Prenatal Development of Cardiovascular Regulation in Avian Species

J. Altimiras, D.A. Crossley II, and E. Villamor

Abstract The pulsatile rhythm of the avian embryonic heart is not under autonomic control until late in development, nor are the blood vessels that nourish the different vascular beds of the growing embryo and fetus. Thus, during early development cardiovascular control is mostly dependent on the release of local or systemic vasoactive and cardioactive molecules. It is only in late development that the rapid reflex regulatory mechanisms that characterize adult cardiovascular control start functioning. The current review focuses on how the transition from an aneural cardiovascular system to a neural adult-like system occurs in the chicken fetus, which is the best (and at times the only) known avian species. First, we review the appearance of the different molecular components of a regulatory loop, i.e., nerve fibers, neurotransmitters or receptors. Second, we take a look at the functional integration and maturation of the different afferent and efferent pathways. Third and last, we offer a general overview of humoral and local effectors of cardiovascular control.

Abbreviations

αAR	α adrenoceptor
ACE	Angiotensin converting enzyme
ANP	Atrial natriuretic peptide
AT	Angiotensin II
AT1R	Angiotensin type 1 receptor
AT2R	Angiotensin type 2 receptor
βAR	β adrenoceptor

J. Altimiras (✉)
Department of Physics, Chemistry and Biology, University of Linköping, SE-58183 Linköping, Sweden, E-mail: jordi.altimiras@liu.se

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BDNF	Brain-derived neurotrophic factor
BNP	B-type natriuretic peptide
BRS	Baroreflex sensitivity
cAMP	Cyclic adenosine monophosphate
cGMP	Cyclic guanosine monophosphate
CAM	Chorioallantoic membrane
CNP	C-type natriuretic peptide
CNS	Central nervous system
CO	Carbon monoxide
CPI-17	Protein kinase c potentiated inhibitor protein-17 kDa
DA	Ductus arteriosus
EC_{50}	Half maximal effective concentration
EHDF	Endothelium-derived hyperpolarizing factor
ET-1	Endothelin-1
ETC	Electron transport chain
HH	Hamburger–Hamilton stage
HPV	Hypoxic pulmonary vasoconstriction
MLC_{20}	Myosin light chain
MLCK	Ca^{2+}-calmodulin-dependent myosin light chain kinase
MLCP	Myosin light chain phosphatase
NO	Nitric oxide
NP	Natriuretic peptide
PGE_2	Prostaglandin E_2
PGI_2	Prostaglandin I_2
PKC	Protein kinase C
pO_2	Partial pressure of oxygen
ROS	Reactive oxygen species
sGC	Soluble guanylate cyclase
SNP	Sodium nitroprusside

1 Introduction

We began conceptualizing this chapter with the ambition of providing a comprehensive overview of the current understanding of cardiovascular regulation during ontogeny and maturation in birds. However, with the exception of the domestic fowl, the information on other species is very incomplete. Therefore, in order to provide the greatest detail available, this chapter will be based exclusively on chicken embryos/fetuses as a model of avian development. The main focus will be on cardiovascular regulatory mechanisms. For a review on the ontogeny of different cardiovascular variables such as heart rate, blood pressure or cardiac output we refer to Tazawa and Hou (1997).

Before we go further it is appropriate to settle a terminological debate, perhaps of little importance, that has long existed in embryological and developmental

Table 1 Relative ages of chicken embryos/fetuses in relation to incubation time and the standard Hamburger–Hamilton stages (Hamburger and Hamilton 1951)

Relative age	Incubation time (d)	HHstages
0	0	1–11
0.1	2	12–22
0.2	4	23–28
0.3	6	29–34
0.4	8	35–36
0.5	10	37–38
0.6	13	39–40
0.7	15	41–42
0.8	17	43–44
0.9	19	45
1.0	21	46

Incubation time (d) has been rounded to the nearest integer value

studies of the chicken as experimental model. That is, should we refer to the prenatal stages of the chicken as embryo or as fetus? Here we adopt the medical terminology that refers to an "embryo" as the organism in the first third of gestation/incubation, while a "fetus" is the organism from the end of the first third until the time of birth/hatching (Larsen 2001). In the chicken the fetal phase starts after the completion of organogenesis by day 8 (HH stage 34, Sissman 1970).

Further, to facilitate fruitful comparisons with other species, we have normalized all incubation stages of the chicken to relative ages (0–1, rounded to 0.05 steps for convenience). Therefore, we refrain from using the Hamburger–Hamilton embryological staging nomenclature (Hamburger and Hamilton 1951), days of incubation or embryonic days. Table 1 presents a simple conversion table that might be of assistance to those more familiar with days of incubation or HH stages. For simplicity, relative age will be often expressed as a simple number without qualifiers such as "relative age" or "incubation time" or "development".

To understand the regulatory mechanisms of the heart and the vasculature and the involvement of the autonomic nervous system, a clear distinction must be made between the actual presence of the different elements of the regulatory pathway (i.e., a nerve fiber or a given receptor) and the functionality of the entire pathway. We start with a review of the time period in which elements of the control mechanisms appear, and continue with a review of the functional onset of tonic control and how this is related to cardiovascular homeostasis. The last part of the chapter is an introduction to the role of humoral and local effectors on the developing chicken, an area in which more research is needed before a thorough revision can be made.

2 Ontogeny of the Control of the Heart Via the Autonomic Nervous System

2.1 Autonomic Innervation of the Heart

The superior cardiac branch and the sinal branch of the vagus nerve (X) reach the truncus and the atria around 0.2 (Kuratani and Tanaka 1990), and contact with all cardiac chambers is reached by 0.35. Sympathetic cardiac nerves projecting from the sympathetic ganglia reach the heart region around 0.5 relative age (Higgins and Pappano 1979; Kirby et al. 1980) and penetrate the myocardium of the fetal heart at 0.75 of development (Verberne et al. 1999). The exact origin of these sympathetic fibers is either from the first pair of the thoracic ganglia (Kirby et al. 1980) or from cervical ganglia (Verberne et al. 1999). Thus, there is a difference in the ontogeny of parasympathetic and sympathetic fibers. The former have a very early onset in comparison with the latter.

Functional studies using field stimulation methods later qualified the conclusions obtained from anatomical studies. Using atrial field stimulation in combination with autonomic drugs, a cholinergic-dependent negative chronotropic response was shown at 0.6. The response appeared earlier (0.5 fetuses) with physostigmine pretreatment (Pappano and Löffelholz 1974). Conversely, an adrenergic-dependent positive chronotropic response to field stimulation was first evident at the time of hatching (Pappano and Löffelholz 1974), but the response could be elicited as early as 0.5–0.6 with tyramine (Crossley 1999; Pappano 1975), a drug that potentiates the release of catecholamines from postganglionic neurons. The effects of tyramine increased with age until 0.9–1 (Crossley et al. 2003b).

These results exemplify the typical sequence of maturation of an autonomic efferent regulatory pathway:

1. Placement of the required regulatory elements: nerve fibers reaching the target tissues and appearance of receptors on the target tissues
2. Maturation of the synaptic coupling between postganglionic neurons and target tissues. In the parasympathetic pathway described above, the blockade of cholinesterases with physostigmine facilitated an earlier onset of chronotropic activity with field stimulation by allowing the accumulation of acetylcholine in the synaptic space. A similar observation can be made for the sympathetic pathway and the increased response to tyramine between 0.5 and 0.9
3. Physiological release of neurotransmitters in response to suitable stimuli

In conclusion, there is a latent period from the time when nerve fibers reach the target organ to the time when nerves are capable of releasing their respective neurotransmitter.

2.2 Cholinergic and Adrenergic Receptors on the Heart

The presence of cholinergic and adrenergic receptors in the pacemaker region of the heart allows the modulation of heart rate (chronotropic effects), while its presence in the ventricle is responsible for changes in the force of contraction (inotropic effects).

Classical studies have demonstrated the existence of muscarinic cholinergic receptors at 0.1–0.15 in the pacemaker areas of the fetal chicken heart (Coraboeuf et al. 1970; Cullis and Lucas 1936; Dufour and Posternak 1960; Hsu 1933; Pappano et al. 1973; Pappano and Löffelholz 1974). When stimulated, these receptors trigger a negative chronotropic effect that is eliminated by pretreatment with atropine, and this occurs early on in the absence of autonomic innervation (reviewed by Pappano 1977).

The timing and localization of postsynaptic β-adrenergic receptors (βAR) in pacemaker regions mirrors that of cholinergic receptors. A βAR-mediated chronotropic response is evident at 0.1 (Berry 1950; Fingl et al. 1952; Hsu 1933; McCarty et al. 1960). The sensitivity of pacemaker tissue to epinephrine is unchanged from 0.6 to 0.85 and decreases from 0.85 to 0.95 (Löffelholz and Pappano 1974). The decrease may be related to the receptor desensitization caused by high circulating catecholamines, which in turn are produced in response to tissue hypoxia and oxygen diffusion limitations in the egg (Crossley et al. 2003b; Wittman and Prechtl 1991). While the exact subtype has yet to be systematically determined, teratological studies suggest that both β_1 and β_2 receptors are present (Lenselink et al. 1994).

In addition to the early presence in atrial and pacemaker tissue, the inotropic response obtained upon pharmacological stimulation suggests that βAR are present

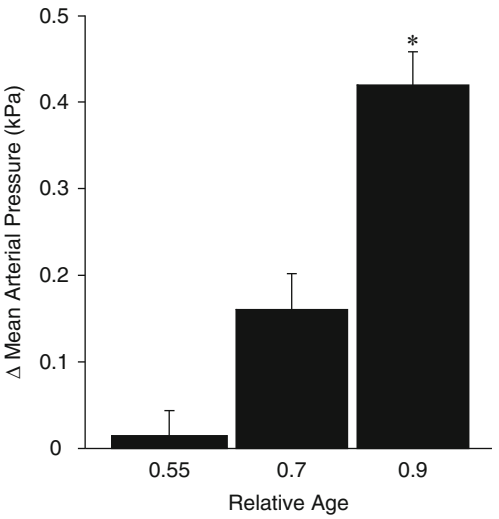

Fig. 1 Change in mean arterial pressure in response to tyramine administration ($10\,\mathrm{mg\,kg^{-1}}$) in fetal chickens at 0.55, 0.7 and 0.9 ($N = 5$). Significant differences indicated by an *asterisk*

by 0.2 of incubation in the fetal ventricle (Frieswick et al. 1979; Higgins and Pappano 1981; McCarty et al. 1960; Shigenobu and Sperelakis 1972). Receptor-binding studies at 0.7 and 0.9 indicate a decrease in the number of βAR from 12 fmol μg protein^{-1} to 8 fmol μg protein^{-1} (Altimiras and Lindgren 2007). Despite the drop in βAR density, the EC_{50} of fetal ventricular tissue to isoproterenol and adrenaline increases from 0.75 to 0.9 before falling prior to hatching (Altimiras and Lindgren 2007; Higgins and Pappano 1981). This is also shown by the increased changes in mean arterial pressure after the administration of tyramine at 0.9, in comparison to earlier stages as shown in Fig. 1 (Crossley 2003b).

3 Ontogeny of the Control of Vascular Contractility

3.1 Developmental Changes in the Mechanisms Controlling Vascular Reactivity

The fetal circulation is designed to meet the requirements of a rapidly growing organism existing at a low pO_2 relative to postnatal life. Therefore, the assembly of a blood vessel into a well-organized and functional structure is essential for organ growth and development. In mature animals, vascular smooth muscle and endothelial cells, both highly specialized cells, have the principal function of regulating blood vessel tone, blood pressure, and blood flow distribution (Owens et al. 2004; Rzucidlo et al. 2007). During development, however, endothelial and vascular smooth muscle cells play a key role in blood vessel morphogenesis. These cells exhibit high rates of proliferation, migration, and production of extracellular matrix, components that make up a major portion of the vessel wall. These processes occur while the newly forming vessels are simultaneously acquiring the capacity to regulate vascular tone (Owens et al. 2004; Rzucidlo et al. 2007). Smooth muscle contraction and relaxation are determined by phosphorylation/dephosphorylation at Ser^{19} of the 20 kDa myosin light chain (MLC_{20}). MLC_{20} phosphorylation is mediated by the Ca^{2+}-calmodulin-dependent MLC kinase (MLCK), which, in turn, is activated by the increase in cytosolic Ca^{2+} (Cogolludo et al. 2007b; Ganitkevich et al. 2002; Somlyo and Somlyo 2003; Webb 2003). Cytosolic Ca^{2+} is increased through Ca^{2+} release from intracellular stores (sarcoplasmic reticulum) as well as entry from the extracellular space through Ca^{2+} channels. In addition to the Ca^{2+}-dependent activation of MLCK, the state of MLC_{20} phosphorylation is further regulated by MLC phosphatase (MLCP), which removes the high-energy phosphate from MLC_{20} to promote smooth muscle relaxation. MLC_{20}, MLCK and MLCP are expressed in the chicken vascular smooth muscle at least as early as 0.5 of incubation (Ogut and Brozovich 2000). At 0.4 incubation, the chicken aorta shows tonic contractile properties in response to an increase in cytosolic Ca^{2+} (Ogut and Brozovich 2000), and the developmental increase in the level of MLC_{20} phosphorylation reaches a plateau from 0.75 onwards (Ogut and Brozovich 2000).

Several G-protein receptor-coupled agonists (including adrenergic agonists) inhibit MLCP, leading to an increase in MLC phosphorylation and contraction without changes in the cytoplasmic Ca^{2+} concentration. This mode of regulation is termed Ca^{2+} sensitization and is an essential process for agonist-induced contraction of smooth muscle (Cogolludo et al. 2007b; Ganitkevich et al. 2002; Somlyo and Somlyo 2003; Webb 2003). At least two signaling pathways are involved in the inhibition of MLCP. First, inhibition via phosphorylation of the MLCP regulatory subunit, MYPT1, which is thought to involve RhoA/Rho-kinase-dependent pathways. The second mechanism of MLCP inhibition is through phosphorylation of the smooth muscle-specific MLCP inhibitor protein, CPI-17 (Protein Kinase C potentiated inhibitor protein-17 kDa, Cogolludo et al. 2007b; Ganitkevich et al. 2002; Kitazawa et al. 2004; Somlyo and Somlyo 2003; Webb 2003). Under physiological conditions, the three mechanisms — Ca^{2+} release, influx, and sensitization — often act in concert. Interestingly, CPI-17 is undetectable in chicken smooth muscles (aorta, mesenteric artery, gizzard and small intestine; Kitazawa et al. 2004), and protein kinase C (PKC) activation does not evoke significant contraction in adult chicken arteries (Kitazawa et al. 2004). In addition, the Rho kinase inhibitors Y-27632 and hydroxyfasudil produce a marked impairment of receptor-dependent and receptor-independent contractions in vessels of chicken fetuses (femoral artery and ductus arteriosus from 0.7 and 0.9 Blanco et al. 2007; Villamor et al. 2008a). The effect of Rho Kinase inhibitors increases with incubation age, suggesting a developmental augmentation in the RhoA/Rho kinase-mediated increase in Ca^{+2} sensitivity of the contractile apparatus. Therefore, the deficiency of CPI-17 in chicken smooth muscle make it a useful model for studying not only the role of CPI-17 but also other potential mechanism(s) regulating Ca^{2+}-sensitivity in smooth muscle contractility.

3.2 Adrenergic Receptors on the Chicken Vasculature

Epinephrine administration increase arterial pressure in the fetus after 0.15 (Girard 1973; Hoffman and Van Mierop 1971) and αAR antagonists trigger hypotension in intact 0.4 fetuses (Crossley 1999), which demonstrates the early presence of adrenoceptors in the fetal cardiovascular system. Additional studies with specific AR agonists and antagonists show that both αAR and βAR are present in the vascular tree as early as 0.3 of incubation (Koide and Tuan 1989; Saint-Petery and Van Mierop 1974). At 0.6 αAR are present in the mesenteric circulation, and they may be present earlier (Rouwet et al. 2000). Between 0.7 and 0.9 incubation, the contractile reactivity to α_1AR and receptor-independent stimulation increases in the femoral and carotid arteries of chicken embryos (Le Noble et al. 2000). In contrast, preductal or postductal pulmonary arteries do not show α-adrenergic-induced contraction at any age (Villamor et al. 2002; Ågren et al. 2007).

Contractile responses to perivascular nerve stimulation have been demonstrated in late-gestation chicken femoral arteries but not in carotid or pulmonary arteries. Because constrictor responses to exogenous norepinephrine are typically obtained

before neurogenic responses (Le Noble et al. 2000), the sympathetic control of arterial vascular resistance is limited to the late phases of fetal life in chickens. βAR relaxation has also been demonstrated in different vessels such as the femoral artery, and the sensitivity and responsiveness increased with incubation age (Blanco et al. 2007).

In summary, the data available to date support the idea that there is a progressive increase in the adrenergic influence on the vasculature that plays a critical role during *in ovo* life, as discussed in Sect. 4.3.

3.3 Cholinergic Receptors and the Endothelial Control of Vascular Reactivity

Possibly the most momentous change in the field of vascular biology in the past 50 years has been the discovery and elucidation of the endocrine/paracrine roles of the endothelium (Alexander and Dzau 2000). In simple but elegant experiments, Furchgott and Zawadzki found that relaxation with muscarinic agonists in precontracted vessels was only possible if endothelial cells were present (Furchgott and Zawadzki 1980). Several endothelium-derived relaxing and contracting factors have been found, including nitric oxide (NO), prostaglandins, thromboxane A_2, endothelin-1 (ET-1), carbon monoxide (CO), and a yet-unidentified factor called endothelium-derived hyperpolarizing factor (EHDF) (Baragatti et al. 2007; Busse et al. 2002). Therefore, it is now widely recognized that the endothelium is not merely a passive, blood-compatible surface but also plays a primary role in the local modulation of vascular function and structure.

Stimulation of muscarinic receptors by acetylcholine evokes an endothelium-dependent relaxation in systemic (aorta, femoral, carotid, mesenteric) and pulmonary arteries of the chicken fetus (Le Noble et al. 2000; Martinez-Lemus et al. 2003; Nishimura et al. 2003; Rouwet et al. 2000; Villamor et al. 2002). The timing of the responses varies between organs, as early as 0.6 in mesenteric arteries but with few changes between 0.7 and 0.9 in femoral or carotid arteries. Endothelium-derived NO appears as the main mediator of this relaxation but EDHF might be also involved (Le Noble et al. 2000; Villamor et al. 2002). Inhibition of the production of prostaglandins (i.e., cyclooxygenase blockade) does not affect acetylcholine-evoked relaxation of systemic or pulmonary arteries (Le Noble et al. 2000; Villamor et al. 2002). Although technical limitations have restricted the studies of endothelium-dependent relaxation to vessels from more mature fetal chickens (starting at 0.6), NO appears as a critical regulator of fetal circulation during earlier stages of development as well. At 0.15 of incubation NO synthase mRNA is expressed in the sinus venosus, ventricle, outflow tract, pharyngeal arch arteries, and aorta of the chicken (Groenendijk et al. 2005). Isolated cardiomyocytes taken from animals at 0.5 relative age respond to both sodium nitroprusside (SNP), an NO donor, and L-arginine, the NO precursor, indicating that the NO/cGMP pathway is functional in the heart at this stage of development (Takahashi et al. 2001; Ungureanu-longrois et al. 1997).

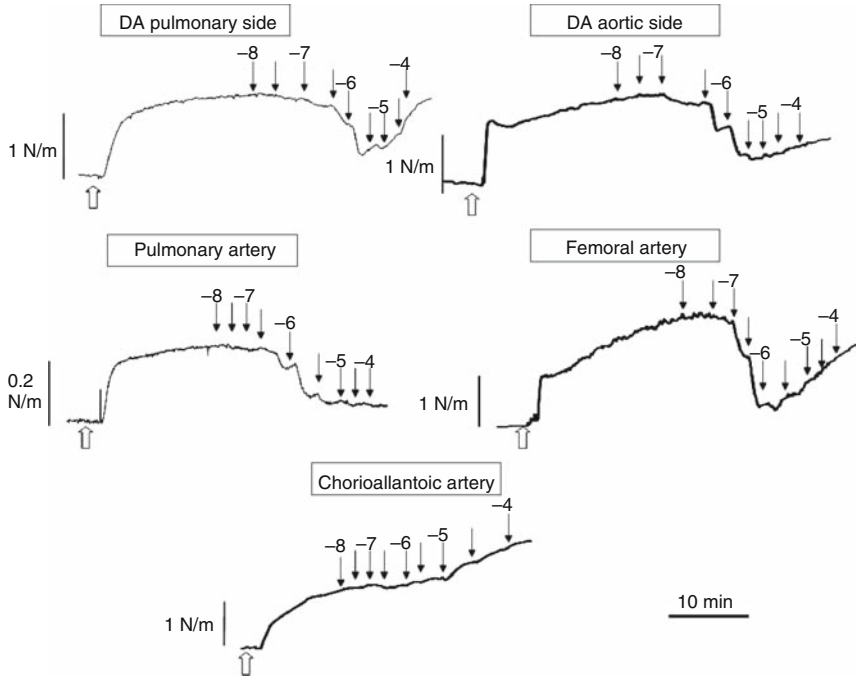

Fig. 2 Typical tracings of isometric tension vs time illustrating the effect of acetylcholine in arteries with intact endothelium from 0.6 fetuses. Vessels were contracted with 62.5 mM KCl (*white arrow*). Concentrations of acetylcholine are shown as log *M*. Note that acetylcholine induced relaxation at low concentrations and (in some vessels) contraction at higher concentrations. In the chorioallantoic artery, no relaxant effects of acetylcholine were observed

NO also causes a clear hyperemia of the CAM vasculature as early as 0.5 (Dunn et al. 2005) and *in vivo* hypotension at 0.45 (Altimiras and Crossley 2000). However, isolated chorioallantoic arteries do not respond to acetylcholine with relaxation but with contraction (Fig. 2). *In vivo* studies have also demonstrated that NO donors elicit a marked decrease in ventricular preload, possibly due to venodilation, without affecting arterial resistance as early as 0.15 (Bowers et al. 1996).

In several mammalian species endothelium-dependent relaxation, particularly in the pulmonary circulation, is reduced during fetal life and transiently compromised after birth (Abman et al. 1991; Boels et al. 1999; Villamor et al. 2003) even if the release of endogenous NO seems necessary for a smooth transition of the pulmonary circulation at birth (Abman 1999; Abman et al. 1990). In the chicken, endothelium-dependent relaxation of pulmonary and systemic arteries remain unchanged during the last phase of incubation, which includes the gradual transition to postnatal life (i.e. during the processes of internal and external pipping) (Le Noble et al. 2000; Villamor et al. 2002). Therefore, the transient impairment of pulmonary endothelial function described in mammalian neonates is absent in the chicken.

Other putative mediators of endothelium-dependent relaxation that regulate vascular tone in fetal chickens have been studied in addition to acetylcholine. Adenosine plays a role in the angiogenic response of the chorioallantoic membrane to hypoxia at 0.5 and 0.65 and decreases whole-body structural vascular resistance in a dose-related manner at 0.5–0.7 (Adair et al. 1989). Thus, the presence of purinergic receptors in the fetal vasculature has at least two roles: to regulate CAM vascularization as well as to regulate vascular tone. The role of other vasoactive compounds acting through the release of endothelial mediators (such as angiotensin II) or other endothelium-derived vasoactive mediators (such as ET-1) is discussed in Sect. 5 of this chapter.

3.4 Vascular Reactivity of the Ductus Arteriosus

All air-breathing vertebrates possess a ductus arteriosus (DA) that connects pulmonary and systemic arterial blood flow. This connection closes permanently at a certain stage in development, or develops the capacity to close and reopen depending on the physiological needs (Bergwerff et al. 1999). Fetal mammals have a single DA while fetal birds have two DA, each acting to shunt a major portion of the cardiac output from the right heart away from the non-ventilated lung into the descending aorta (Bergwerff et al. 1999; Clyman 2006; Smith 1998). Therefore, the *in ovo* or *in utero* patency of DA is essential for prenatal life. Once hatching or birth takes place, the lungs are ventilated, and require an increase in pulmonary blood flow that is achieved through a dramatic decrease in pulmonary vascular resistance and by closing the DA (Bergwerff et al. 1999; Clyman 2006; Smith 1998).

Although the isolated DA is sensitive to a wide range of contractile agonists, the main factors maintaining *in utero* patency of the mammalian DA are low O_2 tension, high levels of circulating prostaglandin (PG)E_2, and locally produced PGE_2 and PGI_2 (Clyman 2006; Clyman et al. 1978; Smith 1998). In addition, the major factor actively stimulating DA contraction at birth is an increase in O_2 tension. This stimulus has a profound effect on the DA, both directly and by modulating its response to vasodilators and vasoconstrictors (Smith 1998; Smith and McGrath 1988, 1993, 1995). The DA acquires vasoactive competence early in development (Bergwerff et al. 1999; Clyman 2006; Smith 1998) and changes in responsiveness with advancing gestational age. These changes have been extensively characterized in numerous mammalian species including man, lamb, mouse, rat, guinea pig, dog, and rabbit (Sutendra and Michelakis 2007). Very recently, the changes in DA reactivity during *in ovo* development and transition to *ex ovo* life have been analyzed in two avian species: the chicken (Ågren et al. 2005, 2007, 2008; Villamor et al. 2008a, b) and the emu (Dzialowski and Greyner 2008). The chicken DA responds to a wide range of vasoactive agonists including O_2, prostanoids, potassium channel blockers, NO, catecholamines, ET-1, adenylate cyclase activators, guanylate cyclase activators, phosphodiesterase inhibitors, and Rho kinase inhibitors (Ågren et al. 2005,

2007, 2008; Villamor et al. 2008a, b). As in the mammalian DA, the multiplicity of vasoactive factors is at odds with the relatively simple physiological role of the DA (Smith 1998). The main vasoconstrictor of the mammalian DA, the postnatal increase in O_2 tension, also plays a relevant role in the closure of the DAs of chicken and emu (Dzialowski and Greyner 2008; Ågren et al. 2007). However, the main vasodilator of the mammalian DA, PGE_2, only triggers weak vasodilation of the chicken and emu DA, and it even stimulates vasoconstriction in the chicken DA at high concentrations (Ågren et al. 2005; Dzialowski and Greyner 2008). In common with mammalian DA, the chicken DA undergoes a process of maturation to prepare the task of postnatal closure. This process is characterized by an increase in the contractile and a decrease in the relaxing capacity of the vessel. Thus, the contractions induced by O_2, membrane depolarization, thromboxane A_2, ET-1 and αAR agonists increased between 0.7 and the end of incubation, whereas the relaxations evoked by acetylcholine, the NO donor sodium nitroprusside, PGE_2, βAR agonists, and adenylate cyclase stimulators decreased (Ågren et al. 2005, 2007, 2008; Villamor et al. 2008a, b).

The endothelium is an important modulator of the vascular tone of the chicken DA during *in ovo* life and during its closure at hatching. Acetylcholine induces a concentration-dependent response in DA in fetal chickens. Low concentrations induce endothelium-dependent relaxation of the chicken DA mediated via NO and EDHF. High concentrations induce an endothelium-dependent contraction (Ågren et al. 2008). Oxygen-induced contraction of the DA is also modulated by the endothelium, a response that increases with inhibition of NO synthase or soluble guanylate cyclase, and decreases in the presence of ET-1 receptor blockers (Ågren et al. 2007). Endothelial damage is common to numerous vascular diseases but, interestingly, occurs as a normal developmental process in the DA. When examined by scanning electron microscopy, the endothelium of the DA from the fetus prior to internal pipping (0.9) shows a smooth and continuous surface. In contrast, the intimal surface of DAs harvested from externally pipped embryos (0.95) has an irregular endothelial lining with protrusion and detachment of endothelial cells, leaving large areas of exposed subendothelial tissue (Ågren et al. 2008). This process of endothelial detachment is accompanied by a marked impairment in NO production and endothelium-mediated relaxation (Ågren et al. 2008).

One of the most relevant features of the chicken DA is the presence of a marked morphological and functional heterogeneity along its path between the pulmonary artery and the aorta (Ågren et al. 2007, 2008; Bergwerff et al. 1996, 1999) (Fig. 3). Specifically, the pulmonary side has the structure of a muscular artery and responds to O_2 with contraction, whereas the aortic segment has the morphology of an elastic artery and relaxes in response to O_2 (Ågren et al. 2007, 2008; Bergwerff et al. 1996, 1999) (Fig. 3). In addition, αAR agonists induce larger contractions when administered to the pulmonary side, while acetylcholine, SNP, and the NO-independent stimulator of soluble gualylate cyclase (sGC) BAY 41-2272 evoke significant larger relaxations in the pulmonary than in the aortic side (Ågren et al. 2008; Ågren et al. 2007). In contrast, the βAR agonist isoproterenol, the adenylate cyclase activator

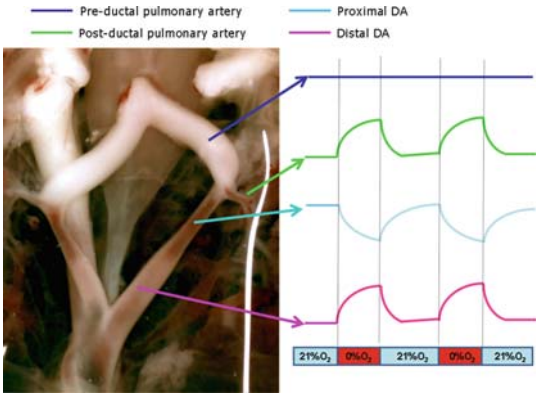

Fig. 3 At 0.9 the chicken fetus presents a series of neighbor vessels with a marked difference in the response to O_2. The pre-ductal extrapulmonary artery does not respond to changes in oxygenation, whereas the post-ductal intrapulmonary arteries contract in response to hypoxia and relax in response to normoxia. The pulmonary side of the ductus arteriosus (DA) contracts in response to normoxia and relaxes in response to hypoxia, whereas the aortic side of the DA shows a similar pattern than the post-ductal pulmonary artery, i.e. hypoxic vasoconstriction and normoxic relaxation

forskolin, and the phosphodiesterase 3 inhibitor milrinone induce larger relaxations in the pulmonary side of the vessel (Ågren et al. 2005). This may indicate that the pulmonary side of the chicken DA is more sensitive to the vasodilators acting through cAMP, whereas the aortic side is more sensitive to cGMP-mediated relaxation.

3.5 Oxygen Sensing in Chicken Fetal Vessels

The DA belongs to a specialized system of O_2-sensitive organs and tissues in the body that includes the pulmonary arteries, the carotid body, and the neuroepithelial body among others. These tissues share striking similarities in their response to changes in O_2 tension (Aaronson et al. 2006; Sutendra and Michelakis 2007; Weir et al. 2002, 2005). The proposed mechanism for DA closure includes an acute phase in which minutes of exposure to postnatal normal O_2 levels result in DA constriction. This mechanism is thought to be intrinsic to the DA smooth muscle cells (Michelakis et al. 2000, 2002; Sutendra and Michelakis 2007; Thebaud et al. 2004; Tristani-Firouzi et al. 1996; Weir et al. 2002, 2005) and, at least in the human or the rabbit DA, it includes a sensor, the electron transport chain of the mitochondria (ETC). The ETC increases production of reactive oxygen species (ROS), particularly H_2O_2, in response to changes in O_2 levels. This mediator (i.e., the freely diffusible H_2O_2) can reach the cell membrane and decrease the opening

of O_2- and redox-sensitive K^+ channels (such as Kv1.5 and Kv2.1). This causes depolarization of smooth muscle, opening of the voltage-gated Ca^{2+} channels, increase in $[Ca^{2+}]_i$ and vasoconstriction (Michelakis et al. 2000, 2002; Thebaud et al. 2004; Tristani-Firouzi et al. 1996; Weir et al. 2002, 2005). The mitochondria-ROS-K^+ channels axis is the basis of O_2 sensing in many other O_2-sensitive tissues (Sutendra and Michelakis 2007; Weir et al. 2005), suggesting the evolutionary preservation of the O_2-sensing mechanism (Cobeño et al. 2008; Cogolludo et al. 2007a; Sutendra and Michelakis 2007). The contraction of the chicken DA to O_2 is markedly blocked by the ETC inhibitors rotenone, myxothiazol and antimycin A, by the H_2O_2 scavenger polyethylenglycol-catalase, and by the Kv channels inhibitors 4-aminopyridine (non-selective) and DPO-1 (Kv1 selective) (Cobeño et al. 2008; Cogolludo et al. 2007a). Furthermore, exogenous H_2O_2 mimicked the responses induced by O_2 (no effect at 0.7, and contraction and relaxation in pulmonary and aortic sides of the DA by 0.9 and 0.95 respectively: Cobeño et al. 2008; Cogolludo et al. 2007a). Altogether, these results indicate that the mitochondria-ROS-K^+ channels are responsible for O_2-induced contraction in the chicken DA. However, and similarly to the situation of the mammalian DA (Hong et al. 2006; Kajimoto et al. 2007), Rho-kinase inhibitors blunt the normoxic contraction of the chicken DA (Villamor et al. 2008a), which means that other pathways such as the calcium sensitization mechanism may be also important in DA closure.

As another O_2-sensitive vessel, the pulmonary arteries, typically contract when exposed to hypoxia (Russell et al. 2008). Hypoxic pulmonary vasoconstriction (HPV) is a highly conserved adaptive physiological mechanism that optimizes oxygen saturation of pulmonary venous blood by increasing pulmonary vascular resistance in poorly aerated lung regions (Aaronson et al. 2006; Michelakis et al. 2004; Moudgil et al. 2005; Russell et al. 2008; Weir et al. 2005; Villamor et al. 1997). In contrast, the systemic vasculature frequently responds to hypoxia with vasodilation in an effort to maintain adequate tissue oxygenation (Russell et al. 2008). For example, femoral arteries of 0.9 fetuses respond to hypoxia with relaxation (Ruijtenbeek et al. 2002). HPV has been demonstrated in adult chicken extrapulmonary arteries pre-constricted with KCl (Russell et al. 2008), although other authors have reported a lack of response without pre-constriction. It is well known that mammalian pulmonary arteries respond very little to hypoxia while at passive resting tension, and that HPV is strongly enhanced by some level of pre-constriction (Aaronson et al. 2006). Interestingly, we have observed a consistent and reproducible response to hypoxia in intrapulmonary arteries of fetal and juvenile chickens at passive resting tension (Villamor, unpublished observations). In contrast, extrapulmonary arteries do not respond to hypoxia under those conditions. Therefore, as illustrated in Fig. 3, the chicken fetus presents a series of neighbor vessels (i.e., the pre- and post-ductal pulmonary arteries, the aortic and the pulmonary sides of the DA) with a marked difference in the response to O_2. The mechanisms that initiate, differentiate and regulate this variety of vascular responses to O_2 warrant further investigation.

4 Functional Integration of Autonomic Cardiovascular Regulation

4.1 Ontogeny of Afferent Pathways

The main sensory areas that trigger cardiovascular reflexes in chickens are:

(1) The carotid bodies involved in chemoreception, and
(2) Specialized mechanosensory nerve endings in the adventitial layer of the aortic arch involved in baroreception

There is no anatomical evidence of carotid sinuses in birds. The homologous region would be the bifurcation of the common carotid with the subclavian artery, but at this location the vessel wall is not thinner and vessel diameter is not expanded as it is in mammals (Ábrahám 1969).

The embryology and maturation of the reflexogenic areas are reasonably well-known from early studies and their innervation patterns have been described. The carotid bodies constitute the primary loci for peripheral chemoreceptors sensing arterial oxygen and carbon dioxide tensions and pH. They are located in the inferior part of the neck that is contiguous with the thoracic cavity, in contrast to mammals, where the carotid bodies are located in a cervical position. This may be due to the elongation of the chicken neck, since other structures located cervically in mammals, such as the bifurcation of the common carotid artery and the nodose ganglion (also called distal vagal ganglion), are also found in the thoracic inlet in the chicken (Wakley and Bower 1981).

The carotid bodies appear around 0.25 and migrate to an adult-like location by 0.4 (Murillo-Ferrol 1967), at which time they consist of mesenchyme-like cells. At 0.6 a large number of granule-containing cells are dispersed in the parenchyma (Kameda 1994), coinciding with a peak for serotonin immunoreactivity (Kameda 1990). These granule-containing cells are denominated glomus cells or Type I cells and they are responsive to chemical stimuli such as the partial pressure of oxygen and carbon dioxide. The first detection of synaptic junctions between long axons and glomus cells is also observed at 0.6 (Kameda 1994). At 0.7 the glomus cells express most of the features found in mature glomus cells (Kameda 1994) but further maturation that extends to the post-hatching period cannot be discarded. In rats, the carotid body increases in size postnatally and glomus cells continue to proliferate after birth (Wang and Bisgard 2005). The responsiveness of glomus cells to hypoxia, as indicated by an increased catecholamine secretion, also increases postnatally and is coupled to a decrease in the constitutive (hypoxia-independent) release (Donnelly 2005). Glomus cells are also found in the wall of the common carotid artery and other vessels of the outflow tract, but their role in chemosensitivity has not been studied (Kameda 2002).

As structures derived from the second–third aortic arch, one would expect the carotid bodies to be innervated from the glossopharyngeal (IX) cranial nerve, as happens in mammals. This is not the case, and the sensory innervation of the chicken

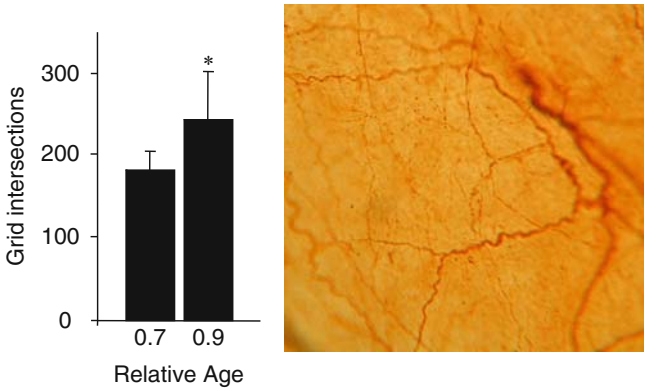

Fig. 4 Innervation density for neurofilament immunostained whole mount sections of the aortic arch of chickens at two stages of development. Innervation density is measured as the number of intersections between nerves and a square grid. A *star* indicates a significant difference between ages

carotid bodies travels along the vagus nerve (X) and one of its branches, the recurrent laryngeal nerves (Jones and Johansen 1972; Murillo-Ferrol 1967). It is possible, however, that some glossopharyngeal nerve fibers move into the vagus via the anastomosis of Staderini that connects the vagal trunk to the petrosal ganglion before it continues caudally, although there is no experimental evidence for it (Whittow and Sturkie 2000).

An additional vagal branch, the aortic nerve (also called depressor nerve, Nonidez 1935), carries axons of sensory neurons to the adventitial layer of the aorta, where the nerve fibers branch to fine free nerve endings patterned as "flower-spray" or "end-net" structures (Ábrahám 1969; Cheng et al. 1997). In rats, these structures are unequivocally identified as baroreceptive nerve endings based on the expression of mechanosensitive channels (γ-subunit of the epithelial sodium channel) in axons from nodose ganglion cells (Drummond et al. 1998, 2001). In chickens, an increase in the innervation density of the aorta from 0.7 to 0.9 has been shown (Altimiras and Crossley 2007), see Fig. 4.

Nonidez's early anatomical study also described a depressor nerve of the carotid in an area homologous to the mammalian carotid sinus (Nonidez 1935). Even if birds have no carotid sinuses, the observation could indicate the existence of other baroreceptive areas independent of the baroreceptors in the aortic arch. However, it is generally accepted that the nerve endings in that location are not mechanosensitive, because the nerve fibers terminate in a plexus that penetrates down to the media of the vessel instead of the adventitial "flower-spray" or "end-net" patterns of typical mechanosensitive nerve endings (Ábrahám 1969).

In contrast to mammals, therefore, the axons of the main cardiovascular afferent neurons are bundled in the vagus nerve and have the cell bodies in a common location, the nodose ganglion. Similar to all peripheral sensory neurons in vertebrates, nodose ganglion neurons are pseudo-unipolar. Their axons bifurcate shortly

after emerging from the cell body. While one branch grows peripherally towards the heart and other viscera, the other branch grows centrally and establishes synaptic connections within the central nervous system (CNS), most importantly with the nucleus of the solitary tract.

Nodose ganglion neurons derive from cells of the nodose placode while Schwann cells and supporting cells of the ganglion are provided by neural crest cells (Harrison et al. 1994). The primordium of the ganglion is visible at 0.15. Following a proliferation period the ganglion reaches the largest number of cells at 0.3, after which time cell numbers will drop to a half by the time of hatching due to programmed cell death (Harrison et al. 1994). The development of the ganglion occurs simultaneously with the projection of neuronal axons to the target tissues and the CNS. When the axons are growing early in development the survival of these neurons is independent of the presence of neurotrophins, but as they reach the target tissues they become dependent on brain-derived neurotrophic factor (BDNF) for survival. Such dependence is reflected in the mRNA expression of the catalytic domain of TrkB, a receptor tyrosine kinase which acts as a BDNF receptor. trkB mRNA increases progressively from 0.15 to 0.2, coinciding with the maximum proliferation of nodose ganglion neurons (Robinson et al. 1996). The neurons are also susceptible to other trophic factors such as nerve growth factor at later stages (0.4, Hedlund and Ebendal 1980).

Altogether it seems that the afferent pathways connecting the reflexogenic areas of the cardiovascular system with the central nervous system are established within the first half of incubation. A maturation of the sensitivity to the time of hatching is probably the case for the carotid bodies, but little information is available for the baroreceptive areas. In the fetal lamb, afferent baroreceptor sensitivity measured from the carotid sinus nerve decreased with age from 0.7 to 0.9, simultaneously with the developmental increase in blood pressure (Blanco et al. 1988). A potential explanation of the results would be that the enhanced sensitivity in earlier fetuses is aimed to stimulate the maturation of the central pathways involved in the reflex, but this hypothesis needs to be tested experimentally.

4.2 Onset of Tonic Control of the Heart

The importance of cholinergic and adrenergic tonic activity to maintain baseline cardiovascular function in fetal chickens has been studied *in vivo* by administering receptor antagonists at different stages of development. Atropine, an antagonist of cholinergic muscarinic receptors, induces no chronotropic effects at any developmental age in White Leghorn chickens (Crossley and Altimiras 2000; Haque et al. 1995; Pickering 1895; Saint-Petery and Van Mierop 1974; Tazawa et al. 1992). Thus, although cholinergic receptors are present in early embryos and the parasympathetic efferent arm is functional by 0.6 (Pappano et al. 1973, see Sect. 2 of this chapter), the White Leghorn fetus develops in the absence of a cholinergic or parasympathetic tone.

It is important to emphasize that the absence of a cholinergic tone does not rule out the possibility that the parasympathetic nervous system can be recruited if baseline cardiovascular function is disturbed. In fact, continuous recordings of instantaneous heart rate in fetal chickens have documented decelerations in fetal heart rate which are probably due to an increase in parasympathetic activity (Akiyama et al. 1999; Höchel 1998; Kato et al. 2002; Tazawa et al. 2002).

A later study in broiler chickens (a chicken strain primarily used for meat production) demonstrated a cholinergic tone on heart rate that started at 0.6 (Chiba et al. 2004), almost at the same time that field stimulation studies can induce changes in spontaneous cardiac contraction frequencies (Pappano et al. 1973). The basis for such strain-specific differences defies explanation but it is worthy of further studies, because it indicates a large degree of plasticity of cardiovascular regulatory mechanisms between strains.

In contrast, a tonic adrenergic stimulation is present throughout fetal development. An adrenergic tone on both heart rate and systemic arterial pressure appears relatively early (Crossley and Altimiras 2000; Koide and Tuan 1989; Saint-Petery and Van Mierop 1974; Tazawa et al. 1992) and is dependent on αAR and βAR with differential effects on cardiac and vascular tissue (Crossley 1999). A βAR-positive chronotropic tone appears at 0.3 incubation (Girard 1973; Saint-Petery and Van Mierop 1974) and is critical to the maintenance of basal baseline function (Crossley 1999; Crossley and Altimiras 2000; Tazawa et al. 1992). βAR chronotropic tone increases in magnitude with fetal development, elevating baseline heart rate 10% at 0.4 to 20% at 0.95 (Crossley 1999). The tone originates entirely from circulating catecholamines, as the elimination of the sympathetic nervous terminals with 6-hydroxydopamine or ganglionic blockade with hexamethonium has no impact on control fetal heart rate (Crossley 1999; Tazawa et al. 1992).

At the same time, αAR antagonists depress heart rate at 0.4 (Crossley 1999) and continue to do so at later developmental stages (Crossley and Altimiras 2000; Koide and Tuan 1989; Tazawa et al. 1992). The magnitude of the αAR tone is maximal from 0.6 to 0.95 but is absent at hatching (Crossley 1999). The bradycardic effects must be due to indirect effects related to the strong αAR-vasodilation that follows phentolamine administration (an αAR antagonist), because αARs are absent from the chicken heart (Chess-Williams et al. 1991). Vasodilation leads to blood pooling in the CAM, reduction in venous return, and decrease in cardiac output and heart rate.

4.3 Onset of Tonic Control of the Vasculature

The total and regional peripheral resistance of the vasculature is primarily regulated by the sympathetic nervous system, which releases catecholamines from both the sympathetic nerve terminals and the adrenal medulla. Because vascular smooth muscle is endowed with both αAR and βAR (Saint-Petery and Van Mierop 1974), the net response to catecholaminergic stimulation will depend on the balance

between the number and sensitivity of vasoconstrictor αAR and vasodilator βAR (Altimiras and Crossley 2001; Guimaraes and Moura 2001).

A powerful αAR vascular tone is present in fetal chickens (0.3) and persists to 0.95 (Crossley 1999; Crossley and Altimiras 2000; Girard 1973; Koide and Tuan 1989; Saint-Petery and Van Mierop 1974; Tazawa et al. 1992). The receptor subtype responsible for maintaining vascular tone seems to be α_1AR because of the similar responses obtained using an non-specific αAR antagonist (phentolamine) and an α_1AR specific antagonist (prazosin, Crossley and Altimiras 2000). As the fetus grows and matures its dependence on αAR-mediated vasoconstriction increases from a meager 10% change in resting arterial pressure to over 55% in the last days of development (Crossley 1999).

αAR vasoconstriction prevails in the skeletal muscles and has limited effects on the heart, intestines, and yolk sac, as shown by the distribution of microspheres away from the carcass with the infusion of αAR agonists (Mulder et al. 2001). Thus, αAR mechanisms contribute to the maintenance of basal vascular tone and to the redistribution of the cardiac output, and these mechanisms are important for defending blood flow to the brain and heart during hypoxemic conditions (Mulder et al. 2001).

The αAR vasoconstrictor tone is opposed by a βAR vasodilator tone that also has an early appearance in embryonic chickens at 0.3 (Saint-Petery and Van Mierop 1974). The magnitude of the βAR vascular tone increases from 0.35 to 0.6 (Crossley 1999; Girard 1973), mostly due to the proliferation and expansion of the extraembryonic vasculature of the CAM, which reaches its maximum by 0.7 (Romanoff 1967). βAR vascular tone remains stable up to 0.75 (Haque et al. 1995; Koide and Tuan 1989; Tazawa et al. 1992) and increases late in development with a maximal expression by 0.9–0.95 (Crossley and Altimiras 2000). A maximal response to βAR antagonists at the same stage has been shown in other chicken strains (Crossley, personal unpublished results) and emus (Crossley et al. 2003a). βAR vascular tone is absent during external pipping (Crossley 1999).

The maximal expression of αAR tone coupled to the absence βAR tone vascular tone in late development is critical to ensure proper delivery of oxygen to embryonic tissues at the time when CAM gas exchange is switched to lung gas exchange, a process that occurs in a rather short period of time (Menna and Mortola 2002).

The αAR and βAR vascular tones are entirely attributed to circulating catecholamines given that neither sympathectomy with 6-hydroxydopamine nor ganglionic blockade with hexamethonium alters resting arterial pressure in fetal chickens (Crossley 1999; Crossley and Altimiras 2000; Crossley et al. 2003b; Tazawa et al. 1992).

4.4 Maturation of Baroreflex Regulation

The baroreflex is the most important short-term regulator of blood pressure in vertebrates. Birds are no exception (Bagshaw and Cox 1986). Nerve activity from barosensitive areas in the common outflow tract relays information of the phasic

(within a cardiac cycle) and tonic (between cycles) changes in blood pressure via nodose ganglion neurons to the CNS, primarily the nucleus of the solitary tract. Baroreceptive and other sensory afferents, e.g., chemoreceptive, are processed and an integrated response is ultimately directed to the heart and the vasculature via the autonomic nervous system.

The cardiac baroreflex response involves parasympathetic vagal efferents and, to a lesser extent, sympathetic efferents that change heart rate and cardiac output in a reciprocal manner to the blood pressure changes. The peripheral response involves sympathetic efferents to the vascular smooth muscle that modify peripheral resistance (see Sects. 3.2 and 4.3 of this chapter). For example, if pressure increases sympathetic activity decreases and vasodilation ensues. The two complementary mechanisms have been denominated the cardiac limb and the peripheral limb of the baroreflex respectively.

Little is known about how the peripheral limb of the baroreflex develops. In adult birds, the peripheral limb contributes to baroreflex regulation by acting in synergy with the cardiac limb, and remains active even when the heart rate response subsides (Jones 1973; Smith and Jones 1992). So, even if changes in peripheral resistance have a longer latency than cardiac responses, the longer activation makes the peripheral limb more effective in blood pressure regulation (Jones 1973). In the fetus, neurogenic constrictor responses in femoral arteries are only observed around the time of internal pipping and the onset of lung ventilation (Le Noble et al. 2000), implying that changes in peripheral resistance mediated by the sympathetic nervous system are limited to the late fetal stages (>0.9). A distinct change in renal sympathetic nerve activity related to blood pressure alterations is also observed in fetal lambs at 0.9 gestation. In fact, peripheral gain in fetuses is several-fold larger than in newborns or 6-week old lambs (Segar et al. 1992).

The ontogeny of the cardiac limb of the baroreflex has been studied in more detail. The standard experimental approach to estimate baroreflex sensitivity (BRS), the so-called Oxford method, is based on the vascular administration of vasoactive substances to elicit blood pressure changes, typically a nitric oxide donor to vasodilate and an αAR agonist to vasoconstrict, so that reflex responses in heart rate can be measured (Smyth et al. 1969). Using this method, the earliest baroreflex responses in White Leghorns are seen at 0.85 (Altimiras and Crossley 2000) but only 17% of the fetuses show a change in heart rate after experimental manipulation of blood pressure. A day later (0.9), the proportion of animals showing a baroreflex response climbs to 33%, and the gain of the baroreflex increases progressively fivefold between 0.9 and hatching (Altimiras and Crossley 2000). In broiler chickens gain at 0.9 is similar, $23\,\mathrm{kPa}^{-1}\ \mathrm{min}^{-1}$ vs $21\,\mathrm{kPa}^{-1}\ \mathrm{min}^{-1}$ in White Leghorns (Altimiras and Crossley 2007).

The Oxford method interferes with the peripheral limb of the reflex (Maloney et al. 1977) and has been criticized for delivering a stimulus to the baroreceptors poorly comparable to physiological blood pressure variations (Parati 2005). This methodological limitation may be of importance during the early onset of the baroreflex when BRS is expected to be lowest. Thus, in a recent study, we followed BRS in fetuses from 0.8 to 0.95 using a sequence procedure of the spontaneous BRS

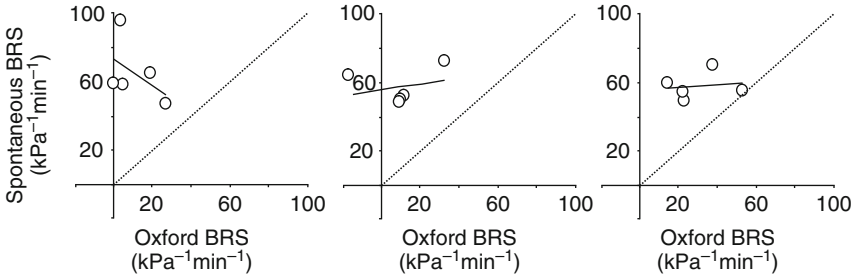

Fig. 5 Correlation between Oxford BRS and spontaneous BRS at different stages of development (0.85, 0.9 and 0.95 from left to right respectively). The *dotted line* corresponds to the line of equality if both methods yielded the same BRS estimate

method (Bertinieri et al. 1985). The procedure is based on the analysis of the correlation between heart rate and blood pressure during episodes (called sequences) when blood pressure changes spontaneously (see Laude et al. 2004 for a comparison of the performance of different procedures). The method also allows a sequential assessment of BRS in the same animal over time, which is not feasible with the Oxford method due to potential cumulative effects of the drugs.

Interestingly, spontaneous BRS is relatively constant from 0.8 to 0.95 and averages 59.8 kPa^{-1} min^{-1}. This value is several-fold larger than the Oxford BRS measured in the same fetuses, which increased progressively from 10.9 kPa kPa^{-1} min^{-1} at 0.8 to 30 kPa^{-1} min^{-1} at 0.95 (Elfwing 2007). The lack of correlation in the alternative BRS estimates at different fetal ages is shown in Fig. 5.

BRS differences with different methods are probably related to the small parasympathetic tone in fetuses (Chiba et al. 2004). While the spontaneous method focuses on heart rate changes that compensate for minute blood pressure changes ($>$0.36 kPa) within a few heart beats ($>$3 beats), the Oxford method elicits larger pressure changes ($>$0.75 kPa) on a longer time scale (60–90 s). Thus, small increases or decreases in blood pressure lead to an enhanced or diminished parasympathetic tone that causes, respectively, a compensatory bradycardia or tachycardia. Because parasympathetic tone in fetuses is small, larger pressure changes such as those induced pharmacologically cannot be defended to the same extent, resulting in a lower BRS.

Thus, the emerging but still speculative picture indicates that baroreflex regulation in chickens is absent during most of fetal life and appears at 0.8 at the latest (Elfwing 2007), a little earlier than previously reported (Altimiras and Crossley 2000). Before the onset of a functional baroreflex, blood pressure homeostasis can be alternatively adjusted by slower mechanisms regulating blood volume such as the renal fluid blood pressure control mechanism (Guyton 1991; Guyton et al. 1972) which in the chicken fetus would imply the transfer of fluid to the allantois, the reservoir that collects waste in the embryo (Hoyt 1979).

The slow time response of the renal mechanism may suffice for the early embryo, which is shielded from gravitational stress by the amniotic cavity. However, the

onset of pseudo-respiratory movements first (around 0.8, Kuo 1937) and proper lung respiration at 0.9 would increase the magnitude of blood-pressure variations, and the parasympathetic nervous system would start contributing more to the buffering of blood pressure changes. Finally, during the proper lung ventilation phase the sympathetic nervous system could set in operation and complete the baroreflex response to the adult-like scenario.

5 Effects of Humoral and Local Effectors: Angiotensin, Endothelin-1 and Natriuretic Peptides

In addition to cholinergic and adrenergic mechanisms, other humoral and local mediators affect cardiovascular function in the fetus. The most studied in fetal chickens are angiotensin II (AT) and local effectors such as ET-1 and natriuretic peptides (NP).

AT is a critical regulator of cardiovascular function in adult chickens (Hasegawa et al. 1993; Nakamura et al. 1982; Nishimura et al. 1982; Stallone et al. 1990). AT injection triggers a biphasic response in which there is an initial vasodilation mediated by NO and a secondary αAR-mediated vasocontriction (Nishimura et al. 1982). However, the function of AT during fetal life is not well understood.

The components of the renin–angiotensin system are present relatively early in fetal chicken development. Angiotensin-converting enzyme (ACE) is measurable in freshly laid eggs and ACE mRNA increases dramatically over the first 54 h of development (Savary et al. 2005). At the completion of the first day of fetal development the yolk sac contains mRNA encoding for ACE, angiotensinogen, renin, and AT receptors (Savary et al. 2005). Thus, the mechanisms for both angiotensin synthesis and signal transduction are present early in fetal chicken development.

AT receptor mRNA is found in cardiac tissue, branchial arch tissue, and mesonephric tissue between 0.15 and 0.2 (Kempf and Corvol 2001). AT triggers an angiogenic response of the CAM vasculature at 0.35 (Le Noble et al. 1991, 1993), and receptor-binding assays have quantified the number of AT receptors in the CAM at 0.5 (Moellera et al. 1996). The enzymatic activity of ACE is present in the chicken aorta as early as 0.5 incubation, and it increases with development (Topouzis et al. 1992). Although AT induces relaxation in isolated aortic rings from 0.9 fetuses (Nishimura et al. 2003), the effects are due to the lack of intact sympathetic nerves, so the vasoconstrictive response is absent. Taken together, these results imply that angiotensin has a role in chicken vascular regulation that needs to be clarified with *in vivo* studies. Preliminary work demonstrates that AT injection induces a clear hypertensive response (Fig. 6) that notably lacks the adult initial vasodilation (Crossley, personal observations).

AT also induces a positive inotropic effect on the heart at 0.85 (Freer et al. 1976) and cardiac hypertrophy from 0.35 to 0.9 (Aceto and Baker 1990; Baker and Aceto 1990; Mathew et al. 2004). The hypertrophic response is due to the activation of the AT type 1 receptor (AT1R) and upregulation of MLC (Mathew et al. 2004).

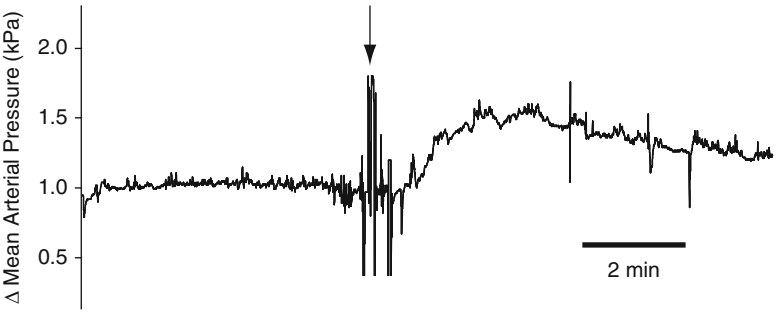

Fig. 6 Effects of an arterial injection of angiotensin II$(1{,}000\,\mu g\,kg^{-1})$ in a chicken fetus at 0.7. The *arrow* indicates the point of injection

Although AT1R is the main receptor type, AT2R is also present in the heart at 0.35 (Rabkin 1996). Cardiac hypertrophy could be coupled to both direct actions of AT on the heart as well as its actions on the fetal vasculature.

ET-1 is also ubiquitously found in the cardiovascular system of chicken fetuses (Kempf et al. 1998). The mRNA for ET-1 receptor subtypes is detected in the wall of the vitelline vessels, the myocardium and the outflow tract as early as 0.15 (Groenendijk et al. 2007), while the converting enzymes for ET-1 can first be detected at 0.2 (Ballard 2002; Hall et al. 2004). The role of ET-1 as a cardiovascular regulator is further confirmed by the hemodynamic alterations that occur after *in vivo* administration of ET-1 receptor antagonists at 0.2 (Groenendijk et al. 2007) and by the positive inotropic effect of ET-1 on cultured cardiomyocytes from 0.5 fetuses (Bézie et al. 1996).

Isolated aortic rings (from 0.7 fetuses) and pulmonary arteries (from 0.9 fetuses) contract in response to ET-1 in a concentration-dependent manner (Martinez-Lemus et al. 2003; Villamor et al. 2002, 2004; Wingard and Godt 2002). Active wall tension in response to ET-1 increases before hatching, and this can be of critical importance for the transition to *ex ovo* life (Martinez-Lemus et al. 2003; Villamor et al. 2004). As mentioned above, ET-1 also plays a determinant role in the reactivity of the DA. Further investigations are necessary to characterize the nature of the receptors and the transduction pathways involved in the vascular responses to ET-1.

The NP family consists of a group of structurally related peptides that are involved in the regulation of sodium and water balance, and cardiovascular homeostasis (Takei 2000; Toop and Donald 2004; Trajanovska et al. 2007) In mammals, three NP subtypes have been isolated, the first two of which are atrial NP (ANP) and B-type NP (BNP), which are produced primarily within cardiac myocytes and released into the circulation in response to a volume overload. The third type is the C-type NP (CNP) that is a paracrine or autocrine factor in the brain and periphery (Takei 2000; Toop and Donald 2004; Trajanovska et al. 2007). Four NP genes have been identified in the chicken genome (Akizuki et al. 1991; Houweling et al.

2005; Trajanovska et al. 2007). These genes encode one BNP, two CNPs and a recently identified NP with an unusual sequence (termed chicken RNP due to its predominant expression levels in the chicken kidney) (Trajanovska et al. 2007). In chicken embryos, NP decreases vitelline arterial pressure and increases vitelline venous diameter as early as 0.2, so NP receptors must be present in the vasculature at that stage (Nakazawa et al. 1990). Further studies of the relaxant effects of chicken NP have been performed using only adult vessels, so our understanding of the fetal response is limited (Trajanovska et al. 2007). Isolated cardiomyocytes also respond to NP at 0.5 (Bézie et al. 1996) and 0.7 (Koide et al. 1996), suggesting that the receptor is present in the ventricle. Further, isolated ventricular cardiomyocytes respond to ET-1 with an increase in the expression of NP mRNA (Bézie et al. 1996). Thus, ET-1 and ANP interact to regulate cardiomyocyte contractility in a paracrine/autocrine fashion, and maintain basal cardiovascular function in fetal chickens.

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