INTRODUCTION

The early view of the human placenta as a passive organ in which blood flow depends only on the arteriovenous pressure difference has been modified by recent evidence that the regulation of vasomotor tone in the vessels of the fetoplacental circulation is important to maintain an adequate blood supply to the fetus (1, 2). As fetoplacental blood vessels lack autonomic innervation, control of vascular tone is mainly influenced by humoral and locally released agents as well as by physical factors such as flow or oxygen tension (3). Accordingly, constriction and relaxation of fetoplacental arteries and veins have been demonstrated in response to a number of agonists and physical stimuli (4-6). Moreover, the fetoplacental vasculature also shows a flow matching mechanism similar to hypoxic pulmonary vasoconstriction (HPV). This mechanism, termed hypoxic fetoplacental vasoconstriction (7-9), would divert blood flow to the placental areas with better maternal perfusion as HPV diverts blood to the better ventilated areas of the lung (9).

The avian homologue of the mammalian placenta is the chorioallantoic membrane (CAM), the principal respiratory organ of the chick embryo during the last two thirds of development (10). At day 6 of incubation (of a total of 21 days), the CAM progressively assumes the gas-exchange function of the area vasculosa, the previous respiratory organ (11). By days 14-15 the CAM capillary volume reaches a maximum and around the end of day 19, the CAM degenerates when lung ventilation is initiated during internal pipping (11). Due to the high rates of vascular proliferation that accompanies its development, the CAM is probably the most widely used in vivo assay for studying angiogenesis (12). However, although this vascular proliferation occurs while the new forming vessels are simultaneously acquiring the capacity to regulate CAM vascular tone, there is no information on the chorioallantoic (CA) vascular reactivity.

The knowledge about the mechanisms which govern CA arterial tone can lead to a better comprehension of the vascular biology of its human analogue, the fetoplacental arteries. In the present study we hypothesized that CA vessels are endowed with vascular regulatory mechanisms similar to those found in human fetoplacental arteries and that CAM vascular responsiveness would be largest at the time when CA vessels reach the largest degree of development. To test our hypothesis, we analyzed the in vitro responsiveness of CA arteries to vasoactive agonists with known activity in the human fetoplacental arteries at two developmental stages: maximal CA vascular expansion (15 of the 21 days incubation period) and prior to hatching. Therefore, we analyzed the reactivity of third order arteries (~200 µm) from the CA membrane of 15 and 19 day chicken embryos. CA arteries contracted in response to K+, the thromboxane A₂ mimetic U46619, endothelin-1, acetylcholine and acute hypoxia, but showed no reaction to α-adrenergic stimulation (phenylephrine). The nitric oxide donor sodium nitroprusside, the adenylyl cyclase agonist forskolin, and the β-adrenergic agonist isoproterenol relaxed CA arteries pre-contracted with K+ or U46619. The contraction evoked by acetylcholine and the relaxations evoked by sodium nitroprusside and isoproterenol decreased with incubation age. In conclusion, CA arteries share many characteristics with human fetoplacental arteries, such as pronounced relaxation to β-adrenergic stimuli and hypoxic vasoconstriction. Our study will be the foundation for future studies to explain disparate and common responses of the CA and fetoplacental vasculature.

Key words: β-adrenergic agonist, chicken embryo, chorioallantoic membrane, hypoxic vasoconstriction, thromboxane A₂, vasoreactivity
The experimental work was performed at the Department of Pediatrics, GROW School for Oncology and Developmental Biology, Maastricht University Medical Center (MUMC+), the Netherlands.

MATERIAL AND METHODS

Egg incubation and vessel isolation

All experiments were performed in accordance with the Dutch law of animal experimentation and were approved by the local Ethical Committee. Fertilized eggs from a pure broiler sire strain were obtained from Breeding Research and Technology Centre van Hendrix Genetics B.V. (Boxmeer, the Netherlands). Eggs were incubated in 37.8°C, 45% relative humidity and turned 90° every hour (Model 25HS, Masalles Comercial). CAM arteries were sampled at 15 and 19 days of incubation (hatching at 21 days). After cutting the roots of the extraembryonic vessels at the point of exiting the abdominal cavity, the egg shell lined with the CAM was rinsed with room temperature Krebs-Ringer Bicarbonate (KRB) buffer and, with the aid of a dissection microscope, tertiary branches of the CA artery (in situ external diameter ~200 μm) were carefully isolated and cut into ~2 mm long rings. Care was taken to keep the endothelium intact. Only one CA artery ring per egg was used.

Recording of arterial reactivity

Chorioallantoic artery rings were mounted between an isometric force transducer (Kistler Morce DSC 6) and a micro displacement device in a myograph (model 610 M, Danish Myotechnology) using stainless steel wires (diameter 40 μm). The myograph organ baths were filled with KRB (38°C and bubbled with 95% O2: 5% CO2). After a 30 min equilibration, the vessels were distended to a resting tension corresponding to a transmural pressure of 1.33 or 2.66 kPa, the mean arterial pressure at 15d and 19d respectively (13). The average final diameter of the vessels at the above transmural pressures did not differ significantly between the age groups (490±28.5 and 590±45.5 μm for 15d and 19d, respectively). After 30 min of stabilization under resting tension, a control contraction was elicited by raising the K+ concentration of the buffer (to 62.5 mM) in exchange for Na+. The preparations were washed three times and allowed to recover before a new stimulation.

Contractile agonists were evaluated under resting tone. Cumulative concentration-response curves to K+ (4.75-125 mM), the thromboxane A2 mimetic U46619 (1 nM-1 μM), endothelin-1 (ET-1; 1 nM-100 nM) and the α1-adrenoceptor agonist phenylephrine (Phe; 10 nM-100 μM) were constructed by increasing the concentration of the drug. In a group of experiments, the responses to K+ and U46619 were analyzed in the presence of the nitric oxide (NO) synthase (NOS) inhibitor Nω-nitro-L-arginine methyl ester (L-NAME, 0.1 mM).

Relaxant agonists were evaluated during contraction induced by 62.5 mM K+. 1 μM U46619. Time controls showed that KCl and U46619 developed a steady-state developed tension over the course of the experiment. Concentration-response curves to acetylcholine (ACh; 10 nM-100 mM), bradykinin (10 nM-0.1 mM), the NO donor sodium nitroprusside (SNP; 10 nM-100 mM), the β-adrenergic agonist isoproterenol (Iso; 1 nM-1 mM) and the adenylate cyclase activator forskolin (Forsk; 1 μM-10 mM) were carried out. In 19 day embryos, concentration-response curves to acetylcholine were also performed after blocking cyclooxgenenase 1 and 2 with indomethacin (Indo; 10 μM).

To study the effects of acute hypoxia, the organ chambers were wrapped in cling film to stabilize oxygen partial pressures, which was checked by a blood gas analyzer (ABL 510 Radiometer). Hypoxia was induced by switching the gas mixture to the organ bath from 95% O2/5% CO2 (yielding a pO2 of 74.7±1 kPa) to 95% N2/5%CO2 (yielding a pO2 of 2.48±0.1 kPa). After 10-20 min of hypoxic exposure, the gas mixture was switched back to 95% O2/5% CO2.

Scanning electron microscopy

In order to discard a damage of the endothelium during the myograph study, the endothelial surface of a subset of vessels (n=8 of each age) was examined by scanning electron microscopy, as previously described (14). After the myograph study, rings were fixed overnight at 4°C in 2.5% glutaraldehyde in 0.13 mol/L cacodylate buffer, pH 7.4. After being rinsed twice in buffer, specimens were postfixed in 1% ostium tetroxide at 4°C for 1.5 hours, rinsed three times in cacodylate buffer, and dehydrated through graded concentrations of ethanol. The specimens were critical point-dried from 100% ethanol with liquid CO2. After the specimens were dried, they were bisected to expose the luminal surface, mounted on stubs, and sputter-coated with gold. The samples were viewed and photographed at 10 kV with a scanning electron microscope (Philips XL30, Eindhoven, the Netherlands).

Immunohistochemistry of the chorioallantoic membrane

Following fixation in 4% paraformaldehyde (60 min) whole mount sections of the chorioallantoic membrane of 19 day old embryos were permeabilized in phosphate-buffered saline (0.1 M, pH 7.2) containing 0.25% Triton X-100 and incubated overnight with a mouse anti-neurofilament antibody (4H6, dilution 1:10, obtained from the Developmental Studies Hybridoma Bank). For immunoperoxidase staining, the membranes were incubated with rabbit anti-mouse IgG (1:50, DakoCytomation Z0259) for 60 min followed with incubation in PAP mouse monoclonal (1:50, DakoCytomation P0850) and reacted with 0.5% 3,3' diaminobenzidine tetrahydrochloride diluted in Tris-HCl buffer (0.1 pH 7.6) containing 0.015% H2O2. Segments of mesenteric membranes from adult chickens were processed in identical fashion and served as positive controls.

Data analysis

All results are presented as mean±S.E. unless otherwise stated. Contractions are expressed as active wall tension (Nm-1) or as percentage of the contraction induced by K+ (62.5 mM) in the same vessel. Relaxations are expressed as percentage of depression of the contractions evoked by K+ or U46619. Potency (expressed as pD2= -log EC50) and efficacy (expressed as maximal effect, Emax) was determined for each artery by fitting individual concentration-response data to a nonlinear sigmoid regression curve and interpolating (GraphPad Prism 4; GraphPad Software Inc.). In the case of Iso, where the drug produced a biphasic response, only the first part of the curve (relaxation) was analyzed. The difference between mean values from 15d and 19d chicken embryos was assessed by Student’s t-test. The fiducial level of significance was set to P<0.05.

Drugs and solutions

The composition of the KRB buffer was (in mM): NaCl 118.5, MgSO4 1.2, K2HPO4 1.2, NaHCO3 25.0, KCl 1.7, CaCl2 2.5 and glucose 5.5. Solutions containing different K+ concentrations were prepared by replacing part of the NaCl with an equimolar amount of K+. Forsk and ET-1 were obtained from Alexis
Biochemicals and U46619 from Cayman Chemicals. All the other drugs were obtained from Sigma-Aldrich. All drugs were dissolved in deionised water, except Forsk and Indo, which were initially dissolved in ethanol and U46619, which was initially dissolved in DMSO. Total DMSO or ethanol added to the organ bath was, at the most, 0.1% v/v and did not affect arterial tone.

RESULTS

Contractile responses

Isolated CA artery rings of 15d and 19d embryos responded with tonic contraction to K+ up to a concentration of 93.75 mM (Fig. 1A and 1B). The response did not differ between ages (Fig. 1D). The effect of all other contractile agents was subsequently expressed as % of the contraction induced by K+ (62.5 mM) in the same vessel. Phe did not evoke any significant change in the tone of the chorioallantoic arteries (Fig. 1C).

ET-1 (Figs. 2A and 2B) and U46619 (Figs. 2C and 2D) induced a concentration-dependent contraction with a threshold concentration of 10 nM. Dose-response curves to ET-1 and U46619 could not be adequately fitted because maximum effect was not reached at the highest concentration of the drug used (Figs. 2E and 2F, respectively). When the contractile effect of equimolar (1 µM) concentrations of U46619 and ET-1 were compared, a higher contractile efficacy was observed for U46619. We observed that vasomotion (example shown in Fig. 2A and 2C) was present in all 15d vessels stimulated with ET-1 and U46619, but absent in all vessels from 19d embryos. The same phenomenon was observed in 100% of 15d arteries and 88% of 19d arteries contracted with U46619 (>0.1 µM). No significant differences between age groups were observed for the magnitude of ET-1- or U46619-induced contraction (Figs. 2E and 2F, respectively). The NOS inhibitor L-NAME (0.1 mM) induced a slight contraction in the 19d CA arteries (7.2±0.9% of the contraction induced by 62.5 mM K+, n=6). In contrast L-NAME-induced contraction was not observed in the 15d vessels. The presence of L-NAME did not significantly affect the contractile response to KCl or U46619 at any age (results not shown).

Response to ACh and bradykinin

In CA arteries contracted with K+ or U46619, ACh induced an additional concentration-dependent increase in wall tension (Figs. 3A, 3B and 4A). In order to discard that the lack of ACh-induced relaxation was due to an inadvertent damage of the endothelium during the experiment, we tested endothelial integrity in a subset of vessels (n=8 of each age) by scanning electron microscopy. We observed that during the experimental procedure the endothelium of the CA arteries was not removed (Fig. 5). The maximal contraction induced by ACh was significantly larger in 15d than in 19d (Fig. 4A). ACh-induced contraction was not affected by the presence of Indo (results not shown). ACh applied under resting tone (i.e. without pre-contraction) had a negligible effect on wall tension and bradykinin did not affect the tone induced by K+ or U46619 in 15 and 19d CA arteries (results not shown).

Relaxation responses

SNP relaxed K+-contracted vessels in a concentration-dependent manner (Fig. 3C, 3D and 4B) without reaching a plateau at the highest concentration tested (0.1 mM). Starting from concentrations higher than 10 µM, the relaxant effect of SNP was significantly higher in 15d than in 19d arteries.
Iso and Forsk also relaxed CA arteries (Figs. 3E-3F and 3G-3H, respectively). Whereas Forsk fully reverted the tone evoked by K+ or U46619 (Figs. 4D and 4F), Iso only induced a partial relaxation (Figs. 4C and 4E) and high concentrations (>1 µM) triggered vasoconstriction (Fig. 3E and 3F). Forsk-induced relaxation was not significantly affected by age or the pre-contractile agent. In contrast, Iso showed a higher relaxant efficacy in the 15d vessels pre-contracted with U46619 than in the 15d vessels pre-contracted with K+. These pre-contraction-related differences in Iso-induced relaxation were not observed in the 19d vessels. No significant differences to the relaxant potency and efficacy of Iso between ages were found (pD2 7.57±0.07 vs 7.25±0.08 for 15d vs 19d K+ pre-contracted arteries, respectively and 8.07±0.24 vs 7.97±0.19 for 15d vs 19d U46619 pre-contracted arteries, respectively). However, as shown in Fig. 4C and Fig. 4E, some particular concentrations of Iso evoked lower relaxation in 19d when compared with 15d.

Response to hypoxia

In non-precontracted CA rings, hypoxia evoked a tonic contraction that was relatively stable after ~5 min in both 15d and 19d vessels (Fig. 6A, top and bottom, respectively). The hypoxia-induced contraction was completely reversed upon reoxygenation. No age-related differences were observed in the response of CA arteries to hypoxia (Fig. 6B).

CAM immunohistochemistry

Positive specific staining of neurofilaments is shown in the mesenteric plexus in Fig. 7A and has also been observed in other embryonic vessels such as in the aortic arch (Altimiras, unpublished results). The absence of innervation of the CAM was confirmed by the absence of specific staining against chicken neurofilaments, as shown in Fig. 7B.
DISCUSSION

Gas exchange organs like the placenta or the postnatal lung possess high flow, low resistance vascular beds in which \( \text{O}_2 \) tension plays a critical regulatory role (9, 15). Our results show important similarities between the vascular reactivity of chicken CA arteries and reactivity reported for human fetoplacental arteries. These include lack of responsiveness to \( \alpha \)-adrenergic receptor stimulation, marked relaxation to \( \beta \)-adrenergic stimulation, no relaxation to known endothelium-dependent vasodilators (i.e. ACh and bradykinin), and presence of hypoxic vasoconstriction. In addition, when arteries from the CAM at its “functional peak” (15d) were compared with those close to the end of its function (19d), we observed selective changes in ACh-
induced contraction as well as SNP- and Iso-induced relaxation. Thus, chorioallantoic arteries display similar vasoactive mechanisms as their human homolog, fetoplacental arteries.

Developmental changes in the reactivity of chorioallantoic arteries

Knowledge about the ontogeny of vasoactivity in human fetoplacental vessels is limited because most research has been conducted on term placentas. Placental trophoblasts are an important bi-interface between the maternal and the fetal compartments because they produce a spectrum of hormones and vasoactive compounds including thromboxane A₂, ET-1, prostacyclin, and NO (16-18), but it is unknown if this local release changes during pregnancy. A developmental increase in K⁺-induced contraction (19) and a decrease in angiotensin-induced contraction (20) of human fetoplacental arteries have been reported and they could serve to protect the uteroplacental

Fig. 4. Concentration-dependent effects of putative relaxant agonists. Effects of increasing concentrations of acetylcholine (ACh, A), the NO-donor sodium nitroprusside (SNP, B), the β-adrenergic agonist isoproterenol (Iso, C, E) and the adenylate cyclase activator forskolin (Forsk, D, F) in isolated chorioallantoic arteries from 15 (open symbols) and 19 (solid symbols) day old chicken embryos (total incubation time 21 days). The vessels were pre-contracted with 62.5 mM K⁺ (A-D) or 1 µM U46619 (E, F). Relaxation is expressed as percentage of K⁺ (A-D) or U46619 (E and F) pre-contraction. n=6 to 9 in all experiments, except for E, in which 15d n=4. Data points are presented as mean±S.E.; * P<0.05 15 day vs. 19 day old embryos.
vasculature from the alterations in maternal vasoactive factors that accompany normal pregnancy (20).

Because the chick embryo lacks maternal influences such a protective mechanism is not needed. Nevertheless, chorioallantoic arteries of 15d chick embryos responded to K+-evoked depolarization and to a variety of agonists indicating the presence of electro- and pharmaco-mechanical coupling. In contrast to the less mature systemic arteries (21), CA arteries can regulate flow in the CAM and the mechanisms are already functional at the time when it reaches a maximal capillary volume (22). As the chick gets closer to hatching, the increments in pulmonary blood flow during internal pipping are coupled to a decrease in chorioallantoic blood flow (23). Thus, the maturation of CA vasoactivity could serve to balance the prevailing vasodilatory tone during development, which could explain the significant drop in SNP- and Iso-induced relaxation.

In vitro investigations of the contractile properties of intact vascular segments are performed using two principally different methods. Vessels can be mounted either as isometric preparations (‘wire-myograph’), when force development is measured at a certain diameter, or cannulated as isobaric preparations where the vessel is exposed to a transmural pressure and allowed to change diameter (‘pressure-myograph’) (24-26). It has been argued that certain characteristics of the vessels are critically dependent on which method is used and that the in vivo situation corresponds more closely to an isobaric condition than to an isometric condition. Nevertheless, in terms of assessing the pharmacological activity of drugs on isolated blood vessels, the use of isometric recording procedures, which were utilized in the present study, are largely considered to be adequate (24-26).

Acetylcholine-induced contraction of chorioallantoic arteries

ACh has been widely used in numerous vascular beds to stimulate endothelium-dependent relaxation (27-29). In the chicken embryo, ACh induces an endothelium-dependent and, at least partially, NO-mediated relaxation of pulmonary and systemic arteries (14, 30-32). In addition, ACh and other endothelium-dependent agonists also induce the release of endothelium-derived contracting factors (EDCFs) such as prostanoids or reactive oxygen species (33, 34). ACh-induced
contraction is interpreted either as a sign of endothelial dysfunction in which EDCFs are insufficiently counteracted by NO or other endothelium-derived relaxing factors (33, 34), but has also been attributed to the direct action on muscarinic receptors of vascular smooth muscle cells in the absence of functional endothelium-dependent vasodilatation. However, in the embryonic chicken ductus arteriosus (35) and CA arteries (present work) we have observed ACh-induced contraction. In the ductus arteriosus, low concentrations of ACh elicited a relaxation, followed by a contraction at higher concentrations (35). ACh-induced contraction was completely endothelium-dependent and involved a cyclooxygenase-1-derived TP receptor agonist (35). In CA arteries we observed that ACh-induced contraction was present in endothelium-intact vessels and was not blocked by Indo, but the exact mechanism behind the ACh-vasoconstriction warrants further investigation. In contrast to ACh, the NO donor SNP relaxed CAM arteries, indicating that the guanylate cyclase pathway of relaxation is active. Nevertheless, it should be noted that SNP showed a low relaxant efficacy when compared with compounds acting through the adenylyl cyclase pathway (i.e. Iso and Forsk).

As ACh arises from parasympathetic activity and is rapidly degraded in the synaptic cleft, circulating levels of ACh are low. Since the CA vasculature lacks innervation, it is not likely to be exposed to significant levels of ACh in vivo. Thus, while the ACh-induced contraction in CM arteries is mechanistically interesting, it might play less of a regulatory role from a physiological point of view. Similarly, receptor-mediated endothelium-derived relaxation is not a major mechanism in the human fetoplacental circulation because fetoplacental arteries do not relax in response to endothelium-dependent agents (such as ACh, bradykinin, or A23187), but they relax in response to NO donors (4, 36). Instead, it has been proposed that flow-induced NO production contributes to the maintenance of low resistance circulation in normal pregnancy (15, 37). This mechanism is also likely in the CAM, because eNOS is present in CA vessels (38) and detectable amounts of NO can be measured across the eggshell during embryonic development (39). In the present work, we observed that the NOS inhibitor L-NAME induced a slight contraction in the 19d CA arteries. This might be taken as an indication of a basal production of NO. However, L-NAME did not contract the 15d CA arteries and did not affect the contractile response to K+ or U46619. Therefore, whether the CA arteries release NO and the stimuli leading to its production is yet to investigate.

Bradykinin induces endothelium-dependent relaxations of many vessels from various species (29). However, we did not observe any relaxant effect of bradykinin in K+- or U46619-contracted chicken CA arteries. Previously, we reported that bradykinin did not relax the chicken ductus arteriosus (a vessel that in contrast to the CA arteries shows ACh-induced relaxation) (14). Nevertheless, it should be noted that bradykinin shows low, if any, affinity for the ornithokinin (the “avian bradykinin”) receptor when compared with the authentic ligand ornithokinin ([Thr6,Leu8]bradykinin) (40).

CA arteries lack α-adrenergic response, but relax to β-adrenergic stimulation

In this study, we demonstrated a lack of innervation in the chicken chorioallantoic arteries (Fig. 7). Just as in the human fetoplacental arteries, this lack of nerve input leaves the control of vascular tone to humoral and locally released factors. In addition to physical factors, eicosanoids, ET-1, and catecholamines have been implicated in the physiological regulation of placental blood flow and the human fetoplacental vessels show a high responsiveness to these agents (4, 41, 42). Accordingly, we observed that chicken CA arteries were highly responsive to the thromboxane A2 mimetic U46619 and, to a lesser extent, ET-1. When we analyzed the response to adrenergic agents, we observed that the β-adrenoceptor agonist Iso induced a marked relaxation, but we were unable to detect an effect of the α-adrenoceptor agonist Phe. Similarly, human fetoplacental vessels do not respond to Phe or norepinephrine (36, 41), but relax in response to Iso and other β-adrenoceptor agonists (6). It has been suggested that the β2-adrenoceptor plays one of the main roles in the regulation of fetoplacental circulation (6).
CA vascular tone, hypoxic vasoconstriction and implications for embryonic circulatory homeostasis

CA arteries respond to hypoxia with contraction, a response shared with vessels from other gas exchange organs such as the lungs and the placenta. It has been suggested that the mechanisms of hypoxia sensing and signalling in human fetoplacental arteries are similar to those in pulmonary arteries (8, 9, 43, 44) so it is likely that similar mechanisms are operational in CA vessels, but their characterization is beyond the scope of the present work.

Hypoxic vasoconstriction in CA vessels would serve the same purpose as in other gas exchangers, i.e. to match ventilation with perfusion. During natural incubation, convection of air around the egg is limited due to the contact with the brood patch and the nest floor, which decreases eggshell conductance (45) and generates an anisotropic pattern of oxygen diffusion in the egg (46), but it is not known if less ventilated areas are also less perfused. Changes in total CA blood flow during generalized hypoxia have been reported, but there is little agreement between studies. Using different flow measurement techniques a decreased CA flow to 10% O₂ has been shown (47), but also a relative increase to 0% O₂ (48) and even no changes in CA flow (49). The disagreement stems from the conflicting direct vasomotor response to low oxygen between embryonic and CA blood vessels and the humoral vasomotor effect of circulating catecholamines released during hypoxia (50). In agreement with the current study low oxygen would cause direct CA vasoconstriction and indirect CA vasodilatation mediated by β-adrenergic receptors, but an integrated understanding of the circulatory changes to hypoxia await further studies.

CONCLUSIONS

In the present study we show that the chicken CA arteries share many contraction and relaxation characteristics with the human fetoplacental arteries. HPV (32), hypoxic systemic vasorelaxation (51), normoxic contraction of the ductus arteriosus (52), and hypoxic contraction of the CAM arteries (present work) are present in the chicken, making this species an attractive model organism for the investigation of putative common vascular mechanisms (53). This first characterization of the CAM artery will serve as the starting point of further investigation to explain the disparate and common responses.

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