Sympathetic control of the cardiovascular response to acute hypoxemia in the chick embryo


Department of Pediatrics, Research Institute Growth and Development, and Department of Pharmacology and Toxicology, Cardiovascular Research Institute Maastricht, Maastricht University, 6202 AZ Maastricht, The Netherlands; and Department of Physiology, University of Cambridge, Cambridge CB2 3EG, United Kingdom

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Hypoxemia in the fetus, resulting largely from placental dysfunction or umbilical blood flow impairment, has been postulated to be a major cause of neurological damage and neonatal morbidity (20, 30). During gestation, fetal cardiovascular responses develop to maintain organ blood flow and minimize damage of sensitive tissues during episodes of hypoxemia (for review see Refs. 11, 12, and 14). The mechanisms mediating the fetal cardiovascular response to hypoxemia are triggered by carotid chemoreceptor stimulation, which elicits bradycardia, hypertension, and a redistribution of the cardiac output in favor of the adrenal gland, the heart, and the brain (10). Neural sympathetic stimulation and endocrine vasopressor substances, such as catecholamines, contribute to peripheral vasoconstriction, prioritizing the fetal cardiac output away from the periphery to the more vital organs (11, 12, 14). Previous studies from our laboratory have demonstrated the importance of catecholamine release in the cardiovascular response to an episode of acute hypoxemia in the chick embryo. At the end of the incubation period, plasma concentrations of catecholamines increase markedly in response to acute hypoxemia (21), and treatment of the chick embryo with the α-adrenergic receptor antagonist phentolamine prevented the redistribution of the cardiac output away from the peripheral circulations (22).

In fetal sheep (4, 5, 27) and neonatal rats (29), at least two mechanisms mediating catecholamine release in response to hypoxemia have been described. First, before functional innervation of the adrenal glands, hypoxemia may stimulate chromaffin cells directly to promote catecholamine release into the circulation. Second, after the establishment of innervation to the adrenal gland, hypoxemia may stimulate adrenal catecholamine release via neurogenic mechanisms. The present study was designed to discriminate between neurogenic and nonneurogenic mechanisms mediating catecholamine release and to determine at which stage of development sympathetic innervation of the adrenal gland contributes to catecholamine release in the chick embryo. The sympathetic ganglion blocker hexamethonium was used to inhibit sympathetic stimulation in response to acute hypoxemia. The distribution of the cardiac output and changes in plasma catecholamine concentrations were determined in response to acute hypoxemia in chick embryos treated with hexamethonium and control chick embryos infused with saline at different stages of development. The study tested the hypothesis that sympathetic blockade has a greater effect on cardiac output distribution and on catecholamine release in response to hypoxemia in chick embryos at the end of the incubation period than in chick embryos at earlier stages of development.
METHODS

Preparation

Fertilized eggs of White Leghorn chickens were maintained in a commercial incubator at 38°C and 60% humidity. At the desired stage of incubation, the eggs were transferred to a clinical infant incubator and catheterized as previously described in detail (23). Briefly, eggs were opened at the air cell and placed in a holder within a Plexiglas box. A polyethylene catheter stretched by heat to a diameter of 100 μm was inserted in a chorioallantoic vein. Clay was used to fix the catheter to the eggsshell. Later, the catheter was used for injections of fluorescent microspheres and for injections of hexamethonium or saline solution. Throughout the procedure, the O₂ concentration in the box was maintained by supplied mixtures of warmed and humidified N₂ and O₂, delivered at a constant flow of 5 l/min.

Experimental Protocol

All experiments complied with the Dutch law for animal experimentation and the “Guiding Principles for Research Involving Animals and Human Beings.” A total of 120 chick embryos were used in this study. On days 11, 15, and 19 of incubation, 10 chick embryos were randomly assigned to a control group and 10 to an experimental group. Cardiac output distribution was determined by injection of 15- to 20-μm fluorescent microspheres (Fluospheres, Molecular Probes, Eugene, OR) suspended in saline and 0.05% Tween 80 (1 × 10⁶ spheres/ml), as previously described (22, 23). Before injection, the microspheres were thoroughly mixed and vortexed. The number of microspheres injected was based on 10⁴ microspheres per sample, which should guarantee a relative error of <5% (25). At 15 min after catheterization, each control and each experimental group, at each stage of incubation, 0.04 ml (4 × 10⁴ spheres) of the suspension of blue-green fluorescent microspheres was injected. Thereafter, each control group was injected with saline (0.9% NaCl), and each experimental group was treated with hexamethonium (hexamethonium bromide, Sigma Chemical) at a dose of 25 μg/g in 5 μl/g of embryo. The dose of hexamethonium (25 μg/g) was three to four times the dose reported to achieve complete blockade of the autonomic nervous system for 6 h in fetal sheep (1). After 5 min, 4 × 10⁴ red fluorescent microspheres were injected to determine the effect of autonomic blockade on basal cardiac output distribution. After 1 min, acute hypoxemia was induced by changing the supplied gas mixture to the box to 100% N₂. Previous studies have shown that this regimen results in a fall in the arterial Po₂ of the chick embryo from 37.7 to 8.7 mmHg (21). After 5 min of hypoxemia, 4 × 10⁴ crimson fluorescent microspheres were injected to determine the effect of autonomic blockade on cardiac output distribution during the hypoxemic challenge. The experiment ended 5 min after normoxia was reestablished. The chick embryos were killed immediately after the end of the experiment by decapitation, and the chorioallantoic membrane, brain, heart, lungs, intestine, liver, and yolk sac were dissected for determination of microsphere distribution.

In another 60 chick embryos, changes in plasma catecholamine concentrations in response to acute hypoxemia were determined before and after treatment with hexamethonium on days 15 and 19 of incubation. Catecholamine concentrations were not measured on day 11, inasmuch as previous studies showed that acute hypoxemia does not increase plasma catecholamine concentrations until day 13 of incubation (21). Arterial blood samples for determination of plasma catecholamine concentrations required ≥0.3 ml of blood. Therefore, only one arterial blood sample was taken from any one chick embryo to minimize blood loss. On days 15 and 19 of incubation, blood samples (0.3–1.0 ml) were obtained from the chorioallantoic artery at 5 min of normoxia (21% O₂ in the Plexiglas box, n = 10 in each group), at 5 min of hypoxemia (100% of N₂ in the Plexiglas box, n = 10 in each group), and at 5 min of hypoxemia after treatment with hexamethonium (n = 10 in each group).

Measurements

Microsphere distribution. Organs and the remaining carcass were dissected in test tubes in a 2 M ethanol-KOH solution. The microspheres were isolated from the homogenate by centrifugation, a method shown to result in >99% recovery of microspheres (36). The dye was extracted with 3 ml of 2-(2-ethoxyethoxy)ethylacelate, and the fluorescence was measured by fluorometry using a fluorospectrometer (model LS-50B, Perkin-Elmer). No correction for spectral overlap was used, since the excitation and emission spectra of the three dyes were well separated. The fraction of cardiac output that was directed to the tissue was expressed as the level of fluorescence, corrected for background, of the sample divided by the sum of fluorescence of all tissues.

Plasma catecholamine concentrations. Chorioallantoic arterial blood samples were collected into heparinized syringes. Blood was drawn into test tubes filled with 25 μl of glutathione-one-heparin solution (10 mg/ml glutathione, 5,000 IU heparin/ml). Samples were centrifuged (8°C, 2,400 g) for 15 min, and plasma was stored at −35°C. Plasma concentrations of epinephrine and norepinephrine were measured using fluorescence high-pressure liquid chromatography, as previously described in detail (34). The lower limit of detection of the assay was 0.46 pg/ml for epinephrine and 0.98 pg/ml for norepinephrine.

Analysis of Data

All data were processed using SPSS statistical software. Data are expressed as median with interquartile range (IQR) or as means ± SE, as appropriate. Statistical significance of comparisons within groups was assessed using one-way repeated-measures ANOVA and for between-group comparisons using the Mann-Whitney U test. Significance was accepted by P < 0.05.

RESULTS

Effect of Autonomic Nervous System Blockade on Basal Cardiac Output Distribution

Basal cardiac output distribution in both groups showed large fractions of the cardiac output directed to the chorioallantoic membrane, yolk sac, and carcass and smaller fractions directed to the heart, lungs, brain, liver, and intestine (Table 1, Fig. 1). After treatment with hexamethonium, small changes in cardiac output distribution were observed. On day 11 of incubation, the fraction of the cardiac output directed to the heart was increased in the hexamethonium-treated group compared with the control group. On day 15, the fraction directed to the intestine was decreased in the hexamethonium-treated group. On day 19, no differences in cardiac output distribution were found between treated and control embryos.
Effect of Autonomic Nervous System Blockade on Cardiac Output Redistribution in Response to Acute Hypoxemia

With advancing incubation time, cardiac output was preferentially distributed to the brain and heart at the expense of the liver, yolk sac, intestines, and carcass during acute hypoxemia (Table 2, Fig. 2). The fraction of the cardiac output directed to the brain increased in response to acute hypoxemia on all 3 days of incubation studied. The fraction directed to the heart increased significantly from day 15 of incubation. In response to acute hypoxemia, the cardiac output fraction directed to the yolk sac decreased on all 3 days of incubation, whereas the fraction directed to the liver and carcass decreased significantly from day 15 of incubation. The cardiac output directed to the intestines increased in response to acute hypoxemia on day 11 but significantly decreased on day 19 of incubation. Interestingly, during acute hypoxemia, the fraction of cardiac output to the heart showed a significant progressive increase from day 11 to day 19 of incubation.

Treatment of chick embryos with hexamethonium did not prevent the redistribution of the cardiac output in favor of the heart and brain during acute hypoxemia at any stage of incubation studied, but it did have an effect on some regional circulations at different stages of incubation. On day 15, the increase in cardiac output distribution toward the heart and intestines was diminished during acute hypoxemia in hexamethonium-treated embryos relative to control embryos (Table 2, Fig. 2). Similarly, on day 19, the fractions of the cardiac output directed to the yolk sac and carcass during acute hypoxemia were significantly greater in hexamethonium-treated embryos than in embryos infused with saline.

Plasma Catecholamine Concentrations

Basal plasma concentrations of epinephrine and norepinephrine were similar on days 15 and 19 of incubation to the heart showed a significant progressive increase from day 11 to day 19 of incubation.

Table 1. Effect of autonomic nervous system blockade on basal cardiac output distribution

<table>
<thead>
<tr>
<th>Organ</th>
<th>Control (n = 10)</th>
<th>Hexamethonium (n = 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart</td>
<td>1.91 (1.14-2.20)</td>
<td>2.75* (2.18-3.20)</td>
</tr>
<tr>
<td>Lungs</td>
<td>0.41 (0.26-0.50)</td>
<td>0.60 (0.40-0.65)</td>
</tr>
<tr>
<td>Brain</td>
<td>3.99 (3.20-4.59)</td>
<td>3.65 (2.20-4.68)</td>
</tr>
<tr>
<td>CAM</td>
<td>46.75 (40.02-52.31)</td>
<td>45.53 (37.30-50.22)</td>
</tr>
<tr>
<td>Liver</td>
<td>1.79 (1.19-2.70)</td>
<td>1.95 (1.72-2.30)</td>
</tr>
<tr>
<td>Intestine</td>
<td>2.00 (1.78-2.90)</td>
<td>2.60 (1.60-3.90)</td>
</tr>
<tr>
<td>Yolk sac</td>
<td>13.44 (12.50-18.76)</td>
<td>15.35 (9.39-17.27)</td>
</tr>
<tr>
<td>Carcass</td>
<td>27.64 (24.07-30.20)</td>
<td>29.48 (24.66-33.20)</td>
</tr>
</tbody>
</table>

Values represent median percentages (with interquartile ranges in parentheses) of cardiac output directed to each organ after injection of saline (control) or hexamethonium. CAM, chorioallantoic membrane. *P < 0.05 compared with control.

Effect of Autonomic Nervous System Blockade on Cardiac Output Redistribution in Response to Acute Hypoxemia

![Fig. 1. Effect of autonomic nervous system blockade on basal cardiac output distribution on days 11, 15, and 19 of incubation. Bars, mean percentage of cardiac output directed to the organ after treatment with saline (control) or hexamethonium; error bars, SE. *Significantly different (P < 0.05) from control.](http://ajpregu.physiology.org/)
During acute hypoxemia, significant increases in plasma epinephrine concentration were observed on day 15 of incubation from 0.78 ng/ml (IQR = 0.3–4.15) to 18.10 ng/ml (IQR = 6.48–26.75) and on day 19 of incubation from 1.71 ng/ml (IQR = 0.55–3.67) to 45.50 ng/ml (IQR = 39.00–68.66; Fig. 3). The increment in plasma epinephrine during acute hypoxemia was much greater on day 19 of incubation (43.79 ng/ml) than on day 15 (17.32 ng/ml, \( P < 0.05 \)). Similarly, plasma norepinephrine concentrations during acute hypoxemia increased significantly only on day 19 of incubation from 14.50 ng/ml (IQR = 9.55–49.90) to 139.31 ng/ml (IQR = 99.49–203.27). Treatment of the chick embryo with hexamethonium had a dramatic effect of catecholamine release during acute hypoxemia on day 19, but not on day 15, of incubation (Fig. 3). On day 19 of incubation, ganglionic blockade markedly attenuated the increase in plasma catecholamines. Plasma epinephrine concentration increased from 1.71 ng/ml (IQR = 0.55–3.67) in the control normoxia group to 14.70 ng/ml (IQR = 9.94–38.40) in the hexamethonium-hypoxia group. Plasma norepinephrine increased from 14.50 ng/ml (IQR = 9.55–49.90) in the control normoxia group to 40.11 ng/ml (IQR = 23.58–111.60) in the hexamethonium-hypoxia group.

**DISCUSSION**

The present study was designed to investigate the contribution of neurogenic and nonneurogenic mechanisms in mediating increases in plasma catecholamine concentrations during acute hypoxemia and to determine at which stage of development sympathetic innervation of the adrenal gland contributes to catecholamine release in the chick embryo. The study tested the hypothesis that sympathetic blockade with hexamethonium has a greater effect on catecholamine release and the distribution of cardiac output during acute hypoxemia in chick embryos at the end of the incubation period than at earlier stages of development.

In human (17) and sheep (15) fetuses and in chick embryos (9), the major sources of circulating catecholamines are the adrenal glands, para-aortic chromaffin tissue, and spillover from postganglionic sympathetic nerve terminals. Jones et al. (15) showed that in fetal sheep after adrenal demedullation the increase in plasma epinephrine was totally abolished and the increase in norepinephrine was reduced 90% in response to hypoxemia. This suggests that in fetal sheep the increase in circulating plasma catecholamines in response to hypoxemia is primarily of adrenal medullary origin. In the adult chicken, epinephrine and norepinephrine are secreted as sympathetic neurotransmitters (8), suggesting that, in the adult chicken, postganglionic sympathetic nerve stimulation might make a greater contribution to the plasma concentrations of epinephrine and norepinephrine. The contribution of neuronal spillover to circulating plasma concentrations of catecholamines is unknown for the chick embryo.

Previous studies in fetal sheep (4, 6) and neonatal rats (29) have demonstrated that catecholamine release in response to a stressor, such as acute hypoxemia, occurs in immature animals before the functional innervation of the adrenal gland is established. This nonneurogenic response is attributed to the direct effect of hypoxia on chromaffin cells (27). When functional innervation of the adrenal gland is established, catecholamine release becomes largely dependent on neurogenic stimulation, and the mature adrenal gland is comparatively insensitive to the direct effects of hypoxia. Evidence for these statements is provided by experiments in late-gestation fetal sheep, in which hexamethonium abolished the increase in plasma catecholamines in response to acute hypoxemia (4). Furthermore, when innervation of the adrenal gland was prevented in neonatal rats by surgical denervation...
Fig. 2. Effect of autonomic nervous system blockade on cardiac output redistribution in response to acute hypoxemia on days 11, 15, and 19 of incubation. Bars, mean percentage of cardiac output directed to the organ; error bars, SE. CAM, chorioallantoic membrane. *Significantly different ($P < 0.05$) from baseline. †Significantly different ($P < 0.05$) from control.
of the adrenals before the onset of splanchnic nerve function, the nonneurogenic ability of the adrenals to release catecholamines was maintained (31). The nonneurogenic ability to release catecholamines in response to acute hypoxemia appears to be an important mechanism for fetal and neonatal survival until the establishment of the sympathetic nervous system. In the present study, treatment of the chick embryo with the ganglion blocker hexamethonium did not influence catecholamine release in response to hypoxemia on day 15 of incubation. This suggests that, at this stage of incubation, catecholamine release into plasma is governed by nonneurogenic direct effects on the adrenal gland in the chick embryo. In contrast, on day 19 of incubation, adrenal catecholamine release was significantly reduced, but not abolished, in chick embryos treated with hexamethonium. This suggests that late in the incubation period, as the chick embryo approaches hatching, its adrenals appear to become more dependent on neural sympathetic stimulation, although they remain sensitive to a direct stimulatory effect of hypoxemia.

The development of the sympathoadrenal system in various species shows remarkable differences in maturation at birth. For example, the lamb is very mature at birth and has a well-developed sympathetic innervation. From 0.86 gestation, adrenal stimulation via the splanchnic nerve results in catecholamine release, which is blocked by hexamethonium (6). Furthermore, at 0.9 gestation, catecholamine release into plasma in response to acute hypoxemia is completely dependent on sympathetic stimulation (4). In contrast, in the rat, sympathetic innervation to the adrenal gland develops, almost completely, after birth. In the rat, splanchnic innervation of the adrenal gland is nonfunctional until postnatal week 1 (32). In 1-day-old rats, hypoxia causes a nonneurogenic depletion of adrenal catecholamines. This response is prevented by sympathetic blockade on day 8 (29). The present study in the chick embryo demonstrates that functional innervation of the adrenal gland takes place from day 19 of incubation, just before the onset of internal pipping and hatching. Compared with the control group, only small changes in basal cardiac output distribution were observed in the chick embryos after treatment with hexamethonium. The fraction of the cardiac output directed to the heart increased only on day 11 of incubation, and the fraction to the intestines decreased only on day 15 of incubation. In late-gestation fetal sheep, hexamethonium is known to reduce blood pressure and heart rate (3); however, no data are available on the effects of ganglionic blockade on regional blood flow for the ovine fetus. In chick embryos between days 12 and 21 of incubation, hexamethonium has no influence on basal heart rate and mean arterial pressure (7; J. C. van Golde, personal communication). Sympathetic tone thus seems to play an unimportant role in the control of basal cardiovascular function in the chick embryo. Because hexamethonium blocks the sympathetic, as well as the parasympathetic, activity, past and present data also suggest that the parasympathetic nervous system does not contribute to the control of basal cardiovascular function in the chick embryo. Accordingly, previous investigations in our laboratory have shown that treatment of the chick embryo with atropine has no effect on basal blood pressure or heart rate (35).

In the present study, it is of interest that treatment of the chick embryo with hexamethonium significantly attenuated the increase in plasma catecholamines during acute hypoxemia on day 19 of incubation and resulted in a greater fraction of cardiac output directed to the carcass compared with the control group during acute hypoxemia. However, cardiac output was still preferentially distributed to the brain and the heart in chick embryos treated with hexamethonium on day 19 of incubation. Previous studies from our laboratory show that absence of peripheral vasoconstriction in response to hypoxemia in phentolamine-treated chick embryos prevents the redistribution of the cardiac output toward the heart and brain. This suggests that peripheral vasoconstriction in response to hypoxemia is essential to facilitate the cardiac output redistribution toward the heart and brain. Furthermore, in day 19 hexamethonium-treated chick embryos, the rise...
in plasma catecholamines, although attenuated, still might be sufficient to induce peripheral vasoconstriction and cardiac output redistribution. In contrast, studies in late-gestation fetal sheep show that sympathetic blockade prevents the increase in fetal arterial blood pressure and results in collapse of the fetal cardiovascular system and death during acute hypoxemia (4). In combination, these findings suggest that the near-term sheep fetus is more dependent than the chick embryo on autonomic functions for the maintenance of perfusion of $O_2$-sensitive circulations during acute hypoxemia (4). In combination, these mechanisms in addition to peripheral vasoconstriction may contribute to the redistribution of the cardiac output toward the brain and cerebral circulations during acute hypoxemia in the chick embryo close to hatching. Potential mechanisms include local vasodilatation in response to release of nitric oxide, adenosine, prostaglandins, and adrenomedullin. In fetal sheep, nitric oxide and adenosine have been shown to contribute to the redistribution of cardiac output toward the brain and heart in response to acute hypoxemia. Blockade of nitric oxide synthase markedly attenuates the redistribution of the cardiac output toward the brain (13, 33) and the heart (26). In addition, the hypoxia-derived vasodilator metabolite adenosine has been shown to dilate cerebral (16) and coronary (26) arteries. Furthermore, studies in newborn pigs demonstrate that prostaglandins may contribute to cerebral vasodilatation in response to hypoxemia (18). Hypoxemia might induce adrenomedullin expression in vascular endothelial cells (24), but the role of adrenomedullin in the fetal response to hypoxemia on regional blood flow is unknown.

Perspectives

These data show that embryonic treatment with the ganglion blocker hexamethonium markedly attenuated the increase in plasma catecholamine concentrations during acute hypoxemia on day 19, but not on day 15, of incubation. In addition, embryonic treatment with hexamethonium diminished the redistribution of the cardiac output away from the carcass and the yolk sac on day 19, but not on day 15, of incubation. However, the effect of hexamethonium on these peripheral circulations on day 19 of incubation did not prevent a significant increase in cardiac output to the brain and heart during acute hypoxemia. In combination, these data support the hypothesis tested in this study and imply that the contribution of neurogenic mechanisms mediating increases in plasma catecholamine concentrations during acute hypoxemia becomes greater by the end of the incubation period in the chick embryo. Simultaneously, nonneurogenic mechanisms are expected to become less important and eventually disappear, as described previously in mammalian species (4, 6, 32). In the chick embryo, it is not reported at what stage of development the nonneurogenic mechanism of catecholamine release disappears. Subsequent studies should address this subject by testing the present protocol on the catecholaminergic response to acute hypoxemia in hatchlings and older chickens. In addition, the mechanisms mediating the maturational changes in the circulatory response to hypoxemia are not fully understood. Glucocorticoid secretion is believed to play an important role in the maturation of essential organ systems and the fetal response to intrauterine stressors (19). Recent studies provide information on the chicken genome (28), which shows the conservation of large syntenic regions between the human and chicken genomes (2). This is important, since it opens the opportunity for further studies on the effects of specific prenatal conditions, such as hypoxemia, on gene expression.

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