LATE GESTATION BETAMETHASONE ENHANCES CORONARY ARTERY RESPONSIVENESS TO ANGIOTENSIN II IN FETAL SHEEP

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Running head: Betamethasone-induced coronary artery AT₁ upregulation
ABSTRACT

Antenatal glucocorticoids are used to promote the maturation of fetuses at risk for preterm delivery. While perinatal glucocorticoid exposure has clear immediate benefits to cardiorespiratory function, there is emerging evidence of adverse long-term effects. To determine if antenatal betamethasone alters vascular reactivity, we examined isometric contraction of endothelium-intact coronary and mesenteric arteries isolated from twin fetal sheep at 121-124 days gestation (term being 145 days). One twin received betamethasone (10 µg/h iv) while the second twin received vehicle (0.9% NaCl) for 48 hours immediately prior to the final physiological measurements and tissue harvesting. Fetuses that received betamethasone had higher mean arterial blood pressures than the saline-treated twin controls (53 ± 1 vs. 48 ± 1mmHg, P < 0.05). Coronary vessels from betamethasone-treated fetuses exhibited enhanced peak responses to angiotensin II (ANG II) (72 ± 17% vs. 23 ± 6% of the maximal response to 120mM KCl, P < 0.05). There was no significant difference in response of the coronary arteries to other vasoactive compounds (KCl, U46619, sodium nitroprusside, 8-Br-cGMP, isoproterenol, and forskolin). Contractile responses to ANG II were similar in betamethasone and control mesenteric arteries (48 ± 17% vs. 36 ± 12% of the maximal response to 10⁻⁶M U46619).

Western blot analysis revealed AT₁ receptor protein expression was increased by betamethasone in coronary but not in mesenteric arteries. These findings demonstrate that antenatal betamethasone exposure enhances coronary but not mesenteric artery vasoconstriction to ANG II by selectively upregulating coronary artery AT₁ receptor protein expression.

Key Words: angiotensin II, betamethasone, coronary artery, vascular smooth muscle
INTRODUCTION

Consensus statements now widely support the routine administration of antenatal corticosteroids to mothers at risk of preterm delivery between 24 and 34 weeks gestation (1, 32). Since the pioneering work of Liggins and Howie in 1972, a multitude of well-designed studies have shown antenatal corticosteroid administration significantly reduces mortality, respiratory distress syndrome and intraventricular hemorrhage in preterm infants (7, 18). In the 1990s, multiple courses of corticosteroids were used in an attempt to maximize fetal health (31). However, recent research has identified progressive growth restriction with repetitive doses (12, 16). Potential adverse effects from a single course of therapy during the late stages of development are now emerging (17, 22, 27).

In animal models, acute glucocorticoid exposure elevates the blood pressure of late gestation fetuses, and ovine small femoral arteries have shown enhanced vasoconstriction to potassium chloride and endothelin-1 immediately following steroid administration (2, 9, 10). Paradoxically, steroid administration has also been associated with enhanced endothelium-dependent vasodilatation to acetylcholine (2). Finally, acute corticosteroid exposure has been associated with increased coronary artery relaxation to bradykinin and nitric oxide, potentially related to increased guanylate cyclase activity (13). The purpose of our study was to clarify whether antenatal glucocorticoid administration alters coronary and mesenteric artery vascular reactivity and to investigate possible mechanisms regulating these effects.

METHODS
**Tissue collection.** 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. Time-dated pregnant ewes with twins at 116-119 days gestation (term being 145 days) were obtained from a local source (*n* = 5). Ewes were anesthetized with 12 mg/kg of thiopental sodium (Abbott Laboratories, Abbott Park, IL), intubated and ventilated with a mixture of halothane (1%), oxygen (33%) and nitrous oxide (66%). After performing an abdominal flank incision, the uterus was partially externalized and opened over the fetal hindlimbs. Polyethylene catheters were inserted into a femoral artery and vein, and a catheter immersed in amniotic fluid was sutured to the fetal skin. The fetal incisions and uterine openings were closed, and the procedure was repeated for the second twin. All the catheters were exteriorized through a subcutaneous tunnel into a cloth pouch on the ewe’s flank. Ampicillin (Sigma, St. Louis, MO) was administered at the completion of surgery (2 g intra-amniotic and 2 g intramuscular to the ewe), followed by intramuscular injections (1 g) to the ewe every 12 hours for 3 days. After surgery, the ewe was returned to an individual pen and allowed free access to food and water.

After a 3-day recovery period, one fetus received betamethasone (10 µg per hour by continuous intravenous infusion over 48 hours; Schering, Kenilworth, NJ) while the control twin received an identical volume of vehicle (0.9% NaCl). The dose and route utilized corresponds to the those used by other investigators to ensure consistent corticosteroid exposure (2), while the use of twin controls was chosen to minimize potentially confounding environmental conditions, such as maternal health and diet. The virtual absence of vascular anastomoses between twin placentae in sheep, coupled with the larger volume of distribution seen in the pregnant ewe compared to the
exposed fetus, assured selective administration to a single fetus (20). Before and after the 48-hour infusion, arterial blood gases and chemistries were sampled using a pH/blood gas/electrolyte analyzer (IL 1640, Instrumentation Laboratory, Milano, Italy), and arterial blood pressure and heart rate were recorded over a one-hour time period, using MacLab software (ADInstruments, Colorado Springs, CO). Immediately following the 48-hour infusion, at 121-124 days gestation, maternal anesthesia was then reinduced and maintained as previously outlined. Fetuses were delivered by cesarean section and euthanized with intravenous pentobarbital sodium (50mg/kg; Abbott Laboratories, Abbott Park, IL). Coronary and second-generation mesenteric arteries were then collected. Artery segments, from which residual blood was expressed and loosely adherent connective tissue removed, were quickly placed in ice-cold, bicarbonate-buffered physiological salt solution (PSS), 4% paraformaldehyde or snap frozen in liquid nitrogen.

**Isolated vessel contractile responses.** Circumflex coronary artery segments were cleansed of adherent connective tissue and sectioned into 3 mm rings on the day of collection. The second-generation mesenteric arteries were stored in chilled (4°C) bicarbonate-buffered PSS overnight, prior to sectioning into 4 mm rings. The endothelium was left intact and the rings were mounted in individual 18 ml isolated organ chambers and connected to an isometric force transducer by 32-gauge stainless steel wire. Contractile responses were recorded with an eight-channel MacLab 8E and stored on a Power Macintosh 8600 computer. The length-tension relationship was defined experimentally to 30 and 90 mM KCl at varying passive stretch. Passive stretch was set at 90% of the tension required to obtain peak responses to KCl (0.7 g for both coronary and mesenteric arteries), and the rings were allowed to equilibrate in PSS at 37°C for 60 min before
the start of experimentation. PSS was aerated with a mixture of 95% O₂ - 5% CO₂; the composition was as follows (in mM): 130 NaCl, 4.7 KCl, 1.18 KH₂PO₄, 1.17 MgSO₄·7 H₂O, 14.9 NaHCO₃, 1.6 CaCl₂·H₂O, 5.5 dextrose and 0.03 CaN₂·EDTA (pH 7.30). The measured osmolality was 293 mosm/kg.

Endothelium intact isolated vessels from twin pairs were studied simultaneously. Initially, the contractile response of each vessel to 120mM KCl was recorded. Subsequent coronary artery contractile responses were then normalized as a percentage of the maximal response. The bath was washed three times with PSS over a 10 min period, and the vessels were allowed to equilibrate again for 20 min before initiation of the dose response protocols. Cumulative concentration-responses to KCl (5 to 90mM), angiotensin II (ANG II, 10⁻¹¹ to 10⁻⁷ M) and the sympathomimetic phenylephrine (10⁻⁹ to 10⁻⁴ M) were conducted with addition of increasing concentration of the agent under study at set intervals (10 minutes for coronary arteries and 5 minutes for mesenteric arteries). The arteries were then re-equilibrated to their baseline with multiple washes of PSS over one hour prior to preconstriction of each vessel with 10⁻⁶ M U46619, a thromboxane A₂ mimetic. Subsequent mesenteric artery contractile responses were then normalized as a percentage of the maximal response to U46619, rather than KCl, given the significant difference in the contractile response to KCl between groups. Cumulative concentration responses to acetylcholine (10⁻¹⁰ to 10⁻⁵ M), the nitric oxide donor sodium nitroprusside (10⁻¹⁰ to 10⁻⁵ M), 8-Br-cGMP (10⁻⁹ to 10⁻⁴ M), the beta-agonist isoproterenol (10⁻¹⁰ to 10⁻⁶ M), and a direct activator of adenylate cyclase, forskolin (10⁻¹¹ to 10⁻⁶ M), were conducted with addition of increasing concentration of the agent at 8 minute intervals. 8-Br cGMP was not utilized with the mesenteric arteries. Microsoft Excel 2000 was used to generate smoothed dose-
response curves for each vasoactive agent. All PSS reagents and vasoactive compounds were acquired from Sigma Chemical (St. Louis, MO) with the exception of U46619, which was supplied by Alexis Corporation (San Diego, CA).

Immunoblotting. Western blot analysis for AT\textsubscript{1} (angiotensin type 1 receptor) and AT\textsubscript{2} (angiotensin type 2 receptor) was performed as previously described, with extrapolation to eNOS (endothelial nitric oxide synthase) analysis (38). Protein concentrations were determined by the method of Lowry, as modified by Peterson (30). Equal protein loading was verified by Ponceau S staining. The AT\textsubscript{1} receptor-specific polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was raised in rabbits against an epitope corresponding to amino acids 306-359 of the human AT\textsubscript{1} receptor. The AT\textsubscript{2} receptor-specific polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was raised in rabbits against an epitope corresponding to amino acids 221-363 mapping the carboxy terminus of the human AT\textsubscript{2} receptor. The eNOS-specific monoclonal antibody (BD Biosciences Pharmingen, San Diego, CA) was raised in mice. Nitrocellulose blots (20 µg protein/lane) were incubated with the primary antibody at a 1:1000 (AT\textsubscript{1} and eNOS) or 1:2000 (AT\textsubscript{2}) dilution for 2 h at room temperature. Blots were rinsed, washed, and then incubated with either a 1:3000 dilution of goat anti-rabbit or a 1:2000 dilution of goat anti-mouse horseradish peroxidase (HRP) conjugated antibody (Sigma) at room temperature for 1 h.

Binding of the secondary antibody was detected using a chemiluminescent system consisting of HRP/hydrogen peroxide oxidation of luminol (Pierce, Rockford, IL). Blots were then exposed to Kodak XAR X-ray film for 1 minute. Films were digitized, and the difference between protein signals and background was quantitated using NIH Image (National Institutes of Health, http://rsb.info.nih.gov/nih-image/).
**Immunohistochemistry.** Isolated vessels were fixed in formalin (10%), embedded in paraffin and sections were mounted on glass slides. Sections were deparaffinized in xylenes and hydrated in an ethanol:physiologic salt solution (PBS) series. After a 5 min PBS rinse, the sections were incubated in \( \text{H}_2\text{O}_2 \) (3% in methanol) for 30 min before rinsing with PBS (x2) and blocking with bovine serum albumin (1% in PBS). Sections were incubated with primary antibody, either rabbit anti-AT\(_1\), 1:100 dilution or goat anti-AT\(_2\), 1:100 dilution (both from Santa Cruz Biotechnology, Inc., Santa Cruz, CA) at room temperature for 60 minutes. The sections were then rinsed two times for 10 minutes per rinse in PBS and stained using a Vectastain Elite kit (Vector Labs, Burlingame, CA). The tissue sections were incubated for 30 minutes in horseradish peroxidase (HRP) conjugated secondary antibody at 1:200 dilution (goat anti-rabbit or donkey anti-goat antibody, as appropriate, both from Santa Cruz Biotechnology, Inc., Santa Cruz, CA), rinsed two times in PBS, then incubated for 45 minutes in avidin-peroxidase reagent. After rinsing two times in PBS the sections were incubated in diaminobenzidine (Sigma Chemical, St. Louis, MO) for 3-5 minutes. Sections were rinsed in PBS twice for 5 minutes, rinsed quickly in distilled water, counterstained with 50% hematoxylin for 2 minutes, dehydrated in an ethanol series, then rinsed with xylenes and mounted with glass coverslips. Incubation with secondary antibody alone was performed for each vessel type to serve as controls. Sections were then viewed under a brightfield microscope (Nikon Optiphot-2) and imaged using a digital camera and Spot software (Diagnostic Instruments, Sterling Heights, MI).

**Data analysis.** Comparison of physiologic parameters was made using two-way analysis of variance, factoring for treatment group and timing in relation to infusion. If the overall analysis
of variance identified significant differences (P < 0.05), pairwise comparisons were made using Tukey's procedure, with P < 0.05 considered significant. Vascular responses and protein expression were compared using Student’s unpaired, two-tailed t-test (with significance at P < 0.05). All analyses were performed using SigmaStat 3.0 for Windows (SPSS Inc., Chicago, IL). All values are presented as mean ± SE, and n refers to the number of animals studied.
RESULTS

Physiologic Parameters. Fetal mean arterial blood pressure was similar between the groups prior to betamethasone administration (Table 1). The mean arterial blood pressure was higher in the betamethasone-treated than the saline-treated fetuses following the 48-hour infusion (53 ± 1 vs. 48 ± 1 mmHg, P < 0.05). There were no significant differences in weight, heart rate, hematocrit, arterial blood gas values or electrolytes between or within either group.

Vascular Reactivity. The maximal vasoconstrictive responses of coronary artery segments to 120mM KCl and 10⁻⁶M U46619 were not significantly altered by betamethasone infusion, while the maximal responses of mesenteric artery segments to 120mM KCl was significantly decreased following betamethasone infusion (Table 2). The dose-response curves for vasoconstriction to increasing concentrations of KCl were similar in betamethasone-infused and saline-infused coronary arteries (Figure 1A). Although the steroid-exposed coronary arteries achieved significantly more vasodilatation to low concentrations of acetylcholine (P < 0.05 vs. control), the vasoconstriction seen at higher concentrations was not significantly different between groups (Figure 1B). The maximal contractile response to angiotensin II was significantly increased in the coronary arteries of the betamethasone-infused versus the saline-infused animals (72 ± 17% vs. 23 ± 6% of the response to 120mM KCl, P < 0.05). However, contractile responses to ANG II were similar in betamethasone and control mesenteric arteries (48 ± 17% vs. 36 ± 12% of the maximal response to 10⁻⁶M U46619). Marked tachyphylaxis to increasing concentrations of ANG II was noted in both coronary and mesenteric arteries (Figures 1C and 1D, respectively).
Fetal mesenteric arteries from the betamethasone-exposed group were significantly less responsive to graded concentrations of KCl than their saline-exposed counterparts (Figure 2A, P < 0.05). There were no statistically significant intergroup differences in vasoconstriction to phenylephrine between the mesenteric arteries (Figure 2B). Responses in the betamethasone-exposed group tended to be reduced, but these responses in general were highly variable. Coronary arteries were completely unresponsive to phenylephrine at concentrations up to $10^{-4}$M (data not shown).

No between-group differences were detected in the coronary vasodilatory responses elicited by sodium nitroprusside, 8-Br-cGMP, isoproterenol or forskolin (Figure 3). The mesenteric artery vasodilatory response to acetylcholine was enhanced following betamethasone administration (Figure 4A, P < 0.05 vs. control), while the response to sodium nitroprusside was attenuated in the steroid-treated group (Figure 4B, P < 0.05 vs. control). There were no significant differences in mesenteric artery response to isoproterenol or forskolin (Figures 4C and 4D, respectively).

**Immunoblotting.** Western blotting consistently demonstrated the presence of AT$_1$ (Figure 5A) and AT$_2$ (Figure 6A) receptor protein in both mesenteric and coronary arteries. The immunoblots probed with the AT$_1$ receptor antibody showed the major band at 67 kDa, as previously reported by Marrero (19), while the AT$_2$ receptor antibody showed the major band at approximately 68 kDa, as previously reported by Servant (39). AT$_1$ receptor protein expression was upregulated by betamethasone infusion in coronary arteries (P < 0.05 vs. control), but not in mesenteric vessels (Figure 5B). There was no difference in AT$_2$ receptor protein expression following betamethasone infusion in either the coronary or mesenteric arteries (Figure 6B). AT$_1$
protein expression was greater in coronary arteries than mesenteric arteries, in contrast to AT₂ protein expression, which was greater in the mesenteric arteries (Figures 5B and 6B).

The immunoblots probed with the eNOS antibody showed the major band at 140 kDa (Figure 7). The increase in eNOS expression following betamethasone infusion did not reach statistical significance in either the coronary or mesenteric arteries (P = 0.12 and P = 0.14, respectively).

**Immunohistochemistry.** Immunostaining localized both AT₁ and AT₂ receptors within the tunica media of each isolated vessel with minimal endothelial staining (Figure 8).

**DISCUSSION**

Emerging information regarding the potential side effects of repeated doses of antenatal corticosteroids has tempered the unbridled use of betamethasone and dexamethasone to promote fetal maturation. In addition to detrimental effects on fetal growth and risk of infection following repetitive administration, the potential exists for cardiovascular maladaptive responses (3, 43). We have shown that direct parenteral administration of betamethasone enhances the coronary, but not mesentery, vasoconstrictor response to ANG II. In addition, betamethasone exposure led to an increase in the expression of angiotensin II receptors, specifically within the coronary arteries.

The direct effects of ANG II are mediated by two distinct receptors, classified as type 1 (AT₁) and type 2 (AT₂) based on selective antagonism by peptide and nonpeptidic ligands (5, 41). The tissue distribution and expression of these receptors are developmentally regulated. In general,
AT_2 receptor expression is high in fetal tissues and decreases with postnatal maturation, while AT_1 receptors appear later in fetal life with expression greatest in tissues regulating cardiovascular and fluid and electrolyte homeostasis (4, 6, 33, 40). The functions of the AT_2 receptor are unclear, although it may exert proapoptotic and vasodepressor effects (15, 46). The functional role of AT_1 receptors in the fetal vasculature has also been incompletely elucidated, although within the cardiovascular system, AT_1 receptor expression is responsive to increased glucocorticoid exposure (23, 25, 36). Furthermore, tissue-specific, local renin-angiotensin systems (RAS) are active in the fetus and are important modulators of circulatory function (8, 35); while later in life, vascular RAS have been shown to play a role in each step down the pathway of coronary artery disease, from endothelial dysfunction to lipid deposition, inflammation, vascular remodeling, apoptosis and thrombosis (24, 34).

In the present study, both AT_1 and AT_2 receptors were present in the coronary and mesenteric arteries. Immunohistochemistry verified the presence of AT_1 and AT_2 receptor protein in both arteries, and localized them predominately within the tunica media. The AT_1 receptors identified were functionally active, as evidenced by arterial contractile responses to ANG II. The detection of functional AT_1 receptors in the fetal coronary arteries complements our previous studies demonstrating AT_1 mRNA and protein are present in isolated fetal ovine renal and mesenteric arteries and that the receptor mediates contraction in response to ANG II (35). The progressive vasoconstriction each vessel displayed to increasing concentrations of ANG II reached a plateau at 10^{-8} M ANG II. Further increases in ANG II concentration resulted in an exponential decline in vessel tone as strong tachyphylaxis evolved. While displaying the same dose-response pattern, the coronary arteries that were exposed to betamethasone achieved peak responses twice
that of their twin controls. This difference may be related to the increased AT₁ receptor density as demonstrated by immunoblotting. The exaggerated coronary artery vasoconstriction to ANG II could predispose towards myocardial ischemia during high renin states or episodes of enhanced myocardial oxygen consumption. This finding may be of particular importance if coronary AT₁ receptor expression remains permanently elevated after antenatal exposure to glucocorticoids.

The vascular responses to potassium chloride-induced calcium channel activation were similar between treatment groups in the coronary arteries and diminished following betamethasone administration in the mesenteric arteries. Voltage-sensitive calcium influx therefore appears to be unrelated to the association between betamethasone administration and vascular hyper-reactivity in the coronary arteries. Likewise, the increase in intracellular calcium release that would have followed phospholipase C activation by U46619, a thromboxane A₂ mimetic, resulted in similar coronary artery contractile responses. These findings supplement the results seen by Anwar et al. in femoral arteries following betamethasone infusion (2). In their study, betamethasone increased the response of small femoral arterial branches to potassium chloride, but no difference in the vascular response to U-46619 were seen. Given the differential roles played by skeletal muscle resistance vessels and visceral conductance vessels, such as the coronary arteries, it is not surprising that they display differential responses to depolarizing potassium chloride concentrations.

While the betamethasone-exposed coronary arteries initially dilated following acetylcholine activation of endothelial nitric oxide synthase, activation of the muscarinic receptors present
within the vascular smooth muscle presumably resulted in overriding vasoconstriction, again involving the activation of phospholipase C. The propensity of coronary arteries to constrict to high concentrations of acetylcholine has been described, and the propensity of vessels exposed to betamethasone to constrict more intensely parallels the results seen by Docherty et al. in cerebral arteries (9, 45). These differences may be a subtle sign of early endothelial dysfunction resulting in overriding vasoconstriction. The ability of ANG II to affect such dysfunction through activation of plasma membrane-bound NADPH oxidase and subsequent production of reactive oxygen species deserves further consideration (14, 46).

Reminiscent of the coronary artery dilatory response to acetylcholine seen only in the steroid-exposed group, the betamethasone-exposed mesenteric arteries displayed significantly enhanced vasodilation to acetylcholine. These results mirror those seen by both Anwar and Molnar in ovine femoral arteries following steroid exposure (2, 21). In the face of accentuated endothelial-dependent vasodilation to acetylcholine following betamethasone exposure, the attenuated response of the same mesenteric arteries to a nitric oxide donor, sodium nitroprusside, raises the possibility that endothelial nitric oxide synthase activity was enhanced following steroid administration. Immunoblots subsequently showed a consistent trend towards increased eNOS expression following steroid-exposure in both the mesenteric and coronary arteries, although the results did not reach statistical significance. This finding is intriguing given the well described protective effect of antenatal corticosteroids on the development of necrotizing enterocolitis, a condition often associated with mesenteric ischemia (7). Highlighting the vessel specific alterations often seen in vascular research, eNOS expression has been shown to be down-
regulated in rat aorta and unchanged in ovine femoral arteries following dexamethasone administration (21, 44).

Unlike the results reported by Anwar et al. (2) showing attenuated femoral artery vasodilation to forskolin following exposure to betamethasone, we found no difference in coronary or mesenteric artery responsiveness to adenylate cyclase activation. Isoproterenol, a beta-agonist that mediates vasodilatation through G-protein coupled activation of adenylate cyclase, and forskolin, a direct activator of adenylate cyclase, produced similar dose-response relationships. These apparently contradictory results again stress the importance of vessel-specific research and highlight the possibility that the pathway connecting corticosteroids and hypertension may be different than that linking antenatal corticosteroid exposure and coronary artery disease. In fact, our own group and others have shown the postnatal increase in mean arterial blood pressure seen with antenatal glucocorticoid treatment is not abolished by AT₁ receptor antagonism, indicating the changes are mediated through mechanisms beyond peripherally accessible AT₁ receptors (29, 37).

Perspectives
There is increasing interest regarding the nonpulmonary effects of glucocorticoid exposure to the fetus. Maternal glucocorticoid administration lessens the incidence of complications associated with prematurity. However, a number of studies have indicated prenatal glucocorticoid exposure may lead to permanent effects on cardiovascular homeostasis (11, 28). In the present study, the amount of betamethasone the fetuses received (0.1 mg/kg/d based on an average fetal weight of 2.4 kg) approximates the equivalent amount of cortisol required at times of stress (roughly five
times the physiologic requirements) (42). Furthermore, this betamethasone dose approximates the fetal glucocorticoid exposure following maternal betamethasone administration to promote fetal maturation prior to preterm delivery, as well as the fetal exposure that results from the normal rise in serum cortisol prior to parturition at the end of term gestation (26). Thus, our findings provide novel information regarding the mechanism of tissue-specific glucocorticoid-induced coronary artery dysfunction following a clinically and physiologically relevant dose of betamethasone. Functionally active angiotensin II receptors mediating vascular tone are clearly present in the fetal cardiovascular system. If the heightened response of the coronary arteries to angiotensin II following betamethasone exposure represents a permanently programmed phenotype, rather than simply premature physiologic maturation, the exaggerated angiotensin responsiveness may provide a link between antenatal glucocorticoid exposure and cardiovascular morbidity. Continued investigation into the effects of corticosteroids on postnatal cardiovascular health is essential if we are to better understand the long-term consequences of antenatal glucocorticoid treatment.
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FIGURE LEGENDS

Figure 1. Cumulative dose responses curves for vasoconstriction (as % of maximal response to 120mM KCl or 10^{-6}M U46619) evoked by KCl (A), acetylcholine (B) or angiotensin II (C and D) in coronary (A, B, and C) or mesenteric (D) arteries from saline-treated (o, n = 5) or betamethasone-treated (●, n = 5) fetuses. Values are displayed as means with vertical lines indicating SE. *Significant difference between betamethasone and saline infused groups (P < 0.05).

Figure 2. Cumulative dose responses curves for vasoconstriction (as % of maximal response to 10^{-6}M U46619) evoked by KCl (A) or phenylephrine (B) in mesenteric arteries from saline-treated (o, n = 5) or betamethasone-treated (●, n = 5) fetuses. Values are displayed as means with vertical lines indicating SE. *Significant difference between betamethasone and saline infused groups (P < 0.05).

Figure 3. Cumulative dose responses curves for vasodilation (as % of pre-induced tone from 10^{-6}M U46619) produced by sodium nitroprusside (A), 8-Br-cGMP (B), isoproterenol (C) or forskolin (D) in coronary arteries from saline-treated (o, n = 5) or betamethasone-treated (●, n = 5) fetuses. Values are displayed as means.

Figure 4. Cumulative dose responses curves for vasodilation (as % of pre-induced tone from 10^{-6}M U46619) produced by acetylcholine (A), sodium nitroprusside (B), isoproterenol (C) or forskolin (D) in mesenteric arteries from saline-treated (o, n = 5) or betamethasone-treated (●, n = 5) fetuses. Values are displayed as means with vertical lines indicating SE. *Significant difference between betamethasone and saline infused groups (P < 0.05).
Figure 5. Western blot analysis for AT₁ receptor protein expression in ovine fetal coronary and mesenteric arteries. Simultaneous analysis was completed for steroid versus control-exposed vessels, and coronary versus mesenteric arteries. AT₁ receptor protein was detected with the major band at ≈ 67 kDa (A, representative immunoblot). There was increased expression of AT₁ proteins in the coronary arteries, accentuated following betamethasone exposure (B, n = 5). Values are displayed as means with vertical lines indicating SE. *Significant difference between betamethasone and saline infused groups (P < 0.05). #Significant difference between coronary and mesenteric arteries (P < 0.05).

Figure 6. Western blot analysis for AT₂ receptor proteins in ovine fetal coronary and mesenteric arteries. Simultaneous analysis was completed for steroid versus control-exposed vessels, and coronary versus mesenteric arteries. AT₂ receptor protein was detected with the major band at ≈ 68 kDa (A, representative immunoblot). There was increased expression of AT₂ proteins in the mesenteric arteries, but expression was not significantly increased following betamethasone exposure (B, n = 5). Values are displayed as means with vertical lines indicating SE. #Significant difference between coronary and mesenteric arteries (P < 0.05).

Figure 7. Western blot analysis for eNOS expression in ovine fetal coronary and mesenteric arteries. Simultaneous analysis was completed for steroid versus control-exposed vessels. eNOS was detected with the major band at ≈ 140 kDa (A and B, representative immunoblots). Expression was not significantly increased following betamethasone exposure (C and D, n = 5). Values are displayed as means with vertical lines indicating SE.
Figure 8. Immunostaining for AT₁ and AT₂ receptor proteins in coronary arteries (first and third rows, respectively) and mesenteric arteries (second and forth rows, respectively). Brown staining depicts positive signal. Left column, saline infused vessels. Middle column, betamethasone infused vessels. Right column, secondary antibody alone. Magnification x 200 for all vessels.
Figure 1.
Figure 2.
Figure 3.
Figure 4.

A

B

C

D

Figure 4.
Figure 5.

A

<table>
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<tr>
<th>Coronary Arteries</th>
<th>Mesenteric Arteries</th>
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<tr>
<td>Betamethasone</td>
<td>Control</td>
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kDa

- 85
- 67
- 42

B

AT1 Receptor Protein Expression (Densitometric Units)

- Betamethasone
- Control

* #
Figure 6.

A

Coronary Arteries
Betamethasone Control

Mesenteric Arteries
Betamethasone Control

kDa
- 89
\( \overset{\leftrightarrow}{68} \)
- 41

B

AT_2 Receptor Protein Expression
(Densitometric Units)

Betamethasone
Control

Coronary Arteries
Mesenteric Arteries

0
50
100
150
200
250
300

#
Figure 7.

A  Coronary Arteries
   Betamethasone  Control  kDa
   210  131  89

B  Mesenteric Arteries
   Betamethasone  Control  kDa
   210  131  89

C  eNOS Expression (Densitometric Units)
   Betamethasone  Control
   150  125  100  75  50  25
   0
   Coronary Arteries

D  eNOS Expression (Densitometric Units)
   Betamethasone  Control
   150  125  100  75  50  25
   0
   Mesenteric Arteries
Figure 8.

Saline Infused
Betamethasone Infused
Negative Control

AT$_1$ receptor proteins
Coronary

Mesentary

AT$_2$ receptor proteins
Coronary

Mesentary
Table 1. Physiologic parameters for ovine fetuses before and after 48-hour continuous intravenous infusion of saline or betamethasone.

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</tr>
<tr>
<td>PaO₂, mmHg</td>
<td>23 ± 3</td>
<td>23 ± 1</td>
</tr>
<tr>
<td>Na⁺, mmol/L</td>
<td>138 ± 1</td>
<td>138 ± 1</td>
</tr>
<tr>
<td>K⁺, mmol/L</td>
<td>4.5 ± 0.2</td>
<td>4.8 ± 0.3</td>
</tr>
</tbody>
</table>

Values are displayed as means ± SE; n = 5. MAP, mean arterial blood pressure; HR, heart rate; bpm, beats per minute.

*Significantly different from postinfusion control value at P < 0.05.
Table 2. Contractile response of isolated coronary and mesenteric artery segments to KCl and the thromboxane mimetic U46619.

<table>
<thead>
<tr>
<th></th>
<th>Coronary Arteries</th>
<th>Mesenteric Arteries</th>
</tr>
</thead>
<tbody>
<tr>
<td>Response to KCl (120mM), gram-force</td>
<td>1.4 ± 0.1</td>
<td>1.8 ± 0.2</td>
</tr>
<tr>
<td>Response to U46619 (10⁻⁶M), gram-force</td>
<td>3.9 ± 0.3</td>
<td>3.2 ± 0.3</td>
</tr>
</tbody>
</table>

Values are displayed as means ± SE; n = 5. *Significantly different from control value at P < 0.05.