Chapter 5

Differential Effects of Endothelin-1 and Endothelin Receptor Antagonists on Chicken Yolk Sac and Embryonic Hemodynamics

Bianca CW Groenendijk, Sandra Stekelenburg-de Vos, Peter Vennemann, Jurij W Wladimiroff, Frans Nieuwstadt, Jerry Westerweel, Robert E Poelmann, Beerend P Hierck, Nicolette TC Ursem.
Abstract

Previously, we have shown that after ligation of the right lateral vitelline vein in chicken embryos (venous clip) cardiac function is altered and morphological malformations develop. Dorsal aortic blood flow and cardiac ET-1 expression were decreased within 5 hours after venous clip. We also demonstrated that infusion of ET-1 or its receptor antagonists results in similar impairment of ventricular function as described in the venous clip model. As ET-1 is altered shortly after vitelline ligation, we determined the direct effects of ET-1 and ET-receptor antagonists on hemodynamics to investigate whether vasoactive capacities of ET-1 can be the cause of the observed ventricular function disturbances at a later stage of development. We infused ET-1, BQ-123 (selective ETA antagonist), BQ-788 (selective ETB antagonist) and PD145065 (non-selective ET receptor antagonist) into the HH18 extra-embryonic chicken vascular system and investigated embryonic hemodynamics via Doppler measurements, and extra-embryonic hemodynamics in vitelline veins by micro-Particle Image Velocimetry (μPIV) measurements. In addition, the expression of ET-1, ECE-1, ETA and ETB mRNA was investigated by in situ hybridisation. μPIV evaluations in vitelline veins showed alterations in hemodynamics and vessel diameter after infusion. Doppler measurements of the embryonic heart, however, did not show any changes. Hybridisation experiments demonstrated that ET-1, ECE-1 and ETB mRNA were present in endothelial and smooth muscle cells of the vitelline vessels, whereas ETA was absent. Our results indicate that hemodynamics in the embryo and vitelline vessels are mechanistically different, and that the μPIV technique is more sensitive than the Doppler ultrasound technique. We postulate that the changes in hemodynamics alter gene expression, and have an effect on the blood flow patterns in the yolk sac circulation. This leads to alterations in intracardiac flow patterns, which result in combination with feedback mechanisms in impaired ventricular function and morphological anomalies that were found at HH24.

Introduction

The development of the heart from an almost straight tube to a four-chambered pump is a complex process in which interactions between genetic and epigenetic influences are involved. Hemodynamic forces have been demonstrated to play an important role in cardiac development\(^1\,^2\). Shear stress is one of those hemodynamic forces, and the expression of many genes changes in response to alterations in shear stress\(^3\,^4\). We have shown that the expression of some of these genes is also shear-related in the developing chicken heart\(^5\).
In the venous clip model the right lateral vitelline vein is ligated in stage HH17 chicken embryos. This model is expedient to study the effects of altered hemodynamics, as venous ligation results in immediate changes in the blood flow patterns through the heart. Furthermore, changes in the dorsal aortic mean and peak blood flow for up to 5 hours after clip have been demonstrated, as well as alterations in shear-related gene expression after 3 hours. At HH21, the cardiac ventricle is less contractile and at HH24 diastolic ventricular filling is disturbed. Over time, the intervention also results in the development of morphological cardiac malformations. Interestingly, knockout mice of components of the endothelin-1/endothelin converting enzyme-1/endothelin-A receptor (ET-1/ECE-1/ETA) pathway demonstrate similar cardiovascular malformations as encountered in chicken embryos after venous clip. We have shown that ET-1 mRNA is down-regulated in the heart after venous clip and that the endothelin-1 pathway is involved in the morphological malformations, and in the functional defects at HH24 that are similar to the ones in the venous clip model (unpublished data, 2005). These functional defects include a decreased passive ventricular filling, which is compensated by an increased active ventricular filling. Kajio and Nakazawa have demonstrated that at HH21 ET-1 has a constrictive effect in vitelline veins even when the endocrine and autonomic nervous systems have not yet been fully developed. In the current study, we investigated the direct hemodynamic effects of ET-1 and its receptor antagonists at HH18, which is the same stage of infusion and venous clip. We performed Doppler ultrasound measurements for hemodynamic parameters in the embryonic dorsal aorta, and micro-Particle Image Velocimetry (μPIV) measurements for determining the vessel diameter and blood flow velocity, and the subsequent calculation of shear stress in the vitelline veins. We compared the experimental embryos with sham-operated ones. To determine the functional mechanism in the vitelline vessels at this stage, we investigated the differences in presence and localisation of the mRNA of ET-1, ECE-1, and the ETA and ETB receptors in the vessel walls of the yolk sac.

Materials and Methods

Animals and Intravenous Infusions
Fertilised White Leghorn chicken eggs (Gallus domesticus) were incubated at 37-38°C and 60-70% relative humidity. Embryos that were dysmorphic, exhibited arrhythmias or overt bleeding were excluded. At Hamburger and Hamilton stage 18 (HH18) the embryos were exposed by creating a window in the shell followed by removal of the overlying shell.
membranes. A glass micropipette was inserted into one of the third order branches of the vitelline vein.

For the Doppler measurements one microliter of the following substances, diluted in Phosphate Buffered Saline (PBS), was slowly infused without using any additional pressure, to keep changes in hemodynamics to a minimum. Endothelin-1 (Bachem) at the concentrations of $10^{-7}$mol/L ($n=5$), $10^{-8}$mol/L ($n=5$), and $10^{-9}$mol/L ($n=6$), the selective endothelin-A receptor antagonist BQ-123 (Bachem) at $10^{-4}$mol/L ($n=8$), $10^{-5}$mol/L ($n=7$), and $10^{-6}$mol/L ($n=7$), the selective endothelin-B receptor antagonist BQ-788 (Sigma-Aldrich) at $10^{-5}$mol/L ($n=7$), $10^{-6}$mol/L ($n=9$), and $10^{-7}$mol/L ($n=8$), and the non-selective endothelin receptor antagonist PD145065 (Sigma-Aldrich) at $10^{-4}$mol/L ($n=7$), $10^{-5}$mol/L ($n=10$), and $10^{-6}$mol/L ($n=7$). Indigo carmine blue (0.25g/mL) was added for visualization of the solution during *in vivo* infusion. The embryos were compared with sham-operated animals ($n=6$) in which only PBS with indigo carmine was infused. During the measurements the temperature of the egg was regulated with a thermoelement at 37/38°C.

For the micro-Particle Image Velocimetry (μPIV) measurements one microliter of the same substances as for the Doppler measurements was infused: Endothelin-1 at $10^{-7}$mol/L ($n=3$), BQ-123 at $10^{-4}$mol/L ($n=3$), BQ-788 at $10^{-5}$mol/L ($n=3$), and PD145065 at $10^{-4}$mol/L ($n=3$), all diluted in PBS with indigo carmine. These embryos were also compared with sham-operated embryos in which only PBS with indigo carmine was infused ($n=3$). During these measurements the temperature of the egg was kept constant at 37/38°C by placement of the egg in a warm water bath.

**Doppler Ultrasound Measurements**
Per embryo 3 dorsal aortic blood flow velocity measurements were performed. Immediately before infusion the first blood flow velocity (baseline) was recorded. The second was performed 5 minutes, and the third 30 minutes after infusion. Dorsal aortic blood flow velocity was recorded using a 20-MHz pulsed Doppler velocity meter (model 545C-4, Iowa Doppler Products, Iowa City, USA), and was measured with a 750-μm piezoelectric crystal positioned at a 45° angle toward the dorsal aorta at the level of the developing wing bud. The Doppler signals obtained from the dorsal aorta were digitised at 24 kHz and stored on hard disk. The maximum velocity waveform was reconstructed from the audio signals using a custom-built analysis program (National Instruments, Austin, TX, USA). A more detailed description of this method has been published previously. We determined peak and mean
systolic velocity, heart rate, peak and mean blood flow, peak acceleration, cardiac output, and stroke volume for each cardiac cycle. For each embryo we analysed 5 consecutive cycles and the data are presented as mean ± standard error of the mean (SEM). The values were standardised by taking the percentage change from baseline level in order to adjust for biological variability. Hemodynamic parameters were compared within and between groups. A paired t-test was performed within each group to compare mean values at consecutive time points with baseline values. Comparison of the different treatment groups at each time point were carried out by a paired t-test. Statistical significance was reached at p<0.05. Calculations were performed with SPSS 11.5 software (SPSS Inc, Chicago, IL).

\[ \mu \text{PIV Measurements} \]

\[ \mu \text{PIV measurements were performed at three time points per embryo: before infusion (baseline), and 5 minutes and 30 minutes after infusion. At each time point, an optically accessible vein (35-105 \mu m), without interference of any underlying arteries, was analysed. The application of in vivo } \mu \text{PIV on embryonic blood flow has been described previously}^{17}. \]

\[ \text{In contrast to point measurement techniques, such as ultrasound Doppler velocimetry, } \mu \text{PIV resolves the spatial velocity distribution (magnitude and direction) in a two-dimensional measurement plane. This allows the derivation of the wall shear stress without the assumption of a certain velocity profile (Equation 1). The velocity gradient perpendicular to the wall, } \frac{\partial u}{\partial n}, \text{ is readily extracted from the velocity field. Multiplication with the viscosity, } \eta, \text{ gives the wall shear stress, } \tau_w. \]

\[
\tau_w = \eta \frac{\partial u}{\partial n}
\]

Volume flow rate, peak, minimum, mean velocity, and heart rate can be derived from sequential \( \mu \text{PIV measurements at sufficient sampling rate. The raw PIV images allow the direct measurement of the vessel diameter.} \]

The tracer particles used in these experiments were the embryonic red blood cells. Compared with artificial, fluorescent tracer particles – that are ten times smaller than erythrocytes –, a lower spatial resolution must be accepted. On the other hand, time-consuming infusion of artificial tracers could be avoided to promote a larger number of experiments.

Each measurement of the current study was based on 500 sequential PIV measurements at 10 Hz repetition rate. This corresponds to 50 seconds measurement duration. The individual evaluation of the measurements with a coarse interrogation window of 64 x 64 pixels was
used to resolve the flow pulsation. Figure 5.1 shows the magnitude of the mean
displacement of red blood cells for each measurement of the time series. The frequency
analysis of this data resolves the heart rate. Afterwards, the individual measurements were
sorted according to their cardiac phase angle. Therefore, the temporal position of the
measurements between the two closest systolic peaks was determined (Fig. 5.2). The cardiac
pulse is now resolved by a dense order of individual measurements. The measurements
were combined into groups of comparable flow conditions by separating the pulse into
twelve segments of 50 ms in length. Each group of individual measurements was evaluated
by means of an ensemble correlation. The ensemble correlation method enabled the
combined evaluation of PIV images to enhance resolution and accuracy in comparison to the
individual evaluation of single PIV images\textsuperscript{18}. With this method, interrogation windows of 32
x 32 pixels (20 x 20 µm) were used (Fig. 5.3).

![Figure 5.1](image1.png)

**Figure 5.1.** Mean displacements (magnitude) of 500 individual PIV evaluations (a). The frequency analysis of the data reveals the heart rate during the measurement (b).

![Figure 5.2](image2.png)

**Figure 5.2.** Systolic peak detection (a). Sub-sample accuracy is achieved by means of a second order polynomial fit. Afterwards, the individual PIV measurements were sorted on the basis of their temporal position between the two closest systolic peaks (b).

The heart rate, vessel diameter, volume flow rate, and the mean wall shear stress in the
vitelline veins were analysed statistically. As the parameters varied largely between the
different sized veins of the different embryos, the changes relative to their baseline values
per parameter per embryo were calculated, which were analysed between groups using the
non-parametric Mann-Whitney test. Values were considered significant when $p \leq 0.05$. Data are presented as the median of the relative change and the 25 and 75 percentiles, showing the variation within the groups.

Figure 5.3. Velocity distribution in a vitelline vessel at twelve successive time points. Each measurement is based on a 50ms time averaged ensemble evaluation. The diameter of the vessel is about 150µm. The peak velocity (red vector color) is 1.6 mm/s. Note that there is some back flow at $t_{10}$ and $t_{11}$. Assuming a circular vessel cross-section, the velocity profile (indicated in yellow) can be used to determine the volume flow rate and shear stress.
Radioactive In Situ Hybridisation

The presence of ET-1, ECE-1, ETA and ETB mRNA in the vitelline vessels was detected using in situ hybridisation (ISH) on HH18 chicken embryos that were fixed in 4% paraformaldehyde (PFA) in 0.1M phosphate buffer and embedded in paraffin, essentially as described before19. 

35S-labelled chicken-specific riboprobes were produced as described previously5,8. In short, a 619-bp (nucleotides 177-796) ET-1 fragment, a 1283 bp (nucleotides 325-1608) ECE-1 fragment, a 845 bp (nucleotides 2256-3101) ETA fragment, and a 440 bp (nucleotides 339-778) ETB fragment were cloned in pCRII (Invitrogen; ET-1, ECE-1, ETB), or pBSK (ETA). After linearisation, sense and antisense cRNA was transcribed in transcription buffer, 0.01mol/L dithiothreitol, 0.25mmol/L G/A/CTP mix, 1.4U/μL RNase-inhibitor, and 1.5U/μL of the appropriate RNA polymerase (T7 for ET-1, T3 for ETA, and SP6 for ECE-1 and ETB) in the presence of 2.31MB 35S-UTP. Concentration of the probes was normalised to 1 x 10^5cpm/μL. All sense probes showed negative hybridisation results (not shown).

Hybridised sections were dehydrated in graded ethanol and air-dried before being coated with Ilford G5 emulsion (ILFORD, Ltd., Mobberley, UK) and were exposed for 7-14 days at 4°C. Slides were developed in Kodak D19 (Kodak, France) for 4 min at room temperature, rinsed, and fixed for 4 min in Ilford fixative (ILFORD Imaging, Ltd., Mobberley, UK). Finally, the sections were counterstained with Mayer’s hematoxylin, dehydrated, and embedded in Pertex (Histolab, Göteborg, Sweden).

Immunohistochemistry

Routine immunohistochemical staining of the 4%PFA-fixed paraffin sections of HH18 vitelline vessels was performed using the primary antibody HHF35 (DAKO, Denmark) against muscle actin20 diluted 1:500 in PBS with 0.05% Tween-20 and 1% ovalbumin as described before5.

Results

Intra-Embryonic Measurements

With Doppler measurements a number of embryonic hemodynamic parameters can be determined, such as the heart rate, dorsal aortic mean flow, peak flow, peak systolic and mean systolic velocity, stroke volume, cardiac output, and peak acceleration. Thirty minutes after infusion of either ET-1 or the receptor antagonists, the heart rate was increased compared with the measurements before infusion. This was observed in all cases, including
Figure 5.4. Mean relative change in heart rate (a) and mean dorsal aortic velocity (b) derived from Doppler ultrasound measurements on the dorsal aorta. The changes at 5 and 30 minutes after infusion of PBS (sham), ET-1, ETA antagonist (BQ-123), ETB antagonist (BQ-788), or the non-selective antagonist (PD145065), are relative to their values before infusion (0' min). Note that none of the substances induced a significant change in heart rate or mean dorsal aortic velocity after 5 or 30 minutes compared with sham. Error bars represent SEM.

Figure 5.5. Median relative changes in heart rate (a), vessel diameter (b), volume flow rate (c), and shear stress (d), obtained from µPIV measurements in vitelline veins. The changes at 5 and 30 minutes after infusion of PBS (sham), ET-1, ETA antagonist (BQ-123), ETB antagonist (BQ-788), or the non-selective antagonist (PD145065), are relative to their values before infusion (0' min). * Significant with p≤0.05. ‡ Trend with p=0.127. Bars show the 25 and 75 percentiles.
the shams. None of the parameters was changed compared with shams after infusion. Figure 5.4 shows the heart rate (Fig. 5.4a) and the mean systolic dorsal aortic velocity (Fig. 5.4b). The parameters showed no differences with either concentration of ET-1 or receptor antagonists (not shown).

Table 5.1. Median of the relative change in hemodynamic parameters (compared with their baseline value) due to infusion of ET-1 or ET-1 receptor antagonists, comparison with sham embryos.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>time point</th>
<th>Sham Δ</th>
<th>ET-1 Δ</th>
<th>p-value</th>
<th>BQ-123 Δ</th>
<th>p-value</th>
<th>BQ-788 Δ</th>
<th>p-value</th>
<th>PD145065 Δ</th>
<th>p-value</th>
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<td>0.050</td>
<td>0.94</td>
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<td>0.87</td>
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<td>0.93</td>
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<td></td>
<td>30</td>
<td>1.05</td>
<td>1.02</td>
<td>0.827</td>
<td>1.03</td>
<td>0.827</td>
<td>0.98</td>
<td>0.513</td>
<td>1.38</td>
<td>0.127</td>
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<tr>
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<td>1.27</td>
<td>0.513</td>
<td>1.14</td>
<td>0.827</td>
<td>0.98</td>
<td>0.513</td>
<td>1.38</td>
<td>0.127</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>1.01</td>
<td>1.31</td>
<td>0.827</td>
<td>1.19</td>
<td>0.513</td>
<td>0.98</td>
<td>0.827</td>
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<td>0.275</td>
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<td>Volume flow rate</td>
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<td>1.04</td>
<td>1.27</td>
<td>0.513</td>
<td>1.42</td>
<td>0.275</td>
<td>0.95</td>
<td>1.00</td>
<td>1.62</td>
<td>0.275</td>
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<tr>
<td></td>
<td>30</td>
<td>0.94</td>
<td>1.88</td>
<td>0.127</td>
<td>1.80</td>
<td>0.513</td>
<td>1.35</td>
<td>0.827</td>
<td>0.88</td>
<td>0.513</td>
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<td>Shear stress</td>
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<td>0.76</td>
<td>0.507</td>
<td>1.34</td>
<td>0.513</td>
<td>0.98</td>
<td>0.275</td>
<td>0.59</td>
<td>0.275</td>
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<tr>
<td></td>
<td>30</td>
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<td>0.275</td>
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<td>0.513</td>
<td>1.15</td>
<td>0.275</td>
<td>0.41</td>
<td>0.046</td>
</tr>
</tbody>
</table>

p-values are compared with sham
0.05: significant
0.127: trend
n=3 per substance per time point

Extra-Embryonic Measurements

The value-range at the first μPIV measurement in vitelline veins, i.e., before infusion, was for the heart rate: 83-122 beats/min with a median of 96 beats/min, for the diameter: 35-105µm with a median of 55µm, for the volume flow: 0.16 – 1.68µL/s with a median of 0.48µL/s, and for the mean wall shear stress: 7.42⋅10^{-7} – 2.94⋅10^{-6} Pa with a median of 1.80⋅10^{-6} Pa. To adjust for biological variability the values were standardised by taking the percentage change from baseline level. These data are summarised in Figure 5.5, the values of this figure in Table 5.1. The heart rate is increased 5 minutes after ET-1 infusion, but is back to sham-levels after 30 minutes (Fig. 5.5a). With blockade of ETA (BQ-123) the heart rate is in a trend-like manner decreased after 30 minutes. Blocking both receptors (PD145065) results in a significant decrease in heart rate 30 minutes after infusion (Fig. 5.5a).
Five minutes after infusion of the non-selective receptor antagonists a trend-like increase of the diameter of vitelline veins with a diameter between 35μm and 105μm was shown, whereas after 30 minutes no difference was measured compared with shams (Fig. 5.5b).

The volume flow rate shows a trend in increase 30 minutes after infusion of ET-1 (Fig. 5.5c). According to the data shown in Figure 5.5c, BQ-123 would also increase the volume flow rate, however, the variation is much larger than that of ET-1, resulting in a p-value of 0.513, indicating that there is no detectable change.

The shear stress in veins is decreased 30 minutes after infusion of the non-selective antagonist PD145065 (Fig. 5.5d).

Infusion of the ETB receptor antagonist (BQ-788) did not result in any changes of heart rate, diameter, shear stress or volume flow rate (Fig. 5.5).

Figure 5.6. Consecutive sections of vitelline vessels of a HH18 embryo, showing expression of ET-1 (a), ECE-1 (b), ETA (c) and ETB (d) mRNA by means of radioactive in situ hybridisation, and an antibody staining against muscle actin (HHF35) (insert in a). Note that ET-1 is expressed in the endothelium (arrows). ECE-1 is primarily expressed in the endothelium (arrows), but also slightly in the media of the vessel wall (arrow head). ETB is strongly present in both the endothelium (arrow) and media of the vessel wall (arrow heads), whereas ETA expression is absent. VV, vitelline vessels. YC, yolk sac. Scale bars = 100μm.
Gene Expression
In the vessels of the yolk sac, the vitelline vessels, ET-1 mRNA expression was shown in the endothelium (Fig. 5.6a). ECE-1 mRNA was expressed in the endothelium and slightly in the media (Fig. 5.6b). ETB showed the same distribution, but with stronger expression in the media (Fig. 5.6d). This expression pattern, and to a lesser degree that of ECE-1, overlaps clearly with the muscle actin (HHF35) staining (Fig. 5.6, insert). Surprisingly, ETA mRNA was undetectable (Fig. 5.6c) both in endothelium and media of vitelline arteries and veins, whereas it was strongly present in the myocardium of the embryo in the same section and weakly in the wall of the dorsal aorta (not shown).

Discussion
ET-1 and its receptor antagonists were infused into the HH18 extra-embryonic circulation to investigate the direct hemodynamic and vasoactive effects of these substances on the heart and vasculature of the chicken embryo and on the vitelline vessels. Previous results showed that at HH24 these substances have an effect on the dorsal aortic mean and peak blood flow velocity, and on cardiac ventricular function that was similar as described in the venous clip model\textsuperscript{10} (unpublished data, 2005).

Embryonic Hemodynamics
The Doppler data of the present study show an increase in heart rate 30 minutes after infusion of all of the substances, including the shams. This increase can be ascribed to growth of the embryo\textsuperscript{21,22}, to the increase in circulating volume with 1\textmu l\textsuperscript{23}, or to the temperature changes that were inevitable in the experimental procedures (see ‘Techniques’ below).

Directly upon infusion of endothelin-1 or its receptor antagonists the Doppler data (determined in the dorsal aorta) did not show any significant changes compared with shams. This may indicate that the changes are below the detection level of this technique, or that there are no direct embryonic dorsal aortic hemodynamic effects at this developmental stage related to the ET-1 pathway. Nevertheless, ET-1 and the receptor antagonists have a long-term influence on cardiac development, since the hemodynamics and the morphology are altered at HH24 and HH35, respectively (unpublished data, 2005). This is probably mediated by changes in gene expression induced by ET-1 or ET-receptor antagonists, which were observed using quantitative RT-PCR from \textit{in vitro} endothelial/muscle cell cultures that
Differential Effects of the Endothelin-1 Pathway

were exposed to these substances. These alterations in gene expression are most likely feedback loop mechanisms (unpublished data, 2005) and can result in a balance-shift in the functional ETA or ETB receptors, leading to altered cardiac function.

Vitelline Circulation
Surprisingly, ETA mRNA is undetectable in the wall of the vitelline vessels at this embryonic stage, whereas ETB is expressed in abundance. This implies that ET-1 can only function through the ETB receptors in these vessels. In the embryo this is different, as both ETA and ETB mRNA are present in the heart (unpublished data, 2005). In the vitelline, or yolk sac, vasculature ET-1 could therefore work through a different mechanism than in the embryonic proper. This explains why the blood flow velocity seems unaltered using Doppler measurements (dorsal aorta) and is increased with the μPIV approach (vitelline veins). The shear stress in vitelline veins is decreased after blocking both the ETA and ETB receptor. This is due to the decreased heart rate that is not compensated by regulation of the vitelline vessel diameter.

Mechanism of the Endothelin-1 Pathway
Activation of the endothelin-1 pathway might lead to apparently conflicting results. This is due to the involvement of two receptors, ETA and ETB. ETB receptor mRNA can be expressed in endothelial and muscle cells, whereas ETA receptor mRNA is only present in muscle cells24 (unpublished data, 2005). These different localisations also involve different actions, as ETA and ETB on smooth muscle cells account for vasoconstriction25, and the ETB receptor located on the endothelium is responsible for vasodilation26,27.

Heterozygous Etb knockout mice, which lack 40-50% of the total ETB receptor protein, show a significantly higher mean arterial blood pressure, which is attributed to the increased influence of ET-1 on the ETA receptors located on resistance vessels28. An increase in resistance would lead to a decrease in flow in veins. In addition, Verhaar et al.27 showed that local administration of the selective ETB receptor antagonist triggers a reduction in blood flow through the brachial artery in healthy subjects. This shows that blood flow is decreased due to the shift toward more activated ETA-receptors after prevention of ETB activation. As reviewed by d’Orleans-Juste et al.29, the endothelial ETB receptors are more sensitive to selective agonists than the receptors on the vascular smooth muscle cells, indicating that ET-1 will mainly bind to the ETB receptors on the endothelium, which will result in vasodilation30. As we have shown that ET-1 is produced and activated (by ECE-1) on site,
but that ETA is not present in the vitelline vessels, ET-1 cannot locally bind to ETA receptors. Therefore, by blocking ETB, a shift toward the ETA-mediated vasoconstriction, and an ETB-mediated NO-release are not to be expected, as are changes in volume flow of the vitelline vessels. However, other pathways of NO-release have been described, e.g., the NOS-3 activation by vascular endothelial growth factor receptor 2 (VEGFR2/Flk1)\textsuperscript{31}. Our results demonstrate that all measured and calculated parameters remain constant after blocking the ETB receptors.

By blocking both receptors, the same is expected as with blocking ETB alone. However, the non-selective antagonist does result in changes in heart rate and shear stress, as determined in the vitelline vessels. This blocker is probably effective in the heart or the vasculature of the embryo proper, where both ETA and ETB are expressed (unpublished data, 2005), altering their inotropic effects\textsuperscript{32,33} and thus the contractility of the heart.

With blockade of the ETA receptor we now expect hemodynamic changes in the vitelline vessels. Indeed, we show a trend in decrease in heart rate. This may also be due to blockade of ETA receptors in the heart or the vasculature of the embryo, as explained with the non-selective antagonist.

When ET-1 is infused it will activate the endothelial ETB receptors resulting in a release of NO and vasodilation and concomitant increased volume flow\textsuperscript{30}. However, our data do not show a significant increase in volume flow and diameter in veins smaller than 100μm, of which the latter is in agreement with Kajio and Nakazawa\textsuperscript{14}. They reported that only veins between 100 and 200μm showed a response to ET-1 in the HH21 chicken embryo. We were not able to confirm this, as with our μPIV technique veins with a diameter larger than 100μm are not optically accessible in HH18 embryos. We were able to confirm the increase in heart rate by ET-1\textsuperscript{14}, which could be detected in the vitelline vessels with μPIV at this embryonic stage.

Interestingly, when we infused ET-1 at a much higher concentration (10^{-4}mol/L, not shown) peripheral vitelline vessels dramatically constricted. The systemic embryonic vessels dilated, possibly because of the high pressure that was built up by the occlusion of the vitelline vessels. The cardiovascular system ruptured at the weakest spot, near the sinus venosus, being the entrance to the heart. These effects indicate that at a high, toxic concentration ET-1 binds not only to the ETB receptors present in the endothelium, but also to the ETB receptors in the smooth muscle cells, resulting in the observed contraction of the vitelline vessels.

The decrease in shear stress caused by the non-selective ET-1 receptor antagonist will have an effect on gene expression in the vitelline vessels, which may result in hampered
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development of the extra-embryonic vascular bed that can lead to an altered yolk sac circulation and to shifted blood flow patterns through the heart. Exogenous ET-1 results in an increased heart rate and volume flow. This may also lead to an altered yolk sac circulation and changed cardiac flow patterns. Furthermore, in vitro data have demonstrated that co-cultures of endocardial and cardiac muscle cells exposed to ET-1, or ETA or ETB blockers, show feedback loop mechanisms in up- or down-regulating ETA and/or ETB receptor mRNA (unpublished data, 2005), showing that gene expression can selectively alter after infusion, independent of hemodynamic changes. Interestingly, at HH24 the passive ventricular filling is decreased after infusion of ET-1, the ETA and non-selective receptor antagonists, but blocking the ETB receptor does not have an influence on this filling. The present study shows that it has no direct effect either on hemodynamics, in contrast with the other antagonists and ET-1. This strongly suggests that the immediate changes in hemodynamics induced by ET-1, ETA receptor or non-selective receptor antagonists lead to the impaired ventricular function at HH24.

**Techniques**

In the present study we used 2 different techniques to investigate the hemodynamic effects. One for measurements in the embryo and the other for measurements in the vitelline veins. Doppler measurements were performed in the dorsal aorta of the embryo. Because the Doppler technique cannot be used in vitelline vessels, due to the small diameter and the other interfering vitelline vessels in the vicinity, the μPIV technique was used for these veins. Vice versa, the μPIV technique cannot be used for the dorsal aorta, as the tissue surrounding this artery is too opaque to optically access the blood cells in the dorsal aorta, which is needed for these measurements. A method to improve the optical accessibility of the dorsal aorta, is to infuse fluorescent particles, which will give a higher resolution, but increases the experimental time. The difference in a significant or non-significant change in heart rate between the dorsal aorta (Doppler) and the vitelline veins (μPIV) seems striking, but is due to differences in the two techniques. Batches of eggs and environmental factors, such as the temperature, humidity, and cooling differences need to be taken into account.

The increase in heart rate 5 minutes after ET-1 infusion, measured with either technique is only 5%. Using the Doppler technique, however, the variation is much larger than with the μPIV technique, resulting in a not statistically significant change. This indicates that μPIV measurements are much more sensitive than Doppler measurements. A drawback of both procedures is that the embryo rapidly cools down through the opening of the egg within the first 10 minutes of data acquisition time, despite the attempts of
keeping the temperature constant at 37/38°C by means of a thermoelement (Doppler) or a warm water bath (μPIV). Previous studies have shown that at 4 minutes the Doppler data will already show a drop in heart rate and blood flow velocity. The cooling of the embryo was taken into account by ensuring that with the first measurement (Doppler or μPIV), the egg was open for 4 minutes. In addition, μPIV measurements are more time-consuming than Doppler measurements. This may explain why the heart rate is not increased 30 minutes after infusion compared with baseline values, as was observed with Doppler measurements. The temperature of the μPIV-embryos may have been slightly lower.

**Future Research**

μPIV is a highly sensitive technique to study blood flow. To get more insight into the hemodynamics after infusion of ET-1 or its receptor antagonists, this will be a perfect method. More determinations in different sizes of vitelline veins (<100μm, 100-200μm, >200μm) will be needed as well as in the arteries, but a measurement in the heart will also be very important, as this will give values of blood flow and shear stress in the heart. In addition, it will be interesting to investigate, both qualitatively and quantitatively, the expression of ET-1, ECE-1, ETA and ETB in heart and extra-embryonic vessels before and after infusion of ET-1 and its receptor antagonists, to investigate whether feedback loop mechanisms are functional in ovo.

**In Conclusion**

The present data show that the mechanism of ET-1-mediated vasoregulation in the vitelline system is different from the embryonic circulation, since ETA receptor mRNA is absent in the vitelline vessels, but is present in the embryo proper. Furthermore, μPIV is more sensitive than Doppler ultrasound, since changes in heart rate, volume flow rate, and shear stress in the vitelline veins were registered by μPIV, and the embryonic hemodynamics did not show alterations upon infusion of either ET-1 or ET-1 receptor antagonists. From the changed heart rate we conclude that the cardiac contractility is altered. We postulate that ET-1-pathway-induced changes in hemodynamics influence gene expression and/or flow patterns in the yolk sac circulation, which results in changes in the intracardiac flow patterns. In combination with the receptor type balance-shift due to feedback mechanisms in receptor mRNA expression by ET-1 or receptor antagonists, this leads to impaired ventricular function and to morphological anomalies, which were observed at HH24.
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References


