The Journal of Experimental Biology 207, 3339-3348
Published by The Company of Biologists 2004
doi:10.1242/jeb.01164

Structural basis for control of secondary vessels in the long-finned eel *Anguilla reinhardtii*

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Accepted 24 June 2004

Summary

Histological sections of primary segmental arteries and associated interarterial anastomoses and secondary vessels from the long-finned eel *Anguilla reinhardtii* were examined by light and transmission electron microscopy. Interarterial anastomoses were found to originate from the primary vasculature as depressions through the tunica intima and media, from where they ran perpendicularly to the adventitial layer, before coiling extensively. From here the anastomoses travelled a relatively linear path in the outer margin of the adventitia to anastomose with a secondary vessel running in parallel with the primary counterpart. In contrast to findings from other species, secondary vessels had a structure quite similar to that of primary vessels; they were lined by endothelial cells on a continuous basement membrane, with a single layer of smooth muscle cells surrounding the vessel. Smooth muscle cells were also found in the vicinity of interarterial anastomoses in the adventitia, but these appeared more longitudinally orientated. The presence of smooth muscle cells on all aspects of the secondary circulation suggests that this vascular system is regulated in a similar manner as the primary vascular system. Because interarterial anastomoses are structurally integrated with the primary vessel from which they originate, it is anticipated that flow through secondary vessels to some extent is affected by the vascular tone of the primary vessel. Immunohistochemical studies showed that primary segmental arteries displayed moderate immunoreactivity to antibodies against 5-hydroxytryptamine and substance P, while interarterial anastomoses and secondary vessels showed dense immunoreactivity. No immunoreactivity was observed on primary or secondary arteries against neuropeptide Y or calcitonin gene-related peptide.

Key words: secondary vessel, interarterial anastomoses, immunohistochemistry, control, structure, organisation, long-finned eel, *Anguilla reinhardtii*.

Introduction

The presence of a secondary vascular system in a number of fishes has been well documented (Vogel, 1981, 1985a,b; Vogel and Claviez, 1981; Steffensen et al., 1986; Lahnsteiner et al., 1990; Dewar et al., 1994; Ishimatsu et al., 1995; Chopin and Bennett, 1996; Olson, 1996; Chopin et al., 1998; Skov and Bennett, 2004). Vascular casts have revealed that secondary vessels originate through interarterial anastomoses that emerge from the majority of the systemic arteries – the caudal aorta, the segmental arteries and the subclavian, carotid, efferent branchial, pseudobranchial and mandibular arteries (Skov and Bennett, 2004). In *Anguilla reinhardtii*, interarterial anastomoses occur at high densities on up to third order branches of the segmental arteries (Skov and Bennett, 2004). Compared with their primary counterpart, secondary vessels arising from branches of the segmental artery run retrograde towards a large secondary vessel that runs in parallel to the main segmental trunk, with which it anastomoses. From here, the secondary vessels run normograde, projecting towards the surface of the animal. It is generally accepted that these vessels give rise to secondary capillary beds in the skin of the trunk and or fins, as has been demonstrated *in vivo* for the glass catfish *Kryptopterus bicirrhis* (Steffensen et al., 1986), through dye injection studies in the Mozambique tilapia *Oreochromis mossambicus* (Vogel, 1985a) and by vascular casting in the eel-tail catfish *Tandanus tandanus* (Chopin and Bennett, 1996). These capillary beds drain into the lateral, dorsal and ventral collecting vessels, which eventually empty into the primary circulation at the caudal heart or *via* the duct of Cuvier (Vogel, 1985a).

The ability of teleost fishes to regulate perfusion of different vascular beds is well documented, and is believed to be mediated predominantly by actions of the autonomic nervous system (for reviews, see Nilsson and Holmgren, 1993; Morris and Nilsson, 1994; Holmgren, 1995; Donald, 1997). The control of the coeliaco-mesenteric circulation has received considerable interest, and is under both adrenergic (Axelsson

et al., 1989, 2000; Sverdrup et al., 1994) and neuropeptidergic control (Holmgren et al., 1985; Jensen et al., 1991; Kågström et al., 1994, 1996; Kågström and Holmgren, 1997, 1998; Domeneghini et al., 2000). The control of the systemic vascular smooth muscle has received little attention in terms of the possible involvement of non-adrenergic non-cholinergic (NANC) transmitters.

The involvement of both neural and humoral aminergic transmitters in the control of the primary systemic vasculature is well documented (Wahlqvist, 1980; Wahlqvist and Nilsson 1980, 1981), while information on the control of the secondary vascular system is very limited. Speculation regarding the regulatory capacity of the secondary vascular system has arisen not only due to difficulties in obtaining high quality vascular casts, but more so in relation the physiological significance of this vessel system. Secondary vessels are currently believed to be under adrenergic control at least, partly because the administration of smooth muscle relaxants appears to have some positive impact on the degree of filling by vascular casting agents (Vogel, 1985a). This was supported by the findings of Chopin and Bennett (1996), who observed tyrosine-hydroxylase immunoreactivity in the vicinity of secondary vessels, which led them to suggest that adrenergic nerves contribute in the regulation of vascular tone in this vascular system.

The aim of the present study was, *via* conventional histological procedures and transmission electron microscopy (TEM), to visualise the structure of the vascular wall of primary and secondary vessels of the systemic circulation. A particular goal was to evaluate the degree of association of smooth muscle cells with interarterial anastomoses and secondary vessels, in order to assess the structural capacity for this vascular system to regulate flow. The possible involvement of a suite of NANC components in the control of this vascular system was also investigated immunohistochemically.

Materials and methods

Six long-finned eels *Anguilla reinhardtii* Steindachner 1867, body mass 1.82–4.38 kg, total length 91–123 cm, were caught by hand-net or hand-line from the lake at the University of Queensland's St Lucia campus, and transported to the laboratory under aeration.

Animals were anaesthetised, heparinised and cannulated as described previously (Skov and Bennett, 2004), except that no adrenergic blocker was administered. In brief, the ventral aorta was cannulated *via* the atrium, and the animal perfused with 300–1000 ml cold (4°C) heparinised (20 IU ml^{-1}) saline (0.9% NaCl) until the effluent was clear of any red blood cells, and subsequently perfusion-fixed with 300–500 ml cold, freshly prepared, 2% paraformaldehyde (PFA). For histological and immunohistochemical examination, cutaneous collecting vessels, skin tissue and segmental arteries and veins with a minimum of surrounding tissue, were dissected free and post-fixed in 4% PFA at 4°C overnight. The following day tissues were rinsed in three 30 min changes of cold 0.1 mol l^{-1} phosphate-buffered saline (PBS 0.9% NaCl, pH 7.2).

Scanning electron microscopy

A number of segmental arteries were carefully dissected free of surrounding tissues for scanning electron microscopy (SEM). Using iris scissors, the vessels were opened along their longitudinal axis, and pinned flat to a piece of balsa wood with the luminal side outwards. Vessels were dehydrated in a series of ethanol (70%, 95% and 3×100% for 1 h each) and critical-point dried by displacement of absolute alcohol with liquid carbon dioxide. Blood vessels were mounted on stubs using double-sided carbon tape, platinum coated and examined by scanning electron microscopy (Jeol 6400, Tokyo, Japan). Pictures were captured digitally at a resolution of 1024×768 pixels.

Transmission electron microscopy

For TEM, tissues containing segmental arteries or lateral collecting vessels (LCV) were fixed overnight in a solution of 2.5% glutaraldehyde and 4% PFA in 0.067 mol l^{-1} cacodylate buffer at 4°C, washed overnight in 0.1 mol l^{-1} sodium cacodylate (pH 7.2). Tissues were then post-fixed in 1:4 (v:v) 4% osmic acid and 0.1 mol l^{-1} sodium cacodylate, washed in several changes of distilled water and stained with 2% uranyl acetate for 30 min, and washed again. Tissues were then dehydrated in a graded series of ethanol (50, 70, 90, 95 and 100% for 10 min each, and then 1 h in 100%), cleared in 3 changes of propylene oxide (10 and 2×20 min), infiltrated in a 1:1 (v:w) mix of propylene oxide and Epon/Araldite mixture, containing 4 g Epon 812, 2 g Araldite, 4.2 g dodecenyl succinic anhydride (DDSA), 2.5 g nadic methyl anhydride (NMA) and 0.2 g benzyldimethylamine (BDMA) overnight, and then in two changes of 100% Epon/Araldite for 7 h each. Tissues were positioned in embedding trays, covered in Epon/Araldite and placed under vacuum at –30 mmHg for 1 h (Labec vacuum chamber, Laboratory Equipment Pty Ltd., Sidney, Australia), and then allowed to polymerise at 60°C for 24 h. Any irrelevant tissue was trimmed away from the block, which was then sectioned at 100 nm on an Ultracut E microtome (Reichert-Jung, Vienna, Austria), mounted on copper grids and stained with Reynolds lead citrate for 2 min using an LKB Ultrastain (LKB, Bromma, Sweden). Sections were examined using a transmission electron microscope (Jeol 1010, Japan), and images were captured digitally.

Histology

For histological examinations, tissues were dehydrated in a graded series of ethanol (70% for 2 h, 2×100% for 2 h and one change of 100% ethanol overnight). Cutaneous tissues were decalcified at room temperature (RT) for 30–60 min, in a 1:1 solution of 20% (w:v) sodium citrate and 45% formic acid (v:v) in distilled water, to reduce fractures in the section caused by cutting mineralised scales. Specimens with large amounts of adipose tissue (skin and cutaneous collecting vessels) were defatted in Carnoy's fixative (60% ethanol, 30% chloroform and 10% glacial acetic acid) for 2–3 h at RT prior to dehydration. All tissues were infiltrated in three overnight changes of glycol methacrylate (Technovit 7100 embedding

kit, Heraeus Kulzer GmbH, Wehrheim, Germany) solution I (Technovit 7100 base with 1 g hardener I per 100 ml), before being embedded in solution II (solution I plus 1 ml hardener II per 15 ml), all at 4°C.

Tissue blocks were sectioned to water at a thickness of 2–4 μm, collected on plain microscope slides, and dried at 60°C overnight. Sections were stained for 5 min in 1% Lissamine Fast Red in 1% acetic acid at RT, rinsed in water and differentiated for 3–5 min in 1% aqueous phosphomolybdic acid at 56°C, rinsed again and counterstained with 1.5% tartrazine in 1.5% acetic acid for 5 min at RT. Other sections were stained in Toluidine Blue for 1 min, and rinsed in running tapwater. Slides were air-dried overnight at RT, coverslips placed on top using DePeX as mounting medium, viewed and photographed on an Axiophot microscope (Zeiss, Oberkochen, Germany) using a Spot Insight colour camera (3.2.0. Diagnostic Instruments, Inc., Sterling Heights, MI, USA). Images were captured digitally using associated software (Spot v. 3.4.2. for MacOSX) at a 1600×1200 pixels resolution.

Immunohistochemistry

For immunohistochemistry tissues were embedded in glycol methacrylate (Technovit 8100 embedding kit; Heraeus Kulzer GmbH). Tissues were postfixed in Carnoy's fixative at 4°C for 2 h before being dehydrated in a series of ethanol (70% 2 h and 3×100% for 2 h each). Following dehydration, tissues were infiltrated with three overnight changes of solution I (Technovit 8100 base plus 0.6 g hardener I per 100 ml) at 4°C. Upon transfer to the last T-8100 solution, tissues were placed under vacuum (–30 mmHg) at RT for 1–2 h, depending on the size of the tissues. This removed any trapped air from within the tissues, ensuring thorough infiltration. Tissues were kept at 4°C overnight, before being embedded in plastic troughs with solution II (solution I plus 1 ml hardener II per 30 ml) and covered with acetate film. Blocks were allowed to polymerise overnight at 4°C, before being bonded onto histoblocks with Technovit 3040 (Heraeus Kulzer GmbH). Blocks were sectioned to water using a LKB 2218 Historange microtome (LKB) in 8 μm thick sections. Test sections were collected on plain slides, dried at 60°C until section had bonded, stained with Toluidine Blue for 1 min, washed in running tapwater, and dried again at 60°C. Sections were viewed using a microscope at 20–40× magnification (Zeiss BH-2) to verify that sections were properly cut and that the desired vessels were found within. If this was the case, ten consecutive sections were collected on SuperFrost Plus slides (Menzel-Gläser, Braunschweig, Germany), drained vertically and air-dried overnight at RT, and sections bordered with wax using a PAP pen (Dako, Glostrup, Denmark). After every tenth section, a test section was cut to verify that desired vessels were still found within the block. Slides that were not immediately processed were stored at 4°C.

Antigen retrieval was performed by treatment with 0.1% trypsin in Tris-buffered saline (TBS) for 30 min at RT. This reaction was stopped by washing in running distilled water for 5–10 min. Endogenous peroxidase was quenched by treating with freshly prepared 0.6% H_2O_2 in distilled water for 15 min at RT, before being washed in 3× 3 min changes of TBS. Sections were then incubated with 2% BSA (w:v) in TBS containing 0.3% Triton X-100 for 30 min at RT, flicked dry and incubated with primary antibodies overnight at RT (Table 1). Slides were washed for 3× 3 min in TBS, before being incubated with biotinylated goat anti-rabbit or goat anti-mouse IgG (Zymed, San Francisco, CA, USA) for 30–45 min at RT. After another three washes in TBS, sections were incubated with TRITC-conjugated streptavidin (Zymed) for 30 min at RT, washed in three changes of TBS, and mounted in carbonate-buffered glycerol (1 part 0.5 mol l^{-1} $NaHCO_3$, pH 8.4: 1 part glycerol). Rat colon, intestine or brain was used as positive controls for the antibodies, while substitution of primary antibodies with TBS served as negative controls. Fluorescence images were collected digitally using a confocal fluorescence microscope (Bio-Rad MRC 1024, Philadelphia, PA, USA).

Results

Critical point dried vessels

Scanning electron microscopy of critical-point dried vessels showed that all interarterial anastomoses are surrounded by numerous microvilli protruding into the lumen of the blood vessel from specialised endothelial cells. In most vessels examined, microvilli were extremely dense (Fig. 1A), completely obscuring the vessel wall. Numerous microvilli, typically 3–7 μm long, originated from a common specialised endothelial cell and protruded nearly perpendicularly into the lumen of the primary artery (Fig. 1B).

Histology

Histological sections of segmental arteries typically contained multiple origins of interarterial anastomoses within

Table 1. *Details of primary antibodies used*

Antibody	Code	Host	Target	Working dilution	Source	Positive control	Product number
Neuropeptide Y	NPY	Rabbit	Human, Rat	1:200	Peninsula Laboratories	Rat intestine	IHC7180
Calcitonin gene-related peptide	CGRP	Rabbit	Human	1:200	Peninsula Laboratories	Rat colon	IHC6012
5-hydroxytryptamine	5-HT	Rabbit	Human	1:50	Chemicon International	Rat intestine	AB938
Substance P	SP	Rabbit	Chicken	1:100	Chemicon International	Rat colon	AB962
Tyrosine hydroxylase	TH	Mouse	Mouse	1:200	Sigma-Aldrich	Rat brain	T 2928

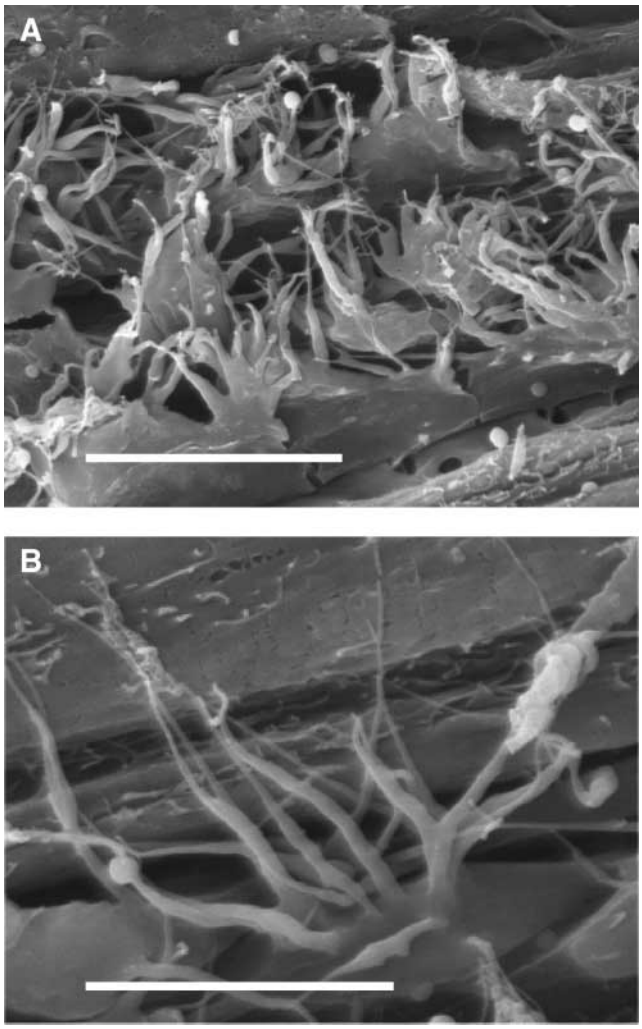

Fig. 1. (A) Scanning electron micrograph showing the dense arrangement of specialised endothelial cells bearing microvilli, which project into the lumen of the primary segmental artery. Scale bar, 10 µm. (B) High magnification illustrating the common origin of several microvilli from a single endothelial cell. Scale bar, 5 µm.

the same section. Each anastomosis originated as a depression through the endothelial and medial layer of the wall of the originating artery (Fig. 2). Within the adventitial layer of the vessel an extensive coiling of the anastomosis was apparent. The anastomosis consequently straightened and ran transversely along the exterior border of the adventitial layer, until it reached a secondary vessel running in parallel with the primary vessel, with which it re-anastomosed. Smooth muscle cells were associated with the opening of the anastomosis, with the most proximal part being continuous with the smooth muscle layer of the primary artery from which it originated (Fig. 2). In the most proximal part of the segmental artery, secondary vessels were observed as narrow-bore vessels running within the adventitia and, as secondary vessels re-anastomosed with each other, large bore vessels lying within a layer of adipose tissue, entirely outside the adventitia of the corresponding primary artery. There was commonly more than one secondary artery associated with a primary vessel. These were occasionally seen to join together (Fig. 3A–D), or divide to form several smaller vessels. Near the surface of the fish, wherever a segmental artery bifurcated, there was a corresponding bifurcation of the associated secondary vessel. After this bifurcation, secondary vessels gradually became spatially removed from the segmental artery, although interarterial anastomoses continued to arise from the primary vessel. These interarterial anastomoses re-anastomosed and periodically sent projections to the secondary vessel. Superficially, below the skin, secondary vessels ran parallel to the exterior surface of the animal.

The skin of *A. reinhardtii* was similar to that of other teleost species, with the dermis comprising a compact layer of dense connective tissue and a spongy layer of loose connective and adipose tissue. The epidermis consisted of a basal layer of germinative cells, covered by goblet and mucous cells and a final layer of covering cells. A dense layer of pigment cells covered the basal side of the compact layer. The compact layer itself was poorly vascularised, but large primary blood vessels could occasionally be seen within. Secondary vessels frequently penetrated the compact layer, traversing the width of the layer between bundles of collagen fibres, to exit between the scales of the spongy layer. The scales of *A. reinhardtii* sit within the spongy layer, and are covered by a dense network of blood vessels, that form a mesh running across the surface of the scales, with occasional hairpin loops projecting up between mucus cells in the vicinity of chloride cells. This vessel system drains *via* numerous collecting veins into the lateral collecting vessels (LCV), which lie in the compact layer of the dermis. The LCV ran within a tube of dense connective tissue made up from two opposed crescent-shaped structures. The gaps between these, as seen in cross-sections, faced dorsally and ventrally.

Transmission electron microscopy

The LCV was made up of a thick endothelial cell layer on an elastic lamina, surrounded by an adventitia composed of elastic fibres and loose connective tissues (Fig. 4A), and was completely free of smooth muscle cells (Fig. 4B).

One or two layers of smooth muscle cells surrounded the primary segmental arteries. The interarterial anastomoses in the adventitial layer, which was otherwise devoid of smooth muscle, were typically associated with a single smooth muscle cell (Fig. 5A), while secondary vessels were surrounded by a single layer of smooth muscle cells (Fig. 5B). Microvillous projections could be seen within the coils of the interarterial anastomoses, demonstrating that they are not restricted to the opening of the interarterial anastomoses or the wall of the originating primary segmental vessel. Segmental arteries, interarterial anastomoses and secondary vessels were all lined with a continuous basement membrane. Endothelial cells all possessed tight junctions. Structures that appeared to be secretory granules could be seen frequently within the wall of interarterial anastomoses.

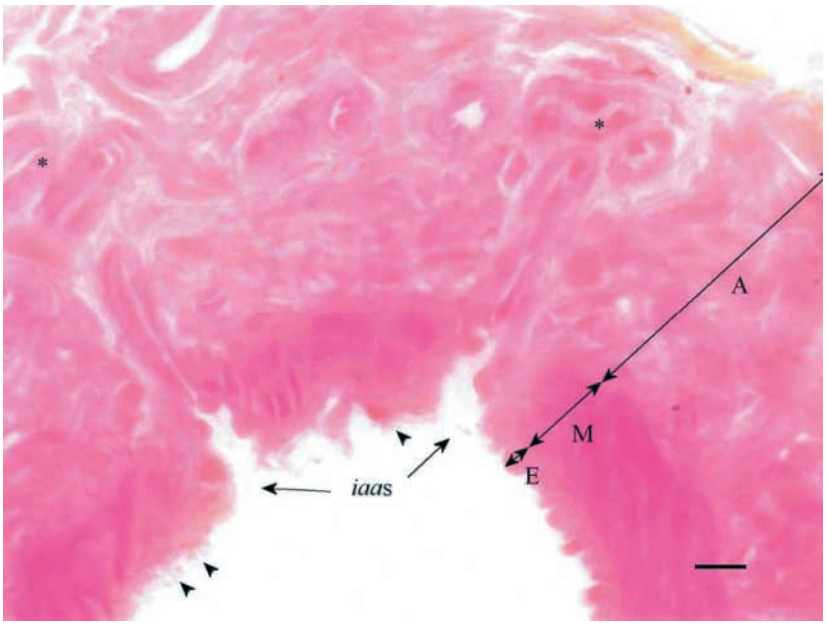

Fig. 2. Light micrograph of the origin of interarterial anastomoses (*iaa*s) from a segmental artery. Anastomoses originate as depressions through the endothelial (E) and medial (M) layer of the primary vessel. Anastomoses follow a linear perpendicular path to the outer margin of the adventitial (A) layer, where they coil extensively (*). Note the numerous microvilli (arrowheads). Scale bar, 10 μm. Lissamine Fast Red, 4 μm section.

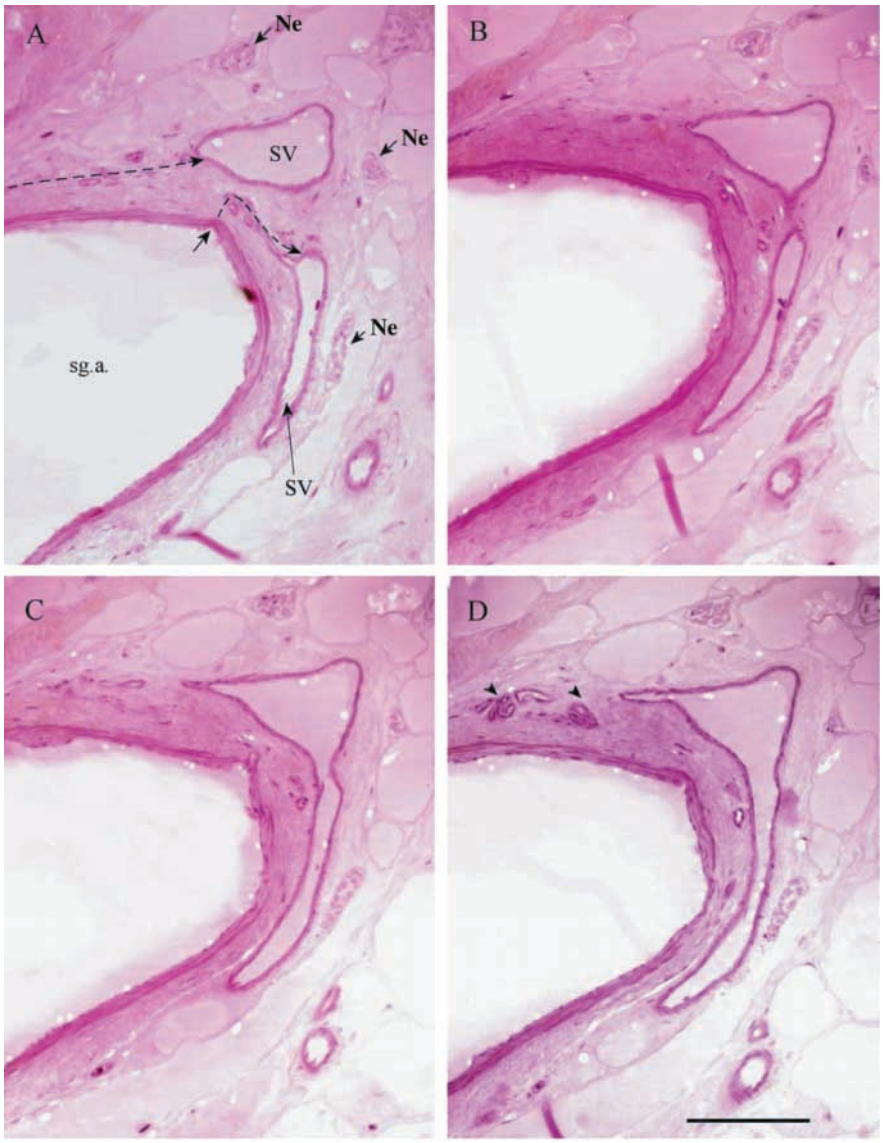

Fig. 3. (A–D) Light micrographs of consecutive sections through a segmental artery (sg.a.) taken 8 μm apart, showing the connection between interarterial anastomoses (*iaa*s) and secondary vessels (SV). The vessel path from the coiled region of *iaa*s (arrowheads in D) is denoted by stippled arrows in A. Note the numerous nerve bundles (Ne) in association with secondary vessels. Scale bar, 100 μm. Lissamine Fast Red, 2 μm section.

Immunohistochemistry

Some substance P-like immunoreactivity (SP-like IR) was observed in the adventitial layer of primary segmental arteries, while both interarterial anastomoses and secondary vessels stained quite heavily. Similarly, 5-HT-like IR was observed around segmental arteries, as well as interarterial anastomoses and secondary vessels (Fig. 6). No IR against any of the antibodies used was observed on segmental veins (Table 2). In addition, no calcitonin gene-related peptide (CGRP) or neuropeptide Y (NPY)-like IR was observed for segmental arteries, interarterial anastomoses or secondary vessels, but CGRP-like IR fibres were observed in association with blood vessels between the epidermis and dermis, above the scales. No IR was observed for SP, 5-HT or NPY in the subepithelial vessels.

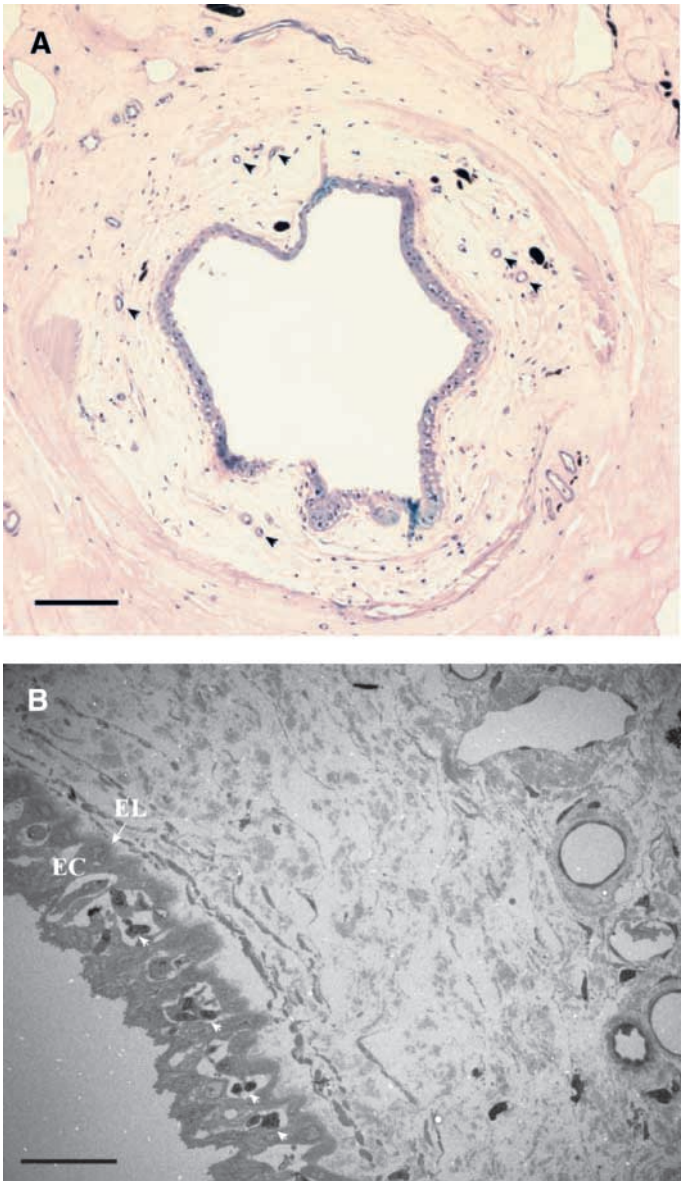

Fig. 4. (A) Light micrograph of lateral collecting vessel, showing a thick endothelial layer, but no distinct medial layer. The vessel sits within a tube of dense connective tissue, formed by two opposed crescent-shaped structures. Numerous blood vessels are readily observed within the 'adventitial' layer (arrowheads). Scale bar, 100 µm. Lissamine Fast Red, 5 µm section. (B) Transmission electron micrograph of the lateral collecting vessel shows that the endothelial cell layer (EC) sits on an elastic lamina (EL), and there is no presence of smooth muscle cells in the vessel. Leukocytes can be seen migrating through the endothelial layer (arrowheads). Blood vessels are frequently seen within the adventitial layer of the vessel wall. Scale bar, 20 µm.

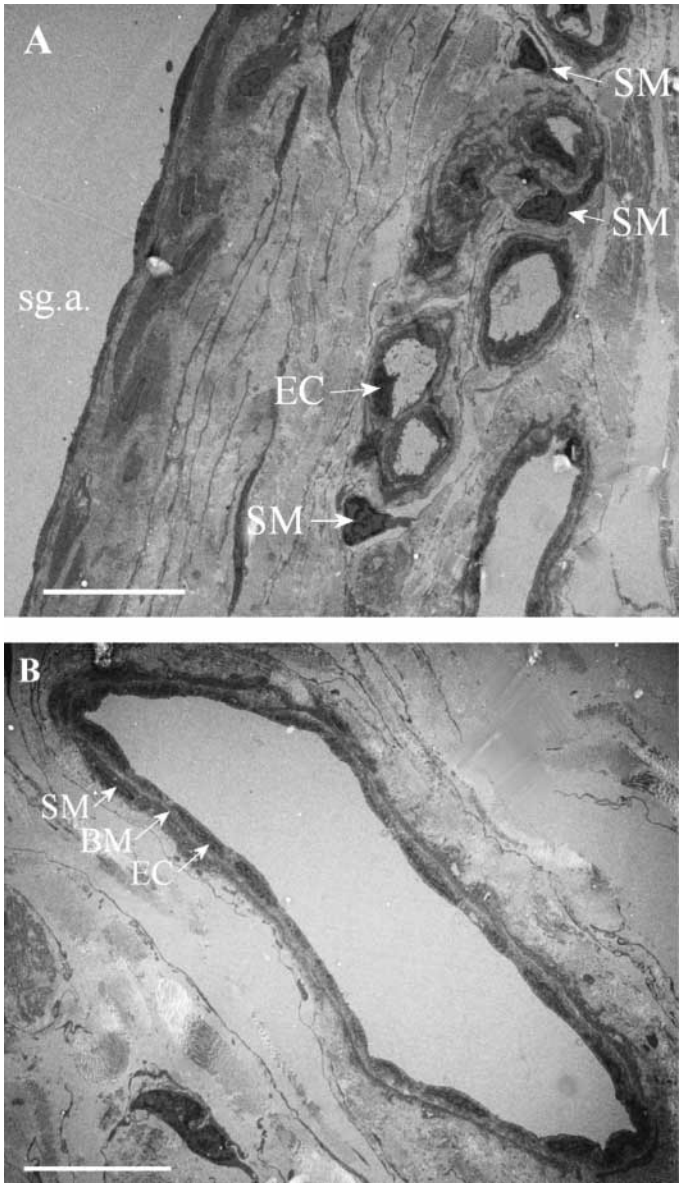

Fig. 5. (A) Transmission electron micrograph of the wall of a primary segmental artery (sg.a.) showing the structure in a coil from an anastomosis. Here, the vessel lumen is lined by a single endothelial cell (EC) on a continuous basement membrane, and is typically only associated with a single smooth muscle cell (SM). Scale bar, 10 µm. (B) This is also the case for secondary vessels, which in all aspects have a structure quite similar to that of primary vessels. Scale bar, 10 µm.

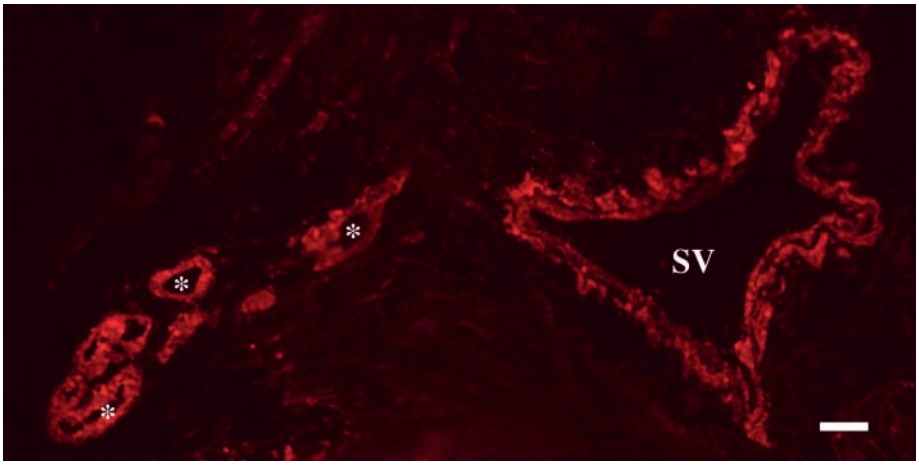

Fig. 6. 5-HT immunoreactivity of interarterial anastomoses *iaa*s (*) and a secondary vessel (SV).

Discussion

Histological sections taken from *A. reinhardtii* showed a vascular organisation of secondary vessels consistent with the findings from vascular casts (Skov and Bennett, 2004). A secondary vessel follows the main segmental trunk within the myoseptum, towards the surface of the animal. As the main vessel bifurcates, so does the secondary vessel, gradually removing itself from the primary vessel, to travel within the adipose tissue beneath the dermis. At regular intervals, secondary vessels project through the dermis into the spongy layer, where they give rise to dense capillary beds overlying the scales. A lack of discrete morphological characteristics at the light microscopy level results in an inability to distinguish between primary and secondary vessels in the periphery, unless as here, the paths of individual vessels are followed through serial sections. An alternative approach to verify this structural arrangement would be desirable.

The presence of specialised endothelial cells bearing numerous microvilli has previously been described in the giant gourami *Osphronemus gorami* (Vogel and Claviez, 1981). In the present study, interarterial anastomoses were so dense that these microvilli completely covered the vessel wall, and were also found within the interarterial anastomoses. The physiological significance of these microvilli has not been unequivocally established, but several proposals have been made. Vogel and Claviez (1981) suggested that the microvilli on the luminal surface of the primary artery might be involved in plasma skimming; a suggestion supported by the *in vivo* observation on the glass catfish *Kryptopterus bichirrhis* (Steffensen et al., 1986). Vogel and Claviez (1981) also suggested that these microvilli were involved in the selective recruitment of leucocytes into the secondary vascular system (SVS), presumably by specialised adhesion molecules. Lahnsteiner et al. (1990) observed that leucocytes appeared to adhere to the microvilli within the segmental artery, and this possible function would explain the presence of microvillous endothelial cells within the *iaa*s.

Table 2. *Observed immunoreactivity on the vascular system of* A. reinhardtii

	Segmental veins	Primary arteries	Interarterial anastomoses	Secondary vessels	Cutaneous vessels
NPY	–	–	–	–	–
CGRP	–	–	–	–	+
5-HT	–	+	+	+	–
SP	–	+	+	+	–
TH	–	–	–	–	–

Immunoreactivity absent (–) or present (+).
For abbreviations, see Table 1.

The organisation of the wall of the primary artery is similar to that of other vertebrates, with an obvious division into a tunica intima, media and adventitia, although the medial layer in *A. reinhardtii* is less well developed than in terrestrial vertebrates. This appears to be a common trait for teleosts, and it has been suggested that because fishes live in a fluid medium of a density similar to that of the body, they have a lower blood pressure than terrestrial vertebrates, thus the degree of muscularity required to maintain or regulate vascular tone is not as great (Satchell, 1991, 1992). Light and transmission electron microscopy showed that smooth muscle cells were associated with all interarterial anastomoses, and that the secondary vessels themselves are surrounded by a layer of smooth muscle, similar to vessels of the primary circulation. This is not in agreement with the findings of Lahnsteiner et al. (1990), who reported that secondary vessels in *Salaria pavo* and *Zosterisessor ophiocephalus* lack smooth muscle cells, and demonstrated that both these components of the secondary vascular system in *A. reinhardtii* possess the structural requirements for regulating flow. This discrepancy in observations may be the result of differences in morphology of the secondary vascular system between the species in question. Secondary vessels are most commonly thought to give rise to capillary beds at the surface of the animal (Vogel, 1981; Steffensen et al., 1986; present study) but in some species it has been reported that they do not (Lahnsteiner et al., 1990; Chopin and Bennett, 1996). The finding by Lahnsteiner et al. (1990) that secondary vessels lack a basal membrane is also in contrast to our observations, where a continuous basement

membrane could be clearly distinguished in transmission electron micrographs of secondary vessels (Fig. 5B).

In theory, there are a number of possible sites for regulation of flow to the secondary vascular system; the interarterial anastomoses, secondary vessels, secondary capillaries or arterioles associated with these, or even secondary venous vessels. An immediate question that arises from viewing the histological sections is – to what extent is flow to the secondary vessels correlated with vascular tone in the corresponding primary artery? As interarterial anastomoses are an integrated part of the vessel from which they originate, both in terms of sharing smooth muscle cells, and also with respect to their physical position within the vessel wall, it seems unlikely that constriction of the segmental artery can occur without a significant reduction in the luminal dimensions of the interarterial anastomoses (see Fig. 2). In contrast, an increased flow through segmental vessels may not necessarily be accompanied by an increase in flow through secondary vessels, if they are able to regulate their luminal diameter independent of primary vascular tone. The regulation of arterial flow by changes in venous tone was, for a long time, not considered to play a significant role in teleost physiology, considering the minute amount of smooth muscle and lack of innervation of venous vessels (Satchell, 1992). Despite this, Conklin and Olson (1994) demonstrated that the duct of Cuvier, the cardinal vein and the intestinal vein from *Oncorhynchus mykiss* were highly responsive to vasoactive substances, in a dose-dependant manner. Thus, flow and perfusion pressure through the arterial system may be affected by a change in venous tone. To what extent this applies to the secondary vascular system was not considered in this study, but the histological observations and TEM of the LCV do not lend support to the idea that any regulation occurs at this level. Although secondary vessels empty into the caudal vein and the duct of Cuvier, any estimate on the effect of contraction of these vessels on the flow through secondary vessels would be speculative at best.

Immunoreactivity against SP and 5-HT observed around primary arteries, interarterial anastomoses and secondary vessels provide direct evidence for the presence of NANC neural transmitters in the control of blood flow through arteries of both the primary and secondary vascular system in *A. reinhardtii*. To our knowledge the presence of SP or 5-HT-like immunoreactivity has not previously been demonstrated for systemic blood vessels in teleosts, although the vascular effects of 5-HT have been widely investigated in a number of species. It appears that 5-HT induces vasoconstriction of the branchial respiratory pathway, while it has a dilatory effect on the systemic vasculature. Vasoconstriction of the branchial vasculature is mediated by either 5-HT_1 or 5-HT_2 receptors, since its effects can be abolished by the administration of methysergide, a general 5-HT_1 and 5-HT_2 receptor antagonist (Fritsche et al., 1992; Sundin et al., 1995, 1998; Janvier et al., 1996; Sundin and Nilsson, 2000). Sundin et al. (1998) showed that branchial vasoconstriction is completely abolished by LY53857, a specific 5-HT_2 antagonist, indicating that this is the sole 5-HT receptor in this vascular bed. However, the receptors and signaling pathways in the vasodilatation of the systemic vasculature is less clear. In the coronary circulation of *O. mykiss*, 5-HT produces a dose-dependant vasodilatation, an effect that could be abolished by the addition of L-NA (N^{ω}-nitro-L-arginine), a nitric oxide synthase (NOS) inhibitor (Mustafa et al., 1997). In contrast, Farrell and Johnson (1995) reported that although 5-HT induced vasodilatation in isolated coronary ring preparations from *O. mykiss*, this effect was endothelium independent. In addition, a number of studies have addressed the physiological effects of 5-HT *in vivo*. The application of methysergide does not abolish the observed drop in systemic resistance, demonstrating that other 5-HT receptor subtypes mediate this vasodilatation. Janvier et al. (1996) showed that a 5-HT mediated drop in systemic resistance in *A. anguilla* could not be abolished by the administration of antagonists for any of the four known mammalian 5-HT receptor subtypes. Similar results have been obtained from *G. morhua* (L. Sundin, personal communication). Thus it appears that teleosts posses a suite of 5-HT receptor subtypes mediating vasodilatation, which are sufficiently different from their mammalian equivalents not to respond to mammalian antagonists (S. Holmgren, personal communication).

The available data on the effects of SP on the teleostean vasculature are limited, and somewhat contradictory. In mammals SP has been shown to decrease vascular resistance in numerous vascular beds (Dockray, 1994). In the gastrointestinal system of the Atlantic cod, mammalian SP has a dual effect, being a potent constrictor of intestinal smooth muscle (Holmgren et al., 1985; Jensen et al., 1987, 1991), thereby increasing gastrointestinal motility, but also a potent vasodilator of intestinal arteries (Jensen et al., 1991). Kågström et al. (1996) examined the *in vivo* effects in *O. mykiss* of trout SP (tSP), which has three amino acid substitutions compared with mammalian SP (Jensen and Conlon, 1992), and found that it caused a significant increase in both systemic and coeliac resistance. In contrast to the effects of mammalian SP on coronary arteries (Farrell and Johnson, 1995), tSP had no vasodilator effect on relaxed or precontracted small gut arteries (Kågström and Holmgren, 1998). Based on this limited data set, it is difficult to interpret the presence of SP-like immunoreactivity or the signalling pathways involved in segmental and secondary vessels in *A. reinhardtii*.

The effects of circulating adrenaline, noradrenaline and isoprenaline on flow rates through the isolated tail of *G. morhua* have been well documented (Wahlqvist, 1980; Wahlqvist and Nilsson, 1980, 1981), as has the neural component of this system (Wahlqvist and Nilsson, 1981). However, immunohistochemistry against tyrosine hydroxylase, the enzymatic precursor to adrenaline, did not reveal any adrenergic innervation. This is in contrast to previous findings by Chopin and Bennett (1996), which showed the presence of tyrosine-hydroxylase immunoreactive fibres in the periphery of secondary vessels in *Arius graeffei*. This cannot necessarily be interpreted as the absence of that vasoactive substance, but may merely imply lack of cross-

reactivity of the antibody (Holmgren, 1995; Kågström and Holmgren, 1998). This could potentially also explain the negative results for NPY, and stresses the need for a perfusion protocol for secondary vessels that would allow for an assessment of the potency and effects of various vasoactive substances.

The presence of CGRP-like IR on capillaries of the secondary vascular system, implies that regulation of flow through the secondary vascular system may also occur at this level. The presence of CGRP has been demonstrated by immunohistochemical methods in a number of systemic, non-visceral, vascular beds from numerous species across divergent phyla (mammals, fishes, amphibians, molluscs etc.). Intradermal injections of CGRP have demonstrated its effects as a potent vasodilator at the microvascular level in human (Brain et al., 1985), rabbit (Brain and Williams, 1985) and rat skin (Chu et al., 2001). Perfusion studies on the rainbow trout gut have shown that CGRP is a potent endothelium-independent vasodilator of adrenaline-precontracted gut arteries from *O. mykiss*, whose effect is mediated by the CGRP-1 receptor (Kågström and Holmgren, 1998).

The findings presented here demonstrate that secondary vessels possess the structural requirements for independent regulation of flow, and are consistent with the hypothesis that a suite of NANC neurotransmitters contribute in the regulation of blood flow through the secondary vascular system. A better knowledge of the control mechanisms of the secondary vascular system would bring us closer to understanding the physiological and physical conditions under which the secondary vascular system may contribute to the overall physiology of actinopterygian fishes.

The Danish Research Agency, the University of Queensland and the Elisabeth and Knud Petersen Foundation are gratefully acknowledged for their financial support of this study. Fish were collected under a Queensland Government Department of Primary Industries, General Fisheries Permit number PRM02030C. The experiments were approved by the University of Queensland Ethics Committee, UEAC permit number ANATDB/468/03/PHD.

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