Contractile and relaxing reactivity in carotid and femoral arteries of chicken embryos

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Le Noble, Ferdinand A. C., Karin Ruijtenbeek, Suzanne Gommers, J o G. R. De Mey, and Carlos E. Blanco. Contractile and relaxing reactivity in carotid and femoral arteries of chicken embryos. Am J Physiol Heart Circ Physiol 278: H1261–H1268, 2000.—In the embryo, hypoxemia causes redistribution of cardiac output from the periphery toward the heart and the brain. In view of this, we investigated developmental changes in the contractile and relaxing properties of the peripheral femoral artery (Fem) and the more central carotid artery (Car) at 0.7, 0.8, and 0.9 of the chicken embryo incubation time. Isolated arteries were studied in myographs and were exposed to norepinephrine or phenylephrine. High K+ (125 mM) and electrical field stimulation (0.25–16 Hz) were used to induce receptor-independent and neurogenic contractions. Relaxing responses to ACh were evaluated in the absence and presence of the nitric oxide (NO) synthase inhibitor Nω-nitro-L-arginine methyl ester (L-NAME) and before and after endothelium removal. α1-Adrenergic contractile responses increased in a time-dependent manner and were significantly larger in Fem than in Car. Neurogenic contractions and adrenergic nerves could only be demonstrated in Fem at 0.9 incubation. ACh caused relaxation in both Fem and Car at 0.7, 0.8, and 0.9 incubation. The NO-independent part of the relaxation was more pronounced in Car than in Fem at all developmental stages. We conclude that the chicken embryo is a useful model to investigate the development of vasomotor control and vascular heterogeneity. The observed regional vascular differences may contribute to cardiac output redistribution during hypoxia in the embryo and might result from endothelial and neurogenic influences on vascular smooth muscle differentiation.

catecholamines; vascular smooth muscle; sympathetic nerves; endothelium; regional heterogeneity; chemoreflex

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lated from 0.7-, 0.8-, and 0.9-incubation chicken embryos (i.e., after 15, 17, and 19 days of the 21-day incubation period). Their responses to adrenergic stimuli, sympathetic nerve stimulation, and ACh were compared. The latter compound is generally considered to be an endothelium-dependent vasodilator (5, 7, 8, 10, 14), but this has not yet been demonstrated in the chicken embryo. The choice of carotid and femoral arteries was justified by their anatomic location, their size, and by the relative ease with which these vessels could be isolated from even the smallest embryos investigated.

**MATERIALS AND METHODS**

Vessel isolation. Experimental procedures followed Dutch laws for animal experiments. Fertilized Lohman-selected White Leghorn eggs were incubated at 38°C and 60% relative air humidity and were rotated once every hour, until day 15, 17, or 19 (0.7, 0.8, or 0.9 incubation time, respectively). The eggs were opened at their blunt side. The eggshell and the outer eggshell membrane were carefully removed using forceps to expose the inner eggshell membrane, which was then superfused with Krebs-Ringer bicarbonate (KRB) solution. An incision of 2 cm was then made in the chorioallantoic membrane. The egg was turned upside down, and the embryo was collected on a petri dish. The extraembryonic membranes were removed, and the embryo was transferred to a petri dish that had been coated with Sylgard (Dow Corning) and filled with KRB. The right carotid and right femoral artery were carefully dissected from the embryo.

Recording of arterial reactivity. The isolated arteries were mounted as ring segments (length 1.7 to 2.0 mm) between an isometric force transducer (Kistler Morce DSC 6, Seattle, WA) and a displacement device in a myograph (model 610M, J.P. Trading, Aarhus, Denmark) using two stainless steel wires (diameter 40 μm). Force was divided by twice the vessel segment length to calculate wall tension. During mounting and experimentation, the myograph organ bath (5 ml vol) was filled with KRB maintained at 37°C and aerated with 95% O2-5% CO2. Each artery was stretched to its individual optimal lumen diameter, i.e., the diameter at which it developed the strongest contractile response to 125 mM K+ (K-KRB), using a diameter-tension protocol as previously described for mammalian small arteries (Ref. 29; Fig. 1).

Reactivity and sensitivity to agonists were examined by constructing concentration-response curves (0.001 to 10 μmol/l). Between experiments the arterial preparations were allowed to recover for at least 15 min. Contractile agonists were evaluated under basal conditions; relaxing agonists were evaluated during contraction induced by 35 mM K+ (K-KRB). The effects of ACh were evaluated in the absence and in the presence of 0.1 mmol/l Nω-nitro-L-arginine methyl ester (L-NAME). The NO synthase inhibitor was added during contractions induced by 35 mM K+, and the effects of ACh were evaluated 30 min later.

In a series of experiments restricted to femoral arteries of 0.9-incubation chicken embryos, the effects of ACh were also evaluated before and after the removal of the endothelium. For this purpose we used two procedures: sliding of human hair (10 cm) through the lumen of the vessel (27), and perfusing the lumen during 60 s with 0.1% Triton X-100 in KRB with 40-mmHg perfusion pressure (8). Using scanning electron microscopy, we observed that both procedures resulted in complete denudation of the luminal endothelial cells.

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**Fig. 1.** Typical tracing of isometric force vs. time illustrating contractile responses in a carotid artery of 0.9-incubation chicken embryo as a function of imposed distension. Isolated arterial segment was mounted in organ chamber, and its lumen diameter was increased in steps of 30 μm from 200 to 410 μm (arrows). At each level of distension, vessel was intermittently stimulated by exposure to 125 mM K+ [between K+ and w (wash)]. Note that maximal contractile response (E_max) was obtained at a diameter of 380 μm.
Organ chamber experiments were terminated by fixing the vessels in phosphate-buffered (pH 7.4) formaldehyde (4%) during 30 min at 37°C.

Morphometry. Fixed vessels were transferred to 70% ethanol and embedded in paraffin. To determine medial cross-sectional area, we performed immunohistochemistry on thin cross sections (4 µm) with antibodies against smooth muscle α-actin (Sigma Chemical, St. Louis, MO). Endogenous peroxidase was blocked by 0.1% H2O2 in methanol for 20 min at room temperature, and the sections were incubated for 45 min with primary antibody (1:4,000). Horseradish peroxidase-conjugated rabbit anti-mouse antibodies (Dakopatts; dilution 1:200) and diaminobenzidine-H2O2 substrate were subsequently used to visualize the immunoreactivity. The area occupied by smooth muscle α-actin was determined using a Zeiss Axioscope (Zeiss, Germany), a standard CCD camera (Stemmer, Germany), and commercially available software (JAVA 1.21, Jandel Scientific, Corte Madera, CA).

We used an actin-based method rather than the more classical Lawson staining of elastin (29), because continuous internal and external elastic laminae could not be observed in the arterial preparations before 0.9 incubation.

Staining of perivascular sympathetic nerves. To demonstrate the presence of norepinephrine (NE)-containing nerves, we stained whole mount vessel preparations with glyoxylic acid (24, 29). Vessel segments were opened longitudinally and were incubated for 30 min at 20°C in 2% glyoxylic acid in 10% sucrose containing phosphate buffer (pH 7.2). Subsequently, the vessel segments were transferred to a mounting glass, air-dried for 30 min, stretched at 100°C for 4 min, and enclosed with Etellan and a coverslip. The presence of glyoxylic acid-induced fluorescence, representing catecholamine-containing nerves, was visualized using fluorescent microscopy (microscope Nikon Diaphot, BA 470-DM 455 filter; Nikon FE2 camera). Photographs of the vessel segments were taken through the microscope (objective Fluoro x10, Nikon) using Kodak 320 ASA film.

Solutions and drugs. The composition of KRB solution (in mmol/l) was as follows: 118.5 NaCl, 4.7 KCl, 1.2 MgSO4 · 7H2O, 1.2 KH2PO4, 25.0 NaHCO3, 2.5 CaCl2, and 5.5 glucose. In K-KRB (125 mmol/l K+), all NaCl was replaced by an equimolar amount of KCl. α-Containing solution (35 mmol/l) was prepared by mixing appropriate volumes of K-KRB and KRB. Phosphate-buffered solution consisted of 0.1 mol/l NaH2PO4 · H2O and 0.1 mol/l Na2HPO4 · 2H2O. ACH, glyoxylic acid, I-NAMe, I-NAME, I-phenylephrine (PE), and prazosin were all obtained from Sigma Chemical. BHT-933 (azepexol) was a multiple comparisons.

RESULTS

Data analysis. Concentration-response curves were analyzed in terms of sensitivity and maximal response by fitting the individual experimental data with a nonlinear sigmoid regression curve (GraphPad Software, San Diego, CA). Maximal contractile responses (E max) were expressed in terms of active wall tension (force divided by twice the segment length; N/m); sensitivities are shown as pD2, where pD2 = log10 (EC50/EC50) (EC50 represents the concentration at which 50% of the maximal responses were observed). Changes and differences in E max and pD2 with time and between types of vessels were evaluated by ANOVA with Bonferroni's correction for multiple comparisons. P < 0.05 was accepted to represent statistical significance. Data are shown as means ± SE.

RESULTS

Femoral and carotid arteries obtained from 0.7- to 0.9-incubation chicken embryos responded to depolarizing high-K+ solution with a contraction (Fig. 1). In both types of vessels, the diameter at which maximal responses were obtained (D opt) and the amplitude of the maximal response (K max) increased significantly with increasing incubation (Figs. 2 and 3). In femoral arteries, D opt averaged 357 ± 8, 409 ± 13, and 449 ± 15 µm and K max averaged 0.26 ± 0.03, 0.73 ± 0.15, and 1.78 ± 0.14 N/m at 0.7, 0.8, and 0.9 incubation, respectively (n = 10–18). In carotid arteries, D opt averaged 368 ± 8, 406 ± 8, and 468 ± 15 µm and K max averaged 0.19 ± 0.02, 0.21 ± 0.04, and 1.01 ± 0.15 N/m at 0.7, 0.8, and 0.9 incubation, respectively (n = 8–13). Despite comparable diameters (Fig. 2), contractile responses to K+ were significantly larger in femoral than in carotid arteries at 0.8 and 0.9 incubation (Fig. 3).

Part of the reason for these results seems to be the significantly larger medial mass in femoral than in carotid arteries (Fig. 2), but statistically significant differences between both vessels persist when contractility was normalized to medial thickness. Active wall stress averaged 69 ± 7 and 41 ± 5 N/m2 at 0.8 incubation and 142 ± 11 and 85 ± 9 N/m2 at 0.9 incubation in femoral and carotid arteries, respectively.

In femoral and carotid arteries at 0.7, 0.8, and 0.9 incubation, high concentrations of relaxing agonists, such as ACh (10 µmol/l), adenosine (1 mmol/l), and papaverine (1 mmol/l) failed to affect basal tension, indicating the absence of spontaneous tone under the experimental conditions.

In all femoral arteries from 0.7 incubation onwards, and in all carotid arteries at 0.9 incubation, NE and the selective α1-adrenergic agonist PE induced concentration-dependent tonic contractile responses (Figs. 3 and 4). At 0.7 and 0.8 incubation, only 30–40% of the carotid arteries investigated responded to the agonists; maximal responses were close to the detection limit of 0.01 N/m. At all time points, E max to the adrenergic stimuli were significantly larger in femoral than in carotid arteries (Fig. 3). At 0.9 incubation, the sensitivity (pD2) for NE was significantly smaller in femoral (6.03 ± 0.8) than in carotid arteries (6.53 ± 0.11), whereas the...
sensitivity for PE was comparable in femoral (5.58 ± 0.09) and carotid arteries (5.27 ± 0.17). Both femoral and carotid arteries failed to contract in response to BHT-933, a selective \( \alpha_2 \)-adrenergic agonist (data not shown), indicating a lack of functional \( \alpha_2 \)-adrenergic receptors on arterial smooth muscle at the developmental stages investigated.

Electrical field stimulation caused frequency-dependent contractions in femoral arteries at 0.9 incubation (Fig. 4) but failed to induce contraction in femoral arteries at earlier developmental stages and in carotid arteries at all stages investigated. Glyoxylic acid staining confirmed the presence of catecholamine-containing nerves in femoral arteries at 0.9 incubation (Fig. 5). In the presence of the \( \alpha_2 \)-adrenergic receptor antagonist prazosin (0.1 \( \mu \)mol/l), contractile responses of 0.9-incubation femoral arteries to electrical field stimulation were reduced by >85% (data not shown).

Embryonic arteries that had been constricted with 35 mmol/l \( K^+ \) responded to ACh with concentration-dependent relaxations (Fig. 6). In 0.9-incubation femoral arteries, the relaxing responses to ACh were abolished by mechanical or chemical removal of the endothelium (Fig. 6). Sensitivity to the cholinergic agonist and its maximal effect did not change significantly between 0.7 and 0.9 incubation and did not differ significantly between femoral and carotid arteries (Fig. 7). L-NAME (0.1 mmol/l) increased the contractile responses to 35 mmol/l \( K^+ \) in both femoral and carotid arteries (Fig. 6) and reduced the sensitivity and the maximal responsiveness to the endothelium-dependent relaxing effects of ACh in both types of vessels (Fig. 7). Although \( K^+ \)-induced contraction was both on an absolute and a relative basis stronger in femoral than in carotid arteries, the L-NAME-resistant component of the relaxing effects of ACh was significantly more pronounced in carotid than in femoral arteries at 0.7 and at 0.9 incubation (Fig. 7). It is noteworthy that the contractile effect of L-NAME in embryonic arteries was not significantly altered by endothelium removal (Fig. 6).

**DISCUSSION**

Between 0.7 and 0.9 incubation, the contractile reactivity to \( \alpha_1 \)-adrenergic and receptor-independent stimulation increased in the femoral and carotid arteries of chicken embryos. Late developmental increases in contractile reactivity, in neurogenic vasoconstrictor responses, and in the responsiveness to endothelium-derived NO were more pronounced in the peripheral femoral artery than in the central carotid artery. Such regional heterogeneity may contribute to the redistribution of cardiac output during hypoxemia in the embryo and may find its origin in neurogenic and endothelial influences on the functional differentiation of arterial smooth muscle.

Previous studies of cardiovascular responses in chicken embryos revealed similarities to those in fetal sheep (17, 26). Most notably, acute hypoxemia results not only in bradycardia but also in a redistribution of cardiac output from the peripheral circulation to the heart and brain in both species. Catecholamines participate in this embryonic chemoreflex in both systems (12, 15, 17, 18, 20, 21, 25, 26, 30). In the present study we analyzed isolated arteries of the chicken embryo. The use of in vitro approaches, which are well established for adult arteries (e.g., 7, 8, 29), allow quantification of responses and sensitivities of individual blood vessels to various vasoactive agents and allow us to quantify neurogenic and endothelium-dependent responses in the absence of modulatory circulating hormones. We compared femoral and carotid arteries as model systems for a peripheral and a central vascular bed. The size and reactivity of the youngest arteries that we studied were only moderately above the limits of the in vitro techniques that are currently available. Vessels further downstream were therefore not included in this study.

In femoral and carotid arteries obtained from 0.7-incubated embryos, direct depolarization induced significant contraction. This indicates that at this developmental stage the arterial smooth muscle cells are already equipped with contractile proteins and an excitation-contraction coupling that most likely in-
volves voltage-operated calcium channels. In both types of vessels, the receptor-independent contractile responses to depolarization increased five- to sevenfold between 0.7 and 0.9 incubation, whereas medial mass, as judged from measurements of medial cross-sectional area, increased only 65–75%. The contractile responses were larger in femoral than in carotid arteries despite comparable lumen diameter, and significant differences persisted after correction for differences in medial thickness. It is most likely that smooth muscle cell differentiation contributes to the developmental increase in arterial contractile reactivity in general and to the difference between femoral and carotid arteries as regards contractile strength in particular.

At all time points investigated, NE and PE induced contraction in the embryonic femoral artery. Adrenergic responsiveness and pharmacomechanical coupling thus develop rather early in the chicken embryo. Previous research (11) established that cardiac adrenergic responses can be observed as early as day 4 of incubation. In sharp contrast, consistent α₁-adrenergic contractile responses could not be obtained in the carotid arteries before 0.9 incubation. With the use of 0.3 nM [³H]prazosin and previously described ligand-binding techniques in intact arterial segments (28), we observed that the density of α₁-adrenergic receptors was comparable in 0.9-incubation femoral arteries (10.8 ± 1.3 fmol/mg total protein, n = 8) and carotid arteries (11.1 ± 1.8 fmol/mg total protein, n = 4). Consequently, aspects beyond the receptors, most likely involving the coupling of these sarcolemmal structures to the contractile apparatus, account for the regional difference. Little is known about the mechanisms that control α₁-adrenergic mechanisms in general and during development in particular. The observed difference between the two types of arteries excludes a major role for circulating factors such as glucocorticoids and catecholamines. Recent findings of our group suggest, on the other hand, that local aspects of smooth muscle cell differentiation and of perivascular sympathetic innervation play a pivotal role in this respect. We demonstrated in adult arteries that dedifferentiation of arterial smooth muscle resulting from balloon injury is accompanied by a marked reduction of α₁-adrenergic receptor density (3) and that the perivascular sympathetic innervation promotes the presence of α₁A-adrenergic receptors while reducing the density of α₁B- and α₁D-adrenergic receptors (28).

In this study we approached perivascular sympathetic nerves histochemically and from a functional point of view. Glyoxylic acid-induced fluorescence of perivascular nerve fibers was prominent in late-incubation chicken femoral arteries. Prazosin-sensitive contractile responses to perivascular nerve stimulation were obtained in late-gestation femoral arteries but not in carotid arteries. In the femoral arteries, constrictor responses to exogenous NE could be obtained before neurogenic responses. In adult arteries, the density of perivascular sympathetic innervation varies considerably between anatomic locations (28). Regionally selec-

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**Fig. 5.** Glyoxylic acid-induced fluorescence in a whole mount preparation of 0.9-incubation chicken embryonic Fem highlighting the presence of NE-containing perivascular nerves. Calibration bar: 280 µm.

**Fig. 6.** Typical tracings of isometric tension vs. time illustrating effects of 35 mM K⁺, ACh, and Nω-nitro-arginine methyl ester (L-NAME) in intact (+endothelium (+E); top) and denuded (+endothelium (−E); bottom) segments of 0.9-incubation chicken embryonic Fem. Removal of endothelium was obtained by perfusion with 0.1% Triton X-100. Concentrations of pharmacological agents are shown as −log M. Horizontal bar: 5 min; vertical bars: 1 N/m.
tive vascular sympathetic innervation during development is brought about by timely secretion by vascular smooth muscle of a mixture of nerve-attracting mediators such as nerve growth factor and nerve-repelling substances. This secretion is restricted to a rather limited period of time and may be limited to an intermediate vascular smooth muscle cell phenotype (9, 16). It may furthermore be more prominent in vascular smooth muscle cells of mesodermal origin than in those that are derived from the neural crest and which primarily populate the blood vessels in the cranial region. In addition to altering the pharmacological properties of the innervated blood vessel (e.g., see 29), the perivascular sympathetic innervation can initially stimulate growth and proliferation of the smooth muscle cells and subsequently promote the development and maintenance of a contractile phenotype (for review, see Ref. 6). We thus propose that the stronger contractility of femoral arteries during late development and their larger responsiveness to $\alpha_1$-adrenergic stimulation are related to their sympathetic innervation. The causal interrelationship between these aspects clearly remains to be established.

Another vessel wall component that plays important roles in the morphogenesis and development of the vascular system and in the control of vasomotor tone is the endothelium. Endothelial cells give rise to the earliest vascular channels and later attract mesenchymal cells to the vessel wall (4). Subsequently, endothelium-derived mediators promote the differentiation of these vessel wall cells into contractile smooth muscle cells (4). Endothelium-derived NO that stimulates cGMP production and protein kinase G activity has been proposed to participate in this differentiating action (2, 23). We approached this mediator with the use of ACh, an agent that induces endothelium-
dependent relaxation in adult arteries of various species (5, 7, 8, 10), including chicken aorta (14). Our experiments with mechanical and chemical endothelium removal demonstrate the endothelium dependency of cholinergic vasodilatation in the chicken embryo. Partial blockade of these relaxations by the NOS inhibitor L-NAME indicates an involvement of endothelium-derived NO. Relaxing effects of endothelium-derived NO could be demonstrated from the earliest developmental stages investigated and were at all stages more prominent in femoral arteries than in carotid arteries. Our current findings do not allow us to attribute the regional difference to a larger endothelial release of NO or to a more elaborated guanylate cyclase-protein kinase G system in femoral than in carotid arteries. Yet, the findings are consistent with a contribution of endothelium-derived NO to the differentiation of arterial smooth muscle (2, 23) and to the developmental increase in arterial contractile reactivity. Future pharmacological intervention studies are, however, needed to strengthen this proposal.

It is noteworthy that in chicken embryonic arteries, as in some adult mammalian systems (5) and in the aorta of mature chickens (14), the endothelium-dependent relaxing responses to ACh could only partly be blocked by L-NAME. A role for an endothelium-derived hyperpolarizing factor (5) seems unlikely because the relaxing effects of ACh were studied during contraction induced by depolarizing high-K+ solution. The exact nature of the L-NAME-resistant component of endothelium-dependent relaxation remains to be established in chicken embryonic arteries. This also applies for the observed endothelium-independency of the L-NAME-induced contraction.

In summary, we observed differences between carotid and femoral arteries of chicken embryos as regards the developmental increase in contractile strength, α₁-adrenergic vasoconstriction, perivascular sympathetic innervation, and endothelium-dependent vasodilatation involving NO. From approximately mid-incubation, the cardiovascular system of the chicken embryo can respond to hypoxemia with a redistribution of cardiac output from peripheral vascular beds to the heart and brain (25, 26). As development progressed, this chemoreflex became more prominent. Based on our observations, the development of arterial contractile reactivity, i.e., its pharmacological control and regional heterogeneity, may participate herein and seems to involve neurogenic and endothelial influences on arterial smooth muscle cell differentiation.

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