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EVIDENCE OF A PROTEIN C - LIKE ANTICOAGULANT SYSTEM IN BONY FISH

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Abstract Evidence is presented, confirming the presence of an anticoagulant system in the plasma of Atlantic salmon (*Salmo salar* L) and rainbow trout (*Oncorhynchus mykiss* Walbaum) (Order: *Teleostei*, Family: *Salmonidae*) that bears striking similarities with the protein C anticoagulant system in mammals; its vitamin K-dependence was documented through a warfarin feeding trial. A potent activator of this system is the protein C activator from the venom of the Central American Moccasin, *Agkistrodon bilineatus*. When activated, the system splits the tripeptide substrate glu-pro-arg-pNa, which is the substrate preferred for the *in vitro* assay of human protein C. It also prolongates the plasma activated partial thromboplastin time, indicating that the system is of clinical relevance. A temperature dependence of the plasma protein C-like activity was evident, the mean activity being 5- to 10-fold higher, but also more variable, in both species in summer and early fall, than it was in winter. There was also a species difference, with rainbow trout having the higher levels. In man, subnormal values of protein C implies an increased risk of thrombosis. Whether this applies to fish is not known. It is, however, a fact that microvascular thromboses are prevalent in farmed Atlantic salmon in winter, whereas thrombotic disease is not a problem in rainbow trout; in the present study plasma protein C-like activity was 30% (of a human reference plasma) in salmon at 4°C compared to a level of 60% in rainbow trout. A complicating factor for the assay of protein C-like activity in salmonid plasma, is the poor stability of the inhibitory system upon storage. Consequently, assays have to be done with freshly prepared citrated plasma.

Protein C is a heterodimeric, vitamin K-dependent plasma zymogen (1), that is activated by a membrane-bound, thrombomodulin-thrombin complex on intact endothelium through limited

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proteolysis of its heavy chain (2). Once protein C is activated it forms a complex with its circulating cofactor, protein S, on the membrane surface (3) where this important coagulation inhibitor exerts its anticoagulant effect through the proteolytic degradation of coagulation factors Va and VIIIa (4,5).

Being of prime importance for the inhibition of localized thrombotic events, the protein C system is crucial for the maintenance of microvascular blood flow. The microcirculation in teleosts (bony fish) is highly developed, and in major organ systems like skin, gills and intestine there is not only one, but two systems of arterioles, capillaries and venules that run in paralel to each other (6,7). The existence of highly developed physiological control mechanisms, like a protein C anticoagulant system, would thus be likely. But the existence of such a system in teleosts has, so far, not been confirmed (8). Preliminary attempts with the purified protein C activator from *Agkistrodon contortrix* (Protac^R), which is a potent activator of human and bovine protein C, but a poor activator of pig protein C (9), gave results which were inconclusive and not reproducible. In this work we present evidence that teleosts of the *Salmonidae* family, Atlanic salmon (*Salmo salar* L.) and rainbow trout (*Oncorhynchus mykiss* Walbaum) have a protein C-like anticoagulant system, and that the protein C activator from the venom of the Central American Moccasin, *Agkistrodon bilineatus*, is a potent activator of this system.

MATERIALS AND METHODS

Snake venoms. Crude venoms from the following snakes were from LATOXAN, Rosans, France: *Agkistrodon bilineatus bilineatus* (Lot PA 973), *A. piscivorus conanti* (Lot PA 339), *A. piscivorus leucostoma* (Lot PA 700), *A. contortrix contortrix* (Lot PA 944), and *Bothrops atrox* (Lot PA 349). Purified protein C activator from *A. contortrix* (Protac^R, Lot 77/113-07), *A. piscivorus* (Lot H39/8-1), *A. bilineatus* (Lot H39/8-2), and the thrombin-like enzyme, batroxobin, from *Bothrops moojeni* (Lot 8/102) were a gift from Dr. K. Stocker, Pentapharm, Basle, Switzerland.

Reagents. Glu-pro-arg-pNa (S 2366) and D-val-leu-lys-pNa (S 2251) were from Chromogenix AB, Mølndal, Sweden. Soybean trypsin inhibitor (SBTI, Lot 40H8200) and Hirudin (Lot 101H 9304) were from Sigma Chemical Co., St. Louis, MO, USA. Normotest (NT, Lot 206037) and Cephotest (Lot 402071) were from Nycomed Pharma AS, Oslo, Norway. Warfarin sodium was from the Norwegian Medicinal Depot, Oslo, and gelatine capsules were filled with 5 mg warfarin and glucose as filler by Ullevål Hospital Pharmacy, Oslo.

Citrated plasma. Pooled citrated plasma from Atlantic salmon (n=15) and rainbow trout (n=15), frozen on solid CO_2 and stored at -80°C, were from AKVAFORSK's research station, Averøy. Pooled human reference plasma (n=32) was from Aker University Hospital, Oslo. Control Plasma Abnormal (Lot 2D3BN, 0.3-0.5 U protein C/ml) was from Nycomed.

The activating capacity of crude snake venoms measured with chromogenic substrates. Assays were done manually at 37°C : To 25 µl citrated plasma diluted 1:8 in 50 mM Tris-HCl pH 8.4 was added 60 µl crude snake venom (625 µg/ml in the same buffer except *A. conanti* venom which had been diluted to 312.5 µg/ml). After 4 min 60 µl 1 mM substrate in buffer was added (S 2366, or S 2366 with 20 U hirudin/ml, or S 2251). The reaction was terminated after 8 min by adding 60 µl 50% acetic acid, and the amidolytic activity was read on a Titertec Multiscan spectrophotometer (Labsystems, Helsinki, Finland) at 405 nm. The same assays were also performed in the abscence of plasma. Since the lys-pNa substrate S 2251 was split to a higher

degree than the arg-pNa substrate S 2366 by all crude venoms except those from *A. bilineatus* and *Bothrops atrox*, and all the venoms themselves split the substrates nearly as efficiently in the abscence of plasma, subsequent assays were made with purified protein C activators.

Effect of purified protein C activators from snake venoms on the contact system, i.e. activated partial thromboplastin time (APTT). APTT tests (Cephotest, Nycomed) were performed manually (on freshly taken plasma only) as described by the manufacturer, with the exception that 0.1 ml of purified protein C activator (1mg/ml in saline) was included during the 6 min incubation stage prior to the addition of 0.1 ml 25 mM $CaCl_2$. Only the activator from *A. bilineatus* venom led to a significant prolongation of the APTT of salmon and rainbow trout plasma. Thus, the purified activator from *A. bilineatus* was chosen for all subsequent assays.

Effect of the purified protein C activator from A. bilineatus *on salmonid plasma and eluate from $BaCl_2$-adsorbed plasma measured with chromogenic substrates.* Preliminary experiments had shown that the protein C-like activity that could be elicited in salmonid plasma by the activator was independent of temperature (range 8-37°C) in the activation stage (results not shown). Assays were performed with fresh and frozen plasma, and with a saline eluate from a $BaCl_2$ adsorbate from frozen plasma (100 µl of a 1 M $BaCl_2$ slurry to 1 ml of plasma) on a Cobas Bio spectrophotometer at 37°C: To 15 µl undiluted eluate or 15 µl of plasma diluted 1:8, 1:16 or 1:32 in 50 mM Tris-HCl pH 8.4 was added 20 µl diluent (distilled water) and 40 µl protein C activator from *A. bilineatus* (0.5 mg/ml in the same buffer). After 120 s 40 µl 1 mM substrate was added (S 2366, or S 2366 with 25 U hirudin/ml, or S 2366 with 10 mg SBTI/ml, or S 2251, all in the same buffer). The reaction was then followed for 15 min at 405 nm with read-out every 30 s. Neither substrate was split by the activator alone (in Tris). Substrate S 2251 was not split, while S 2366 showed high amidolytic activity that could be inhibited to 20-30% by SBTI, but not by hirudin or Tris, or by the activator itself. Thus, S 2366 was the substrate chosen for further experiments. The activator did not elicit any protein C-like activity in frozen plasma from either species. When incubated with eluate from frozen plasma, a high amidolytic activity was seen on S 2366 that was inhibited to more than 90% by hirudin. Subsequent assays were, therefore, done with freshly taken plasma only.

Depletion of vitamin K-dependent coagulation enzymes in salmon and rainbow trout: feeding trial with warfarin. Twenty Atlantic salmon and 20 rainbow trout were randomly allocated, 10 of each species, to two 27 m^3 net pens in sea water; fish were netted one at a time, and five individuals of each species, i.e. every fourth fish, was anaestethized in well-aerated water containing 80 mg/l tricaine methane sulphonate (MS 222) and placed on a table ventral side up. Citrated blood was drawn from the caudal vein with Venoject ^{R} tubes and centrifuged at 2000 g for 10 min. Plasma was pipetted off (with disposable plastic pipettes) into plastic tubes within 15 min. Prothrombin time and APTT were measured manually according to the manufacturer's recommendations (Nycomed AS). Factor X was determined, after RVV-activation, as described previously (10). The protein C-like system was activated manually as described, with the exception that crude venoms were replaced with purified *A. bilineatus* activator (25 µl 0.5 mg/ml in buffer, final concentration 0.1 mg/ml). Amidolytic activity on S 2366 was determined on a Titertec Multiscan spectrophotometer as described. All assays were performed within 2 hours. For the accuracy and precision control of the analyses an abnormal control plasma was included in each series of analyses.

Because of low (4°C) and rapidly declining water temperatures, fish were hand-fed with semi-moist pellets for 6 months before the feeding trial could be started. The fish were then fed three

times a day with freshly prepared semi-moist pellets, each containing a gelatine capsule with 5 mg warfarin, at an estimated dose of 5-10 mg per kg live body weight (mean weight of salmon was 1.8 kg, of rainbow trout 2.2 kg); mean calculated daily dose the first 7 days was 9.8 and 7.9 mg/kg in pen A and 8.8 and 7.3 mg/kg in pen B for salmon and trout, respectively. The calculated dose declined only negligibly the second week, but rapidly during the third week, when the fish also started to die, with salmon dying first. Mortalities were recorded daily, and dead fish were all submitted to post mortem examination. On day 19 the remaining fish were anaestethized and citrated blood was drawn from the caudal vein with Venoject^R tubes, after which fish were killed and examined for gross pathological signs.

To control the effect of the ambient sea water temperature, which had risen to 13.7°C, on coagulation and vitamin K-dependent coagulation enzymes, blood was also drawn from five salmon and five rainbow trout of the same year-class as the test fish. Prothrombin time, APTT, FXa, and protein C-like activity were determined within 2 h as described.

Protein C-like activity in salmon and rainbow trout plasma at different water temperatures and durability of activity. In one experiment, at which time the water temperature had been slowly declining from a maximum of nearly 18°C four weeks previously to 14.5°C, Atlantic salmon (n=25) and rainbow trout (n=25) were netted one at a time, anaestethized, and citrated blood was drawn from the caudal vein. To prevent proteolytic degradation samples were immediately placed on ice, handled at 4°C through centrifugation and pipetting, and assayed for protein C-like activity with purified *A.bilineatus* activator on S 2366 as described. Remaining (fresh) plasma was incubated with an $Al(OH)_3$ slurry (0.2 ml 6% $Al(OH)_3$ to 1 ml of plasma), and the saline eluate was then assayed with the same procedure.

In a second experiment citrated blood was taken from 5 fish of each species at a surrounding water temperature of 8.4 °C; the temperature had fluctuated between 9.4 and 7 °C for the last few weeks. Samples were placed on ice and handled at 4°C as described. Plasma was assayed for protein C-like activity, and in the APTT test (Cephotest) system with and without the activator, immediately after sampling, and after storage on ice for 3 h. Another two tubes of citrated blood were taken from each of 3 individuals from each species; one tube was handled on ice all the way, while the other tube was handled at room temperature. Both samples from each fish were assayed for protein C-like activity, and in the APTT test (Cephotest) system with and without the activator.

RESULTS

Effects of purified protein C activators from snake venoms on the activated partial thromboplastin time are shown in Table 1.

The protein C-like activity elicited by the purified activator from *A. bilineatus* and determined on substrate S 2366 was definitely vitamin K-dependent, being severely depleted in warfarin treated fish (Table 2). Likewise, did warfarin treatment lead to depletion of factor Xa and to a prolongation of APTT and prothrombin time.

Necropsy findings in warfarin treated fish were identical in both species: Gills were extremely pale. Some fish exhibited petechiae of the skin on the ventral body surface. There was serous fluid in the abdominal cavity and the pericardium was distended with serous fluid. In most fish

the carcass had a pale appearance. The liver was either mottled or extremely pale with subcapsular petechial haemorrhages. The spleen was enlarged. There were subserosal petechiae in the gastrointestinal tract. The stomach was filled with mucous fluid, and all fish had a number of more or less disintegrated gelatin capsules in the intestine.

TABLE 1

Effect of purified protein C activators from snake venoms on activated partial thromboplastin time in salmonids and man. Concentration of activator during the activation stage 0.33 mg/ml, final concentration 0.25 mg/ml. Details see text.

Activator	*A. piscivorus*	*A. bilineatus*	*A. contortrix*	*B. moojeni*	NaCl
Species	Activated partial thromboplastin time (s)				
salmon	22	45	23	sp.c.*	23
trout	15	26	16	sp.c.	16
man	240	210	54	sp.c.	34

* spontaneous coagulation

TABLE 2

Effect of oral warfarin treatment of salmon and rainbow trout on vitamin K-dependent coagulation enzymes and on coagulation tests. Protein C-like activiy, PCa; activated factor X, FXa; activated partial thromboplastin time, APTT; prothrombin time, NT. Values are given as mean and s.e. Details see text.

Source of plasma	PCa(%)*	APTT(s)	NT(s)	FXa(%)*
salmon, controls (n=5)	65.6±32.0	25.6±0.8	74.8±1.9	5.2±0.8
warfarin treated (n=2)	29.0±2.0	30.7±5.6	4200±180	1.4±0.3
trout, controls (n=5)	155.8±35.7	19.8±0.6	56.6±0.9	89.5±3.3
warfarin treated (n=8)	35.9±3.3	184±90.9	600±0	6.5±1.1

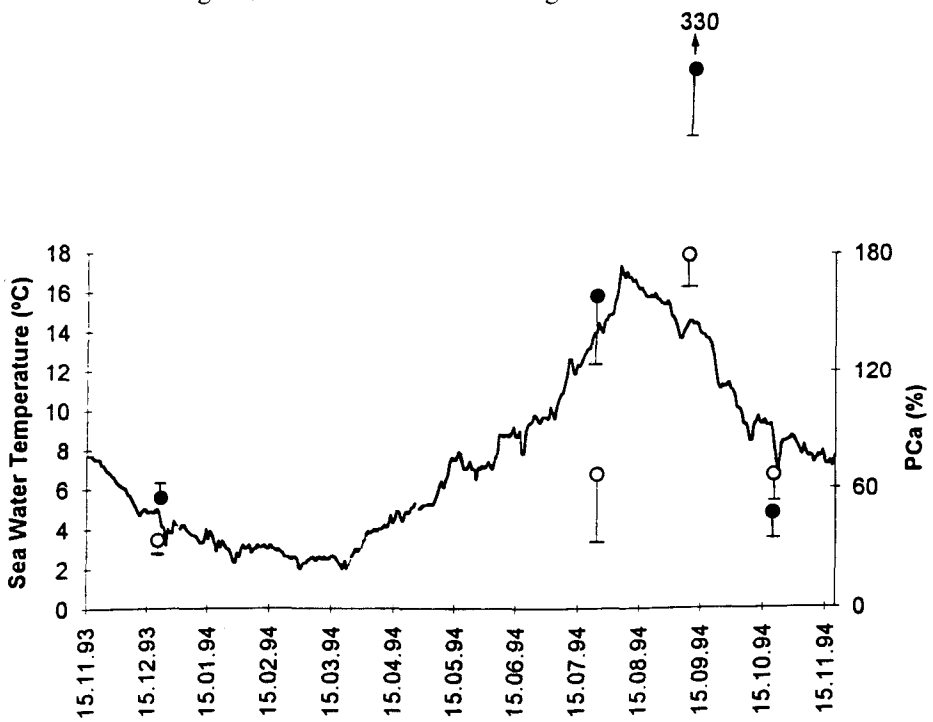
*per cent of a human reference plasma

The association between plasma protein C-like activity and sea water temperature is illustrated in FIG. 1. Levels were high particularly in summer and early fall, as was the variation; at the highest level in September protein C-like activity was 328.7±33.6% and 178.2±16.3% (mean and s.e., n=25) in rainbow trout and in salmon, respectively, while corresponding mid-December values were 55.8±2.6 and 36.4±2.1, all in per cent of a human reference plasma. The level of

protein C-like activity covaried with factor X activity in rainbow trout, but were at the same low level in salmon, both in December, when declining water temperatures had reached 4°C (data not shown), and in July, when water temperatures had been steadily increasing to 13.7°C (Table 2, controls).

FIG. 1

Protein C-like activity in salmon (open circles) and rainbow trout plasma (filled circles) superimposed on a sea water temperature graph which spans an entire year. Values are given as mean activity, and bars represent one s.e.(n=5 except for September values where n=25). Purified protein C activator from *A. bilineatus*; concentration during activation 0.23 mg/ml; final concentration 0.1 mg/ml. Details see text.



The stability of protein C-like activity in salmon plasma was marginally better than that of rainbow trout plasma. After 3 h on ice the s.e. of the protein C-like activity was twice as high in rainbow trout; the increase could not be inhibited by hirudin or SBTI so excluding the activation of prothrombin or factor X in the samples. Incubation of frozen plasma with $BaCl_2$ and subsequent elution with saline activated prothrombin, while eluate from $Al(OH)_3$ -adsorbed fresh plasma showed considerable amidolytic activity on S 2366 that could not be identified. Neither eluate contained protein C-like activity. What we had is a well-known problem with fish plasma, namely a quickly increasing autolytic activity which precludes the determination of a number of, but not all (11), single factors even in frozen plasma. This is further illustrated by the fact that plasma cannot be used to determine an APTT value in a manual system (Cephotest) after freezing and thawing. Whether or not the citrated blood samples were put on ice immediately and handled at 4°C, or handled at ambient (room) temperature, did not matter as long as assays were performed within 2 hours.

DISCUSSION

There is a vitamin K-dependent, anticoagulant system in teleost plasma that bears striking similarities with the protein C anticoagulant system in mammals. A potent activator of this system is the protein C activator from the venom of the Central American Moccasin, *Agkistrodon bilineatus*. When activated the system splits the tripeptide substrate glu-pro-arg-pNa, which is the substrate preferred for the *in vitro* assay of human protein C, and this activity is inhibited to 20-30% by SBTI but not by the specific thrombin inhibitor hirudin, so excluding thrombin as the source of amidolytic activity. Moreover, when activated it prolongates the plasma activated partial thromboplastin time in much the same way as does Protac^R -activated protein C in human plasma, indicating that the system is of clinical relevance.

The vitamin K-dependence of the system was clearly established in salmon and trout that had been fed 5-10 mg warfarin per kg live body weight and day for 2-3 weeks; both species suffered a severe depletion of protein C-like activity that was accompanied by a simultaneous depletion of factor X activity. There was also a species difference in plasma protein C-like activity, like the one previously reported for factor X (10) and confirmed in this work, with rainbow trout having the higher levels.

A temperature dependence of the plasma protein C-like activity was also evident, the mean activity being 5- to 10-fold higher, but also more variable, in both species in summer and early fall than it was in winter. Since there were no signs of thrombosis in any species at any time (prothrombin time, APTT and FXa were all normal), this variation is probably physiological, reflecting a lowered production of the coagulation inhibitor in winter. In man the winter levels of around 30% protein C-like activity that were observed in salmon would imply an increased risk of thrombosis (12). Whether this applies to fish is not known. It is, however, a fact that microvascular thromboses are prevalent in farmed Atlantic salmon in winter (13,14), whereas thrombotic disease is not a problem in rainbow trout where the protein C-like activity was 60% at 4 °C (and still declining) water temperature. This suggests the presence of a highly developed anticoagulant system of major importance for the maintenance of microvascular flow.

From an evolutionary point of view it was interesting that the protein C activator from *A. bilineatus* was the better activator in salmonid fish. In snakes, polyvalent venoms have evolved that serve to immobilize possible prey species in their natural habitat. Sea snakes of the *Elapidae* family produce neurotoxins to this end (15). Venom proteins that interfere with blood coagulation proteins in prey animals are widely distributed in terrestrial snakes. Such venom proteins act in a species specific manner with prey proteins, but will often have an additional, narrow action on a single protein from other species than prey animals (8). The protein C activator from *A. piscivorus*, the terrestrial, fish eating relative of *A. contortrix*, did not cause any prolongation of the APTT in salmonids, but the activator from the terrestrial *A. bilineatus* evidently had this "side effect". In a recent work Kogan and associates (16) found this activator to be a potent activator of human protein C but a poor activator in the rat. In this respect the *A. bilineatus* activator was similar to Protac^R, but Protac^R is evidently a poor activator in salmonid fish. This probably mirrors recently reported inter-species structural similarities between snake venoms (17), but also inter-species and inter-class structure analogies between protein C molecules.

A complicating factor for the assay of protein C-like activity in salmonid plasma is the poor stability of the inhibitory system: it is inactivated by freezing and thawing, and it is inactivated during adsorption to insoluble barium (or aluminium) precipitates and elution. Thus, assays have

to be done with freshly prepared citrated plasma. As long as the stability problem is not solved, this again would mean that future studies on the protein C system in teleosts will have to be conducted at, or near, the fish farm.

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