Evidence for microvascular dysfunction after prenatal dexamethasone at 0.7, 0.75, and 0.8 gestation in sheep

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Received 22 January 2002; accepted in final form 8 May 2002

The National Institutes of Health advises routine administration of a single course of antenatal betamethasone or dexamethasone (DM) to pregnant women at risk of premature delivery at 24–32 wk gestation (35a). Treatment consists of two doses of 12 mg betamethasone given intramuscularly 24 h apart or four doses of 6 mg DM given intramuscularly 12 h apart. Antenatal corticosteroid therapy results in a substantial decrease in neonatal morbidity and mortality as well as substantial savings in health care costs. At the present time, there are insufficient data to support routine use of repeat courses of antenatal corticosteroids in clinical practice. However, multiple doses have been given to some patients in the past (35b).

Several studies conducted in different species indicate the possibility of unwanted effects of glucocorticoids (GCs). For example, excess GCs in utero cause growth retardation (5, 10, 21). Importantly, low birth weight is correlated with the risk for developing cardiovascular disease later in life (3).

In vivo studies clearly show that both single and repeated courses of GC administered to either the mother or directly to the fetus increase blood pressure acutely (1, 8, 13, 14, 16, 52). In addition, the mechanisms underlying GC-induced hypertension remain poorly understood. However, there is evidence for the presence of GC receptors on endothelial cells and vascular smooth muscle cells (37, 38). Therefore, direct actions of GCs on the vasculature are possible.

Effects of GCs on vascular resistance (13) have been explained, in part, by an increased constrictor and/or decreased dilator response. For example, vascular endothelial cells play a key role in cardiovascular regulation by producing a number of potent vasoactive agents, including the vasodilator nitric oxide (NO) and the vasoconstrictor peptide endothelin (ET)-1. A dysfunction of the vascular endothelium has been implicated in the pathophysiology of a number of cardiovascular diseases, particularly essential hypertension (17, 47, 49). Decreased NO availability (impairment of synthesis or increased inactivation by superoxide radicals) may also account for the increased peripheral vascular resistance associated with GC exposure. Similarly, increased ET synthesis, or increased smooth muscle sensitivity to ET, could account for many of the features of hypertension.

GCs downregulate endothelial NO synthase (eNOS) expression in cell culture (52). Mangos et al. (34) showed impaired endothelium-dependent vasodilation in cortisol-exposed healthy subjects. GCs have an effect on ET production, binding, and receptor activity (28). Vascular responsiveness in fetal sheep in vitro is altered by in vivo GC administration (1, 14).
The purpose of this paper is to examine the effects of repeated courses of DM administered to the mother on the fetal peripheral vasculature. We hypothesized that repeated maternal administration of DM, used at gestational age similar to that at which treatment is recommended in human pregnancy, would alter the function of the l-arginine/NO pathway and vasoconstrictor responses to ET in the fetal femoral vasculature. The femoral vascular bed was chosen as it represents skeletal muscle. Skeletal muscle is one of the largest vascular beds in the body receiving 25% of cardiac output in the ovine fetus at 0.9 gestation (26).

**METHODS**

**Animals.** Rambouillet-Columbia crossbred ewes (Ovis Ar- 

cies n = 14) carrying single fetuses of known gestational age were studied. The experimental protocol was approved by the Institutional Animal Care and Use Committee at Cornell University College of Veterinary Medicine. All facilities were approved by the American Association for the Accreditation of Laboratory Animal Care.

**Prenatal DM treatment.** DM (2 mg) or vehicle was admin-

istered intramuscularly to pregnant ewes as a course of four injections at 12-h intervals. Three courses were given, start-

ning on days 103, 110, and 117 of gestation (term = 149 days). The dose of DM was chosen following delivery trials in which 2, 4, and 6 mg were administered without progesterone coverage. Delivery within 48 h of receiving the first four injection courses occurred in 84 and 100% of animals in the 4- and 6-mg groups, respectively (n = 6/group). All animals in the 2-mg group delivered at term. We previously showed that the administration of a course of 2 mg DM increases fetal blood pressure with each injection and that the increases in blood pressure to the first course at 103–105 dGA (23%) and to the second course at 110–112 dGA (30%) (30) were similar to that induced by direct infusion of DM to the fetus, which achieves an increase of 24% (13).

**Tissue collection.** All ewes underwent Caesarean section under halothane anesthesia on 119 dGA, 12 h after the last injection of the third course. All fetuses removed were euthanized by exsanguination while still under anesthesia and underwent immediate necropsy for tissue retrieval. Ewes were euthanized with an overdose of 10 ml of 10% pentobar-

itrate (B. Braun, Aarhus, Denmark). One of these wires was attached to a movable micrometer, by which the diameter of the arterial lumen could be varied, while the other was attached to an isometric force transducer. This arrangement enables wall tension to be measured at a predetermined internal circumference. Vessels were bathed in PSS at 37°C and aerated continuously with 5% CO₂-95% O₂ to achieve pH of 7.4. After a 30-min equilibration period, the resting wall tension-internal circumference relationship was determined. The arteries were then set to an internal circumference of 90% of IC100, which has been shown to be the optimum calibrer for studies of the peripheral vasculature (15).

**Protocol.** The start-up protocol and evaluation of vessel viability were conducted as described previously (1). Briefly, each vessel was stimulated with NEK (5 μM norepinephrine (NE) in 125 mM potassium-substituted PSS (KPSS)), 5 μM NE alone, and KPSS alone and again with NEK. Vessels were washed several times with heated and oxygenated PSS after reaching a plateau to allow full relaxation. The functional integrity of the endothelium was assessed in each artery by the addition of ACh (5 × 10⁻⁶ M) following con-

traction with NE. The endothelium was considered intact if ACh evoked a relaxation of greater than 60% of the NE-induced tone.

Concentration-response curves (CRCs) were performed to the receptor-dependent vasoconstrictor ET (10⁻¹¹ to 10⁻⁶ M) in the absence and presence of the NO synthase inhibitor N³-nitro-l-arginine methyl ester (l-NAME); 100 μM for 1 h), to the endothelium- and receptor-dependent agonist ACh (10⁻⁹ to 10⁻⁵ M) in the absence and presence of l-NAME (100 μM) and bradykinin (BK; 10⁻¹ⁱ to 10⁻⁶ M), and to the receptor- and endothelium-independent NO donor sodium nitroprusside (SNP; 10⁻¹⁰ to 10⁻⁵ M). Responses to vasodila-

tor agents were determined following a stable contraction to 5 μM NE. The molarity is expressed as the final concentra-

tion in the bath. Increasing concentrations of agonists were added only once a plateau had been reached. The experimenter was unaware of treatment groups during data collection.

**Drugs.** All PSS and KPSS reagents and ACh (A-6500), NE (A-9512), BK (B-3259), l-NAME (N-7571), and SNP (S-0501) were acquired from Sigma Chemical (St. Louis, MO). ET (H-6995) was obtained from Bachem (Torrance, CA). Stock solutions were made up in distilled H₂O. ET stock solution was dissolved in ethanol. Stock solutions were frozen as aliquots at −20°C and thawed as required. Dilutions were made in PSS on the day of each experiment and kept on ice before use. DM (Azium) was obtained from Schering, NY.

**Data analysis.** Data are presented as means ± SE. Signific-

ance was assessed by t-test for parametric data at P < 0.05. Wall tension is expressed as milliNewtons per millimeter of artery length (mN/mm). Relaxation is given as percent relax-

ation of induced tone to NE. Sensitivity to the agonist is expressed as the negative log of the effective concentration required to produce 50% of maximum effect. Sensitivity was calculated from each CRC by fitting the Hill equation using Prism (GraphPad Software, San Diego, CA).

\[ E = E_{\text{min}} + (E_{\text{max}} - E_{\text{min}})/(1 - e^{-cM - E_{\text{50}}}) \]

Where E is the effect of the agonist, k is the slope of the curve, M is the molar concentration of the agonist, EC₅₀ is the molar concentration of the agonist that elicits a half-

maximal response, and Eₘₐₓ is the maximal tension caused by the agonist.

**RESULTS**

**Body weights.** DM-exposed fetuses were significantly smaller than saline-exposed [control (CTR)] fetuses (CTR: 2.56 ± 0.07 kg vs. DM: 2.25 ± 0.09 kg; P = 0.01).
Artery diameter. There was no significant difference in the length (CTR: 1.68 ± 0.1 mm vs. DM: 1.72 ± 0.10 mm) and in the calculated internal diameter (CTR: 365.22 ± 23.20 µm vs. DM: 355.97 ± 28.03 µm) of femoral vessels in the control and DM-exposed groups.

Constrictor responses. Tension developed in response to 5 mM NE was not different between groups (CTR: 1.66 ± 0.38 mN/mm vs. DM: 1.81 ± 0.21 mN/mm). Maximum tension to 125 mM KPSS (which causes receptor-independent contraction by depolarizing the vascular smooth muscle) was enhanced in the arteries from DM-exposed fetuses (CTR: 1.19 ± 0.12 mN/mm vs. DM: 1.83 ± 0.22 mN/mm; *P = 0.02). Therefore, tension development to the agonists was not calculated as a percentage of the potassium contraction. Correction of tension development is a common practice to control vascular reactivity for variations in vessel size. In this case, plotting sensitivity against internal diameter revealed zero slope regression lines. Therefore, there was no need for corrections. All arteries produced concentration-dependent vasoconstriction to ET. The maximum tension developed and sensitivity to ET were enhanced in femoral arteries obtained from DM-exposed fetuses (Fig. 1). With 1-h incubation of one vessel in each group with 100 µM L-NAME, a competitive inhibitor of NO synthase, an ET CRC was constructed and compared with a CRC to ET performed on a separate vessel without L-NAME preincubation (higher concentrations of L-NAME did not produce greater inhibition in preliminary experiments). L-NAME increased sensitivity and maximum tension to ET in controls but not in the DM-exposed group (Figs. 2 and 3).

Relaxation responses. The tension developed to NE before relaxation curves was similar in vessels from CTR and DM-exposed animals (CTR: 1.8 ± 0.34 mN/mm vs. DM: 1.63 ± 0.1 mN/mm).

Endothelium-dependent relaxation. ACh, SNP, and BK produced concentration-dependent relaxation. Arteries from both groups relaxed to baseline tensions. Relaxation to ACh was similar in arteries from DM-exposed animals both in terms of maximal relaxation and sensitivity of relaxation curves (CTR: 8.64 ± 0.28 vs. DM: 8.38 ± 0.09). Relaxation to BK was enhanced in vessels from DM-exposed fetuses (CTR: 9.5 ± 0.13 vs. DM: 9.89 ± 0.06; *P = 0.03) (Fig. 4). Arteries from control fetuses were more sensitive to BK than ACh (ACh: 8.64 ± 0.28 vs. BK: 9.5 ± 0.13; *P = 0.01). ACh relaxation response curves were also constructed after one vessel in each group was incubated with 100 µM L-NAME and compared with ACh-induced relaxation in the absence of L-NAME (Fig. 5). L-NAME shifted the CRC to the right in vessels from the CTR group, indicative of a 10% increase in sensitivity, but it had no
effect in the DM-exposed group. L-NAME did not change maximal relaxation in either treatment group.

**Endothelium-independent relaxation.** Relaxation to the NO donor SNP was similar in both groups.

**DISCUSSION**

In the present study, we demonstrated that maternal administration of three courses of DM at weekly intervals, starting at 103 dGA, decreases fetal body weight and alters vascular responsiveness in the fetal femoral vasculature in vitro at 119 dGA. With the use of the technique of small vessel wire myography, we showed that DM administered in this manner results in an increase in the maximal tension developed in response to potassium, increased sensitivity, and maximum tension in response to the potent vasoconstrictor ET and enhanced relaxation to BK but not to ACh. In the control but not treated animals, we found that, when NO synthase is inhibited with L-NAME, the vasoconstrictive effect of ET is enhanced and the sensitivity to ACh is reduced.

Slotkin et al. (43) showed a dose-dependent effect of DM in the rat that induces fetal growth inhibition at high doses, whereas at low doses enhancement of noradrenergic synaptogenesis occurs without growth inhibition. Furthermore, the same low-dose DM to the pregnant rat has a promotional effect on kidney development, whereas the higher growth-inhibiting doses do not (44). Furthermore, a lower dose (0.2 mg/kg) betamethasone has been shown to lack the lung maturational effects of a higher dose (0.5 mg/kg) in sheep (39). The dose of DM we used does not appear to qualify as "low dose" in the sense that we showed a pronounced (12%) growth restriction after repeated administration before 119 dGA. It therefore seems reasonable to assume that the dose of DM we used is not exerting its effects via promotion of cell differentiation or synaptogenesis. In the absence of studies on lung function, however, we cannot comment on whether the dose we used impacts lung development. Furthermore, because we demonstrated growth inhibition in our model, the changes in vascular development following repeated DM exposure may result from the steroid-induced growth inhibition and/or from direct action of the steroid on gene expression. Further studies to elucidate these differences are required, and therefore, extrapolating of our results to clinical practice must be undertaken with caution.

Because our data are from the fetus, we cannot reach conclusions regarding the life-long consequences of the vascular changes we describe. With the use of the same dose of DM in a different group of sheep, we showed delayed parturition by 2 days with no difference in birth weight (31). These observations suggest that fetuses exposed to repeated 2 mg of DM that produces growth retardation at 119 dGA also exhibit catch-up growth if allowed to deliver naturally. Catch-up growth is a well-documented phenomenon and is associated with several metabolic and physiological changes that have long-term deleterious consequences (5). We are currently investigating animals of various postnatal ages that were born of pregnancies in which our DM-dosing regimen was used.

Our cardiovascular data are consistent with the results of experiments using both higher maternal doses and direct fetal infusion. In our hands, route of administration does not alter fetal blood pressure, heart rate, and baroreflex response to DM. In addition, the changes in vascular function we describe here are similar to those we previously showed following direct fetal administration of DM (14). In addition, Berry et al. (9) reported route-independent enhancement of kidney function following both direct fetal and maternal administration of betamethasone. Moss et al. (35) showed that only maternal (not direct fetal) repeated betamethasone administration causes postnatal changes in cardiovascular function. Interestingly, the lambs from their protocol exhibited a relative hypotension at 3 mo of age that later normalized. Given that the repeated beta-

![Fig. 4. Bradykinin (BK) relaxation response curves (means ± SE) in femoral arteries from DM (●) and saline-exposed (CTR; □) sheep fetuses at 119 dGA (n = 4,5). Sensitivity: CTR 9.5 ± 0.13 vs. DM 9.89 ± 0.06; *P = 0.03 (P = 0.05).](image-url)

![Fig. 5. Sensitivity to ACh in femoral arteries from DM- and saline-exposed (CTR) fetal sheep at 119 dGA with (hatched bars) or without preincubation with L-NAME. Data are presented as means ± SE, n = 6,6. *Different from CTR at P < 0.05.](image-url)
methasone group in this study (35) experienced significant complications including abortion, postnatal mortality, and difficulties in feeding, it is difficult to draw generalized conclusions on postnatal cardiovascular function from the data given.

The vascular endothelium makes a major contribution to local vascular tone and blood flow through the release of both vasodilator (NO, prostacyclin, and endothelium-derived hyperpolarizing factor) and vasoconstrictor agents (ET and cyclooxygenase products) (11, 20, 24, 25, 36, 41). Any disturbance in the balance between constrictor and dilator systems may produce homeostatic changes that result in raised blood pressure. Furthermore, many agonists that cause constriction in healthy arteries may also contribute to vasodilatation through the release of NO from the endothelium. Vessels in which this dilatory capacity is compromised by virtue of having dysfunctional endothelium may have augmented vasoconstrictive responsiveness in response to such complex agonists with mixed vasoconstrictor and vasodilator function. Endothelial cell dysfunction may therefore alter both the basal vascular tone and the response of blood vessels to circulating endocrine factors and pharmacological agents that, in turn, contribute to increased peripheral resistance and raised blood pressure.

Several experimental findings suggest that ET is involved in different forms of hypertension. In the deoxycorticosterone acetate hypertensive rat, nonselective ET-receptor blockade reduces blood pressure (42). ET is involved in the maintenance of high blood pressure and cardiac hypertrophy in malignant hypertension in the stroke-prone spontaneous hypertensive rat (45). Furthermore, in salt-sensitive (low renin) hypertension, the ET system is activated and ET antagonism significantly reduces blood pressure (4). In contrast, other experimental models of hypertension do not appear to involve ET, because endothelins do not contribute to the development of hypertension or the vascular hypertrophy seen in young spontaneous hypertensive rats (33). ET is released by damaged or hypoxic endothelium, and increased circulating levels of ET are associated with several vascular diseases including some forms of hypertension (46, 51, 53, 54).

In vivo administration of ET has been shown to produce systemic and pulmonary hypertension in late-gestation fetal sheep (27).

We used wire myography, which is a powerful tool for evaluating in vitro vascular resistance artery function, for two major reasons. First, it demonstrates functional performance of the vessels in a way that is not done by traditional techniques. Second, by isolating the vessel from in vivo homeostatic responses, the direct effects of agonists on vascular responsiveness can be evaluated. We showed that sensitivity and maximum tension generated in response to ET are increased in fetuses exposed to DM. Binding to either the ETA or ETB receptor on vascular smooth muscle cells can lead to vasoconstriction (23). Upregulation of either receptor subtype could therefore have occurred in our DM fetuses. To modulate net effect of ET, however, binding to ETB receptors on endothelial cells results in the release of NO and dilator prostanoids that offset the vasoconstrictive effect of ET (41). ETB also functions to remove ET from the circulation (19). Upregulation of the ETB-receptor subtype therefore seems unlikely unless such upregulation targets only those ETB receptors on vascular smooth muscle cells. Inhibition of ETB activity, and the decrease in NO release that would result, may play a role in the DM-induced effects we observed.

NO acts to inhibit ET-stimulated receptor signaling, promotes ET dissociation from the ETA receptor, and disrupts calcium mobilization as part of its vasodilatory effect in response to ETA-receptor binding (2). Inhibition of NO production augments the vasoconstrictive property of ET (32). To evaluate the effects of DM administration on the role played by the NO system in the fetal vascular response to ET, we assessed vascular sensitivity and maximum tension developed to ET in the presence L-NAME. Blocking NO production had no effect on basal vascular tone in either group. Sensitivity and maximum tension generated to ET increased in the presence of L-NAME in controls but not in the DM-exposed group, indicating blunted ET-induced NO synthesis following DM exposure. We propose that, in addition to upregulating ETA-receptor expression (14), DM may also downregulate eNOS and/or ETB-receptor expression and postreceptor signaling. Taken together, these findings suggest that glucocorticoid-induced hypertension in the fetus is characterized by increased ET vasoconstrictive tone and that this alteration in vascular responsiveness may depend on decreased endothelial ETB receptor-mediated NO production resulting from impaired NO availability. In such conditions, endothelial ETB-induced vasodilation may no longer be able to compensate for the direct vasoconstrictor effect mediated by smooth muscle cell ETA and ETB receptors.

Alteration in endothelium-dependent relaxation in hypertension is not uniform and depends on the model of hypertension as well as the vascular bed studied. ACh-induced NO release in the forearm circulation is reduced in patients with essential hypertension and renovascular or endocrine hypertension (18). In addition, the plasma levels of NO are decreased in patients with essential hypertension (18). In contrast, similar vasodilation in response to ACh, carbachol, and isoproterenol has been reported in normotensive controls and patients with essential hypertension (12). In some vascular beds, ACh has been associated with the release of vasoconstrictor cyclooxygenase products and endothelium-derived hyperpolarizing factor from endothelial cells (29, 50). In our study, maximum relaxation to ACh was similar in femoral arteries from control and DM-exposed fetuses. Furthermore, maximum relaxation was not affected by blocking NO production with L-NAME in either group. Although sensitivity to ACh was increased in the presence of L-NAME in controls, sensitivity was unchanged in the DM group. We conclude that NO synthesis in the ovine fetal femoral vasculature is not the only pathway mediating vasodi-
ylation in response to ACh. Furthermore, our results indicate that fetal DM exposure may blunt NO-induced vasodilation and/or enhance endothelium-derived vasoconstriction.

We demonstrated that DM exposure enhances vasoconstriction resulting from potassium-induced entry of calcium into vascular smooth muscle cells via voltage-gated calcium channels. Calcium influx in cultured vascular smooth muscle cells is also increased by DM exposure (22). These observations suggest that, in addition to alteration in ET action, DM administration to the ovine fetus may upregulate voltage-gated calcium channels, increase extracellular calcium stores, and/or alter the signaling pathways involved in smooth muscle contraction. However, these observations may also indicate that the number of muscle fibers increased, thereby producing increased force of contraction.

With the use of SNP, an NO donor, we investigated the relaxation response downstream from NO synthesis. NO generated by NO synthase in endothelial cells freely diffuses to the underlying smooth muscle cells and stimulates guanosine 3'5'-cyclic monophosphate (cGMP) by binding to soluble guanylate cyclase. cGMP, in turn, has actions on protein kinases, ion channels, and possibly other proteins leading to reduced calcium responses and eventually vasodilation. Because relaxation to SNP was similar in both groups of fetuses, we conclude that differences in the ability of the fetal femoral vasculature to dilate lie upstream from NO and the vascular smooth muscle cell.

Similar to ACh, BK mediates vasodilation by binding to a receptor on endothelial cells and stimulating the release of NO (40). BK has also been implicated in the release of prostacyclin and endothelium-derived hyperpolarizing factor (48). Because BK-induced relaxation was enhanced in the DM-exposed group, we suggest that BK sensitivity may be enhanced to offset the DM-induced elevation in vascular tone. Because there is an NO-independent vasodilation to BK in patients with essential hypertension that possibly involves endothelium-dependent hyperpolarization (48), we propose that the differences between ACh- and BK-induced relaxation we describe may derive from BK-induced hyperpolarization rather than in the release of NO.

Perspectives

We conclude that three courses of maternal DM repeated at weekly intervals between 0.7 and 0.8 of gestation in sheep have a direct effect on fetal femoral vasculature that increases vascular tone while recruiting compensatory dilatory mechanisms. The increase in ET-mediated vasoconstriction suggests altered ET-receptor function in the endothelium and/or smooth muscle. Increased responsiveness to potassium suggests that modification of second messenger systems may be part of the increased ET response, although DM-induced increase in smooth muscle cell number cannot be ruled out. The combination of enhanced ET-induced vasoconstriction, abnormal endothelium-dependent relaxation, and normal endothelium-independent relaxation indicates microvessel dysfunction following antenatal DM administration. We cannot differentiate between the direct effect of GCs and the direct effect of growth restriction at this age. Nonetheless, because such dysfunction is generally documented in various forms of hypertension in adults, similar findings in the fetus following glucocorticoid exposure underscore the importance of investigating the mechanisms by which these steroids cause fetal hypertension and whether there are long-term consequences of antenatal exposure for adult cardiovascular health.

This work was supported by National Institutes of Health Grant HL-21350.

REFERENCES


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