

Nitric oxide and vascular reactivity in developing zebrafish, *Danio rerio*

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Fritsche, Regina, Thorsten Schwerte, and Bernd Pelster. Nitric oxide and vascular reactivity in developing zebrafish, *Danio rerio*. *Am J Physiol Regulatory Integrative Comp Physiol* 279: R2200–R2207, 2000.—We used a newly developed digital motion analysis video technique to study the effects of nitric oxide (NO) and epinephrine on the early larval arterial and venous vasculature of zebrafish. Application of the NO donor sodium nitroprusside resulted in a significant increase in both the venous and arterial vessel diameters, whereas *N*^G-nitro-L-arginine methyl ester caused a significant decrease in the same diameters. Thus our results show that both the venous and arterial vasculature of the 5- and 6-day-old zebrafish larvae are influenced by endogenously produced NO. By use of immunohistochemistry, NO synthase immunoreactivity was demonstrated in endothelial cells of the dorsal vein. Local application of epinephrine onto the dorsal artery had no effect on vessel diameter. However, if the embryos were preincubated with *N*^G-nitro-L-arginine methyl ester, addition of epinephrine resulted in a significant reduction in both arterial and venous vessel diameters. Thus this study provides increasing evidence that before a functional autonomic innervation of the peripheral vascular system, vascular tone in larval tissue is regulated by a complex interaction of vasoactive substances that are produced locally by vascular endothelial cells.

ontogeny; nitric oxide synthase; epinephrine; cardiac; immunohistochemistry

INDEPENDENT OF SPECIES, the cardiovascular system is the first functioning component of the developing vertebrate embryos. Most studies have focused on the genetic cascade responsible for development of the heart as well as gene mutations leading to visible cardiovascular abnormalities. However, insights into the relationships between embryonic cardiovascular structure and function are poorly understood. Although the basic changes in hemodynamics during development have been described for representative species from several vertebrate groups (see Ref. 4 for reviews), information on the development of cardiovascular control systems is scarce. The mature cardiovascular system is precisely regulated by both extrinsic and intrinsic mechanisms to meet the metabolic need of the tissues. The extrinsic control systems include 1)

the autonomic nervous system, 2) the endocrine system, and 3) physicochemical factors in the blood, whereas the Frank-Starling mechanism represents an intrinsic control system.

In many vertebrate embryos, different cardiovascular receptors appear to develop before the appropriate nerves (19, 27, 29, 31, 33). In the amphibians *Xenopus laevis* and *Rana temporaria*, adrenergic agonists stimulate the heart long before the adrenergic nerves develop (19, 31). Furthermore, an adrenergic tonus exists on the larval heart of *Xenopus*, which is devoid of adrenergic nerve fibers. This tonus has been suggested to be due to epinephrine released from intrinsic cardiac adrenergic cells in *Xenopus* (19). It has been suggested that regulation of embryonic circulation before functional innervation is achieved mainly by the Frank-Starling mechanism and by factors as temperature (25) or oxygen availability (9).

It is interesting to notice that many of the regulatory peptides that are involved in cardiovascular control in adult animals are present in endocrine cells in embryonic tissue (12). Therefore, involvement of these peptides in regulating processes is likely. The endothelial cells are known to produce several vasoactive products, including prostacyclin, nitric oxide (NO), ATP, and endothelin, which contribute significantly to vascular tone in adult animals (3, 23). Because the endothelium is present in all vessels as soon as they develop, these factors could also be possible candidates for vascular control in embryos.

NO plays a key function in many physiological processes such as control of vascular tone, neurotransmission, macrophage activity, and angiogenesis (13, 30). The fact that NO can be released from endothelial cells and has effects on the vascular tonus makes it an attractive candidate for cardiovascular control in embryos and larvae also early in development before the peripheral nerves are functional. NO synthase (NOS) immunoreactivity (IR) has been localized by using immunohistochemical techniques in the gut of *Xenopus laevis* tadpoles (A. Holmberg, U. Hägg, R. Fritsche, and S. Holmgren; unpublished observations), and NO is present and causes vasodilation in the pulmonary and

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systemic arteries of newborn guinea pigs (5). Recently, a study on rainbow trout alevins showed that long-term exposure to the NO donor isosorbide dinitrate resulted in dilation of the vitelline vein, reduced heart rate, and increased cardiac output (7).

Thus it is known that NO regulates vascular tone and influences cardiovascular function in neonatal and mature circulations (1, 5, 8). However, the ontogeny and functional development of NO reactivity during early organogenesis have not been investigated. Our experiments were performed on 3-, 4-, 5-, and 6-day-old zebrafish (*Danio rerio*) larvae. At these stages the heart has only primitive atrioventricular cushions that function like valves and no functional autonomic innervation. We have studied the following: 1) the presence of NOS in the embryonic cardiovascular system, 2) the vascular effects of locally administered sodium nitroprusside (SNP; NO donor), 3) N^G -nitro-L-arginine methyl ester (L-NAME; NOS inhibitor), as well as 4) the interaction of the NO and adrenergic control systems in developing zebrafish. The results show that NOS is present in endothelial cells of the dorsal vein and that NO has vascular effects already early in development when nervous control is scarce or lacking.

MATERIALS AND METHODS

The experiments were performed using the almost-transparent larval stages of the zebrafish. Zebrafish larvae were obtained from our own breeding colony. Because of a better transparency, poorly pigmented mutants of the zebrafish (Albino, Brass) were used. Parent animals to start the breeding colonies were either obtained from a local supplier or generously provided by Dr. Frohnhofer from the Max-Planck Institute for Developmental Biology in Tübingen and A. Y. Loos from the University of Konstanz. Breeding colonies and larvae were kept in small aquariums at a temperature of 25°C. Experiments with zebrafish larvae were performed at 25°C.

Immunohistochemistry

A total of 40 zebrafish embryos (3–6 days old) was used for immunohistochemistry. The animals were removed from the aquarium and anesthetized in 0.01% 3-aminobenzoic acid ethyl ester (MS-222, Sigma). Anesthetized larvae were fixed at 4°C in formaldehyde (4%) for 4 h. The formaldehyde-fixed samples were washed in phosphate-buffered saline (PBS, 2% NaCl) for 30 min and quickly rinsed in PBS (0.9% NaCl with 30% sucrose) and placed at least overnight in the same sucrose buffer. Whole animal preparations were prepared by incubating the entire larvae with normal donkey serum (1:10; Jackson Immuno Research) for 60 min before incubation

with the primary antibody (see Table 1 for details) for 3–4 days in a moist chamber at room temperature. Excess amount of the primary antibody was removed by rinsing three times for 5 min in PBS (2.0% NaCl). After washing was completed, excess PBS solution was removed and the larva was incubated with the secondary antibody for 1 day followed by incubation with the conjugate SACy3 (streptavidine indocarbocyanine; 1:400; Jackson Immuno Research) for 1 day in a moist chamber at room temperature. The larvae were mounted in carbonate-buffer glycerol and viewed with an Olympus fluorescence microscope. Control preparations were prepared by omitting the primary antibody from the procedure above.

Mounting of Zebrafish Larvae

For the physiological experiments, embryos and larvae of the zebrafish from 3 to 6 days postfertilization were used. For the experiments the animals were anesthetized by adding 50 mg/l of phosphate-buffered tricaine (MS222; pH 7.0) to the water. After 1 min of being exposed to the anesthesia, the animals were removed and carefully mounted in the experimental chamber with a thin layer (100–200 μ m) of 1% low-melt agarose (Sea Plaque; gelling point 26–30°C) containing 50 mg/l phosphate-buffered tricaine. The gel was covered with 1 ml of air-equilibrated water containing 50 mg/l phosphate-buffered tricaine. This chamber was transferred into the temperature-controlled microscope desk of an inverted microscope (Zeiss Axiovert 25CF). The inverted microscope allowed for a plane view of the ventral or lateral side of the larvae. The left side of the animal provided the best view of the central cardiac system.

Imaging System

To reduce vibratory movements, the inverted microscope was placed on a solid, heavy-weight steel plate. The illumination could be set to infrared light with a wavelength of 780 or 913 nm to prevent light-induced stress reactions of the animals. The microscope was equipped with a 2/3" charge-coupled device camera (Hamamatsu C-2400 without infrared cut-off filter), which in turn was connected to the luminance input of a SVHS videorecorder (Sony S-9500). The VCR was remote controlled via the RS232 serial communication port. The setting of the video recorder as well as the recorded images were digitized by a monochrome frame grabber card (Imagenation PX-610) with a personal computer (PIII 450 MHz).

Measurement of Vessel Diameter by Digital Motion Analysis

A cast of the vascular bed was obtained by summing up the visualized shifting vectors of moving erythrocytes (32). Briefly, by subtracting the 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

Table 1. Details of the primary and secondary antibodies used

	Animals Raised In	Conjugate	Supplier	Code	Dilution
Primary antibodies					
Anti-eNOS	Rabbit		Transduction laboratories	N30030	1:500
Anti-nNOS	Rabbit		Transduction laboratories	N31030	1:100
Secondary antibody					
DaR-bio	Donkey	Biotin-SP	Jackson	711.065.152	1:400

eNOS, endothelial nitric oxide synthase; nNOS, neural NOS.

shifting vectors, generated by this subtraction, represented a direct measurement for the velocity of a moving particle, i.e., an erythrocyte in the vascular system. By accumulation of shifting vectors generated from several subsequent video frames, a complete trace of the routes, on which erythrocytes moved, was obtained (Fig. 1). The diameter of the vessels in a defined region of interest (ROI) was determined automatically with the software package Optimas (Media Cybernetics). With the use of a "rectangle-fit" algorithm, the best-fitting rectangle covering the blood vessel in the ROI was defined, and the major-axis length was extracted as a direct measurement of the diameter of the vessel (Fig. 1). The length of the short axis of the rectangle was given by the size of the ROI.

To compensate for minor movements of the animal, a reference point was determined by detection of the branching point of the dorsal artery and the intersegmental artery in the tail region of the fish. This reference point was automatically relocated before every determination of the vessel diameter, and the ROI was kept in a fixed position from the

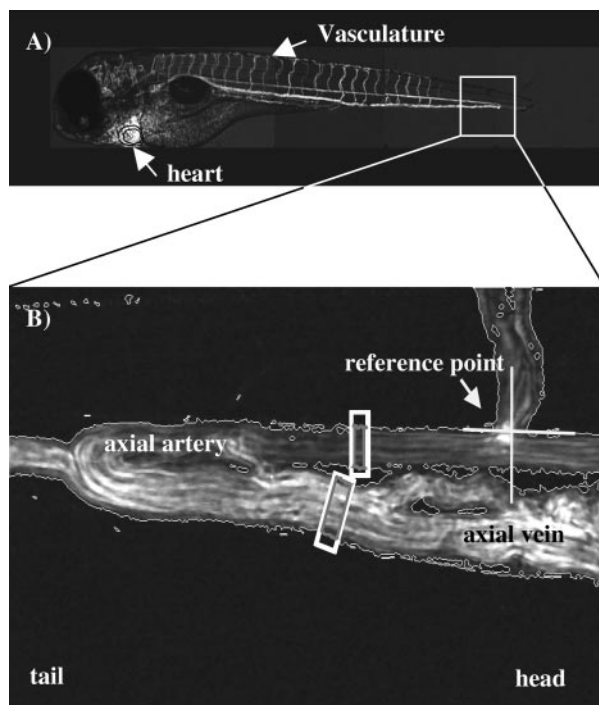


Fig. 1. A: video image showing a cast of the vascular bed of a zebrafish larva using the digital motion analysis technique. In the anesthetized larva, the moving objects are the erythrocytes (giving a cast of the vasculature) and the beating heart. The field of investigation in this study is indicated by the square. The top vessel in the square is the dorsal artery and the bottom vessel is the dorsal vein. The vein has a mean diameter of $12.8 \mu\text{m}$ and the artery $9.0 \mu\text{m}$. B: magnification of the tail vasculature shown in A. The area surrounded by the straight line was the defined area of interest. By defining a threshold for the gray scale value, the "rectangle-fit" algorithm automatically defined the best-fitting rectangle covering the blood vessel within this region of interest (uneven inner line). The length of the long axis of this rectangle was taken as vessel diameter. A reference point was determined at the center of the last intersegmental artery, which splits from the dorsal artery. This reference point was automatically relocated before each determination of the vessel diameter, and the region of interest was kept in a fixed position from the reference point by the computer. Thus the vessel diameter was determined at a fixed position from this reference point (see MATERIALS AND METHODS for details).

reference point by the computer. In consequence, the vessel diameter was determined at a fixed position from this reference point (Fig. 1B). The images used for measurement of vessel diameter were recorded using a $\times 40$ phase-contrast objective resulting in a resolution of $0.336 \mu\text{m} \times 0.359 \mu\text{m}$ per image pixel.

Measurement of Cardiac Output Using Digital Motion Analysis

Determination of cardiac output using digital image analysis basically followed the method described by Hou and Burggren (16). Video sequences of the ventricle were grabbed to the computer memory. The perimeter of the ventricle during end diastole and during end systole was outlined manually using a graphic tablet. The determination of cardiac output in these small larvae is based on the assumption that the ventricle has a shape that is close to a prolate spheroid (9, 16, 21, 24, 26). The perimeter was analyzed with a "fit-to-ellipse" algorithm, which calculated the best-fitting ellipse and directly transferred the major and minor axis into a Microsoft Excel worksheet. From these data end-systolic and end-diastolic volume were calculated using the formula for the volume of a prolate spheroid $V = 4/3 \cdot \pi \cdot a \cdot b^2$. Stroke volume was obtained by subtracting end-systolic volume from end-diastolic volume. For each determination, six diastoles and systoles were analyzed.

Erythrocyte Count Using Digital Motion Analysis

The gray scale value of any given pixel or of a defined number of pixels in the picture generated by digital motion analysis increases from 0 to 255, depending on the number of erythrocytes passing it, and Schwerte and Pelster (32) indeed observed a linear relationship between the gray scale value of a pixel within a blood vessel and the number of erythrocytes passing this pixel. Although the depth of gray scale display on the screen is limited to 8 bit, the actual range for the calculations was extended to 24 bit. Thus the erythrocyte distribution could automatically be recorded in defined blood vessels. A calibration of the signal was obtained by correlating the gray scale values of a defined area with the number of erythrocytes passing this area as counted by the conventional frame-to-frame technique.

Experimental Protocol

Effect of SNP. After the animals were mounted, the animal was allowed to settle down for about 3 min followed by 5 min for registration of resting values. Before the end of the fifth minute, an ejection capillary was placed on top of the dorsal artery and vein very close to the anal fin to inject 800 nl of 10^{-3} M SNP at the beginning of the sixth minute. This injection resulted in a concentration of 10^{-6} M in the animal chamber. The registration of vascular parameters continued for 15 min. For control experiments the SNP solution was exchanged by Ringer, and the same procedure was repeated on a separate group of animals.

Effect of epinephrine after preincubation with L-NAME. After the animal was mounted, it was allowed to settle down for about 3 min followed by 3 min for registration of resting values. Before the end of the third minute, the ejection capillary was placed on top of the dorsal artery and vein very close to the anal fin to inject 800 nl of 10^{-3} M L-NAME at the beginning of the fourth minute. This injection resulted in a concentration of 10^{-6} M in the animal chamber. Three minutes after the injection, the vascular parameters were re-

corded. After a nonrecorded incubation time of 27 min, we again recorded for 3 min. At the end of the 33rd minute, the injection electrode was replaced by one with 10^{-2} M epinephrine to inject 800 nl at the beginning of the 34th minute. Vascular parameters were recorded for the following 5 min. For control experiments, the L-NAME solution was exchanged by Ringer and the same procedure repeated on a separate group of animals.

Effect of epinephrine on the cardiac output. After the animal was mounted, it was allowed to settle down for about 5 min followed by 5 min for registration of resting values. Before the end of the fifth minute, the ejection capillary was placed on top of the dorsal artery and vein very close to the anal fin to inject 800 nl epinephrine at the beginning of the sixth minute, resulting in a concentration of 10^{-5} M in the animal chamber.

Statistics

Statistically significant differences in the observations were evaluated using a one-way ANOVA followed by a multiple-comparison procedure (Bonferroni, SigmaStat). Significance was accepted when $P < 0.05$. Data are presented as means \pm SE.

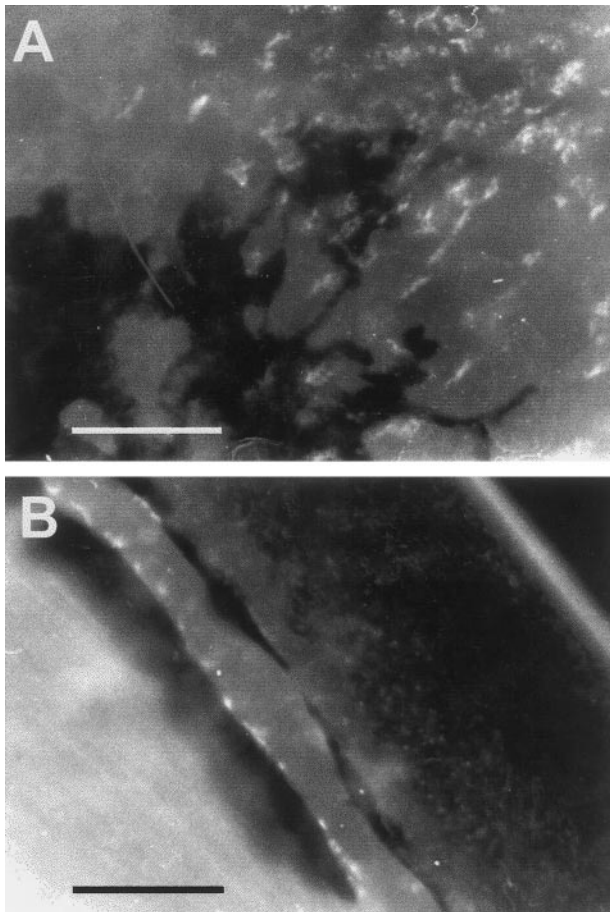


Fig. 2. A: endothelial nitric oxide synthase (eNOS)-immunoreactivity (IR) in the cardiomyocytes of a 3-day-old zebrafish; B: eNOS containing endothelial cells in the dorsal vein of a 5-day-old zebrafish. The black areas in the photographs are melanophores. Bar is 50 μ m.

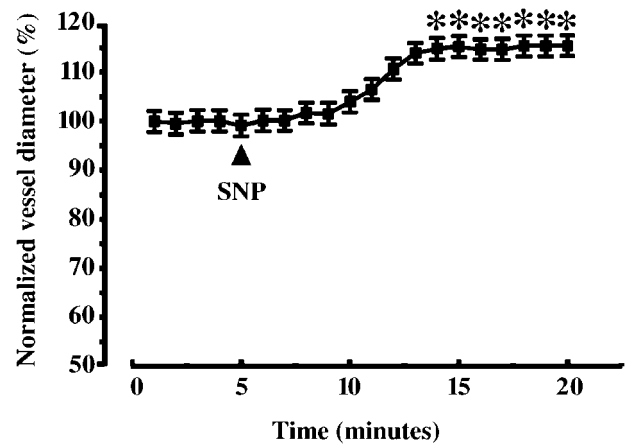


Fig. 3. Effect of sodium nitroprusside (SNP) on the diameter of the dorsal artery (normalized to the diameter in the control situation) in 5- and 6-day-old zebrafish larvae. Values are means \pm SE. *Significantly different from the control period.

RESULTS

NOS Immunoreactivity

Endothelial NOS (eNOS)-IR was demonstrated in the whole animal preparations at all investigated ages in endothelial cells of the dorsal vein (Fig. 2B), whereas the distribution of eNOS in the artery was more diffuse and could not be localized to specific cells. The heart also showed IR to eNOS (Fig. 2A). Brain NOS (i.e., neural; nNOS)-IR could not be detected anywhere in the peripheral tissues. Preparations where incubation with the primary antibody was excluded did not show any IR.

Effect of SNP and L-NAME on Vessel Diameter in 5- and 6-Day-Old Zebrafish Larvae

Administration of SNP onto the dorsal artery and vein resulted in a significant increase in the artery vessel diameter from 9.04 to 10.41 μ m (Fig. 3). The diameter of the dorsal vein increased from 14.3 to 15.7 μ m, but this increase was not significant. The diameter of the intersegmental vessel was unaffected. Vasodilation started \sim 5 min after administration of SNP and lasted during the whole experiment (at least 15 min after administration) (Fig. 3). The number of erythrocytes passing in the intersegmental artery increased from 285 to 326 erythrocytes/min 10 min after administration of SNP (Fig. 4). Injection of the L-arginine analog L-NAME (NOS inhibitor) resulted in a significant vasoconstriction of both the dorsal vein and the dorsal artery (Fig. 5, A and B). Two minutes after administration, the arterial diameter had decreased significantly from 9.32 to 8.85 μ m and the venous diameter from 14.53 to 13.82 μ m. The decrease was further reduced 31 (vein) and 32 (artery) min after administration of L-NAME (Fig. 5, A and B).

The vasoconstrictory effect of L-NAME was also reflected in the reduced number of erythrocytes (from 285 to 265 erythrocytes/min) passing into the intersegmental artery from the dorsal artery (Fig. 4).

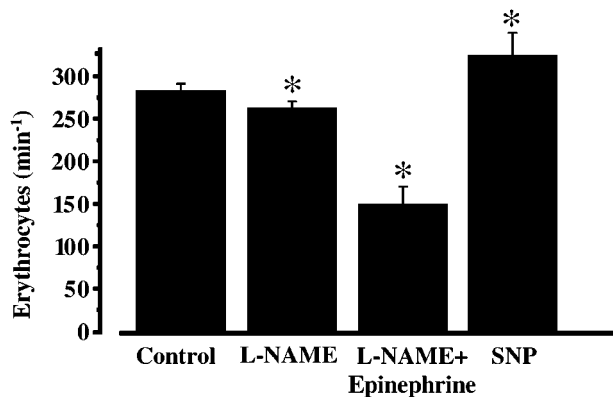


Fig. 4. Effect of *N*^G-nitro-L-arginine methyl ester (L-NAME), L-NAME + epinephrine, and SNP on the number of erythrocytes passing from the dorsal artery into the intersegmental artery each minute in the 5- and 6-day-old zebrafish embryo. Values are means \pm SE. *Significantly different from the control period.

Effect of Epinephrine on Heart and Vasculature in 3- and 4-Day-Old Zebrafish Larvae

Suffusion of epinephrine onto the dorsal artery and vein did not affect the vessel diameter or the erythro-

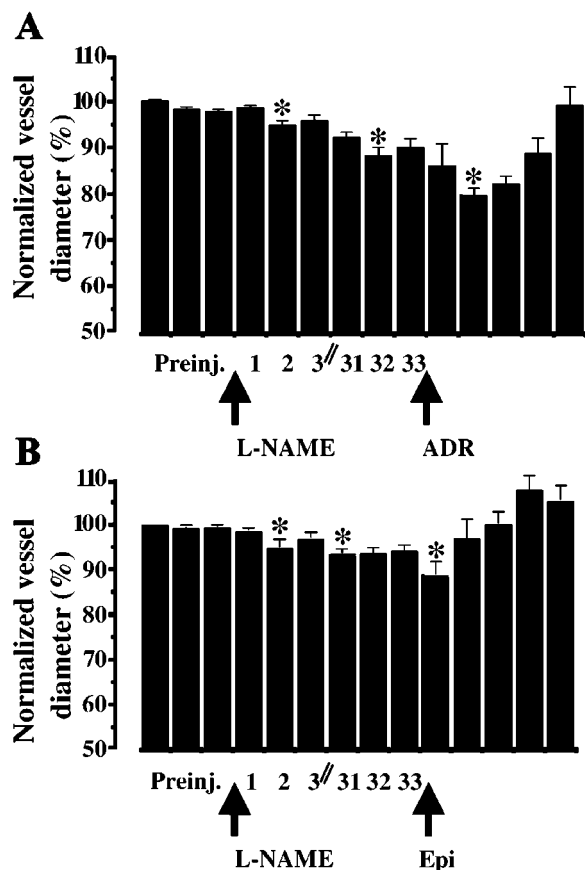


Fig. 5. Effect of L-NAME and L-NAME + epinephrine (Epi) on normalized diameter of the dorsal artery (A) and normalized diameter of the dorsal vein (B) in 5- and 6-day-old zebrafish embryos. The effect of L-NAME is shown 1, 2, 3, 31, 32, and 33 min after the injection. Epi was injected 33 min after the injection of L-NAME, and the effect continued for an additional 5 min. Values are means \pm SE. *Significantly different from the control period.

cyte count (data not shown) at any age investigated (days 3, 4, 5, and 6). However, application of epinephrine onto the heart resulted in a significant increase in cardiac output in both 3- and 4-day-old larvae (Fig. 6). This increase was due to a significant increase in heart rate, whereas stroke volume remained unchanged (data not shown).

Interaction Between NO and Epinephrine

Application of epinephrine after incubating the animal with L-NAME for 30 min resulted in significant reductions in the diameter of both the dorsal artery and vein. This vasoconstriction lasted only 1–3 min after application of epinephrine and was followed by a rapid increase in diameter (Fig. 5, A and B). The effect of epinephrine after incubation with L-NAME was also reflected in a large reduction in the number of erythrocytes passing from the dorsal artery to the intersegmental artery (Fig. 4).

DISCUSSION

Embryos and larvae of many fish and amphibian species hatch as free-living embryos/larvae in the external environment and are often transparent during a large portion of development. These characteristics make them excellent models for studies of early embryonic functions of the cardiovascular system. For example, the heart and vasculature can easily be observed with a microscope without any physical disruption to the animal. Studies on vascular reactivity are traditionally performed on isolated vessels or by recording blood pressure and calculating vascular resistance. In small fish embryos such as the zebrafish, neither of these techniques can be successfully used. Although blood pressure has been recorded in young amphibian larvae (9, 16) and 3- to 5-day-old zebrafish larvae (28), long-term recordings of pressure in newly hatched embryos are too invasive and technically extremely difficult. We used a recently developed video microscopic technique to determine vascular reactivity of the blood vessels. Our results show that the dorsal artery and the dorsal vein of 5- and 6-day-old zebrafish

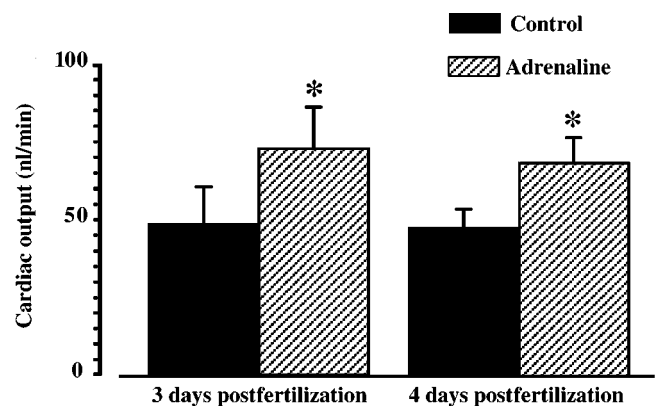


Fig. 6. Effect of epinephrine on cardiac output in 3- and 4-day-old zebrafish larvae. Values are means \pm SE. *Significantly different from the control period.

larvae vasodilate in response to NO released from SNP *in vivo*. In addition, application of the L-arginine analog L-NAME resulted in a decreased diameter of the dorsal artery and vein, suggesting that an endogenous NO production is affecting the larval vasculature and that there are NO-sensitive second-messenger systems in smooth muscles of the main dorsal blood vessels. Indeed our immunohistological studies revealed the presence of NOS in the endothelial cells of the dorsal vein at these early stages.

The importance of NO during development has been demonstrated for processes including angiogenesis (13, 30) and uterus implantation (14). The influence of NO on vascular tone and cardiovascular function has previously only been described for much later developmental stages such as the neonatal circulation of mammals (1, 5). In 3.5-day-old (stage 21) chick embryos, the effects of SNP on ventricular volume and pressure has been studied by Bowers et al. (2). Their data suggest that NO reduces cardiac preload due to reduced venous return caused by venodilation, but afterload was not affected, suggesting that arterial tone was unaltered by NO in these embryos. However, no direct measurements of arterial tonus were made and therefore an effect on the arterial vasculature cannot be excluded. The involvement of NO in cardiovascular development has been studied in another fish larvae, the rainbow trout (7). However, in the experiments by Eddy et al. (7), the entire alevin was exposed to isosorbide dinitrate (NO donor) for up to 4 wk, and therefore the effects on the vascular and cardiac parameters could be indirect effects on the cardiovascular, respiratory, or other physiological systems.

In mammals, the low resistance of the pulmonary vasculature before birth is mediated by the release of NO from the endothelium (8, 34). In the fetal lamb, for example, inhibition of the endogenous production of NO leads to marked physiological changes that are similar to persistent pulmonary hypertension of the newborn (8). In contrast, an excess production of NO can lead to circulatory shock due to abnormal vasodilation of the systemic vasculature. Nevertheless, we do not know how a chronic increase in NO production will affect the morphological or physiological development of the systemic vasculature. NO could also be important in regulating the cardiovascular system already early in development when other control systems are scarce or lacking.

No effect of epinephrine on the dorsal vein and artery diameter was observed in 5- or 6-day-old zebrafish larvae. The lack of response to epinephrine could be due to (apart from the lack of adrenergic receptors) a high vasodilatory tonus on the vessel. Because we found a strong NO-mediated vasodilation on the zebrafish vasculature, we tested whether an effect of epinephrine could be seen after blocking this vasodilation. The preincubation with L-NAME (blocking the NO-mediated vasodilation) before injecting epinephrine resulted in a vasoconstrictory effect of epinephrine in both the dorsal vein and artery (Fig. 7).

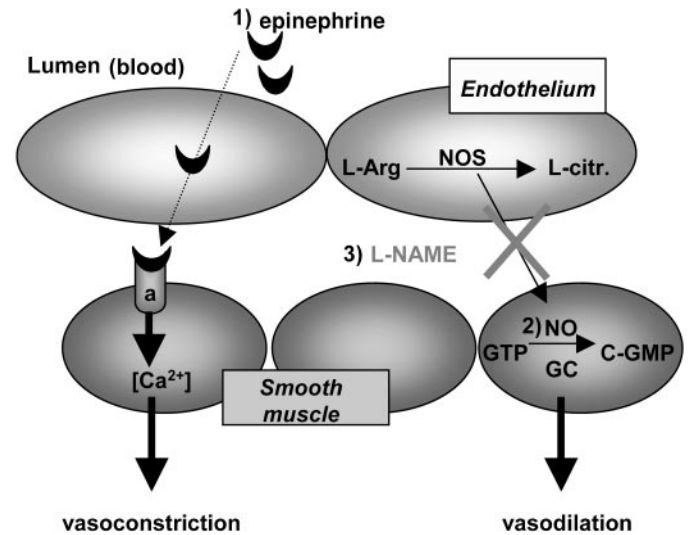


Fig. 7. Schematic drawing showing the possible action of NO and epinephrine on vascular smooth muscle of the larval zebrafish before a functional innervation of the peripheral vascular system. 1) Epinephrine possibly released from chromaffin cells in the larvae can act in a paracrine or endocrine way on α -receptors (a) on the smooth muscles thereby causing vasoconstriction. 2) NOS contained in the endothelium can synthesize NO from L-arginine (L-Arg), and NO diffuses to the neighboring smooth muscle cells and causes vasodilation. 3) L-NAME blocks the effect of endogenously produced NO by competing with L-Arg for NOS. L-Cit, L-citrulline; GC, guanylate cyclase. See text for further discussion.

Epinephrine, after preincubation with L-NAME, also produced a strong reduction in the number of erythrocytes passing into the intersegmental artery. Whereas L-NAME caused a 7% reduction in the number of erythrocytes passing into the intersegmental vessel, subsequent application of epinephrine resulted in a decrease by almost 50%. Compared with this effect on the number of erythrocytes passing into the segmental vessel, the decrease in the diameter of the dorsal artery was small. It therefore appears to be unlikely that the decrease in vessel diameter could explain the changes in erythrocyte distribution in the segmental vessel, especially if the concomitant increase in cardiac output is taken into account. In older larvae of the zebrafish (8 days old), the existence of a "sphincter" at the entrance of the intersegmental vessel, which significantly constricted after application of the α -adrenergic agonist phenylephrine, has been demonstrated (32). Although changes in diameter of this "sphincter" were not obvious in our experiments on earlier stages, our results show that the peripheral vascular system of the zebrafish does respond to adrenergic stimulation.

The observed increase in cardiac output in 3- and 4-day-old zebrafish larvae is in contrast to the results presented for larval *Xenopus* (19). Whereas an adrenergic tonus on the heart was also shown to exist in Nieuwkoop Faber stage 40 *Xenopus* larvae, application of epinephrine caused no significant changes in cardiac activity in this species. In zebrafish the epinephrine induced increase in cardiac output was solely due to increases in heart rate, whereas stroke volume remained unchanged. Catecholamines are widely distrib-

uted in vertebrate animals and are present during the early stages of development in all species studied up to date (6, 12, 17, 18, 20, 22). Thus it is likely that epinephrine stored in chromaffin cells as well as NO released from endothelial cells are involved in regulating the vascular tonus and cardiac activity in developing animals.

In conclusion, we have shown that both the venous and arterial vasculature of the 5- and 6-day-old zebrafish embryos are influenced by endogenously produced NO and that NOS immunoreactivity is present in the endothelial cells of the dorsal vein and in the heart. In addition, our results demonstrate the presence of epinephrine-sensitive receptors on the vasculature as well as on the heart. Thus this study provides increasing evidence that early in development when autonomic nerve fibers are scarce or lacking, vascular tone in larval tissues is regulated by a complex interaction of vasoactive substances that are produced locally by vascular endothelial cells and by circulating or endogenous catecholamines.

Perspectives

Studies on cardiovascular function in embryos have only recently become possible thanks to improvement and development of different microtechniques. With the recent identification of a vast number of genes possibly involved in cardiovascular development, an enormous future need to understand the function of these genes is obvious. We have used a novel model, the zebrafish (*Danio rerio*), to study functional development of the cardiovascular system. Most gene sequences in this species are known, and there are numerous cardiac mutants available in which the relationship between a changing morphology and the physiology can be studied. However, before we can determine the consequences of a changing morphology on the physiology, we need to understand the "normal" functional cardiovascular development. With the use of the technique described in this paper, the functional development of the cardiovascular system and its control system can be readily investigated.

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