Vascular control in larval *Xenopus laevis*: the role of endothelial-derived factors

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Summary

We used novel digital contrasting methods to measure the effects of endothelin-1 (ET-1, 10^{-6} to 10^{-8} mol l^{-1}), the nitric-oxide-releasing agent sodium nitroprusside (SNP, 10^{-2} mol l^{-1}) and N_ω-nitro-L-arginine methyl ester hydrochloride (L-NAME, 10^{-4} mol l^{-1}), a non-selective inhibitor of nitric oxide synthase, on the diameter of tiny arteries and veins of the head in anaesthetized *Xenopus laevis* tadpoles (stage NF 50–53). Perfusion of the main artery and vein supplying the head with ET-1 caused an immediate, significant and dose-dependent vasoconstriction. While preincubation with L-NAME caused no significant change in vessel diameter, the constriction provoked by subsequent endothelin administration was significantly enhanced. Application of SNP caused a vasodilation that was significant only after preconstriction with ET-1 (10^{-6} mol l^{-1}). Our results provide strong evidence that the vasculature of developing *Xenopus laevis* tadpoles (NF stage 50–53) is influenced by endogenously released nitric oxide and endothelin. Vasoactive mediators released from vascular endothelial cells could be particularly important in vascular control in early embryos when the autonomic innervation is undifferentiated, poorly developed or even absent.

Key words: video image analysis, digital motion analysis, microcirculation, cardiovascular system, development, endothelin, nitric oxide, *Xenopus laevis*, endothelial cell.

Introduction

In all vertebrate embryos, blood circulation starts early in development, and the heart needs to function before it is completely differentiated and matured. Despite the early onset of the heart beat and blood circulation, their physiological role in oxygen transport has been questioned (Pelster, 1999; Pelster and Burggren, 1996). In small larvae such as those of *Xenopus laevis* and zebrafish, diffusion of oxygen through the body surface alone is considered adequate to meet the metabolic needs of the animal (Territo and Burggren, 1998).

There is, however, increasing evidence that the haemodynamic force generated by blood flow could be an important factor in the developmental process of angiogenesis (Risau, 1997). Furthermore, it is interesting to note that blood pressure is quite predictable at any stage of development, suggesting that some sort of control exists at an early stage.

In mature animals, regulation of the cardiovascular system is achieved mainly by the autonomic nervous system, circulating hormones and physicochemical factors. Cardiovascular control in embryos was thought to be driven exclusively by intrinsic characteristics of the heart (Wagman et al., 1990) and abiotic environmental factors such as temperature (Nakazawa et al., 1986; Pelster, 1999). There are, however, several recent studies showing that cardiovascular control systems are active early in development (Fritsche et al., 2000; Schwerte and Pelster, 2000).

In many vertebrate embryos, a variety of cardiovascular receptors appear to develop before the appropriate nerves (Jacobsson and Fritsche, 1999; Pappano, 1977; Pelster et al., 1993; Protas and Leontieva, 1992; Petery and Van Mierop, 1977). In the amphibians *Xenopus laevis* and *Rana temporaria*, adrenergic agonists stimulate the heart long before the adrenergic nerves develop (Jacobsson and Fritsche, 1999; Protas and Leontieva, 1992), and an adrenergic tonus exists on the non-innervated larval heart of *Xenopus laevis*. This tonus appears to be due to release of adrenaline from intrinsic cardiac adrenergic cells (Jacobsson and Fritsche, 1999; Fritsche and Jacobsson, 2000).

At early developmental stages, cardiac and vascular nerves are few or undifferentiated (A. Kloberg and R. Fritsche, unpublished data). It is therefore likely that other mechanisms such as hormones (A. Kloberg and R. Fritsche, unpublished data), intrinsic mechanisms (Warburton and Fritsche, 2000) and factors released from endothelial cells (Fritsche et al., 2000) could be important for homeostasis of circulation. The endothelium produces a number of vasoactive substances, both vasoconstrictors and vasodilators (Vane and Botting, 1992). The endothelium can synthesize and release an endothelium-derived relaxing factor, nitric oxide (NO) (Palmer et al., 1987; Moncada et al., 1988). Nitric oxide is formed from L-arginine (Moncada et al., 1989) and has been shown to cause

vasodilation in different vessels in a great variety of species. It is involved in a variety of physiological processes such as neurotransmission, platelet aggregation and adhesion and macrophage activity (Ignarro et al., 1999; Moncada, 1994).

Endothelin-1 (ET-1) is also secreted by the endothelium (Vane and Botting, 1992) and exhibits a variety of responses: vasoconstriction and/or vasodilation (Wong et al., 1993), depending on the development of the different receptor types and the maturation of the regional circulation and vascular tone (Wong, 1997). The endothelium can regulate the local concentration of endothelin by enzymatic degradation (Jackman et al., 1993) and by activation of the endothelin-converting enzyme. The haemodynamic effects are very complex and are initiated by activation of several receptor subtypes located on the surface of the endothelial and smooth muscle cells (Wong et al., 1994).

The present study was undertaken to increase our understanding of the role of endothelial-derived factors in vascular control in a developing animals. Although the main function of blood circulation in small larvae such as those of *Xenopus laevis* is not oxygen transport, precise regulation is probably important for other processes such as angiogenesis. It is also possible that increased cardiovascular performance is of importance during periods of increased metabolic demand, such as 'exercise' and feeding. We have studied the influence of (i) endothelin-1 ET-1), (ii) sodium nitroprusside (SNP; a NO donor) and (iii) N_ω-nitro-L-arginine methyl ester hydrochloride (L-NAME; a nitric oxide synthase inhibitor) and (iv) the interactive effects of NO and endothelin on the main head artery of larval *Xenopus laevis*.

In addition, immunohistochemistry was used to demonstrate the presence of ET-1 in the endothelial cells of the main head artery and vein of larval *Xenopus laevis*.

Materials and methods
Animal preparation

Adult *Xenopus laevis* adults were bred in the laboratory, and the larvae were reared at 20 °C and fed daily with commercial *Xenopus laevis* diet (Blades Biological, Kent, UK). All studies were performed on Nieuwkoop–Faber (NF) stages 50–53 larvae (Nieuwkoop and Faber, 1956).

Surgery and experimental arrangement

Prior to surgery, larvae were immersed in 0.05–0.1 % tricaine methanesulphonate (MS222, Sigma) buffered to pH 7.8 with 1 mol l^{-1} NaOH. The larvae were removed from the anaesthetic when they lost their righting reflex and were then placed ventral side up in a clear glass dish with a transparent silastic bottom layer. Amphibian Ringer at 20 °C equilibrated with 100 % oxygen and containing 0.05–0.1 % MS222 was superfused over the larvae throughout the experiment. Each larva was held in place using a 'cage' of insect pins, and the thoracic cavity was surgically opened in a caudal–cranial direction. This open thoracic preparation is the

reason that the animal was maintained in Ringer's solution rather than tap water. The main artery supplying the head was carefully exposed, and a borosilicate glass micropipette was placed close to the vessel. Exposure of the artery also allowed unobstructed perfusion of test solutions. Test solutions were injected into the animal chamber close to the desired vessel using a nanolitre injector (WPI model Micro-1).

Immunohistochemistry

Ten *Xenopus laevis* tadpoles (NF stages 50 and 52) were used for immunohistochemistry. The animals were removed from the aquarium and anaesthetised in 0.01 % MS222 (Sigma). Anaesthetised larvae were fixed in Zamboni's fixative (15 % saturated picric acid, 2 % formaldehyde in 0.1 mol l^{-1} phosphate-buffered saline, pH 7.2) for 24–48 h at 4 °C. The fixed samples were rinsed and dehydrated in ethanol, treated with xylene for 30 min, rehydrated and quickly rinsed in phosphate-buffered saline (PBS; 0.9 % NaCl) with 30 % sucrose as a cryoprotectant and placed, at least overnight, in the same sucrose buffer. Animals were then treated as described below for sectioning.

Preparations from the ventral half of the head region, including the gills and heart, were removed from buffer and embedded in an agarose/sucrose solution (1.5 % low-melting-point agarose, 5 % sucrose). The warm liquid agarose–sucrose solution was poured into a plastic well, and the larva was placed in the desired orientation. The well was left at room temperature to solidify (melting point approximately 26 °C). The embedded larva was placed at 4 °C in a 30 % sucrose solution until the agarose block had sunk to the bottom. The animals were then frozen in isopentane prechilled in liquid nitrogen and cut into 14 µm sections on a cryostat. The preparations were incubated with normal donkey serum (diluted 1:10; Jackson Immuno Research, USA) for 60 min before incubation for 1 day at room temperature with the primary antiserum to endothelin-1 (diluted 1:200; code 4113-0915; raised in rabbit; Biogenesis). The preparations were washed three times for 5 min in hypertonic PBS (2 % NaCl) and incubated for 1 h with a 1 in 100 dilution of the appropriate secondary antiserum (donkey anti-rabbit; Jackson Immuno Research, USA) conjugated to fluorescein isothiocyanate. The preparations were mounted in carbonate-buffered glycerol and viewed with an Olympus fluorescence microscope. Micrographs were taken with an Olympus camera using Kodak T$_{MAX}$ 400 print film. Controls were performed by omitting the primary antibody.

Immunohistochemistry was used to examine the main head arteries and veins. These blood vessels are arranged in two parallel pairs, with the veins lying more ventral and outside the arteries. They are clearly discernible through the ventral body wall. To identify the vessels of the cross-sectioned tadpoles on the slides, a careful comparison was made with an anaesthetized tadpole of the same developmental stage.

We will use the term endothelin-1-like immunoreactivity to describe the localisation of the peptide since we employed antibodies raised against mammalian antigens.

Measurement of vessel diameter by digital motion analysis

A cast of the vascular bed was obtained by summing the visualized shifting vectors of moving erythrocytes. By subtracting two fields of a video frame, any movement that occurred within the 20 ms necessary for the acquisition of one field was visualized. The length of the shifting vectors, generated by this subtraction, represents a direct measure of the velocity of a moving particle, e.g. an erythrocyte in the vascular system. By accumulating shifting vectors generated from several subsequent video frames, a complete trace of the routes of moving erythrocytes was obtained. The diameter of the vessels in a defined region of interest was determined automatically using a custom-made program for the software package Optimas (Media Cybernetics). Using a 'rectangle-fit' algorithm, the best-fitting rectangle covering the blood vessel in the region of interest was defined, and the length of the minor axis length was extracted as a direct measure of the mean diameter of the vessel along the major axis, which was determined by the size of the region of interest (Fig. 1).

The images used for measurement of vessel diameter were recorded using a 4.9× objective, giving in a resolution of 2.5 μm×2.2 μm per image pixel (see Schwerte and Pelster, 2000).

Statistical analyses

The acquired data and the extracted data were automatically exported into an ASCII file for statistical analysis. After preprocessing the data in Microsoft Excel, statistical analyses were performed using appropriate software packages. Statistically significant differences were evaluated using a Friedman non-parametric repeated-measures test followed by Dunn's test. Vascular diameters were normalized to the mean value over a 2 min control period before treatment. Significance was accepted at $P<0.05$. Data are presented as the mean ± the standard error of the mean (S.E.M.).

Experimental protocol

In all experiments, animals were allowed to settle for approximately 5 min to allow cardiovascular parameters to stabilize. The injection electrode was then put into place, and a period of 2 min was allowed for resting values to be recorded.

The effects of endothelin

At the end of the second minute, 2000 nl of endothelin (at 10^{-6} mol l^{-1}, 10^{-7} mol l^{-1} or 10^{-8} mol l^{-1} in three separate groups of animals) were injected. Recording of vascular parameters continued for another 12 min. In a separate group of animals, the head vein was kept in focus for digital measurements of vessel diameter. In this group, the effect of 10^{-7} mol l^{-1} endothelin was investigated. Control experiments were performed by replacing the endothelin solution with Ringer's solution and repeating the same procedure on a separate group of animals.

Endothelin treatment after preincubation with L-NAME

At the end of the second minute of recording resting values, a solution of L-NAME was added to the animal chamber to give a final concentration of 10^{-4} mol l^{-1}. The animal was incubated for 20 min. After 22 min, 2000 nl of endothelin (10^{-7} mol l^{-1}) were injected, and the vascular parameters were recorded for 12 min. For control experiments, the endothelin solution (10^{-7} mol l^{-1}) was given to a separate group of animals preincubated with pure Ringer's solution instead of Ringer containing L-NAME.

Sodium nitroprusside treatment

At the end of the second minute of recording resting values, 5 μl of SNP (10^{-2} mol l^{-1}) was injected from the micropipette After the injection, data were recorded for 15 min.

The effects of sodium nitroprusside after preincubation with endothelin

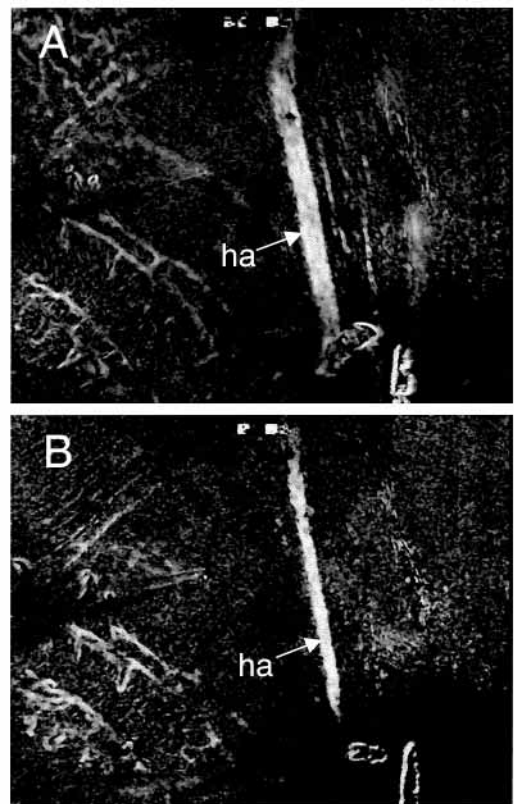Before the end of the second minute of recording of resting

Fig. 1. (A,B) Video images showing a cast of the vascular bed of the main vessels in the head of a *Xenopus laevis* tadpole using the digital motion-analysis technique. In the anaesthetized tadpole, the movements of the erythrocytes are digitally contrasted. The arrows point to the location where vessel diameter measurements were made by defining a threshold for the grayscale value and applying the 'rectangle-fit' algorithm. This automatically defined the best-fitting rectangle covering the blood vessel within this region of interest. The length of the short axis of this rectangle was taken as vessel diameter (see Materials and methods for details). (A) Before endothelin application; (B) after endothelin (ET-1, 10^{-6} mol l^{-1}) application. ha, head artery. Image width, 1.6 mm.

values, 2000 nl of endothelin (10^{-6} mol l^{-1}) were injected. Vascular parameters were recorded for 33 min. After 24 min, 5 µl of SNP (10^{-2} mol l^{-1}) were injected. For control experiments, Ringer's solution was used instead of SNP solution in a separate group of animals.

Results

Endothelin immunoreactivity

Endothelin-1-like immunoreactivity (ET-1 IR) was present within a small area estimated to be 100–200 µm long in the endothelium of the paired arteries close to the truncus
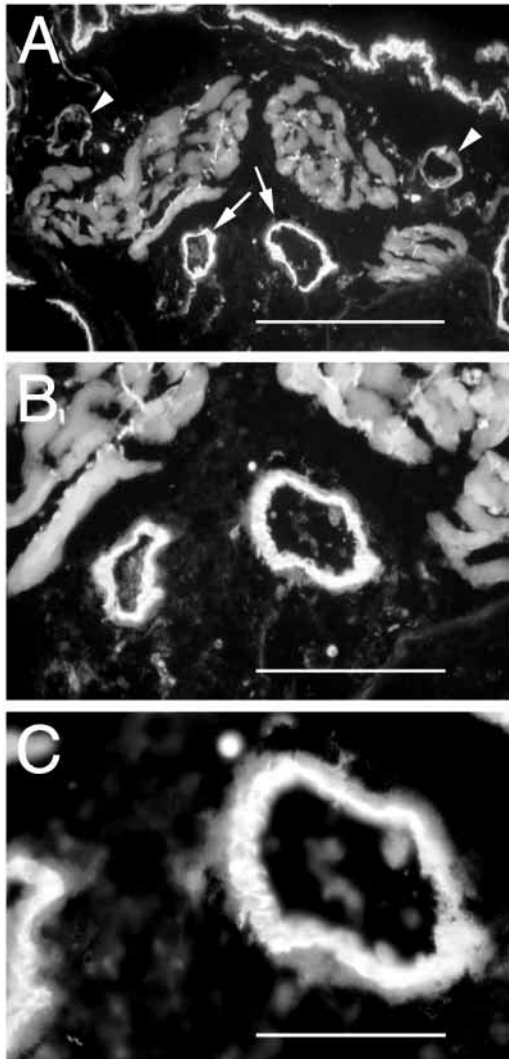

Fig. 2. (A–C) Endothelin-1-like immunoreactivity (ET-1 IR) in *Xenopus laevis* NF stage 50 tadpoles. Cross sections of the head region cranial to the heart and truncus arteriosus with the ventral side at the top of the figure. (A) The immunoreactive endothelial cells of the main head arteries (arrows). Note that the two main head veins lack ET-1 IR (arrowheads). The body wall showed nonspecific labelling (top), which was also present in control sections. Scale bar, 400 µm. (B) The two main head arteries with the endothelium, showing ET-1 IR. The smooth muscle layer, the connective tissue and the melanophores covering parts of the blood vessels cannot be seen in this micrograph. Scale bar, 200 µm. (C) A higher-power view of one of the main head arteries. Scale bar, 100 µm.
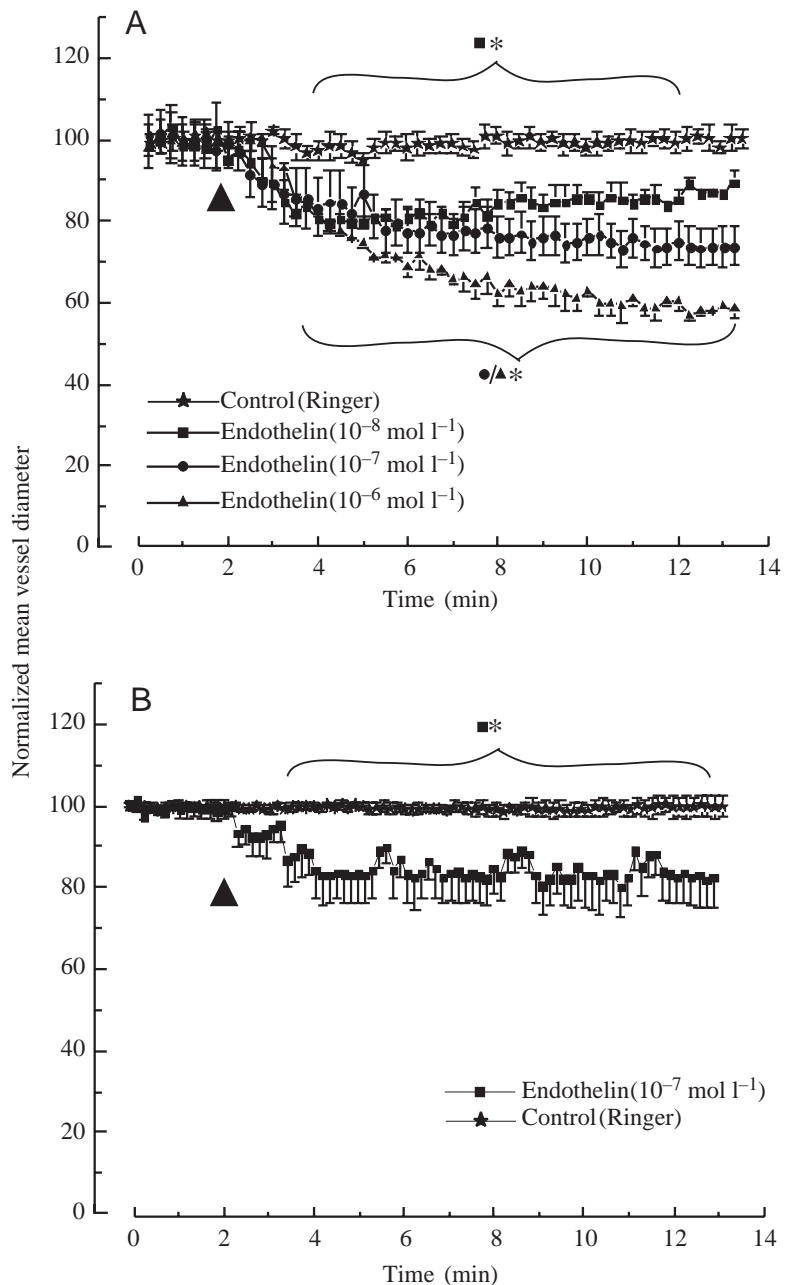

Fig. 3. (A) Effects of endothelin (ET-1) on the diameter of the main artery to the head (normalized to the diameter under control conditions) in *Xenopus laevis* tadpoles (N-F stages 50–53). Values are means ± s.e.m. (*N*=6); the symbols and asterisks indicate significant differences from the control period. (B) Effect of endothelin (ET-1) on the diameter of the main vein from the head (normalized to the diameter under control conditions) in *Xenopus laevis* tadpoles. Values are means ± s.e.m. (*N*=6). Arrowheads indicate the point at which endothelin was applied.

arteriosus. All the stained cells showed a very high density of ET-1 IR (Fig. 2). No immunoreactivity was found in the head veins in this study.

Effects of endothelin on vessel diameter

Application of endothelin to the main artery supplying the head resulted in a significant, dose-dependent decrease in vessel diameter. Typical data are presented in Fig. 3A,B.

The normalized mean arterial diameter decreased by 20% within 2 min after application of 10^{-8} mol l^{-1} endothelin. Higher concentrations provoked a stronger vasoconstriction, 26% by 10^{-7} mol l^{-1} and 40% by 10^{-6} mol l^{-1} endothelin (Fig. 3). The arterial diameter (mean 85.4±3.9 μm; N=6) began to recover only slowly approximately 4 min after application of 10^{-8} mol l^{-1} endothelin, and there was no recovery at higher concentrations (Fig. 3A). The diameter of the main head vein was also significantly reduced (by 16.7%) after application of 10^{-7} mol l^{-1} endothelin (Fig. 3B). The smaller vessels (10–32 μm) in the vicinity of the focused artery showed no significant changes in diameter. The ventral aorta was also unaffected by application of endothelin (data not shown).

Interaction between NO and endothelin

To investigate whether the artery was influenced by a dilating tonus resulting from endogenously produced nitric oxide, the animal was incubated with the non-selective nitric oxide synthase inhibitor L-NAME. Preincubation with 10^{-4} mol l^{-1} L-NAME for 20 min caused no significant change in vessel diameter. However, this experimental group showed a significantly greater decrease (10%) in vessel diameter (mean 67.3±4.03 μm) after subsequent application of 10^{-7} mol l^{-1} endothelin than the control group incubated with Ringer instead of L-NAME (Fig. 4). The smaller vessels in the vicinity of the focused artery (10–32 μm) showed no significant changes in diameter.

Effects of SNP on vessel diameter in Xenopus laevis NF stage 50–53 tadpoles

The application of 5 μl of SNP (10^{-2} mol l^{-1}) had no significant effect on the diameter of the main head artery (data not shown).

Interaction between endothelin and the nitric oxide donor sodium nitroprusside

Application of 10^{-2} mol l^{-1} SNP did not significantly affect the diameter of the main head artery (data not shown). However, when the vessel was preconstricted by application of 10^{-6} mol l^{-1} endothelin (65% of resting diameter), subsequent administration of 5 μl of SNP (10^{-2} mol l^{-1}) resulted in a significant vasodilation to a vessel diameter comparable with the resting vessel diameter within 5 min of application (Fig. 5). The mean vessel diameter was 62.8±3.5 μm (N=6). The smaller vessels in the vicinity of the focused artery
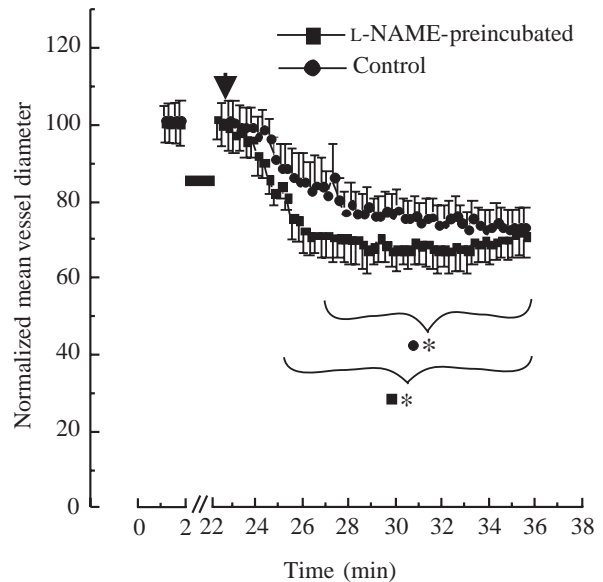

Fig. 4. Effects of 10^{-7} mol l^{-1} endothelin (ET-1) on the normalized diameter of the main artery to the head (normalized to the diameter under control conditions) in *Xenopus laevis* tadpoles. One group of tadpoles was preincubated for 20 min (black bar) with L-NAME (10^{-4} mol l^{-1}) in Ringer's solution prior to injection of endothelin. The other group was preincubated with Ringer's solution alone for 20 min (black bar) prior to injection of endothelin. The arrowhead indicates the time of endothelin injection. Values are means ± S.E.M. (N=6); symbols and asterisks indicate significant differences from the control period.
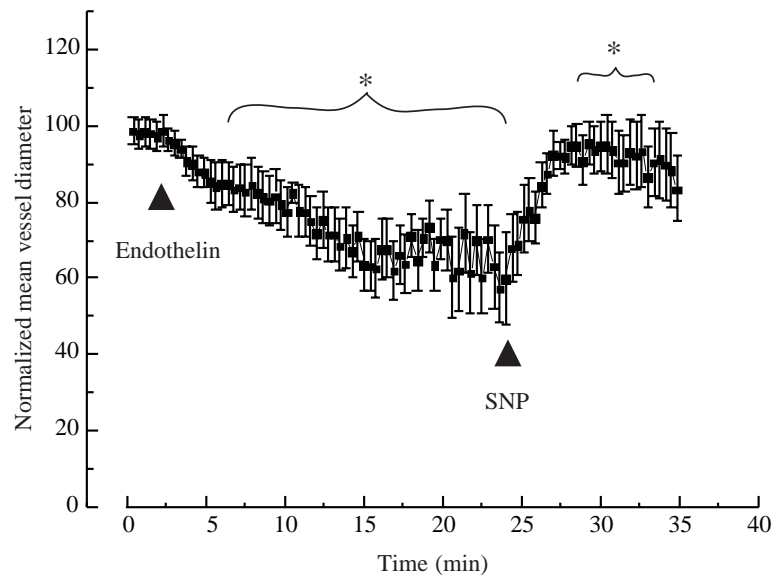

Fig. 5. Effects of 10^{-6} mol l^{-1} endothelin (ET-1) and 10^{-2} mol l^{-1} sodium nitroprusside (SNP) on the normalized diameter of the main artery to the head (normalized to the diameter under control conditions) in *Xenopus laevis* tadpole. Arrowheads indicate the time of drug injections. Values are means ± S.E.M. (N=6). Asterisks indicate significant differences from the control period.

(10–32 μm) showed no significant changes after endothelin application but a small, but non-significant, vasodilation after application of SNP.

Discussion

Fish and amphibian embryos and larvae are excellent models for studying the development of the cardiovascular system because they are free-living, very often transparent and breeding procedures for many species are well established. In addition, our rapidly increasing knowledge of the genome of *Xenopus laevis* and zebrafish makes them ideal models for physiological studies in individuals that have mutations affecting the cardiovascular system, the first functional organ system. Traditionally, experiments on the vascular system are performed on isolated vessel strips or using Doppler flowmeters and micropressure techniques. However, most of these techniques are unsuitable for long-term investigations because they are too invasive or are extremely difficult to perform in tiny animals such as *Xenopus laevis* tadpoles or fish larvae. Recent studies (Fritsche and Burggren, 1996; Mirkovic and Rombough, 1998; Hou and Burggren, 1995; Schwerte and Pelster, 2000; Fritsche et al., 2000) have demonstrated the usefulness of video imaging techniques for cardiovascular research in larvae and embryos. In earlier studies, the outer diameter of vessels was analyzed during microscopic inspection (Nakazawa and Kajio, 1997; Fung, 1997). The artery investigated in this study was found to be surrounded by a melanophore-pigmented sheath, which could easily be confused with the outer diameter of the vessel. For this reason,

we used a recently developed digital video technique that contrasts moving erythrocytes with the non-moving background (Schwerte and Pelster, 2000).

Although endothelin is well-known as a potent vasoconstrictor in adult vertebrates, information about its role during the early stages of cardiovascular development is scarce. The main head artery and vein showed a vasoconstriction in response to endothelin, an effect that was dose-dependent and enhanced by preincubation with the arginine analogue L-NAME. This suggests that endogenous NO production is affecting the larval vasculature and that NO- and ET-1-sensitive second-messenger systems are present in vascular smooth muscles.

Immunohistology revealed the presence of ET-1 in the endothelial cells. Endothelin has been shown to be involved in vascular regulation in newborn mammals (Wong et al., 1993), and in adult rats it acts in cooperation with NO (Filep, 1997). It has also been demonstrated that both endothelin and NO are involved in angiogenesis (Pipili-Synetos et al., 1993; Goligorsky et al., 1999b). The effects of ET-1 and NO on angiogenesis are often analyzed in endothelial cell cultures in studies that concentrate on the focal adhesion of these cells (Goligorsky et al., 1999a).

The physiological effects of endothelin on isolated rat and pig heart were a transient vasodilation and a vasoconstriction of the coronary vessels combined with a positive inotropic action (Baydoun et al., 1990). Endothelin had a positive chronotropic and inotropic effect on cultured chick embryo heart cells, and it appears to be one of the most potent inotropic agents in cultured cardiac myocytes (Concas et al., 1989).

Fig. 6. (A) Schematic drawing showing the possible mechanism of regulation of vessel diameter of the main head artery in *Xenopus laevis* tadpoles before functional innervation of the peripheral vascular system. Endothelin (ET-1) is known to be a potent and long-lasting endothelium-derived vasoconstrictor. Nitric oxide (NO) can also be produced by the endothelium and has a dilatory effect. NO is known to be released in response to shear stress induction. Recent studies (see text, for references) provide evidence for a functional coordination and cooperation between NO and ET-1. The time course is indicated on the axis in B. The numbers in circles indicate the corresponding points in B. (1) Endothelin, possibly released by the endothelium, provokes a strong vasoconstriction, which in turn indirectly induces the release of NO (3) by increasing shear stress (2). NO acts directly on the vascular smooth muscle (vsm) to dilate the vessel (5). In addition, it acts as a 'physiological brake' on endothelin function by decreasing the affinity of ET-1 for its receptor (4), which enhances the vasodilation (5). L-NAME is a competitive inhibitor of the endothelial nitric oxide synthase. (6) The effect of preincubation with L-NAME. (B) Typical pharmacological experiments demonstrating the possible coordination and cooperation between NO and ET-1. SNP, sodium nitroprusside.
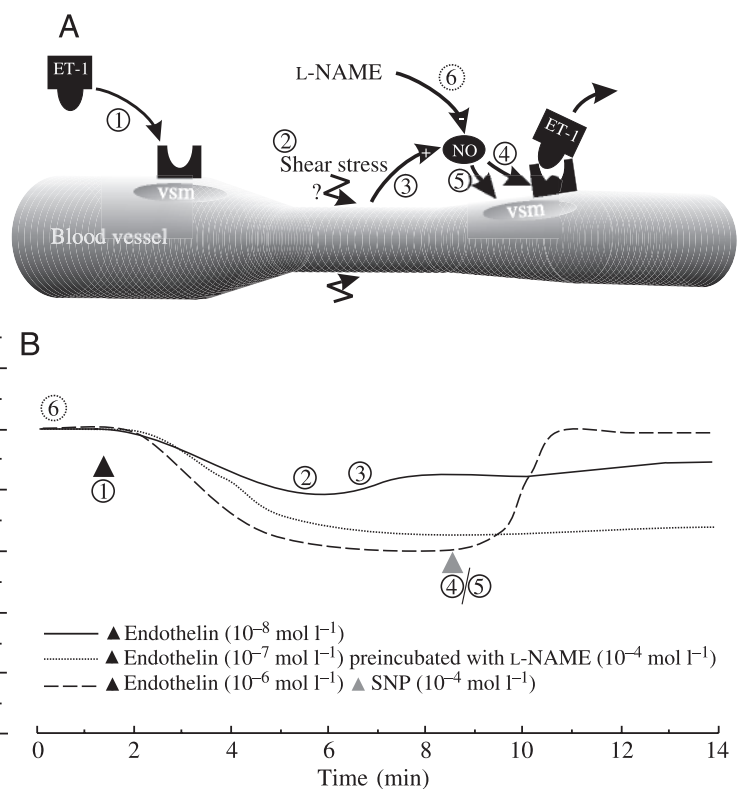
Kajio and Nakazawa (1997) found a positive chronotropic effect and an increase in blood pressure in stage 21 chick embryos, while a vasoconstrictory effect of endothelin was observed in both arteries and veins in foetal lambs (Wang and Coceani, 1992). In the present study, we observed a dose-dependent vasoconstrictory effect on arteries and veins with a mean diameter of 65.4 μm. The largest response found was a decrease in vessel diameter by 40 % using a local application of 10^{-6} mol l^{-1} endothelin. The effect appeared within 30 s after the application. Larger veins (diameter 100–200 μm) in the vicinity of our region of interest were also affected by the endothelin application. This suggests that endothelin receptors are present in the larger vessels in the head (diameter 65–200 μm) but not in the smaller arterioles and venules (diameter 10–32 μm). These results are similar to the findings of Kajio and Nakazawa (1997), who demonstrated that endothelin-1 had the greatest effect (reduction in diameter of 20 %) on middle-sized veins (100–200 μm in diameter), while smaller vessels did not show any significant change in diameter. In the truncus arteriosus close to the heart, no vasoactive effect of endothelin was observed.

The fact that endothelin and nitric oxide are both produced and released by the endothelium suggests the possibility that they interact to regulate vascular tone. As previously demonstrated in zebrafish larvae, nitric oxide release was associated with a dilating tonus in larvae already 5 days post-fertilization (Fritsche et al., 2000). To test whether a sufficient level of permanently released nitric oxide to cause a dilating tonus was present in *Xenopus laevis*, a non-specific antagonist of nitric oxide synthase, L-NAME, was used. Even after 20 min of incubation with L-NAME, however, the vessel diameter did not decrease (Fig. 4). This suggests that a NO-derived dilating tonus is of minor importance for the homeostasis of the vessel diameter. The fact that SNP did not provoke any vasodilation compared with control values suggests that the main head artery is fully relaxed at the beginning of treatment and throughout the control experiments.

In many animals, stress or anaesthesia can provoke a general vasodilation. We found no evidence for this in *Xenopus laevis* larvae. Control values did not change significantly over a 60 min experiment. During experiments with adrenergic agonists and blockers, we have found strong evidence for a dominant vasoconstrictory action for adrenaline (A. Jacobsson, T. Schwerte and R. Fritsche, in preparation). To check the hypothesis that blocking the chronic dilating tonus effect of NO could enhance the constricting effect of lower endothelin concentrations, we applied 10^{-7} mol l^{-1} endothelin to vessels that had been preincubated with L-NAME. The results show a significantly greater decrease (10 %) in vessel diameter compared with the group incubated in Ringer alone. From this, it can be concluded that NO is released by ET-1 application and can modify the constrictory effect of ET-1. In addition, the fact that application of SNP caused an immediate relaxation of the vascular smooth muscles in ET-1-preconstricted vessels clearly demonstrates a possible interaction between NO and ET-1 in regulating vascular diameter.

Interactions between endothelin and nitric oxide have also been described for human arteries (Luscher et al., 1990). In this study, NO was shown to reverse the vasoconstrictive effect of endothelin. One question that arises is whether SNP acts directly on smooth muscle or interacts with the ET-1 cascade. The fact that incubation with L-NAME provoked only minor changes in obviously relaxed vessels and the observation that blocking NO production strengthens the effect of endothelin-derived constrictive action are strong evidence for the counteracting effects of ET-1 and NO. As mentioned above, SNP caused an immediate relaxation of ET-1-preconstricted vessels. This effect can be explained either by a strong NO-mediated vasodilation, which overrides the constrictive action of endothelin, or by an interaction in the second-messenger pathway, as described by Goligorsky et al. (1994). These authors demonstrated that NO acts as a physiological brake on ET-1 signalling and also provided strong evidence that endothelial NO can enhance the dissociation of ET-1 from its appropriate receptors and interfere with the intracellular pathway for Ca^{2+} mobilization. Given the speed of vessel relaxation, a combination of both actions of NO, blocking ET-1 signalling and causing vascular smooth muscle relaxation, is a possible model for blood vessel diameter homeostasis in *Xenopus laevis* larvae.

However, specific sensor/effector mechanisms have not been directly demonstrated in the present study, and further observations are necessary to elucidate the mechanisms involved in the endothelium-derived hormonal system in homeostasis of the embryonic circulation.

Physiological significance

Although embryos and young larvae often lack a fully developed innervation of the cardiovascular system, adaptability of the circulation can be of importance for processes such as angiogenesis and organogenesis and to meet the metabolic demands of developing tissues.

The primary function of the embryonic heart in small larvae such as *Xenopus laevis* is probably not only oxygen transport, since chemical or physiochemical inactivation of embryonic haemoglobin–oxygen transport does not impair metabolism in small zebrafish larvae (Pelster and Burggren, 1996). Instead, blood pressure may be important for the highly ordered process of angiogenesis (Karimu and Burton, 1994; Risau, 1997). Even though the specific effector pathways have not been demonstrated directly, an endothelium-derived vascular regulation is apparent. Fig. 6A indicates how the interaction between NO and ET-1 could take place. When endothelin is released, the vasoconstriction provoked increases in shear forces on the vessel wall by increasing flow resistance. It is well known that NO is released when shear stress on the endothelium is increased. Nitric oxide interferes with the ET-1 second-messenger pathway (see above) and indirectly limits the constriction of the endothelin. As a direct effect, NO can also cause vasodilation. Fig. 6B shows an overview of the vascular behaviour observed during the experiments described in the present study.

In summary, it appears that, during the early larval development of amphibians, local endocrine processes are more important for cardiovascular regulation than the autonomic nervous system, which matures much later. The complex interaction between substances released by the endothelial cells may drive developmental processes and enable the metabolic demands for these early developmental stages to be met. The 'onboard' autoregulation of the endothelial cells may allow the cardiovascular regulation to retain flexibility for the restructuring that occurs during metamorphosis, thus minimizing developmental expenses compared with a reorganization of a 'fixed-wired' nervous system.

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