Development of fetal vascular responses to endothelin-1 and acetylcholine in the sheep

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Development of fetal vascular responses to endothelin-1 and acetylcholine in the sheep. Am J Physiol Regulatory Integrative Comp Physiol 280: R554–R562, 2001.—Responses to K⁺, endothelin-1 (ET-1), and acetylcholine (ACh) of isolated adrenal, femoral, middle cerebral, and renal arteries from fetal [110–145 days gestational age (dGA, term −148 dGA)] and 0- to 24-h newborn (NB) lambs were evaluated using the technique of wire myography. Responses at distinct developmental ages for each vascular bed were compared. In all arteries sensitivity to K⁺-induced vasoconstriction was similar at all fetal age points examined. In contrast, sensitivity to ET-1 increased with increasing fetal age in arteries from all vascular beds. The magnitude of the maximal vasoconstriction was positively correlated with GA for K⁺ in adrenal, femoral, and cerebral arteries and for ET-1 in femoral, cerebral, and renal arteries. Cerebral arteries showed a greater sensitivity when compared with the other systemic arteries to K⁺ and ET-1 at all fetal ages and to K⁺ in NB. ACh evoked relaxatory responses in fetal and NB femoral and adrenal arteries. However, renal arteries relaxed comparatively less in response to ACh, and no vasodilation was noted in middle cerebral arteries at any age points examined. For femoral arteries ACh-induced vasorelaxation decreased with increasing GA but was restored in arteries from NB lambs. In summary, the responsiveness of isolated resistance arteries varies with developmental age in the fetal and perinatal sheep and these effects are both agonist and vascular bed specific. The augmented sensitivity in response to ET-1 of middle cerebral compared with other systemic arteries may reflect the importance of cerebral blood flow control during this critical developmental period.

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mid-trimester values. As a result, it has been suggested that ET-1 may contribute to fetal hemodynamic changes (32). In vivo administration of ET-1 has been shown to produce systemic and pulmonary hypertension in the late gestation fetal sheep (24). Evidence exists for postnatal age-related changes in ET-1-induced responses in pulmonary (14) and systemic (23) arteries.

The endothelial-derived agonists ET-1 and nitric oxide (NO) influence the effects mediated by one another. For example, NO inhibits the production of ET-1 in vivo and in cultured vascular endothelial cells (8). ET-1 can stimulate the release of NO via activation of endothelial ET\(_R\) receptors. However, ET-1 was shown to inhibit NO synthesis via activation of ET\(_A\) receptors in rat vascular smooth muscle cells (22).

Because altered vascular responsiveness with fetal development may reflect changes in the endothelium, we also examined responses of preconstricted arteries to the endothelium-dependent relaxatory agent acetylcholine (ACh). Numerous reports indicate a role of NO during fetal life in various vascular beds. For example, basal production of NO was shown to maintain baseline renal blood flow in third trimester fetal sheep (6). As with studies of vascular contractility, most previous studies examining development of relaxatory responses compare only postnatal age points (3, 33, 36).

There is a lack of information regarding development of vascular responsiveness during the final third of gestation, a critical period of fetal development, when fetal blood pressure is increasing. In the present study, we sought to determine the in vitro functional response of fetal [110–145 days of gestational age (dGA)] and NB (<24 h) sheep resistance arteries from adrenal, femoral, renal, and cerebral vascular beds to the known vasoconstrictor agents K\(^+\), ET-1 and the endothelium-dependent relaxatory agent ACh. We chose these specific fetal vascular beds because the cerebral and adrenal circulations are “protected” at times of oxygen deficiency and undergo vasodilation and increased flow (10). In contrast, the renal and femoral beds are unprotected, acting as reservoirs of peripheral vascular resistance to maintain perfusion pressure in protected vascular beds. In addition, to date no longitudinal prenatal ontogenic study has been performed in several vascular beds from the same fetuses. Therefore, in addition to examining ontogenic changes in arteries from a given vascular bed, we also aimed to compare effects in these functionally different fetal circulations at particular fetal age points.

### MATERIALS AND METHODS

#### Animal Preparation

Mature Rambouillet-Columbia cross-bred ewes (*Ovis ar- ries*) carrying a fetus of known GA after exposure to the ram at a single estrus were studied. Fetal sheep were removed from ewes at cesarean section under halothane general anesthesia. Fetuses at 110 (n = 4), 125 (n = 5), 138 (n = 6), and 145 (n = 6) dGA (term ~148 dGA) and 0- to 24-h NB (n = 7) lambs were euthanized by exanguination under halothane anesthesia. Fetal or neonatal brain, left kidney, left adrenal, and femoral muscle from the left side were collected in ice-cold physiological salt solution (PSS). The experimental protocol was approved by Institutional Animal Care and Use Committee at Cornell University for College of Veterinary Medicine. All facilities were approved by the American Association for the Accreditation of Laboratory Animal Care.

#### Wire Myography

Second-order middle cerebral, renal arcuate, adrenal cortical, and femoral arteries were immediately dissected from the collected tissue. Isolated arteries (~2 mm in length) were mounted on two 40-μm tungsten wires in a small vessel wire myograph as previously described (2). Vessels were equilibrated in PSS at 37°C and continuously aerated with 95% O\(_2\)-5% CO\(_2\) (pH 7.4) for 30 min. Vessel dimensions were then normalized individually by using their resting wall tension internal circumference curve. This determined the point on the fitted exponential curve corresponding to the internal circumference that the artery would have attained in situ when relaxed and exposed to a transmural pressure of 13.3 kPa (100 mmHg), termed \(L_{100}\). Preliminary studies showed that the active tension development is maximal in ovine fetal resistance arteries when set to 0.9 \(L_{100}\) (Fig. 1). Thus experiments described in this study were performed at 0.9 \(L_{100}\).

After several washes and a further 30-min equilibration, vessel responsiveness was tested by stimulation with NEK [5 μM norepinephrine (NE) in 125 mM potassium-substituted PSS (KPSS)], KPSS alone, 5 μM NE alone, and a second exposure to NEK. Cumulative concentration response curves were performed to KCl (2–125 mM) and ET-1 (10 μM) in PSS. The relaxing effects of the cumulative addition of ACh (1 μM–10 μM) were assessed after preconstriction with 5 μM NE for femoral and renal arteries. Because NE-induced vasoconstriction was not maintained in adrenal and middle cerebral arteries, 1 μM 5-hydroxytryptamine and 50 mM KCl were used to constrict these arteries, respectively. Additions were performed after a plateau in the response had been attained to the preceding concentration.

#### Drugs and Solutions

PSS was of the following composition (in mM): 119 NaCl, 4.7 KCl, 1 KH\(_2\)PO\(_4\), 0.9 MgSO\(_4\), 2.5 CaCl\(_2\), 11.1 glucose, 2.5 NaHCO\(_3\), and 0.021 EDTA. KPSS was prepared by equimolar substitution of NaCl by KCl in PSS. Chemicals for PSS and NE bitartrate salt, 5-hydroxytryptamine, and ACh were
purchased from Sigma (St. Louis, MO). ET-1 was obtained from Bachem (Torrance, CA).

Data Analysis

Tension is expressed as millinewtons per millimeter artery length or as a percentage of the maximal response to K\textsuperscript{+}. Relaxation is expressed as a percentage of the preinduced tension. Concentration-response curves were constructed by fitting data to the logistic sigmoid equation (log agonist concentration vs. effect) using the GraphPad Prism program (San Diego, CA). Sensitivity (pEC\textsubscript{50}) to the agonists is expressed as the negative log of the effective molar concentration of the agonist required to elicit 50% of the maximum response.

Statistical tests were performed using the SigmaStat program (Jandel, San Rafael, CA). Magnitude of responses and pEC\textsubscript{50} values were compared between groups by one-way ANOVA, followed by Tukey’s post hoc test. To examine the possibility of correlations in the responses with GA, the data were first subjected to a normality test. Data sets for which the residuals were normally distributed were subjected to the Pearson product moment correlation test. The Spearman correlation test was used to analyze data that failed the normality test. Correlation tests were performed on scatter plots of individual values obtained in each artery. Differences were considered statistically significant at \( P < 0.05 \). Power analysis tests were performed on statistical results. All results are presented as means ± SE, and \( n \) refers to the number of animals studied.

RESULTS

Normalized Internal Diameters

All arteries were normalized to 0.9 \( L_{100} \). The calculated internal diameters for all arteries within each age group are shown in Table 1. Arteries within each vascular bed were of a similar internal diameter regardless of the age point of study. For each particular age point, the only significant difference was seen between renal and middle cerebral arteries from 138 dGA fetuses (Table 1).

Responses to KCl

Affect of GA. Sensitivity to K\textsuperscript{+} was similar in middle cerebral arteries at all age points studied (Fig. 2C). Renal (Fig. 2D) and femoral (Fig. 2B) arteries from NB fetuses.
lambs were significantly less sensitive than those from 110, 125, and 138 dGA fetal sheep. Adrenal and renal arteries from NB lambs were less sensitive to K⁺ than those from 145 dGA fetal sheep.

Analyzing individual values for each artery showed that there was a significant positive correlation of the magnitude of the response to 125 mM K⁺ with dGA in femoral (Fig. 3B, P = 0.02) and middle cerebral arteries (Fig. 3C, P = 0.00005) but not in adrenal (Fig. 3A, P = 0.08) or renal (Fig. 3D, P = 0.33) arteries.

Comparing K⁺-induced vasoconstriction in NB vs. fetal arteries, the response in femoral and renal arteries from NB lambs was not significantly different from those in femoral and renal arteries from fetal sheep. Vasoconstriction to K⁺ was greater in middle cerebral arteries from NB lambs (2.28 ± 0.56 mN/mm) compared with 110 dGA fetuses (0.61 ± 0.12, P < 0.05). NB lamb adrenal arteries had a markedly attenuated K⁺ response compared with those isolated from 145 dGA fetuses (0.86 ± 0.15 vs. 1.78 ± 0.22, P < 0.05).

Comparison of vascular beds. Data in Fig. 2 allow comparison of K⁺ sensitivity in arteries from the different vascular beds for each developmental age. Potency of K⁺ was similar in all vascular beds at 110 and 125 dGA. Middle cerebral arteries were more sensitive compared with adrenal and renal arteries at 138 dGA and adrenal and femoral arteries at 145 dGA. K⁺ sensitivity was greater in middle cerebral arteries from NB lambs compared with all others. In isolated vessels from 145 dGA fetuses, renal arteries were more sensitive to K⁺ than femoral arteries. At <24 h after birth, renal arteries were significantly less sensitive than all others (Fig. 2A). The magnitude of the vasoconstriction to 125 mM K⁺ was similar in all isolated vessels at each age point examined.

Responses to ET-1

Affect of GA. Sensitivity of adrenal arteries to ET-1 was greater at 145 dGA compared with 125 and 138 dGA and in NB adrenal arteries compared with those from 125 dGA fetuses (Fig. 4A). Femoral arteries from 138 dGA fetuses were markedly more sensitive than those from 110 and 125 dGA fetuses and NB lambs; sensitivity was also greater at 145 compared with 110 dGA (Fig. 4B). Middle cerebral arteries from NB lambs were less sensitive to ET-1 compared with arteries from 138 and 145 dGA fetuses. The potency of ET-1 was also reduced at 110 and 125 dGA compared with 145 and 138 dGA, respectively (Fig. 4C).

There was a significant positive correlation of the magnitude of the maximum ET-1-induced response (in mN/mm) with dGA in femoral (Fig. 5B, P = 0.02) and middle cerebral (Fig. 5C, P = 0.0004), and renal arteries (Fig. 5D, P = 0.03) but not in adrenal arteries (Fig. 5A, P = 0.12). The maximal ET-1 response in NB arteries was similar to those in term fetal arteries from all vascular beds.
The maximum ET-1-induced vasoconstriction was significantly greater in renal (1.46 ± 0.2 mN/mm) compared with femoral (0.76 ± 0.2 mN/mm) and middle cerebral (0.61 ± 0.1 mN/mm) arteries at 110 dGA ($P < 0.05$). At all other GAs there was no significant difference in ET-1-induced maximum contractions, expressed as millinewtons per millimeter, between artery types.

Analyzing the ET-1 maximum as a percentage of $K^+$ maximum for each artery showed a significantly greater vasoconstriction in adrenal and renal arteries compared with middle cerebral arteries at 125 dGA (adrenal 149.2 ± 11.4% and renal 151.1 ± 8.2% vs. middle cerebral 90.8 ± 7%, $P < 0.01$) and <24 h after birth (adrenal 159.8 ± 3.1% and renal 142.7 ± 16.1% vs. middle cerebral 86.8 ± 8.1%, $P < 0.05$). The mag-
nitude of the ET-1 maximum was also greater in femoral compared with middle cerebral arteries from NB lambs (168.9 ± 15.2% vs. 86.8 ± 8.1%, P < 0.01).

Responses to ACh

Affect of GA. Sensitivity of adrenal (pEC50 ~ 7.1) and femoral (pEC50 ~ 7.2) arteries to ACh-induced relaxation did not alter with developmental age. Maximal relaxatory responses were comparable in adrenal arteries at all GAs, being ~65% of the induced tone (Fig. 6A). However, in femoral arteries the maximal relaxatory response was greater at 125 (16.2 ± 0.7% of induced tone) compared with 145 dGA (66.7 ± 13.2%, P < 0.05). A marked ACh-induced relaxation was restored 0–24 h after birth, as the maximal relaxatory response of femoral arteries from NB lambs (16.4 ± 6.6%) was greater than that noted at 138 (60.9 ± 15.1%) and 145 dGA (P < 0.05, Fig. 6B).

ACh did not evoke a relaxatory response in renal arteries from 125-dGA fetuses and 0- to 24-h NB lambs (Fig. 6D). Renal arteries from 138 and 145 dGA fetuses relaxed ~50% of the induced tone (Fig. 6D). ACh concentrations higher than 0.3 μM evoked a contractile response. No relaxatory response was noted in middle cerebral arteries from fetal or NB sheep. Indeed, increasing ACh concentrations tended to augment the vascular tone (Fig. 6C).

Comparison of Vascular Beds

The maximal ACh-induced response was similar in adrenal, femoral, and renal arteries examined from 138 and 145 dGA fetuses. Because of the small or negligible relaxation of renal and middle cerebral arteries, the maximal vasorelaxation of femoral arteries was greater than that of renal and middle cerebral arteries at 125 dGA (P < 0.01) and 0–24 h after birth (P < 0.001). Relaxatory response of adrenal arteries was greater than that of 1) middle cerebral arteries from 125 dGA (P < 0.05) and NB (P < 0.001) preparations and 2) renal arteries from NB lambs (P < 0.001).

DISCUSSION

K+-Induced Responses

Adrenal, femoral, and renal arteries from NB lambs were less sensitive to K+ compared with fetal arteries, whereas NB lamb middle cerebral arteries remained unchanged. Other investigators suggest that vascular maturation involves important shifts in the mechanisms mediating vascular pharmamechanical coupling. In particular, in the cerebral vasculature it has been proposed that normal development may involve a reduction in the Ca2+-sensitizing effects of agonists (1). Furthermore, under pathophysiological conditions it is possible that failure of this proposed shift may lead to maintained vascular hyperreactivity in adult vessels.

Although K+ sensitivity of middle cerebral arteries was unaltered in the transition from fetal to NB life, the magnitude of the maximal K+-induced vasoconstriction was augmented. An earlier study on isolated ovine common carotid, basilar, posterior communicating, and middle cerebral arteries also showed an increase in maximum contractile tension during the transition from fetal to NB life (35). The increase in tension with age ranged from 18% in middle cerebral arteries to 82% in the common carotid. In contrast, Wagerle et al. (41) reported a reduction in the magnitude of NE response in ovine middle cerebral arteries...
of a larger diameter than we examined with GA (105
dGA, 7 days postnatal). Akopov et al. (1) reported that
K⁺ evoked a similar contractile tension and cytosolic
Ca²⁺ response in basilar arteries from 138–141 dGA
fetal and adult sheep. Hence a possible explanation for
differing findings between studies with regard to the
effect on maximal tension generated may be related to
methodological factors such as differences in diameter
of arteries under investigation and set-up procedures.
Baseline Ca²⁺ sensitivity of ovine cerebral arteries was
shown to vary with artery size and age, being greater
in immature than in mature arteries, correlating in-
versely with artery size (1). Inositol 1,4,5-trisphos-
phate [Ins(1,4,5)P₃] receptor density increased from
140 dGA to NB and adults in ovine common carotid but
not in posterior, middle, or anterior cerebral arteries
(43).

Sensitivity to K⁺ was unaltered (manifested by sim-
ilar pEC₅₀ values) in all arteries over the GA period we
examined. However, the magnitude of the maximal
vasoconstriction was positively correlated with GA in
femoral and middle cerebral arteries, possibly because
of increase in vascular smooth muscle mass. Differences
in responsiveness with GA and between arteries of
different origin could reflect dissimilarities in vas-
cular smooth muscle phenotype. Expression of smooth
muscle proteins and myosin heavy chain isoforms have
been shown to be developmentally regulated and tissue
dependent in the sheep (9). Studies in perinatal and
adult sheep arterial smooth muscle show that signifi-
cant maturational changes occur in the isotonic and
isometric mechanical properties of vascular pulmonary
and systemic smooth muscle (4).

ET-1-Induced Responses

ET-1 evoked a potent concentration-dependent vaso-
constriction in isolated resistance arteries from several
ovine systemic beds, including renal arcuate arteries.
A contractile effect of ET-1 has been shown in isolated
renal arteries from several species, including adult rat
(13) and human (26). However, in vivo ET-1 has a
vasodilatory effect in the renal circulation of the fetal
sheep. ET-1 activates two different receptors, ETₐ and
ETᵦ. In this earlier study (7), ET-1 was shown to act
primarily via ETᵦ receptors, producing vasodilation;
however, ETₐ receptors, mediating vasoconstriction,
were shown to contribute to renal vascular tone. Such
disparities in findings may be due to differences in
experimental conditions, such as in vivo vs. in vitro,
vessel size investigated, and/or species variability, fac-
tors that have been shown to alter ET receptor popu-
lations (30).

In adrenal, femoral, renal, and middle cerebral ar-
teries cumulative addition of ET-1 resulted in tachy-
phylaxis at concentrations exceeding 0.1–0.3 µM. This
desensitization was specific for ET-1 because it was not
observed with K⁺. Such a tachyphylactic phenomenon
has been reported previously for ET-1-induced re-
sponses (29) and is also commonly observed in studies
involving angiotensin II (25).

In contrast to our finding with K⁺, sensitivity to
ET-1 increased with fetal age in all vascular beds. ET-1-induced maximal vasoconstriction increased with
GA in femoral, middle cerebral, and renal arteries but
not adrenal arteries. Human fetal ET plasma levels are
significantly higher than maternal (27), and develop-
mental changes also occur in fetal plasma ET-1 levels
(16). Endo et al. (16) measured serum NO metabolites
and ET-1 in healthy human neonates at birth, at 12
and 24 h, and at 5 days postnatally. The lowest con-
centration of NO metabolites was observed at birth
and increased with age, whereas the highest concentra-
tion of ET-1 was observed at birth and decreased with age
(16). These findings together with our own indicate an
important role for ET-1 as a vasoactive agent involved
in the transition from fetal to extraterterine life.

Middle cerebral arteries from ovine fetuses showed
the greatest sensitivity to K⁺ and ET-1 compared with
the other systemic arteries we investigated. Porcine
cerebral arteries also have been shown to be more
sensitive to ET-1 compared with coronary and renal
arteries (17). Enhanced responses of canine cerebral
and coronary arteries to ET-1 compared with mesen-
teric arteries was shown to be dependent on Ca²⁺ influx
through voltage-dependent Ca²⁺ channels (34). ET-1
also induced greater accumulation of inositol
bisphosphate (InsP₂) in rat mesenteric arteries than in
thoracic aorta, suggesting that turnover of Ins(1,4,5)P₃
may be faster in the former than in the latter (34).

The ability to regulate blood supply to the fetal and
neonatal brain very precisely according to prevailing
conditions is critical for survival of the challenges of
perinatal life. Cerebrovascular homeostasis is depen-
dent on the responsiveness of the cerebral arteries to
vasoactive agents. The importance of precise control
of cerebral blood flow is reflected in our finding of signif-
icantly enhanced contractile responsiveness of middle
cerebral compared with adrenal, femoral, and renal
arteries.

ACh-Induced Responses

We utilized the endothelial-dependent agent ACh to
examine the role of NO in fetal and NB arterial respon-
siveness. Concentration-dependent relaxations were
noted in all femoral and adrenal arteries. A notable
relaxation of renal arcuate arteries was only evident in
vessels from 138 and 145 dGA fetuses when tension
was reduced to ~50% of the preconstricted tone. Such
ACh infusion in third trimester fetal sheep caused a 60% reduction in baseline tension (6). Hence
in vitro and in vivo studies indicate a regulatory role of
NO (basal and/or agonist induced) in renal blood flow.

In contrast to the other arteries, second-order middle
cerebral arteries from fetal and NB sheep did not relax
to ACh. Similarly, examination of endothelium-depen-
dent relaxations in isolated arteries from adult rabbits
and dogs demonstrated a reduced effect of ACh in
cerebral compared with extracerebral (e.g., femoral)
arteries (31). Hayashi et al. (19) reported that isolated
cerebral artery strips from premature and NB baboons
showed a marked contractile response to ACh, whereas arteries from adult baboons showed little response. In contrast, in vivo studies of cerebral arterioles of 90–111 dGA, 128–143 dGA, and NB sheep showed an increase in pial arteriolar diameter in response to ACh, thus demonstrating a sensitive dilator response in immature fetuses (39). Such discrepancies may be related to the specific cerebral arterial branch being examined. For example, vascular segments in the fetal sheep lung have been shown to differ anatomically and functionally from one another. Immunostaining for soluble guanylate cyclase showed very weak to no staining in large arteries but intense staining in distal arteries, implying a greater role for NO-mediated vasodilation in smaller arteries (11). Because vessels were precontracted with K+ depolarization, we are not able to comment on endothelium-derived hyperpolarizing factor-dependent mechanisms.

Sensitivity of femoral and adrenal arteries to ACh did not change with developmental age. However, the mean maximum relaxation in femoral arteries was greater at 125 compared with 145 dGA. The reduction in relaxation with increasing GA may indicate a relative attenuation in NO generation. Such a change could contribute to the physiological rise in fetal blood pressure of ~1 mmHg/day, which occurs from ~125 dGA (38). ACh-induced relaxation was restored in femoral arteries 0–24 h after birth. The increased ACh responsiveness may be indicative of a greater role of basal and/or agonist-induced NO in femoral artery tone at 125 dGA and <24 h after birth compared with the other GA examined. Interestingly, the ACh relaxation was greatest in femoral arteries in the groups that corresponded to the age points in femoral arteries, which showed the lowest ET-1 sensitivity. Moreover evidence exists for the interaction of these two endothelial-derived agents (8, 22).

Other investigators have examined postnatal changes in ACh-induced relaxation of femoral arteries. The relaxatory response to ACh was shown to be similar in femoral arteries from NB and 7-day-old piglets. Interestingly, ACh response was not influenced by ET-1. These observations, together with those described above, provide evidence for the interaction of these two endothelial-derived agents (8, 22). However, the precise mechanisms underlying these interactions are not fully understood.

In summary, previous studies examining ontogeny of vascular responsiveness have focused on postnatal changes and those occurring with adult maturation. To our knowledge, this is the first study that examines reactivity of resistance arteries from several vascular beds, both protected and unprotected, of the same species across the last third of gestation and the 24 h after birth. The overall findings are that the responsiveness of isolated ovine resistance arteries varies 1) with fetal developmental age and 2) between four distinct and critical vascular beds, both protected and unprotected, and 3) these effects are agonist specific.

Information on such fundamental differences in vascular reactivity to both vasoconstrictor and vasodilator agents between vascular beds and the variation with fetal developmental age is essential to our knowledge of regulation of vascular function at this critical stage of development during which correct vascularization of developmental tissue is vital to normal tissue growth and development.

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REFERENCES

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