Effects of sex and estrogen on chicken ductus arteriosus reactivity

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Flinsenberg TWH, van der Sterren S, van Cleef ANH, Schuurman MJ, Ågren P, Villamor E. Effects of sex and estrogen on chicken ductus arteriosus reactivity. Am J Physiol Regul Integr Comp Physiol 298: R1217–R1224, 2010. First published February 17, 2010; doi:10.1152/ajpregu.00839.2009.—Sex hormones have an important influence on cardiovascular physiology and pathophysiology and sex differences in vascular reactivity have been widely demonstrated. In the present study we hypothesized 1) the presence of sexual dimorphism in chicken ductus arteriosus (DA) responsiveness to contractile and relaxant stimuli and 2) that estrogens are vasoactive in the chicken DA. In vitro contractions (assessed with a wire myograph) induced by normoxia, KCl, 4-aminopyridine, norepinephrine, phenylephrine, U46619, or endothelin-1, as well as relaxations induced by ACh, sodium nitroprusside, BAY 4-aminopyridine, norepinephrine, phenylephrine, U46619, or endothelin-1, were not significantly different between males and females. The estrogen 17β-estradiol elicited concentration-dependent relaxation of KCl-, phenylephrine-, and oxygen-induced active tone in male and female chicken DA. The stereoisomer 17α-estradiol showed less relaxant effects, and the selective estrogen receptor (ER) agonists 4,4′,4′′-((4-propyl-[1H]pyrazole-1,3,5-triyl)tris-phenol (ERα) and 2,3-bis(4-hydroxyphenyl)-propionitrile (ERβ) did not show any effect. There were no sex differences in the responses to estrogen. Endothelium removal or the presence of the soluble guanylate cyclase inhibitor ODQ, the K+ channel blockers tetraethylammonium, glibenclamide, and charybdotoxin, or the ER antagonist fulvestrant did not modify 17β-estradiol-induced relaxation. CaCl2 (30 μM–10 mM) induced concentration-dependent contraction in DA rings depolarized by 62.5 mM KCl or stimulated with 21% O2 in Ca2+-free medium. Preincubation with 17β-estradiol or the L-type Ca2+ channel blocker nifedipine produced an inhibition of CaCl2-induced contractions. In conclusion, there are no sex-related differences in chicken DA reactivity. The estrogen 17β-estradiol induces an endothelium-independent relaxation of chicken DA that is not mediated by ER activation. This relaxant effect is, at least partially, due to inhibition of Ca2+ entry from extracellular space.

sex differences; chicken embryo; estradiol; oxygen signaling

When compared with females, human males are at higher risk of prematurity, as well as pulmonary, neurological, gastrointestinal and cardiovascular prematurity-related conditions, including failure of closure of the ductus arteriosus (DA) (18, 19, 50). This “male disadvantage” with respect to neonatal morbidity and mortality has been recognized for more than three decades (8, 34), but the contributing biological mechanisms are poorly understood and likely to be multifactorial. It has been proposed that the male disadvantage begins in utero, when gonadal steroid production already differs strongly by sex (19). Considerable evidence indicates that sex hormones have an important influence on cardiovascular physiology and pathophysiology, and sex differences in systemic and pulmonary vascular reactivity and pathology have been widely demonstrated (17, 27, 35, 38, 39, 44, 57, 60). Estrogen, progesterone, and testosterone receptors are expressed in endothelial and vascular smooth muscle cells, and it is now apparent that these hormones participate in the control of vascular tone (38). Specifically, free estrogens diffuse through the plasma membrane and form complexes with cytosolic/nuclear receptors, resulting in the induction of genomic effects (7). Estrogens also bind to plasma membrane receptors in cells and induce rapid nongenomic events (7, 26). Estrogen-induced, endothelium-dependent and -independent relaxation has been demonstrated in numerous vascular beds from several species (see Ref. 57 for review). However, neither the presence of sex differences in vascular reactivity nor the vascular effects of sex hormones has been so far investigated in the DA.

In recent years, the chicken embryo has emerged as a suitable model for the study of DA vascular biology (1–4, 13, 14, 21, 32, 53, 54). Genotypic mechanisms control sex determination in birds like in mammals but, in contrast with mammals, the avian female is heterogametic (ZW) and the male homogametic (ZZ) (6, 9, 52). In addition, sexual differentiation occurs in opposite manners in birds and mammals. In the latter, the presence of testosterone and other androgens is a necessary condition for development of male characteristics, but in the absence, development takes the female course (6, 9, 52). In contrast, birds develop a masculine phenotype in the absence of estrogens. In the avian female, the W chromosome positively controls early aromatase synthesis and consequently estrogen production, which is crucial not only for gonadal sex differentiation but also for subsequent hormonal and phenotypical sexual differentiation (6, 9, 52). In the chicken, plasma levels of steroids start to differ between males and females as early as 7.5 days of incubation (9). Therefore, estrogen-dependent sexual differentiation makes the chicken embryo also a suitable organism for testing the potential effects of gonadal steroids during development. In the present study we hypothesized 1) the presence of sexual dimorphism in chicken DA responsiveness to contractile and relaxant stimuli and 2) that estrogens are vasoactive in the chicken DA. To test these hypotheses, we analyzed, using wire myography, the reactivity of DA rings from male and female chicken embryos. METHODS

Egg incubation and vessel isolation. All experimental procedures were carried out according to the regulation of the Dutch Law on Animal Experimentation and the European Directive for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (86/609/EU), and were approved by the Committee on Animal Experimentation of the University of Maastricht. Fertilized eggs of White Leghorn chickens were incubated at 37.8°C, 45%
humidity and rotated once per hour (Incubator model no. 25HS; Masalles Comercial, Barcelona, Spain). Noninternally pipped embryos incubated for 15, 17, or 19 days of the 21-day incubation period were studied. The embryos were taken out of the egg and immediately killed by decapitation; a midline laparotomy and sternotomy were then performed. The sex of the chicken embryos was determined by the in situ microscopic examination of the gonads. In female chickens, only the left gonad develops as an ovary, and on day 15 and onward, the female urogenital already presents a severe dissymmetry (the left ovary is developed and functional, whereas the right gonad is clearly atrophic) (6). In contrast, the male reproductive tract is a paired system and the two testicles and the two ductus deferens can be clearly visualized by 15 days and onward in chicken embryos (6). After sex was determined, the right and the left DA were carefully dissected free from surrounding tissue, severed distal to the takeoff of the right or left pulmonary artery and proximal to the insertion into the aorta, and divided into two segments referred to as the pulmonary side and aortic side. The boundary between pulmonary and aortic side was determined on the basis of the marked differences of diameter observed between the two DA (1).

**Recording of DA reactivity.** Two stainless-steel wires (diameter 40 μm) were inserted into the lumen of the DA, which was mounted as a ring segment between an isometric force transducer and a displacement device in a myograph (Danish Myo Technology A/S model 610M, Aarhus, Denmark). The myograph organ bath (5 ml) was filled with Krebs-Ringer bicarbonate buffer (KRB) buffer maintained at 39°C and continuously aerated with 95% N2-5% CO2 (Po2 19.16 kPa, SD 0.52, n = 12, measured with an ABL 510 blood gas analyzer, Radiometer Copenhagen, Denmark). Each DA was stretched to its individual optimal lumen diameter, i.e., the diameter at which it developed the strongest contractile response to 62.5 mM K+, using a diameter-tension protocol as previously described (1, 54). The response of the DA rings to normoxia was assessed by bubbling the organ chamber with 21% O2-74% N2-5% CO2 (Po2 19.16 kPa, SD 1.15, n = 12), as previously described (1). The other contractile and relaxant agents were studied under 5% O2-90% N2-5% CO2 (Po2 6.96 kPa, SD 0.34, n = 12), as previously described (1). The other contractile and relaxant agents were used under 5% O2-90% N2-5% CO2 (Po2 6.96 kPa, SD 0.32, n = 12). This level of oxygenation was chosen because it corresponds with the arterial Po2 of 15–19-day chicken embryos (~7 kPa) (51). Concentration-response curves to KCl (4.75 mM–125 mM), the voltage-gated K+ channel (Kv) inhibitor 4-aminopyridine (4-AP, 1 mM–10 mM), the nonselective adrenergic agonist norepinephrine (NE, 10 nM–0.1 mM), the α1-adrenergic agonist phenylephrine (Phe, 10 nM–0.3 mM), the thromboxane A2 mimetic 9,11-dideoxy-11α,9-epoxyethyleno-PGF2α (U46619, 10 nM–10 μM), and endothelin-1 (ET-1, 0.01 nM–0.1 μM), were constructed. The responses to O2, 4-AP, and KC were also analyzed in DA rings from 15–17-day embryos. The other contractile and relaxant agents were studied only at one age (19 days). Relaxant agonists were evaluated during contraction induced by 62.5 mM K+. Concentration-response curves for ACH (10 nM–30 μM), the nitric oxide (NO) donor sodium nitroprusside (SNP, 10 nM–0.1 mM), the NO-independent stimulator of soluble guanylate cyclase BAY 41–2272 (10 mM μM), PGE2 (10 nM–10 μM), the β-adrenergic agonist isoprotrenol (0.1 nM–3 μM), the adenylyl cyclase activator forskolin (10 nM–10 μM), and the Rho-kinase inhibitors Y-27632 (1 mM–30 μM) and hydroxyfasudil (1 mM–30 μM) were constructed. The relaxations to PGE2 were studied in the presence of the thromboxane/PGF2α (TP) receptor antagonist SQ 29,548 (10 μM) as previously described (1, 12). The responses to 17β-estradiol (10 nM–0.1 mM) and its stereoisomer 17α-estradiol (10 nM–0.1 mM) were studied in pulmonary side DA rings precontracted with 21% O2, 62.5 mM KCl (under 5% O2), or 10 μM phenylephrine (under 5% O2) and in aortic side DA rings precontracted with 62.5 mM KCl (under 5% O2). To gain insights into the putative mechanisms of estrogen-induced relaxation, some experiments were performed in endothelium-denuded vessels (endothelium was removed by gentle rubbing of the vessel lumen with a horse tail hair, and its absence verified by the absence of relaxation to 10 μM ACh) or in the presence of one of the following pharmacological tools: the soluble guanylate cyclase (sGC) inhibitor 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ; 10 μM), the nonselective K+ channel inhibitor tetraethylammonium (TEA; 5 mM), the ATP-sensitive K+ channel (KATP) inhibitor glibenclamide (10 μM), the long-conductance Ca2+-activated K+ channel (BKca) inhibitor charybdotoxin (10 μM), and the estrogen receptor (ER) antagonist fulvestrant (formerly ICI 182,780, 1 μM). To test the functionality of ERs in our DA preparations, concentration-response curves to the selective ERα agonist 4,4′,4″-(propyl-[1H]pyrazole-1,3,5-triyl)trisphenol (PPT, 0.01–10 μM) and the selective ERβ agonist 2,3-bis(4-hydroxyphenyl)-propionitrile (DPN, 0.01–10 μM) were performed. Finally, in another set of experiments, the ability of 17β-estradiol to modulate Ca2+-entry was assessed by obtaining concentration-response curves to CaCl2. For this purpose, the DA rings were washed three times (5-min interval) with Ca2+-free medium containing 1 mM EDTA. Then, DA rings were washed twice with Ca2+-free medium (without EDTA), incubated with vehicle, 17β-estradiol (10 μM), or another L-type Ca2+ channel (CaL) blocker nifedipine (10 μM) and stimulated with 21% O2 or KCl (62.5 mM) before adding cumulative concentrations of CaCl2 (10 μM–10 mM).

**Drugs and solutions.** KRB contained (in mmol/l): 118.5 NaCl, 4.75 KCl, 1.2 MgSO4, 7H2O, 1.2 KH2PO4, 25.0 NaHCO3, 2.5 CaCl2, and 5.5 glucose. Solutions containing different concentrations of K+ were prepared by replacing part of the NaCl by an equimolar amount of KCl. BAY 41–2272, forskolin, and ET-1 were obtained from Alexis Biochemicals (Uithoorn, The Netherlands). U46619, PGE2, and SQ 29,548 were obtained from Cayman Chemical (Ann Arbor, MI), and ODQ was from Tocris (Ballwin, MO). All of the other drugs were obtained from Sigma (St. Louis, MO). Drugs were initially dissolved in distilled deionized water (except forskolin and nifedipine, which were dissolved in ethanol, charybdotoxin that was dissolved in 0.9% NaCl, and BAY 41–2272, SQ 29,548, PGE2, U46619, estradiol, fulvestrant, PPT, and DPN that were dissolved in DMSO) to prepare stock solutions, and further dilutions were made in KRB. Total DMSO added to the organ bath for preincubations or concentration-response curves was, at the most, 0.34% vol/vol and did not affect arterial tone. Total ethanol was, at the most, 0.1% vol/vol and produced a slight contraction of the DA, as previously described (1).

**Data analysis.** Results are shown as means (SD) of measurements in n embryos. For clarity, results are shown in the figures as means ± SE. The contractions generated upon the addition of agonists are expressed in terms of active wall tension (N/m), calculated as the force divided by twice the length of the segment, while the relaxant responses are expressed as the percentage of reduction of the precontraction. Sensitivity (expressed as pD2 = –log EC50) to agonists was determined for each artery by fitting individual concentration-response data to a nonlinear sigmoidal regression curve and interpolating (GraphPad Prism ver. 5.01; GraphPad Software, San Diego, CA). Differences between mean values were assessed by one-way ANOVA followed by Bonferroni post hoc test. Unpaired t-test was used if only two groups were compared. Differences were considered significant at a P < 0.05. All analyses were performed using a commercially available statistics package (GraphPad InStat ver. 3.00).

**RESULTS**

**Lack of sex-related differences in chicken DA reactivity.** The egg mass at day 0 of incubation (male: 63.75 g, SD 4.24, n = 120; female: 62.36 g, SD 3.53, n = 120), and the body mass of the 15-day (male: 13.15 g, SD 1.48, n = 30; female: 12.96 g, SD 1.43, n = 30), 17-day (male: 19.58 g, SD 1.78, n = 30; female: 19.32 g, SD 1.73, n = 30), and 19-day (male: 29.07 g, SD 2.11, n = 120; female: 28.89 g, SD 2.07, n = 120) chicken embryos did not vary with sex. The chicken DA (pulmonary side) contractions induced by O2 (Fig. 1A), 4-AP (Fig. 1B),
KCl (Fig. 1C), NE, Phe, U46619, and ET-1 (Fig. 2, A and B), and the relaxations (Figs. 2, C and D) induced by ACh, SNP, BAY 41–2272, PGE2 (in the presence of SQ 29,548), isoproterenol, forskolin, Y-27632, and hydroxyfasudil were not significantly different between male and female 19-day chicken embryos. When the reactivity of the DA from 15- and 17-day embryos was analyzed, we observed a developmental increase in the contraction induced by O2 (15-day < 17-day < 19-day embryos, Fig. 1A), 4-AP (15-day = 17-day < 19-day embryos, Fig. 1B), and KCl (15-day = 17-day < 19-day embryos, Fig. 1C) but without sex-related differences.
Estrogen-induced relaxation of chicken DA. The estrogen 17β-estradiol fully relaxed the 19-day chicken DA (pulmonary side) precontracted by 21% O2 (Fig. 3, A and B; pD2 male: 4.81, SD 0.07, n = 6; pD2 female: 4.70, SD 0.13, n = 7), 62.5 mM KCl (Fig. 3C, pD2 male: 4.67, SD 0.17, n = 7; pD2 female: 4.74, SD 0.16, n = 8), or 10 μM Phe (Fig. 4A; pD2 male: 4.94, SD 0.24, n = 10; pD2 female: 4.98, SD 0.16, n = 8). As shown in Fig. 3D, 17β-estradiol also induced a full relaxation of KCl-contracted DA rings from the aortic side (pD2 male: 4.81, SD 0.21, n = 6; pD2 female: 4.70, SD 0.19, n = 6). The stereoisomer 17α-estradiol also relaxed chicken DA rings (pulmonary and aortic sides) with similar potency than 17β-estradiol but with a lower efficacy (Figs. 3 and 4). No sex-related differences in estrogen-induced relaxation were observed (Figs. 3 and 4).

Relaxation to 17β-estradiol was not significantly modified by mechanical rubbing of the endothelium (Fig. 4B) or by incubation of DA rings with the sGC inhibitor ODQ (Fig. 4B), the nonselective K+ channel blocker TEA (not shown), the selective KCa channel blocker charybdotoxin (Fig. 4C), the selective KATP channel blocker glibenclamide (Fig. 4C), or the ER antagonist fulvestrant (Fig. 4C). The selective ERα agonist PPT and the selective ERβ agonist DPN did not induce relaxation of phenylephrine-contracted DA rings (not shown).

Cumulative concentrations of CaCl2 (10 μM–10 mM) induced concentration-dependent contraction in DA rings (pulmonary side) stimulated with 21% O2 or depolarized by 62.5 mM KCl (under 5% O2) in Ca2+-free medium (Fig. 5). As shown in Fig. 5A and previously described (13), O2 induced a slight contraction (0.025 N/m, SD 0.002, n = 10) in the absence of extracellular Ca2+. Preincubation with 17β-estradiol (10 μM) or the L-type Ca2+ channel blocker nifedipine (10 μM) produced a partial inhibition of CaCl2-induced contraction (Fig. 5, B and C).

**DISCUSSION**

The participation of different sex hormones on vascular tone regulation has been widely described (38). To our knowledge, however, the present study is the first attempt to evaluate sex-related differences in DA reactivity and DA responsiveness to estrogens in any species. We did not observe significant sex-related differences in chicken DA response to contractile or relaxant stimuli but demonstrated that 17β-estradiol elicits an endothelium and estrogen receptor-independent relaxation that appears to be mediated, at least partially, by the inhibition of Ca2+ entry from extracellular space.

Absence of sex-related differences in DA reactivity. In the last two years, the vascular biology of the chicken DA has been characterized by our group and another laboratory. The chicken DA responds to a wide variety of vasoconstrictors and vasodilators and undergoes, in general, a developmental increase in its responsiveness to constrictors and a developmental decrease in its responsiveness to relaxant agonists (1–3). Similarly to the mam-
malian DA, closure of the chicken DA occurs in two stages: an initial occlusion, mediated by constriction of smooth muscle in the pulmonary side, followed by anatomical vessel remodeling, leading to luminal obliteration (4). The timing of the closure of the DA correlates with an increased contractile response of the vessel to O₂, and the mechanisms underlying O₂ sensing and

Fig. 4. A: Relaxant effects of 17β-estradiol and 17α-estradiol in 10 μM phenylephrine-contracted DA rings (pulmonary side; PO₂ 7 kPa) from male and female 19-day chicken embryos. *P < 0.05 for difference in maximal relaxation from 17β-estradiol. B: lack of effect of endothelium removal (E-) or preincubation with the soluble guanylate cyclase ODQ on the relaxation induced by 17β-estradiol in chicken DA rings (pulmonary side). C: lack of effect of the KCa channel blocker charybdotoxin, the KATP channel blocker glibenclamide, and the estrogen receptor antagonist fulvestrant on the relaxation induced by 17β-estradiol in chicken DA rings (pulmonary side).

Fig. 5. Effects of 17β-estradiol (10 μM) and the L-type Ca²⁺ channel blocker nifedipine (10 μM) on the contraction induced by CaCl₂ in 19-day chicken DA (pulmonary side) previously incubated in a Ca²⁺-free medium. A: representative tracing of active wall tension vs. time showing the response of an O₂-stimulated DA ring (pulmonary side) to CaCl₂. The numbers above the tracing indicate log [CaCl₂] (M). B and C: mean effects of CaCl₂ in DA rings stimulated with 21% O₂ (PO₂ 19 kPa, B) or 62.5 mM KCl (at a PO₂ of 7 kPa, C). *P < 0.05 for difference from control.
signaling appear to be very similar in chickens and mammals (1, 4, 13, 21). In contrast, the main vasodilator of the mammalian DA, PGE$_2$, is a weak relaxant agent of the chicken DA, which even induces contraction through TP receptor stimulation (2, 21).

Herein, we report that there are no sex-related differences in DA reactivity. However, it should be noted that the majority of our study was performed in 19-day chicken embryos. The 19-day chicken embryo is close to initiate the transition to ex ovo life (i.e., internal pipiling) and presents, therefore, a relatively “mature” DA (1–4, 13, 21). For that reason, we decided to investigate whether sex-related differences in DA reactivity were present in DAs from less mature chicken embryos (i.e., 15- and 17-day embryos). We confirmed that although the 15-day DA contracts in response to high K$^+$-depolarizing solution and K$_e$ channel inhibition, it is almost unresponsive to O$_2$ (1–13). In contrast, O$_2$ elicited a tonic contraction in the 17-day DA that was ~25% of the contraction observed in the 19-day DA. The reactivity of 15-day and 17-day DAs was similar between males and females, suggesting that the developmental trajectory of the DA is not sex specific. Therefore, our first hypothesis, i.e., the presence of sexual dimorphism in chicken DA reactivity, was not supported by our results. It could be argued that the chicken is not a good model for studying sex-related differences in cardiovascular (patho)physiology. The information about sexual dimorphism in chicken cardiovascular (patho)physiology is scarce when compared with the data available from mammalian species. However, it has been demonstrated that male chickens are more prone to show elevation of blood pressure, neointimal plaque formation in the abdominal aorta, and aorta hardening than females (35, 44). In addition, male chickens present a higher incidence of pulmonary hypertension (60) and heart failure (37) than females. Although these alterations do not take place in early life and a direct role for sex hormones has not been demonstrated, the above data suggest that the chicken is a species in which sex hormones might influence cardiovascular development.

**Responsiveness to estrogen of chicken DA.** Our second hypothesis, i.e., that estrogens are vasoactive in the chicken DA, was supported by our results. However, it should be noted that chicken DA relaxation was induced with concentrations of estrogen far greater than the high picomolar-nanomolar levels of free hormone found in the plasma of chicken embryos at this developmental stage (56). In many mammalian studies, vascular effects of estrogen in vitro require ~1,000 times higher concentrations of free hormone than are typically present in circulating plasma (57). Therefore, effects of micromolar estrogen in vitro are often considered as mere pharmacological curiosities rather than potentially relevant responses (57). Nevertheless, it has been argued that free plasma levels of estrogen may far underestimate actual effective concentrations in the vicinity and inside target cells and that estrogen can act in a paracrine or even autocrine manner in vascular smooth cells (24, 57). Thus, as proposed by White (57), it appears no longer accurate to assume that only responses to nanomolar concentrations of estrogen have physiological relevance.

Numerous studies demonstrated that the response and the specific mechanisms responsible for the vascular effects of estrogens are species, age, sex, and vessel dependent (10, 11, 16, 22, 23, 25, 29, 33, 41–43, 45, 49, 57–59). Several studies strongly support that estrogen-evoked relaxation is mediated via endothelium-derived NO, but endothelium-derived prosta-cyclin has also been proposed as a mediator (10, 16, 27, 38, 41, 43, 45, 48, 49, 57). Other authors demonstrated an endothelium-independent effect of estrogen through activation of neuronal NOS, or by modulation of Ca$^{2+}$/and/or K$^+$ channel activity (11, 22, 23, 29, 33, 42, 43, 57–59). By using pharmacological tools, we evaluated the participation of some of these mechanisms in the ductal effects of estrogen. We observed that estrogen-mediated DA relaxation was endothelium independent and was unaffected by the presence of the sGC inhibitor ODQ, or the K$^+$ channel blockers TEA (nonselective), glibenclamide (K$_{ATP}$ selective), or charybdotoxin (BK$_{Ca}$ selective). Therefore, three of the mechanisms most frequently involved in estrogen-induced relaxation of mammalian vessels (i.e., the activation of the NO-sGC-cyclic GMP pathway and the opening of BKCa or K$_{ATP}$ channels) (57–59) appear not to be involved in the chicken DA relaxation.

The majority of data from mammalian studies indicates that estrogen lowers intracellular Ca$^{2+}$ concentration in vascular smooth muscle cells (57). One study indicated that estrogen may reduce sarcoplasmic reticulum Ca$^{2+}$ release (23), but other investigators have found that estrogen-induced vascular relaxation does not involve significant Ca$^{2+}$ release from intracellular stores or changes in Ca$^{2+}$ sensitivity of the contractile apparatus (15, 30, 57). Rather, estrogen attenuates extracellular Ca$^{2+}$ influx (15, 57) and/or stimulates Ca$^{2+}$ efflux (40). The inhibition of extracellular Ca$^{2+}$ influx by 17β-estradiol is supported by our results showing inhibition of the contraction elicited by CaCl$_2$ in O$_2$-or KCl-stimulated chicken DA rings. These data raise functional evidence for the Ca$^{2+}$ antagonistic effect of 17β-estradiol in chicken DA and are in line with a similar effect reported in rabbit carotid (46), cerebral (47), and coronary (28) arteries. In addition, electrophysiological evidence of inhibition of Ca$^{2+}$ currents by 17β-estradiol has been reported in several mammalian vascular smooth muscle cells (36, 62). Interestingly, we observed that when 17β-estradiol was compared with the dihydropyridine nifedipine (a classic Ca$_{Ca}$ channel blocker), both compounds showed, at high micromolar concentrations, a similar functional antagonism against CaCl$_2$-induced contraction. In contrast to our results, dihydropyridines showed a higher Ca$^{2+}$ antagonistic potency than 17β-estradiol in mammalian vessels (46, 47). Compared with other chicken vascular beds (like the pulmonary arteries, authors’ unpublished observations), the DA appears to be less sensitive to dihydropyridines as evidenced by the low potencies of the Ca$_{Ca}$ agonist BAY K8644 to induce contraction and of nifedipine to relax KCl- or O$_2$-induced contraction (13). However, it should be noted that, although an important part of the normoxic DA contraction relies on Ca$^{2+}$ entry through Ca$_{Ca}$ channels, inhibition of O$_2$-induced contraction in human (31) or lamb (12, 55) DA also required relatively high concentrations of nifedipine (10 µM).

**Estrogens and the two sides of chicken DA.** An extremely interesting feature of the chicken DA is the presence of morphological and functional heterogeneity along its path between the pulmonary artery and the aorta. Thus, the pulmonary side shows the structure of a muscular artery and responds to O$_2$ with contraction, whereas the aortic part shows the morphology of an elastic artery and relaxes in response to O$_2$ (1, 4, 14). In addition, the two parts of the DA show marked differences in their responsiveness to contractile and relaxant agents (2, 3). Bergwerff et al. (5) demonstrated that the distal elastic part of the chicken DA is mesodermal in origin and is
the result of the incorporation of dorsal aorta tissue, whereas the muscular pulmonary side was shown to consist almost exclusively of neural crest-derived cells. Interestingly, estrogen methylation rate appears to be different in vascular smooth muscle cells depending on the mesodermal or the neural crest origin (61). However, in the present work, we observed that estrogen-induced relaxation was uniformly present in both segments of the chicken DA, suggesting a similar sensitivity to estrogen in these two populations of vascular smooth muscle cells.

Estrogen receptors and estrogen-induced relaxation of chicken DA. Numerous studies demonstrate that mammalian vascular endothelium and smooth muscle cells express both ERα and ERβ, but this expression seems heterogeneous with regard to species, vascular bed, and sex (57). Herein, we observed that chicken DA relaxation induced by 17β-estradiol was not affected by the presence of the ER antagonist fulvestrant, and neither the specific ERα agonist PPT nor the specific ERβ agonist DPN showed any relaxant effect in the chicken DA. These considerations lead us to postulate that 17β-estradiol induces endothelium-independent relaxation of chicken DA via a mechanism that does not require the participation of the classical estrogen receptors. However, it shows steroselectivity because 17α-estradiol also relaxed the chicken DA with similar potency but lower efficacy. This steroselectivity may indicate that a putative, receptive, specific structure is responsible for the relaxant effect. It has been suggested that estrogens could act through direct binding to regulatory subunits in the CaL channel (47). Alternatively, other receptors for the estrogen-induced relaxation were uniformly present in both origin (61). However, in the present work, we observed that estrogen-induced relaxation was uniformly present in both segments of the chicken DA, suggesting a similar sensitivity to estrogen in these two populations of vascular smooth muscle cells.

Perspectives and Significance

The mammalian placenta provides a highly estrogenic environment for both female and male fetuses. In contrast, in ovo development of the chicken takes place under a marked sex-related difference in estrogen levels (6, 9, 20). Our present data do not suggest that such a difference influences DA reactivity. However, the chicken embryo still appears as a suitable natural model to study the influence of early estrogen exposure in other vascular beds, like the coronary circulation, particularly susceptible to the hormone. In addition, manipulation of the sex steroid hormonal environment of the chicken embryo can be easily performed by injection of estrogens or inhibitors of estrogen synthesis into the albumen of fertilized eggs (6, 9, 20). Thus, a single treatment with an aromatase inhibitor, which blocks the synthesis of estrogen from testosterone, at an embryonic stage when gonads are bipotential, causes genetic females to develop a permanent male phenotype (20). On the contrary, the embryos that are exposed to estradiol in ovo hatch as phenotypic females regardless of their genotypic sex (6, 9). Whether these hormonal manipulations influence developmental vascular biology warrants further investigation.

GRANTS

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DISCLOSURES

No conflicts of interest are declared by the authors.

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