known. However, because VSMC growth in culture is clearly independent of endothelial influences, the programming stimulus appears to induce a VSMC-specific change in proliferative capacity. Because of accumulating evidence that nonphagocytic NADPH oxidase-generated ROS may directly or indirectly serve as an important signaling molecule in VSMC, regulating migration, proliferation, and contraction, the expression of Nox1 and Nox4 was also evaluated (15, 33). Five distinct Nox isoforms have been identified, although only two, Nox1 and Nox 4, have consistently been identified in both human and rodent VSMC (4). The specific roles of the Nox isoforms have not been fully elucidated, although Nox 4 appears to be constitutively expressed and responsible for basal ROS production (4). In an elegant series of studies, Clempus et al. (4) demonstrated in rat aorta VSMC that Nox 4-derived ROS is required for differentiation marker gene expression and the maintenance of contractile-type actin fibers. Using early (1–2) and late (6–13) passage VSMC as a model of differentiated and dedifferentiated VSMC phenotypes, these investigators found that Nox 4 was downregulated in late-passage cells, whereas as Nox 1 was markedly upregulated in late passage. Of note, our studies were performed in early-passage VSMC. The decrease in Nox4 expression that we observed in cells from dex-exposed animals would be consistent with these cells being maintained in a dedifferentiated, or proliferative state, the state that VSMC are in during early neointimal formation (31). Additional studies on the role of Nox isoforms have identified Nox 1 to be an important regulator of VSMC migration and neointimal formation (14, 28). Given these findings, we postulate that coronary VSMC from dex-exposed newborn lambs may remain in a relatively dedifferentiated state and may be “primed” for proliferation and/or neointimal formation. Such a condition, if present in humans after exposure to an adverse perinatal environment, may contribute to the increased incidence of atherosclerosis and death from cardiovascular disease seen in former low birth weight infants.

The absence of difference in VSMC proliferation rates obtained from mesenteric arteries of control and dex-exposed animals provides additional evidence for the vessel-specific
effect of the programming stimulus. Interestingly, we previously described in newborn lambs that mesenteric vascular reactivity to ET-1 was not altered following early gestation exposure to dexamethasone, a sharp contrast to that observed in coronary arteries from the same animals (30). Thus, both the VSMC proliferation and vascular reactivity programming effects of antenatal corticosteroids appear to be vessel specific. This finding likely relates to the different embryological origins of the vessels, the heterogeneity in phenotypes of VSMCs within the arteries, and epigenetic modifications of the genome in response to glucocorticoid exposure (34).

We acknowledge a number of limitations of the present study. First, sheep are an unusual species to examine factors contributing to coronary vascular dysfunction. Although sheep, to our knowledge, do not develop coronary atherosclerosis, underlying pathways and biology can still be examined. Second, although our results suggest an important role of reduced endothelial NO function, we did not directly measure levels of NO in the isolated vessels. Third, because of a paucity of reagents for the species, we were unable to quantify protein levels of Nox1 and Nox4. It also should be recognized that far more characterization and phenotyping of the VSMCs from dexam-exposed animals is needed to make conclusions regarding the effect of the in utero environment on these cells. However, the model also provides a number of strengths. For example, because of the size of the lamb heart and coronary arteries, we were able to perform the wire myography and isolate coronary VSMC from the same animals. In addition, the tissues were obtained from newborn lambs before the development of the overt hypertensive phenotype. Thus, alterations in vascular function and VSMC proliferation and gene expression are primary changes related to early gestation dexamethasone exposure and not secondary changes related to the presence of hypertension.

Perspectives and Significance

The present studies provide novel information regarding early-gestation dexamethasone exposure-induced alterations in coronary artery function. We have demonstrated that early-gestation dex exposure elicits coronary endothelial dysfunction, resulting from an apparent reduction in endothelial NO and EDHF bioavailability. Ex vivo coronary VSMC proliferation was also selectively enhanced. These changes are present prior to the development of an overt in vivo vascular phenotype, suggesting that these alterations in physiology are a primary response to the programming stimulus and not secondary to the development of hypertension, atherosclerosis, or other disease state.

Early-gestation dexamethasone exposure, or any other “programming” event is unlikely to directly cause coronary artery disease, just as in humans, low birth weight does not cause atherosclerosis. Rather, a suboptimal intrauterine environment may predispose to, or increase the risk for development of coronary artery disease in response to known risk factors for heart disease. Such a hypothesis has not, to our knowledge, been assessed in a programming model. Future studies are needed to build upon our findings regarding the contribution of ROS and various signaling pathways in the induction of coronary vascular dysfunction and the VSMC phenotype. Animal models in which atherosclerosis can be induced, such as apoE and LDLr null mice, may allow assessment of whether the in vitro abnormalities identified in our studies contribute to the development of programmed cardiovascular disease. Only through such studies will we be able to develop strategies to alleviate and potentially prevent the adverse contributions of early developmental effects on postnatal cardiovascular health.

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DISCLOSURES

No conflicts of interest are declared by the authors.

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