Acute defense mechanisms against hemorrhage from mechanical gill injury in rainbow trout

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Sundin, Lena, and Göran E. Nilsson. Acute defense mechanisms against hemorrhage from mechanical gill injury in rainbow trout. Am. J. Physiol. 275 (Regulatory Integrative Comp. Physiol. 44): R460–R465, 1998.—By cutting gill filaments in anesthetized rainbow trout (Oncorhynchus mykiss), observing the bleeding through a stereomicroscope, and using blockers of various known endogenous filament artery vasoconstrictors, we have here attempted to characterize hemostatic mechanisms in gills. The immediate hemostatic response to a cut in a gill filament artery was a local vasoconstriction, stopping the hemorrhage within ~20 s. In heparinized fish, the hemorrhage recommenced after ~8 min, suggesting that the vasoconstriction soon subsides and blood clotting becomes responsible for the hemostasis. Antagonists of acetylcholine, adenosine, and serotonin receptors were unable to block the hemostatic vasoconstriction. Also, tetrodotoxin was without effect, indicating a nonnervous origin. By contrast, indomethacin significantly affected the measured bleeding times, suggesting that eicosanoids play a significant role in this process (possibly by stimulating vasoconstriction and/or by inducing local thromboocyte aggregation). By possessing several hundred virtually identical filaments with readily observable vasculature, the fish gill appears to be a good experimental model for studying hemostatic mechanisms.

Materials and Methods

Acute defense mechanisms against hemorrhage from mechanical gill injury in rainbow trout. These are serotonin (5-HT), AcH (17, 19), and adenosine (1, 18). Other possible mechanisms include the release of endothelium-derived constricting factors such as cyclooxygenase products of arachidonic acid metabolism (eicosanoids) and endothelin. With regard to the former, an effective vasoconstricting eicosanoid in mammals as well as fish is thromboxane A2 (2, 7), and another is prostaglandin F2α, which in fish has been shown to constrict coronary arteries (3, 4). Endothelin is less likely to be involved in the protection against gill hemorrhage. Although endothelin has been found to constrict some blood vessels in rainbow trout (14, 16), it does not constrict filament arteries in trout gills (Sundin and Nilsson, unpublished observations).

The present study was undertaken to examine the hemostatic events of the branchial vasculature in response to mechanical trauma in anesthetized rainbow trout, and efforts were made to resolve the mechanisms involved.

MATERIALS AND METHODS

Animals

Rainbow trout (Oncorhynchus mykiss), body mass 110–370 g, were obtained from a fish farm near Oslo. They were kept indoors on a 12:12-h light-dark cycle in tanks continuously supplied with dechlorinated Oslo tap water (5–8°C) at the Department of Biology in Oslo for at least 4 mo before the experiments, which were conducted in January and February.

Drugs

The drugs used were 8-cyclopentyltheophylline (CPT) (from Research Biochemicals International, Natick, MA), methyseryglide dimaleate (from Sandz, Basel, Switzerland), and benzocaine, tetrodotoxin (TTX), indomethacin, and atropine (all from Sigma).

Experimental Setups

In vivo experiments. The fish (n = 42) were anesthetized by addition of benzocaine (as a 5% solution in ethanol) to the water to a final concentration of 100 mg/l. They were then transferred to the experimental room (5–7°C) and placed on their sides in a cradle made of chicken wire suspended inside a semicircular Plexiglas box. Each fish was respired with aerated tap water (5–7°C) from a closed system (containing 50 mg benzocaine/l). The water was flowing over the gills at a rate of 400 ml/min through a tube that was inserted into the mouth. The outer one-half of the left operculum was removed, and the gills were observed through a Leica MZ12 stereomicroscope (the view being exemplified in Fig. 1). Mechanical gill damage was induced by cutting off the tips of gill filaments with a pair of microsurgery scissors. The water...
carried the flow of blood away from the cut filament, making it easy to determine diminished and recommenced bleeding. The animals were left for ~20 min before any experiments were conducted. Care was taken to keep the fish moist.

To obtain the high magnification pictures shown in Fig. 2, we observed the gills through a Leitz Ortholux/Ultrapak epi-illumination microscope, as described previously (20). Because of the short working distance of this microscope, the objective had to be retracted from the fish during cutting. Therefore, we had to use the stereomicroscope (see above) to record bleeding times after cutting filaments. A Sony digital video camera (DCR-PC7E) was attached to both microscopes, and video frames were digitized with Avid VideoShop 3.0.2 and processed with Adobe Photoshop 3.0 on a Macintosh 7500/100.

Possible changes in ventral aortic blood pressure were monitored in three animals to be treated with indomethacin, an antagonist that we have not previously used in anesthetized rainbow trout. A cannula (PE-50) filled with heparinized (100 IU/ml) 0.9% NaCl was inserted into the left afferent branchial artery of the third gill arch for measurement of afferent branchial blood pressure. The cannula was attached to a Gould Statham P23 Db pressure transducer calibrated against a static column of water. The transducer was connected to a Grass bridge amplifier.

In situ perfused branchial basket. The fish (n = 4) were anesthetized and injected with heparin (0.3 ml/kg, 100 IU/ml) into the caudal vein. The outer one-half of the left operculum was removed, and the fish was placed in the cradle to allow observation of the gills in exactly the same way as described in the in vivo experiments. The heart was exposed and transected between the ventricle and atrium. A flanged cannula (PE-160) was inserted into the ventricle, advanced forward into the ventral aorta, and secured with a suture. The cannula was attached to a container filled with Cortland salmonid saline (23) and raised to create a 4-kPa pressure head. The saline was colored with Evans blue (1 g/l) to allow the observation of saline flowing out from a cut filament. This preparation lasted for ~30 min, whereupon a significant reduction of flow in the filament arteries was seen, coinciding with a formation of blue granules that eventually occluded most of the microvasculature.

**Experimental Protocol**

Before most in vivo experiments, the fish were injected with heparin (0.3 ml/kg, 100 IU/ml) in the caudal vein to prevent blood clotting from having an effect on bleeding. About 3 mm of the tip of a filament was cut, and the time from cutting until bleeding stopped was denoted time to constriction. The time elapsing from this initial stop in bleeding until the bleeding recommenced was denoted duration of constriction. The bleeding stopped and recommenced at approximately the same time from afferent and efferent filament arteries, and no effort was made to separately record afferent and efferent bleeding. Eight filament tips were cut off from each fish in the control and treatment groups. It should be mentioned that a rainbow trout of the present size has ~1,000 gill filaments, so our experimental manipulations are unlikely to have had any systemic effects on the fish. The antagonists used were atropine (3 µmol/kg) to block muscarinic ACh receptors, methysergide (2.5 µmol/kg) to block 5-HT receptors, CPT (40 nmol/kg) to block adenosine A1 receptors, and indomethacin (14 µmol/kg) to block cyclooxygenase-mediated synthesis of eicosanoids. These doses have been found to be effective in previous studies on rainbow trout (15, 18–20). TTX (200 nmol/kg) was used to block voltage-gated Na⁺ channels and thus neurotransmission. TTX has a very high affinity for voltage-gated Na⁺ channels, the dissociation constant being around 1–10 nM (6). Thus 200 nmol TTX/kg should have a strong inhibitory effect on nervous conduction.

The time allowed to elapse from the injection of an antagonist to the cutting of filaments was 30 min for atropine,
methysergide, and indomethacin and 10 min for CPT. Previous studies using the listed antagonists (18–20) and ventral aortic pressure recordings from indomethacin-treated fish in this study (data not shown) show that the cardiovascular parameters have stabilized and returned to preinjection values during these time spans. Also, TTX was injected 10 min before cutting. After an initial slow down or stop in the gill circulation (as seen through the stereomicroscope), blood flow had resumed after 10 min. All antagonists were injected into the caudal vein in a volume of 1 ml/kg (110–370 µl/fish). The antagonists were dissolved in 0.9% NaCl, and controls were injected with this vehicle 30 min before the cutting of filaments. However, indomethacin had to be injected in 40% ethanol. A control group (n = 5) given 40% ethanol (1 ml/kg) 30 min before the cutting did not differ significantly from NaCl controls with regard to time to constriction (19.5 ± 1.8 s) or duration of the constriction (398 ± 76 s). This was expected because fish rapidly lose ethanol over the gills (22), and this group was therefore included in the control group.

Instead of using each fish as its own control, we used an independent control group to avoid possible effects of a prolonged blood loss from already cut filaments due to antagonist treatment of heparinized fish. However, because only the time to constriction was measured in the TTX-treated group of fish, these were not heparinized and therefore eight filaments were sequentially cut before and after TTX treatment, making each fish its own control. In addition, in six of the fish used in the control group, filaments were cut before the heparin treatment to find out whether heparin by itself affected the time to constriction.

For the in situ perfused branchial basket, six filaments were cut in each of four preparations to test whether the constriction occurred without the participation of blood. Because some eicosanoids are involved in thrombocyte aggregation in rainbow trout (9), we examined whether the fluidity of the blood was altered after indomethacin treatment. Therefore, at the end of the cutting experiments described above, ~3 ml of blood were drawn from the caudal vein in seven controls and in seven fish given indomethacin. The blood was poured into a glass Pasteur pipette vertically positioned with two marks placed 50 mm apart in the narrow portion of the pipette (inner diameter = 1.0 mm). The speed (in mm/s) by which the top of the blood column traveled this distance (recorded with a stopwatch) was taken as a index of fluidity and was plotted against the hematocrit (determined with a hematocrit centrifuge).

Data Treatment and Statistics

The time to constriction as well as the duration of constriction was calculated for each fish from the average times recorded from six to eight cut filaments. Data presented show means and SE, where one fish was regarded as one observation. Evaluations of statistically significant differences were made using ANOVA followed by Bonferroni adjusted unpaired Student’s t-tests (paired when testing for the effects of TTX and heparin, where each fish served as its own control). P < 0.05 was considered significant. Difference between

Fig. 2. Vasoconstriction of efferent filament arteries after a cut as shown by video frames taken through the epi-illumination microscope. In A (30 s after a cut), a portion of an artery has constricted. B and C (6 and 8 min after a cut, respectively) show how the same artery starts to relax, finally leading to a recommenced hemorrhage through the cut. In D, a different filament is shown 30 s after a cut. Vasoconstricted portion of this artery is very short, thereby allowing blood to flow through lamella closest to the cut. Width of each picture is 880 µm.
bleeding starting after this was always the case in heparinized fish, the recommenced after the initial vasoconstriction, whereas we could only in a few occasions observe that bleeding recommenced bleeding. In non-heparinized trout, recording the time elapsing between the arrested and measure the duration of filament artery constriction by the cut. The fish were heparinized to allow us to filament artery was seen in the immediate proximity to after some 20 s. At that time, a vasoconstriction of the blood was flowing out of the cut filament arteries was soon reduced (within 10 s) and the hemorrhage stopped.

The results show that trout have very effective means of preventing excessive hemorrhage inflicted by mechanical injury to the gill filaments. This is accomplished by a rapid vasoconstriction of both the afferent and the efferent filament arteries, which totally halted the bleeding within some 20 s. This appears to be a very fast hemostatic response. For example, the bleeding time in humans (from 1-mm-deep forearm incisions) is generally 4–5 min.

**RESULTS**

Cutting and observing the gill filaments through the stereomicroscope showed that the rate by which the blood was flowing out of the cut filament arteries was soon reduced (within 10 s) and the hemorrhage stopped after some 20 s. At that time, a vasoconstriction of the filament artery was seen in the immediate proximity to the cut. The fish were heparinized to allow us to measure the duration of filament artery constriction by recording the time elapsing between the arrested and the recommenced bleeding. In non-heparinized trout, we could only in a few occasions observe that bleeding recommenced after the initial vasoconstriction, whereas this was always the case in heparinized fish, the bleeding starting after ~8 min. Thus blood clotting becomes responsible for the hemostasis after the initial vasoconstriction has subsided. Figure 1 shows a typical experiment, as seen through the stereomicroscope: an initial vasoconstriction stops the bleeding 20 s after the filament has been cut. In Fig. 2, A–D, the vasoconstriction is illustrated at high magnification by images taken through the epi-illumination microscope. Fig. 2, A–C, shows a constricted filament artery, where the constricted portion starts to relax 6–8 min after a cut. Figure 2D shows a filament artery where the vasoconstriction is very narrow, actually allowing blood to flow through the lamellae closest to the cut (all illustrations from heparinized fish).

A second reason to heparinize the fish was to prevent blood clotting from affecting the measurements of the time taken for the vasoconstriction to stop blood from flowing out of the cut filament arteries (the “time to constriction”). However, in an initial experiment, we found that heparinization did not significantly affect the time to constriction. Thus the time to constriction was 17.6 ± 1.2 s before heparinization and 19.4 ± 2.2 s after heparinization in a group of six fish (NS, paired Student’s t-test). This suggests that blood clotting has no effect on the initial stop in bleeding from a cut filament artery. In fact, no other mechanism than vasoconstriction appears to be needed for stopping the bleeding initially, because vasoconstriction also occurred in the Cortland saline-perfused gills, the time to constriction being 21.9 ± 1.1 s (n = 6) in that preparation.

Figure 3A shows that indomethacin significantly prolonged the time to constriction from 19.0 ± 0.8 s (control) to 24.3 ± 2.3 s (P = 0.018). By contrast, none of the antagonists against three known endogenous constrictors of the filament arteries had any effect on the time to constriction. These antagonists were atropine (blocking muscarinic ACh receptors), methysergide (blocking 5-HT receptors), and CPT (blocking adenosine A1 receptors) (Fig. 3A). Even when the trout were injected with TTX, the vasoconstriction was not significantly affected, the time to constriction being 14.1 ± 0.9 and 13.2 ± 1.0 s before and after TTX injection, respectively (n = 4). This result appears to exclude the participation of nerves in the vasoconstriction.

The duration of the constriction was 435 ± 39 s in controls (Fig. 3B), and indomethacin was the only drug tested that significantly affected this measure, reducing it to 197 ± 41 s (P = 0.0006). Indomethacin is an inhibitor of eicosanoid synthesis (blocking cyclooxygenase). Because eicosanoids may play a role in aggregation of thrombocytes in fish (9), we tested whether or not indomethacin treatment could increase blood fluidity and thereby affect our measures of constriction times. Nevertheless, indomethacin treatment had no effects on blood fluidity. The fluidity was linearly correlated to the hematocrit, and the regression lines did not differ significantly between indomethacin-treated fish and controls (Fig. 4).

**DISCUSSION**

The results show that trout have very effective means of preventing excessive hemorrhage inflicted by mechanical injury to the gill filaments. This is accomplished by a rapid vasoconstriction of both the afferent and the efferent filament arteries, which totally halted the bleeding within some 20 s. This appears to be a very fast hemostatic response. For example, the bleeding time in humans (from 1-mm-deep forearm incisions) is generally 4–5 min.

The regression lines was tested using GraphPad Prism 2.0 for Macintosh.
sine, acting through A1 receptors, causes a general vasoconstriction which is effective as a vasoconstrictor in efferent, but not afferent, filament arteries (19). However, adenosine, acting through A1 receptors, causes a general vasoconstriction of both efferent and afferent filament arteries (18). Still, its involvement in the hemostatic response appears to be excluded by the fact that the A1 receptor blocker (CPT) was without effect on the hemostatic parameters measured.

In mammals there exists a sympathetic reflex compensation in shock that stimulates sympathetic vasoconstriction of arteries in most parts of the body. Still, the filament vasoconstriction persisted even after TTX treatment, which indicates that nerves are unlikely to be integrated in the response. Also, other circumstances argue against a nervous involvement. A sympathetic reflex compensation against hemorrhage from fish gills is unlikely because the branchial arterial vasculature mainly contains humorally stimulated dilatory adrenergic receptors (12, 21). Moreover, the constriction occurs even after only a minute blood loss from 1 out of ~1,000 gill filaments, an injury that could not cause a fall in blood pressure and thereby sympathetic activation. Finally, we saw no signs of vasoconstriction in filaments adjacent to the cut filament, and it seems impossible that every filament would be under separate nervous control from the autonomic system.

As mentioned in the introduction, endothelin-1 does not constrict trout filament arteries (Sundin and Nilsson, unpublished observations), making it unlikely that it is involved in the observed hemostatic vasoconstriction. It should also be mentioned that the endothelin-1 receptors located in the gill microvasculature differ pharmacologically from mammalian receptors (10), so unfortunately there are presently no known blockers for trout endothelin-1 receptors that we could use.

On the other hand, pretreatment with the cyclooxygenase inhibitor indomethacin significantly extended the time to constriction (Fig. 3A) and also shortened the duration of the constriction (Fig. 3B). This strongly indicates that eicosanoids, possibly released from the endothelium, in some way are involved in the hemostatic mechanism. Indeed, prostaglandin F2a produces powerful constrictions of coronary arteries in fish (3, 4), and it would be interesting to find out whether prostaglandin F2a has similar effects on the branchial vasculature. Another possible eicosanoid that may be involved is thromboxane A2, which is a potent vasoconstrictor in trout (2). This compound, released from platelets, may be partly responsible for the vasoconstriction induced by damage to the vascular wall in small mammalian vessels (8). Indeed, biosynthesis of eicosanoids has been shown to occur in trout thrombocytes, which may function as platelet equivalents (9). Eicosanoids also stimulate aggregation of trout thrombocytes (9). Although we could not detect any increase in blood fluidity after indomethacin treatment (Fig. 4), such an effect may be very local. Thus it is possible that the ability of indomethacin to affect our measures of bleeding at least in part was caused by an inhibition of thrombocyte aggregation in the injured area.

Neither indomethacin treatment nor the exclusion of blood-borne factors in our saline-perfused preparation prevented the vasoconstriction. There is relatively little knowledge about vasoconstrictory mechanisms involved in hemostasis in mammals, and the vasoconstriction is probably partly dependent on local myogenic
constriction of the blood vessels initiated by direct damage to the vascular wall (8). Indeed, we have observed that some filamental arteries constrict when being touched, showing that they are sensitive to mechanical forces.

To conclude, the present results show that the immediate hemostatic response to a cut in a gill filament artery is vasoconstriction, which totally stops the hemorrhage within ∼20 s. After some 8 min, blood clotting has taken over the responsibility for the hemostasis and the vasoconstriction subsides. Eicosanoids appear to play a significant, but maybe not crucial, role in this process, although it is uncertain whether they do this by stimulating vasoconstriction or by inducing local thrombocyte aggregation (or both). By contrast, known constrictors of the filament arteries (ACH, adenosine, and 5-HT) do not appear to be involved in the hemostatic response in trout gill filaments.

Perspectives

The present study shows that fish, for good reasons, have very effective means for stopping gill hemorrhage. Hemostatic mechanisms may be similar among vertebrates, and we suggest that cutting gill filaments is a convenient in vivo model for basic studies of hemostatic responses in small arteries. The filament arteries are readily observable under a stereomicroscope (due to a thin epithelial coverage), and because a rainbow trout has ∼1,000 filaments in its gills cutting a few of these will have little or no systemic effects. Moreover, the high number of filaments makes it possible to use each fish as its own control.

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