Vascular reactivity in intrapulmonary arteries of chicken embryos during transition to ex ovo life

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Villamor, Eduardo, Karin Ruijtenbeek, Victor Pulgar, Jo G. R. De Mey, and Carlos E. Blanco. Vascular reactivity in intrapulmonary arteries of chicken embryos during transition to ex ovo life. Am J Physiol Regulatory Integrative Comp Physiol 282: R917–R927, 2002; 10.1152/ajpregu.00369.2001.—The present study aimed to characterize pulmonary vascular reactivity in the chicken embryo from the last stage of prenatal development and throughout the perinatal period. Isolated intrapulmonary arteries from non-internally pipped embryos at 19 days of incubation and from internally and externally pipped embryos at 21 days of incubation were studied. Arterial diameter and contractile responses to KCl, endothelin-1, and U-46619 increased with incubation but were unaffected by external pippering. In contrast, the contractions induced by norepinephrine, phenylephrine, and electric field stimulation decreased with development. No developmental changes were observed in endothelium-dependent [acetylcholine (ACh) and cyclopiazonic acid] or endothelium-independent [sodium nitroprusside (SNP)] relaxation. These relaxations were abolished by the soluble guanylate cyclase inhibitor 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one. Endothelium-dependent relaxation was unaffected by blockade of cycloxygenase or heme oxygenase but was significantly reduced by nitric oxide (NO) synthase inhibitors. Reduction of O2 concentration from 95 to 5% produced a marked reduction in ACh and SNP-induced relaxations. Chicken embryo pulmonary arteries show a marked endothelium-dependent relaxation that is unaffected by transition to ex ovo life. Endothelium-derived NO seems to be the main mediator responsible for this relaxation.

DURING PRENATAL LIFE the lungs do not participate in gas exchange. This function is assumed by the placenta in the mammalian fetus and by the chorallantoic membrane (CAM) in the avian embryo (27). Consequently, during this period, the lungs receive only a small proportion of the cardiac output, to ensure pulmonary growth and development (3, 30, 32, 54). Thus the prenatal pulmonary circulation exists as a high-resistance/low-flow circuit that transits to a low-resistance/high-flow circuit at the onset of pulmonary respiration (3).

Despite extensive investigations, mechanisms that contribute to the maintenance of high vascular tone in the mammalian fetal lung are not completely understood but include a different balance, compared with the postnatal lung, between vasoconstrictor and vasodilator mediators (3). Numerous studies demonstrated marked differences in the contractile and relaxing responses of isolated pulmonary vessels from fetal and neonatal mammals when compared with adults (e.g., 2, 7, 22, 24, 43).

Several mechanisms contribute to the normal fall in pulmonary vascular resistance at birth, including the establishment of a gas-liquid interface in the lung, increased O2 tension, rhythmic distension of the lung, and shear stress (3). These physical stimuli act, at least partially, through the production of vasoactive products, such as nitric oxide (NO) and prostacyclin, by the pulmonary vascular endothelium (3). Unlike the rapid transition from an intraterine to an extraterine environment displayed in most mammals, bird hatching from eggs is an event that may take place over several days (17). O2 demand increases exponentially during development, and it exceeds the capacity of the CAM gaseous diffusion by the end of the avian incubation period (33). At this point, around day 19 of incubation in the chicken, the beak of the embryo penetrates the air cell, air enters the lung, and breathing is initiated. This process is termed internal pippering and is followed by external pippering when an opening of the shell is achieved and ambient air is breathed for the first time. Chicken embryos spend ~9.5% of their 21-day incubation time pippering (17). During the pippering period, the gas exchange of the CAM declines while that of the lung increases rapidly. In parallel, the relative blood...
flow to the CAM declines, whereas blood flow to the lungs increases (32). This leads to the final hatching act.

Because of its isolation from maternal influences and the relative separation of nutrient and respiratory transport, the chicken embryo is an attractive model to study cardiovascular responses during development, under physiological and pathological conditions. This is particularly relevant for the pulmonary vasculature because, in mammals, adverse intraterine events appear to be related to failure of the lung circulation to undergo a normal transition at birth (3). In previous works of our laboratory, we have characterized the reactivity of isolated systemic arteries from chicken embryos (21) and evaluated the effects of exposure to chronic hypoxia during development on this vascular reactivity (37). In the present study, we aimed to characterize the contractile and relaxant properties of the pulmonary arteries from chicken embryos during late gestation and to analyze how they are influenced by its gradual transition to ex ovo life, i.e., by the processes of internal and external pipping.

**METHODS**

**Vessel isolation.** Experimental procedures followed Dutch laws for animal experimentation. Fertilized eggs of White Leghorn chickens were incubated at 38°C, 21% O2 with a relative air humidity of 60% and were rotated hourly. Embryos incubated for 19 and 21 days of a 21-day incubation period were studied. The 19-day-old embryos were defined as non-externally pipped embryos, as verified by candling. The 21-day-old embryos were defined as internally but non-externally pipped embryos, as verified by candling and the presence of an intact eggshell, or as externally pipped embryos, when an opening of the eggshell was observed by careful inspection. The embryos were taken out and immediately killed by decapitation, and the heart and lungs were removed en bloc and immersed in ice-cold Krebs-Ringer bicarbonate (KRB) solution. With the aid of a dissecting microscope, main axial intrapulmonary arteries were carefully dissected free of surrounding tissue and cut into rings of 1.7–2 mm of length.

**Recording of arterial reactivity.** The isolated arteries were mounted between an isometric force transducer (Kistler Morex DSC 6, Seattle, WA) and a displacement device in a myograph (model 610M, J. P. Trading, Aarhus, Denmark) by using two stainless steel wires (diameter 40 μm). 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+ (using a diameter-tension protocol as previously described (21)).

**Contractile responses.** Contractile agonists were evaluated under basal tone. Concentration-response curves to K+ (4.75–125 mM), the thromboxane A2 mimetic 9,11-dideoxy-11α,9-epoxymethano-prostaglandin F2α (U-46619; 10–8 M–10–5 M), endothelin-1 (ET-1; 10–9 M–3 × 10–7 M), nor epinephrine (NE; 10–8 M–3 × 10–5 M), and phenylephrine (Phe; 10–8 M–3 × 10–4 M) were constructed by increasing the organ chamber concentration of the drug by cumulative increments after a steady-state response had been reached with each increment. Sympathetic neuroeffector mechanisms were studied by using electrical field stimulation (EFS; 0.25–16 Hz, 2 ms, 85 mA) via two platinum electrodes that were placed in the axial direction of the blood vessel. Constant-current pulses were delivered by a stimulator (Technical Services, Universiteit Maastricht, The Netherlands).

**Relaxing responses.** Relaxing agonists were evaluated during contraction induced by 125 mM K+. Some specific protocols were performed during 40 mM K+– or 10–7 M ET-1-induced contractions (see RESULTS for further explanation). Concentration-response curves for acetylcholine (ACH; 10–9 M–10–4 M), the NO donor sodium nitroprusside (SNP; 10–8 M–10–4 M), and the Ca2+-ATPase inhibitor cyclopiazonic acid (CPA; 10–7 M–10–5 M) were constructed. Some experiments were performed in the presence of the cyclooxygenase inhibitor indomethacin (10–5 M), the NO synthase inhibitors N’-nitro-l-arginine methyl ester (l-NAME; 10–3 M), and S-methyl-l-thiocitrulline (l-SMTC; 10–4 M) (18), the soluble guanylate cyclase inhibitor 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ; 10–5 M) (8), or the heme oxygenase inhibitor tin protoporphyrin IX (SnPP-IX; 10–5 M) (25). These drugs were added after the precontraction had reached a steady-state response, and the effects of the relaxing agonists were evaluated 20 min later. Relaxing responses were also studied in endothelium-denuded arteries. For this purpose, the endothelium was removed by rubbing the inside of the mounted vessel with a human hair as previously described (21). In another group of experiments, responses to ACh and SNP were studied under lower oxygenation conditions (5% instead of 95% O2). In these experiments, arteries were mounted and stabilized under 95% O2, and bubbling gas was switched to 5% O2-5% CO2-90% N2 15 min before 125 mM K+ precontraction and maintained during the concentration-response curve to the relaxant agonists. The PO2 values were measured by a blood gas analyzer (ABL 510 Radiometer, Copenhagen, Denmark).

**Evaluation of basal production of NO.** Cumulative concentration-response curves for l-NAME were obtained in pulmonary arteries under basal tone or after contraction with 125 mM K+. Contractile responses were taken as an indication that there was attenuation of tone by endogenous NO. In some experiments, l-arginine (10–5 M), d-arginine (10–5 M), or ODQ (10–6 M) was included in the organ chamber 20 min before the l-NAME. Some of the experiments that involved ODQ were performed after contraction with 40 mM K+ instead of 125 mM K+.

**Data analysis.** Results are given as means ± SE of measurements in n embryos/arteries (only 1 arterial ring of each embryo was used). Contractile responses are expressed in terms of active wall tension (force divided by twice the segment length; N/m). Relaxations are expressed as a percentage of the preexisting tone. Individual cumulative concentration-response curves were analyzed by fitting the experimental data to a nonlinear sigmoidal regression curve (GraphPad Software, San Diego, CA). When a drug produced a biphasic response (e.g., ACh, which produces relaxation at low concentrations and contraction at high concentrations), only relaxant concentrations were taken in account for the regression curve. Maximal relaxant effect (Emax) and EC50 were calculated from the fitted concentration-response curves for each ring. EC50 is expressed as negative log molar (pD2). The significance of differences between mean values was assessed by Student’s t-test or one-way ANOVA followed by Bonferroni post hoc t-test (for parameters normally distributed) or by the Mann-Whitney U test (for parameters nonnormally distributed). Differences were considered significant at P < 0.05.

AJP-Regulatory Integrative Comp Physiol • VOL. 282 • MARCH 2002 • www.ajpregu.org
**EMBRYONIC PULMONARY ARTERIAL REACTIVITY**

**Drugs and solutions.** KRB buffer 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. Arterenol bitartrate (NE), indomethacin, L-NAME, ET-1, and U-46619 (methyl acetate solution) were obtained from Sigma Chemical (St. Louis, MO); ACh chloride was from Janssen Chimica (Beerse, Belgium); SNP was from Acros (Geel, Belgium); ODQ was from Toeris Cookson (Bristol, UK); and CPA, SnPP-IX, and L-SMTC were from Alexis (Bingham, UK). All the drugs were dissolved initially in distilled deionized water (except ODQ and CPA in DMSO, indomethacin in ethanol, and SnPP-IX in 0.1 M NaOH titrated with 0.1 M HCl to pH 7.4) to prepare a 10−2 M stock solution, and further dilutions were made in KRB. Experiments involving SnPP-IX were carried out in a darkened room because metalloporphyrins are light sensitive.

**RESULTS**

**Contractile responses.** Pulmonary arteries isolated from chicken embryos at 19 and 21 days of incubation responded to depolarizing high-K+ solution with a tonic contraction. The diameter at which maximal responses were obtained (19 days: 239 ± 5.6 μm, n = 32; internally pipped 21 days: 514 ± 12 μm, n = 34; externally pipped 21 days: 521 ± 10 μm, n = 32; P < 0.01, 19 days vs. both groups of 21 days) and the amplitude of the response (Fig. 1A) increased significantly with increasing incubation. The responses to U-46619 and ET-1 also increased between 19 and 21 days of incubation (Fig. 1, B and C, respectively). However, no differences were found in arterial diameter and in the responses to K+, U-46619, or ET-1 between the 21-day-old internally pipped and the 21-day-old externally pipped embryos. The contractile responses to NE, to the α1-adrenergic agonist Phe, and to EFS were very small and significantly reduced in the 21-day-old embryos compared with the 19-day-old embryos (Fig. 2). Constrictor responses to EFS are attributed to stimulation of perivascular sympathetic nerves in several vascular beds (31). Because of the weak contraction obtained in chicken embryo pulmonary arteries, this effect of EFS has been assumed for these vessels but not further investigated by blockade of sympathetic receptors.

**Relaxing responses.** Figures 3 and 4 illustrate the effect of ACh on pulmonary arteries precontracted with 125 mM K+. Concentration-response curve parameters are summarized in Table 1. In endothelium-intact arteries, ACh induced concentration-dependent relaxations that were similar in 19- and 21-day-old embryos. Mechanical removal of endothelium did not affect the level of 125 mM K+-induced contraction but almost abolished the relaxant response to ACh in both age groups (Fig. 3). Neither indomethacin nor SnPP-IX affected ACh-induced relaxation. However, the NO synthase inhibitors L-NAME and L-SMTC significantly reduced the relaxing activity of ACh, whereas the soluble guanylate cyclase inhibitor ODQ completely abolished it (Table 1 and Fig. 4). The presence of indomethacin did not interfere with the effects of L-NAME or L-SMTC (Table 1). As L-NAME, L-SMTC, and ODQ significantly increased the contractile tone induced by 125 mM K+, some experiments were performed after precontraction with 40 mM instead of 125 mM K+. Combination of L-NAME, L-SMTC, or ODQ with 40 mM K+ produced a contraction nonsignificantly different from that produced by 125 mM K+. Under these experimental conditions L-NAME, L-SMTC, and ODQ showed similar inhibitory effects of ACh-induced relaxation (data not shown).
In endothelium-intact segments from 19- and 21-day-old embryos, CPA elicited concentration-dependent relaxations of 125 mM KCl-induced tone (Fig. 5). No significant differences of CPA-induced relaxation were observed among the three experimental groups studied. The absence of endothelium or the presence of L-NAME or ODQ abolished CPA-induced relaxation in the three experimental groups (Table 1 and Fig. 7; in Fig. 7 only the data from ACh in 19-day-old embryos and from SNP in externally pipped 21-day-old embryos are shown).

The NO donor SNP produced a similar pattern of relaxation in pulmonary arteries from 19-day-old, 21-day-old internally pipped, and 21-day-old externally pipped chicken embryos (Fig. 6). SNP-induced relaxations were unaffected by endothelium removal (data not shown) but were completely abolished by the presence of ODQ.

Bubbling the organ chamber with 5% O2 (PO2 13.1 ± 0.1 kPa) instead of 95% O2 (PO2 74.3 ± 1 kPa) markedly reduced the relaxant effects of ACh and SNP in the three experimental groups (Table 1 and Fig. 7; in Fig. 7 only the data from ACh in 19-day-old embryos and from SNP in externally pipped 21-day-old embryos are shown).

Arteries stimulated with ET-1 (10^{-7} M) showed a level of contraction similar to that of arteries stimulated with 125 mM KCl (e.g., 21-day-old internally pipped embryos: 0.89 ± 0.19 vs. 0.75 ± 0.12 N/m). However, ET-1-stimulated arteries showed a markedly increased relaxant response to ACh and SNP (Table 1 and Fig. 8; in Fig. 8 only the data from ACh in 19-day-old embryos and from SNP in externally pipped 21-day-old embryos are shown).

Contractile effects of NO synthase inhibition on pulmonary artery preparations. Addition of l-NAME to pulmonary arteries under basal tone resulted in concentration-dependent contractions (Fig. 9). The detectable threshold concentration for this contractile effect of l-NAME was 10^{-4} M. In addition, l-NAME increased 125 mM KCl-induced contractions, and this effect was observed from a concentration of 10^{-6} M (Fig. 9). The contractions to l-NAME were abolished by L-arginine (10^{-3} M, Fig. 9) but were unaffected by D-arginine (10^{-3} M, not shown). The presence of the soluble guanylate cyclase inhibitor ODQ (10^{-6} M) completely inhibited l-NAME-induced contractions. ODQ increased 125 mM KCl-induced contractions in a manner similar to that observed for l-NAME. To exclude the possibility that this ODQ-induced increase in 125 mM KCl-induced contraction may be responsible for the inhibition of l-NAME-induced contraction, some experiments were performed after stimulation of the pulmonary arteries with 40 mM KCl. The additive effects of

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ODQ and 40 mM K\textsuperscript{+} were not significantly different from the contractile effects of 125 mM K\textsuperscript{+} (e.g., 21-day-old, 0.72 ± 0.14 vs. 0.83 ± 0.14 N/m). Under these conditions, ODQ also inhibited L-NAME-induced contraction (Fig. 9).

DISCUSSION

Over the past several years, numerous studies focused on developmental changes in pulmonary vascular reactivity from several mammalian species. Maturational differences in vasoconstriction and endothelium-dependent and -independent relaxation have been described in fetal and neonatal lambs (2, 12, 20, 47), pigs (7, 22, 50), rabbits (10, 28), and guinea pigs (6, 46). The purpose of these studies was to achieve a better understanding of 1) the hemodynamically unique situation of the fetal pulmonary circulation, when the gas-exchange function of the lung is dormant, and 2) the mechanisms that regulate the pulmonary circulatory transition that occurs at birth. To the best of our knowledge, the functional properties of avian embryonic pulmonary vessels and the influence of their particular transition to pulmonary respiration over these properties have not been studied before. We demonstrated that avian embryonic pulmonary arteries have viable effector mechanisms for contraction and relaxation.

Contractile properties. We observed that pulmonary arteries from chicken embryos responded, in vitro, to receptor-independent contraction (K\textsuperscript{+}-induced depolarization), to a prostanoid (U-46619), a polypeptide (ET-1), and, very slightly, to adrenergic agents (NE and Phe) and perivascular sympathetic nerve stimulation (EFS). The contractile responses to K\textsuperscript{+}, U-46619, and ET-1 increased with embryonic age, concomitantly with the increase in weight of the embryos and in diameter of the pulmonary arteries. In contrast, the weak contractile response to adrenergic stimulation and perivascular nerve stimulation decreased with embryonic age.

The autonomic nervous system may modify adult pulmonary blood flow under physiological conditions and may be involved in the pathophysiology of pulmonary vascular diseases (4). However, the absence of axons in the terminal pulmonary arterioles of adult chickens contrasts with the profuse innervation of mammalian terminal arterioles (19). Neurohumoral mechanisms do not appear to contribute to basal pulmonary vascular tone in the mammalian fetus, but the ability to respond to adrenergic and cholinergic stimuli is present early in maturation and may modulate pulmonary vascular resistance during stress (3). Developmental changes in adrenoceptor-mediated pulmonary vasoconstriction and relaxation have been described. Thus the response to β-adrenergic stimulation increased in the fetal lamb between 75 and 90% of gestation but did not change between 90% of gestation and the postnatal period (38). On the other hand, α\textsubscript{1}-adrenergic receptor density was less in fetal ovine intrapulmonary vascular smooth muscle compared with adult animals (41). In contrast, third-generation fetal ovine pulmonary arteries were more sensitive to NE than neonatal vessels, suggesting the presence of a mechanism to harmonize the dramatic drop in pulmonary vascular resistance with the high levels of catecholamines released into the circulation during the birth process (12). Basal plasma epinephrine and norepinephrine concentrations by the end of the incubation period were much higher in the chick embryo than values reported for mammalian fetuses during late gestation (29). In contrast, we have found, in pulmo-
nary arteries from chicken embryos, a very weak contractile response to adrenergic stimulation. Moreover, this response decreased with embryonic age. In a previous work, we described that the contractile reactivity to α1-adrenergic stimulation increased with development in the femoral and carotid arteries of chicken embryos (21). However, the magnitude of this contractile response was even higher than the contraction reached in response to 125 mM K+ in the femoral and carotid arteries of chicken embryos (21). Therefore, the process of internal pipping affected both endothelium-dependent and -independent relaxation.

Endothelium-dependent relaxation is achieved by combined vaso dilator effects of endothelium-derived prostanol, NO, carbon monoxide, and endothelium-derived hyperpolarizing factor (EDHF), among others (25,49). Contribution of these factors in relaxation varies across species, vascular beds, and also with the age of chicken embryos. In fact, the α-receptor blocker phentolamine did not change the percentage of cardiac output diverted to the lungs in 19-day-old chicken embryos (Mulder AL and Blanco CE, unpublished results).

Relaxing properties: contribution of NO, prostanol, carbon monoxide, and endothelium-derived hyperpolarizing factor to endothelium-dependent relaxation. Focusing on the relaxing properties of chicken embryo pulmonary arteries, we described the presence of endothelium-dependent relaxation to Ach and CPA and of endothelium-independent relaxation to the NO donor SNP. Very interestingly, neither the process of internal nor external pipping affected both endothelium-dependent and -independent relaxation.

Table 1. Concentration-response curve parameters for acetylcholine in chicken embryo pulmonary arteries

<table>
<thead>
<tr>
<th></th>
<th>19-Day-Old</th>
<th>21-Day-Old, Non-Externally Pipped</th>
<th>21-Day-Old, Externally Pipped</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pD2</td>
<td>Emax</td>
<td>n</td>
</tr>
<tr>
<td>Control</td>
<td>7.07 ± 0.1</td>
<td>74.99 ± 5.9</td>
<td>10</td>
</tr>
<tr>
<td>Indo</td>
<td>7.06 ± 0.1</td>
<td>72.11 ± 6.3</td>
<td>8</td>
</tr>
<tr>
<td>L-NAME</td>
<td>6.54 ± 0.12*</td>
<td>37.8 ± 3.6*</td>
<td>10</td>
</tr>
<tr>
<td>L-SMTC</td>
<td>6.63 ± 0.1*</td>
<td>34.7 ± 3.9*</td>
<td>7</td>
</tr>
<tr>
<td>SnPP-IX</td>
<td>7 ± 0.15</td>
<td>70.9 ± 9.1</td>
<td>6</td>
</tr>
<tr>
<td>Indo + L-NAME</td>
<td>6.61 ± 0.09*</td>
<td>40.12 ± 4.3*</td>
<td>10</td>
</tr>
<tr>
<td>Indo + L-SMTC</td>
<td>6.68 ± 0.1*</td>
<td>32.21 ± 4.1*</td>
<td>8</td>
</tr>
<tr>
<td>Indo + L-NAME + L-SMTC</td>
<td>5.92 ± 0.1*</td>
<td>19.5 ± 4.8*</td>
<td>8</td>
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<tr>
<td>Indo + SnPP-IX</td>
<td>6.82 ± 0.2</td>
<td>72.71 ± 11</td>
<td>6</td>
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<tr>
<td>Indo + SnPP-IX + L-NAME</td>
<td>6.42 ± 0.15*</td>
<td>36.14 ± 6.1*</td>
<td>6</td>
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<tr>
<td>Precontraction with ET-1</td>
<td>6.92 ± 0.1</td>
<td>97.4 ± 5.1*</td>
<td>8</td>
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<tr>
<td>5% O2</td>
<td>5.98 ± 0.1*</td>
<td>38.36 ± 4.8*</td>
<td>8</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = no. of embryos (arteries). Indo, indomethacin; L-NAME, Nω-nitro-L-arginine methyl ester; L-SMTC, S-methyl-L-thiocitrulline; SnPP-IX, tin protoporphyrin IX; ET-1, endothelin-1; pD2, –log M; Emax, maximal relaxant effect. *P < 0.01 vs. control (95% O2 and precontraction with 125 mM KCl).

Fig. 5. Concentration-dependent relaxant effects of the Ca2+ -ATPase inhibitor cyclopiazonic acid (CPA) in endothelium-intact pulmonary arteries from non-externally pipped 19-day-old (●), internally pipped 21-day-old (●), and externally pipped 21-day-old (▲) chicken embryos (total incubation time 21 days). The effects of endothelium removal (○), the presence of L-NAME (●), or the presence of ODQ (□) are shown only in internally pipped 21-day-old embryos. Each point represents mean ± SE of 6–10 embryos. **P < 0.01 vs. age-matched control. Significance is only shown at the maximal relaxant dose.

Fig. 6. Concentration-dependent relaxant effects of sodium nitroprusside (SNP) in endothelium-intact pulmonary arteries from non-externally pipped 19-day-old (●, □), internally pipped 21-day-old (●, ○), and externally pipped 21-day-old (▲, ◦) chicken embryos (total incubation time 21 days). Arteries were precontracted with 125 mM KCl. Experiments were performed in the absence (solid symbols) or in the presence (open symbols) of the soluble guanylate cyclase inhibitor ODQ. Each point represents mean ± SE of 6–10 embryos.
agent used to stimulate the endothelium (45). In the present work, we aimed to compare the relative role of these agents in endothelium-dependent relaxation in chicken embryo pulmonary arteries. Neither inhibition of the cyclooxygenase nor the heme-oxygenase pathways, by indomethacin and SnPP-IX, respectively, affected endothelium-dependent relaxation. This suggests a lack of involvement of prostacyclin and heme-oxygenase-produced carbon monoxide (25). Moreover, the majority of the experiments that involved relaxant responses were performed after contraction of the pulmonary arteries with very high K\textsuperscript{+} concentrations (125 mM). Under these conditions, endothelium-dependent relaxation was maintained, suggesting a lack of involvement of EDHF. However, ACh-induced relaxation was lower in K\textsuperscript{+}-precontracted arteries than in ET-1-precontracted arteries, but the same was observed for SNP-induced, endothelium-independent relaxation. It has been shown that the nonspecific inhibitory effect of depolarization on smooth muscle relaxation is due to an inhibition of cGMP formation by high K\textsuperscript{+} concentrations (34).

ACh- and CPA-induced relaxations were endothelium dependent and markedly reduced by the presence of the NO synthase inhibitors L-NAME or L-SMTC. These facts suggest that the main endothelial vasodilator released by ACh and CPA in the chicken embryo pulmonary artery is NO, which would stimulate the

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Fig. 7. Effects of O\textsubscript{2} concentration on acetylcholine- (A) and SNP-induced (B) relaxation in endothelium-intact pulmonary arteries from non-internally pipped 19-day-old (A) and externally pipped 21-day-old (B) chicken embryos (total incubation time 21 days). Organ chambers were bubbled with 95% O\textsubscript{2} (○) or 5% O\textsubscript{2} (●). Each point represents mean ± SE of 6–10 embryos. *P < 0.01, 5% O\textsubscript{2} vs. 95% O\textsubscript{2}.

Fig. 8. Effects of precontraction induced by 125 mM K\textsuperscript{+} (●) or ET-1 (○) on acetylcholine- (A) and SNP-induced (B) relaxation in endothelium-intact pulmonary arteries from non-internally pipped 19-day-old (A) and externally pipped 21-day-old (B) chicken embryos (total incubation time 21 days). Each point represents mean ± SE of 6–10 embryos. *P < 0.05, **P < 0.01, K\textsuperscript{+} vs. ET-1-contracted arteries.

Fig. 9. Concentration-dependent contractile effects of L-NAME in endothelium-intact pulmonary arteries from non-internally pipped 19-day-old chicken embryos (total incubation time 21 days). Arteries were under basal tension (○) or precontracted with 125 mM KCl (●), 2 mM or 42 mM KCl (□). Experiments were performed under control conditions or in the presence of L-arginine (△) or ODQ (○). Each point represents mean ± SE of 6–10 embryos.
cGMP formation by activation of soluble guanylate cyclase. Such an assumption is supported by the fact that ODQ, an inhibitor of this enzyme, abolished the relaxation caused by ACh and CPA. The ability of ACh to induce pulmonary vascular relaxation through the release of endothelium-derived NO has been very well known for many years (16). CPA is known to block endoplasmic Ca$^{2+}$-dependent ATPase and promote the entry of extracellular Ca$^{2+}$ (55). The influx of Ca$^{2+}$ is thought to elevate endothelial Ca$^{2+}$ concentration and therefore stimulate the release of endothelium-derived NO.

The presence of agonist-stimulated production of NO has been previously demonstrated in systemic arteries from chicken embryos (21) and in pulmonary arteries from adult chickens (26). Chicken embryonic femoral and carotid arteries responded to ACh, and this response was abolished by removal of the endothelium and partly reduced by the presence of L-NAME (21). Sensitivity to ACh and its maximal effect did not change significantly between 16 and 19 days of incubation (21). Additionally, Martinez-Lemus et al. (26) demonstrated the presence of ACh- and A23187-induced relaxation on pulmonary arteries from adult chickens and inhibition of this response by L-NAME. The pattern and magnitude of response that they report (26) are very similar to that presently described in embryonic arteries. Therefore, the maturational changes in endothelium-derived NO activity described in mammalian pulmonary arteries (2, 7, 24) seem not to be present in the chicken. Very interestingly, Martinez-Lemus et al. (26) also reported impairment of pulmonary endothelium-dependent relaxation in broiler compared with Leghorn chickens. Broiler chickens are highly susceptible to pulmonary hypertension, and reduced endothelium-derived NO activity may contribute to its pathophysiology (26).

In addition to agonist-stimulated production of NO, we also studied basal NO production. We demonstrated that chicken embryo pulmonary arteries developed sustained contractions in the presence of the NO synthase inhibitor L-NAME. Moreover, the force of contraction induced by K$^+$ was also significantly increased in the presence of L-NAME, and this effect was abolished by the presence of L-arginine, the substrate for NO production, or by ODQ-induced inhibition of soluble guanylate cyclase. All these facts highlight a significant contribution of NO to vascular tone in chicken embryo pulmonary arteries. Therefore, these vessels exhibited both basal and stimulated release of NO.

**Transition to postnatal life and vascular reactivity.**

In several mammalian species, numerous investigations demonstrated that endothelium-dependent relaxation is reduced in the fetal life and transiently compromised immediately after birth. In the ovine lung, endothelium-dependent relaxation in response to ACh, ADP, and A23187 is minimal in utero and at birth and increases rapidly during the first week of life (2, 43). Endothelium-dependent relaxation to ACh is absent in porcine pulmonary arteries immediately after birth (7, 22, 24) but present in fetal animals (7) or after 12–24 h of postnatal life (50). Similar findings have been reported in rabbit pulmonary arteries (28). In contrast with this perinatal impairment of endothelium-dependent relaxation, increased release of endogenous NO seems to be necessary for a smooth transition of the pulmonary circulation at birth (1, 3). In the present study, we described that endothelium-dependent and -independent relaxation remained unchanged during the gradual transition to postnatal life of the chicken embryo, i.e., during the processes of internal and external pippering. Therefore, the transient impairment of endothelial function described in neonatal mammalian species does not seem to be present in the chicken.

The transition to ex utero/ex ovo life, and the consequent beginning of pulmonary respiration and exposure to atmospheric O$_2$, is prepared during late gestation in the mammalian fetus and in the avian embryo in a very similar way. However, some differences seem to be present. During the final 10–20% of gestation in the fetal lamb, rat, rabbit, hamster, and guinea pig, lung antioxidant enzyme activity and particularly superoxide dismutase activity rises sharply in parallel with the development of the surfactant system (13, 52). In the chicken embryo, pulmonary lung superoxide dismutase enzyme activity increased ~2.5-fold between days 16 and 18 of incubation, i.e., before internal pippering, but no further changes occurred afterward (48). In addition, the pattern of development of the surfactant lipids in the embryonic chicken was similar to that of mammals (17). However, chicken surfactant did not attain a completely mature composition until after pulmonary ventilation had been established, and it has been suggested that, after internally pippering, birds might rely partly on their CAM for gas exchange while titrating their surfactant and aerating the tiny air capillaries and parabronchi (17). Finally, exposure to O$_2$ occurs more gradually in the chicken embryo than in the mammalian fetus. Partial pressure of O$_2$ in the air cell of the egg is ~35 mmHg lower than the atmospheric Po$_2$ (33). Therefore, during internal pippering, the chicken embryo breathes a relatively hypoxic gas mixture, and only when external pippering is started are the lungs exposed to atmospheric O$_2$.

Pulmonary adaptation to ex ovo or extraterine life consists of a rapid sequence of integrated morphological and functional changes. Experimental studies, in mammalian species, suggest that the immediate postnatal period is characterized by rapid recruitment of small alveolar duct and wall vessels, which appear to be functionally and structurally closed in the prenatal period (14). In the chicken embryo, in ovo onset of pulmonary respiration, i.e., internal pippering, initiates a rapid development in number and length of the capillaries between arterioles and venules (11). Therefore, the decrease in pulmonary vascular resistance at birth is also explained partly by an increase in the cross-sectional area of pulmonary microvasculature that occurs in either mammalian fetuses or chicken embryos.
Changes in endothelium-dependent relaxation with oxygenation. NO has a dual relationship with O2. On the one hand, O2 is a substrate for NO production, because NO synthase is a dioxygenase that catalyzes the reaction between molecular O2 and L-arginine (36). Thus it has been demonstrated that low PO2 decreased NO production in fetal ovine pulmonary endothelium (40, 42). On the other hand, NO is destroyed rapidly by the reduced species of molecular O2, superoxide anion, leading to loss of its vasodilator activity (5). Thus the response to ACh in rabbit pulmonary arteries was absent in neonatal animals but restored by the presence of the superoxide anion scavenger superoxide dismutase (28). Therefore, low fetal O2 concentration has been proposed to explain reduced endothelium-dependent relaxation during fetal life (3, 40, 42), and the postnatal exposure to a much more O2-enriched environment has been proposed to justify the transient impairment of endothelium-dependent relaxation immediately after birth (28).

In the present work, we described that a reduction in O2 tension from 74 to 13 kPa impaired ACh-induced relaxation in chicken embryo pulmonary arteries, suggesting the presence of a critical level of molecular O2 for NO production. The purpose of selecting these two levels of PO2 was to provide a wide spectrum of in vitro oxygenation and not to attempt to mimic levels of oxygenation that yield cardiovascular effects in vivo (39). O2 tension is an insensitive indicator of O2 availability in vivo, as it does not reflect the vast majority of blood O2 content bound to hemoglobin (39). Our results in the chicken embryo contrast with findings by Morecroft and MacLean (28) in pulmonary arteries from rabbit fetuses. They demonstrated that, at 90% of gestation, 95% O2 impaired ACh-induced relaxation, which was restored by the use of 16 or 3% O2. However, at 70% of gestation, both extreme oxygenation conditions, i.e., 95 and 3% O2, impaired the activity of ACh.

Additionally, we observed that reduction of O2 in the organ chamber also affected SNP-induced relaxation, suggesting a possible inhibition of soluble guanylate cyclase under low O2. However, Wanstall (53) demonstrated, in rat pulmonary arteries, that in vitro hypoxia attenuated the relaxant effects of SNP but not relaxation induced by other NO donors such as sodium nitrite. This suggests that the reduced vasorelaxant effect of SNP under lower oxygenation conditions could be due to a diminished ability of SNP to generate NO.

Reduction of O2 tension in the organ chamber did not produce any direct contractile or relaxant effect in chicken embryo pulmonary arteries. O2 tension is a determinant regulator of pulmonary vascular tone through the presence of hypoxic pulmonary vasoconstriction (HPV), a rather unique response specific for the pulmonary vascular bed by which circulating blood is diverted to better ventilated alveoli, optimizing the ventilation/perfusion matching (4). Isolated pulmonary artery rings from several species contract in response to hypoxia (4, 51). However, this contractile response generally requires a more dramatic reduction of O2 in the organ chamber and some level of active tone not being observed in arteries at resting tension. Moreover, it has been argued that these experiments in isolated vessels may not reflect the physiological mechanisms of HPV (4, 51). In fact, in vitro hypoxic contraction has been demonstrated in several systemic arteries (4, 51), whereas HPV is unique to pulmonary vessels.

Perspectives

Recent evidence from animal studies and preliminary evidence in humans suggest that an imbalance between fetal demands and supply leads to an adaptive series of stress responses that appear to permanently alter neuroendocrine development (15). Thus adverse environmental events occurring prenatally or early in life are currently receiving progressive attention as predictors of disease in later stages of life. Focusing on the pulmonary circulation, adverse intrauterine stimuli, such as chronic hypoxia or hypertension, have been related to persistent pulmonary hypertension of the newborn, a clinical syndrome that reflects the incompetence of the lung vasculature to adapt to extraterine life (3). Moreover, it has been suggested that brief hypoxic exposure during critical periods of lung growth may alter the course of normal pulmonary development and leaves persistent changes in lung structure and/or function that cause an exaggerated response to adverse stimuli later in life (44). The chicken has historically been the mainstay of developmental biologists. It develops within the confines of a rigid eggshell that is directly exposed to an environmental atmosphere. Therefore, maternal humoral, neurogenic, and cardiovascular responses do not have to be taken into account. These facts convert the chicken embryo into an invaluable tool for the study of the effects of prenatal adverse environments on vascular reactivity. In fact, our group recently demonstrated that chronic in ovo hypoxia led to sympathetic hyperinnervation of the chicken embryo systemic arterial system (37) and that chronic in ovo exposure to tobacco smoke extract resulted in impairment of endothelium-dependent relaxation in chicken embryo pulmonary arteries (9). In the present work, we have described the normal pattern of reactivity of isolated pulmonary arteries from chicken embryos during late gestation and have found similarities and differences with pulmonary vascular reactivity in the mammalian fetus. We believe that this information constitutes a good starting point for further investigations involving the influence of adverse prenatal stimuli on pulmonary circulation in this experimental model.

REFERENCES


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