Effect of prostanoids and their precursors on the aggregation of rainbow trout thrombocytes

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Hill, D. J., M. B. Hallett, and A. F. Rowley. Effect of prostanoids and their precursors on the aggregation of rainbow trout thrombocytes. *Am. J. Physiol.* 276 (*Regulatory Integrative Comp. Physiol.* 45): R659–R664, 1999.—The role of prostanoids and their precursor fatty acids in the aggregatory response of thrombocytes (platelet equivalents of fish) from the rainbow trout, *Oncorhynchus mykiss*, was studied. Aggregation of these cells was induced by the thromboxane mimetic U-46619 or arachidonic acid (AA) in the presence of human or trout fibrinogen. The production of $TXB_{2/3}$ by thrombocytes in response to stimulation with AA was inhibited by aspirin, ibuprofen, and indomethacin. However, thrombocyte aggregation in response to AA stimulation was not significantly altered by these agents at the concentrations tested (10–100 μM), with the exception of indomethacin at 20 and 40 μM. Effects on cytosolic calcium concentration have been suggested as an alternative mechanism for the inhibitory action of indomethacin on human platelet aggregation. The present study, however, failed to identify this as a mechanism for the inhibition of U-46619-induced trout thrombocyte aggregation by indomethacin. The polyunsaturated fatty acids docosahexaenoic acid and eicosapentaenoic acid both exhibited an inhibitory effect on U-46619-induced thrombocyte aggregation similar to that observed with mammalian platelets. Unlike the case in mammalian hemostasis, prostacyclin inhibited thrombocyte aggregation only at high concentrations (>5 μM). Prostaglandin E_2, however, inhibited thrombocyte aggregation at much lower concentrations (>0.01 μM), suggesting that it may be the major inhibitory eicosanoid in trout.

eicosanoids; platelet equivalent; hemostasis; fish

SINCE THE DISCOVERY OF PROSTANOIDS, such as thromboxane (TX) A_2, prostaglandin (PG) E_2, and prostacyclin (PGI_2), the role of eicosanoids in mammalian platelet function has been well characterized (8, 18). TXA_2 is the most potent platelet activator (28), whereas PGE_2 has been shown to exert a biphasic effect on these cells (34). At low concentrations, it potentiates platelet aggregation in response to various stimuli, whereas at high concentrations, it has an inhibitory effect (34). The strongest naturally occurring inhibitor of platelet aggregation is PGI_2. Synthesis of this compound occurs not in platelets but mainly in the endothelial cell layer of the vasculature. The inhibitory effect of PGI_2 is brought about by an increase in cAMP levels in platelets that decreases the cytosolic calcium concentration and thus prevents activation of calcium-dependent processes (31). PGD_2 is also a potent inhibitor of platelet function but is produced in relatively small amounts and therefore not considered a major contributor to hemostatic regulation (5).

Precursor fatty acids also play a role in platelet regulation in the hemostatic response. All the aforementioned prostanoids are derived from the polyunsaturated fatty acid (PUFA) arachidonic acid (AA). This PUFA has no direct effect on aggregation; rather its action is exerted via conversion to proaggregatory prostanoids such as TXA_2 (32). It has been noted that Eskimos have a much lower incidence of thrombosis than is found in many Western cultures. This observation has been attributed to the high dietary intake of fish oils, which contain high levels of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (4, 33). Dietary trials have been performed in which volunteers were given higher levels of EPA and/or DHA for a period of weeks. In all cases, a higher proportion of these PUFAs were incorporated into the membrane phospholipids of the subject's platelets at the expense of AA (15, 19, 24). A decrease in platelet aggregation in response to collagen and thrombin stimulation occurred, and TXB_2 (nonenzymatic hydrolysis product of TXA_2) production was reduced in platelets of subjects on the high-EPA diet (12, 15, 30). EPA competes with AA as a substrate for the enzyme cyclooxygenase and is metabolized to generate the three series eicosanoids, TXA_3, and PGH_3, which have little if any biological activity (29). PGI_3, however, is equipotent to PGI_2 in inhibiting platelet function (27). It is possible that DHA can act synergistically with EPA to inhibit platelet function (3). Recently, however, it has been indicated that diets rich in DHA or AA alone had no significant effect on the function of platelets from these subjects (20, 21).

Fish blood lacks platelets but possesses nucleated cells termed thrombocytes, which are thought to serve a similar hemostatic function (10). In mammalian systems, the predominant PUFA used as substrate for the biosynthesis of eicosanoids is AA. In fish, however, higher levels of DHA and EPA, rather than AA, are present in the major phospholipids (23), and hence a wider range of eicosanoids are generated in such animals (23). The prostanoid-generating capacity of thrombocytes from different species of fish has been shown to be variable. For example, thrombocytes from flounder, red sea bream, black sea bream, and black rockfish have been reported to convert AA to $PGF_{2\alpha}$, PGE_2, and PGD_2, whereas EPA was only converted to PGE_3 and PGD_3 by black rockfish thrombocytes (16). Furthermore, DHA was not converted to eicosanoids in any of

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the four species studied (16). Leucocytes from the dogfish, *Scyliorhinus canicula*, have been shown to generate $PGF_{2\alpha}$, PGE_2, and PGD_2 on stimulation with the calcium ionophore A-23187, and smaller amounts of TXB_2 were also produced (25). Purified thrombocytes from carp have also been reported to synthesize $PGF_{2\alpha}$, PGE_2, and PGD_2; however, no TXB_2 generation was observed (13). Conversely, whole blood and purified thrombocytes from the rainbow trout, *Oncorhynchus mykiss*, generated more TXB_2 than other eicosanoids on stimulation with calcium ionophore (14, 22). Given that TXA_2 is the most potent inducer of human platelet aggregation, the lack of generation in the blood cells of certain fish may indicate that it is not as important in inducing thrombocyte aggregation in those species.

Although our understanding of the hemostatic mechanisms in fish is improving (see Ref. 26 for review), little work has been performed regarding the role of eicosanoids and their precursors in this process. The present study was therefore undertaken to study the effect a range of prostanoids and their precursor fatty acids have on the hemostatic response of thrombocytes from the rainbow trout, *O. mykiss*.

MATERIALS AND METHODS

Fish and chemicals. Adult rainbow trout, *O. mykiss*, were obtained from Lliw Mill Trout Farm, Pont Lliw, South Wales. They were maintained in large external tanks and fed ad libitum on Mainstream expanded trout diet (BP Nutrition, Cheshire, UK). The fatty acids AA, DHA, and EPA, the TX mimetic U-46619 (9,11-dideoxy-9α,11α-methanoepoxy $PGF_{2\alpha}$), and PGE_2 were obtained from Cascade Biochem (Reading, UK). PGI_2 was supplied by Alexis Biochemicals (Nottingham, UK). Fura 2 acetoxymethyl ester (AM) was purchased from Molecular Probes (Eugene, OR). All other chemicals were of the highest grade available commercially.

Thrombocyte isolation. Before blood collection, fish were given terminal anesthesia by immersion in MS-222 (final concentration 0.1 g/l) for 10–15 min. Blood (~7 ml) was collected from the caudal vessel into a syringe containing heparin (final concentration ~10 IU/ml). A two-step Percoll density gradient centrifugation procedure was used to isolate thrombocytes from the peripheral blood of rainbow trout, as described previously (9, 14). Each fish yielded $5–7 \times 10^7$ thrombocytes of ~86% purity. The contaminating cells were mainly lymphocytes, neutrophilic granulocytes, and erythrocytes.

Thrombocyte aggregation studies. The aggregatory response of trout thrombocyte suspensions purified on Percoll gradients was measured turbidimetrically by a method similar to Born (1), using a Payton Minigator II aggregometer (Payton Associates, Scarborough, ON, Canada). Data were collected by a Macintosh LCII computer via a MacLab/2e interface using Chart software (ADInstruments, London, UK). Aggregometers measure cell aggregation by changes in light transmittance. As cells aggregate, the light transmittance through a cell suspension increases, which is measured by a detector in the aggregometer. Thrombocyte suspensions [500 μl aliquots; 1×10^7 cells/ml in Ca^{2+}-Mg^{2+} containing Hanks' balanced salt solution (HBSS) with 5 mM MOPS] were aggregated by the addition of the TXA_2 mimetic U-46619 (0.5 μM) in the presence of trout or human fibrinogen (400 μg/ml). The percentage aggregation was determined against a cell-free blank. To assess the involvement of various nonsteroidal anti-inflammatory drugs (NSAIDs), aspirin, ibuprofen (both 0–100 μM), and indomethacin (0–40 μM) were preincubated with thrombocyte suspension for 15 min before the induction of aggregation with U-46619 and fibrinogen. The effect of the AA, DHA, and EPA (0–10 μM) was studied by their addition immediately before the induction of aggregation. To assess the effect of PGE_2 and PGI_2 on aggregation, these prostanoids (0.001–1.0 and 0.1–20 μM, respectively) were added to thrombocyte suspensions alone or immediately before the induction of aggregation with U-46619 and fibrinogen. Because PGI_2 is unstable in aqueous media, particularly in the presence of cells, it was prepared immediately before its addition and all experiments performed within a few hours of its preparation.

TXB_2 EIA. $TXB_{2/3}$ production by thrombocyte preparations was determined using a Biotrak enzyme immunoassay (EIA) system (Amersham Life Sciences, Little Chalfont, UK). Assay reagents were prepared and used according to the manufacturer's instructions. The sensitivity of this assay was 3.6 pg/ml, with cross-reactivity of 100% with TXB_3, 60.5% with 2,3-dinor TXB_2, 0.18% with PGD_2, and <0.01% with PGE_2.

Measurement of cytosolic Ca^{2+} concentration. Thrombocytes (~1×10^7 cells/ml) were loaded with fura 2 by incubation with fura 2-AM (1 μM) for 40 min at 12°C. The cells were then centrifuged and resuspended in Ca^{2+}-Mg^{2+}-containing HBSS (1.8 mM $CaCl_2$) and kept at 4°C before use. Dual-excitation fluorescence measurements at 340 and 380 nm were achieved using a Spex Fluorolog dual-wavelength fluorimeter (Glen Spectra, Stanmore, UK) on cell suspensions maintained at 18°C challenged with U-46619 (0.3–20 μM) in the presence of indomethacin (10–40 μM) or ethanol (1 μl) as a control. Maximum and minimum 340/380 ratio values were determined in every experiment using digitonin (50 μM) and EGTA (20 mM), respectively, and the cytosolic free Ca^{2+} concentration was calculated assuming a dissociation constant of 183 nM, as described previously (7).

Statistical analysis. Statistical analyses were performed with Instat2 (Graphpad Software) using Student's *t*-tests or the Student-Newman-Keuls multiple comparison test as appropriate. All experiments reported were performed using at least three different batches of thrombocytes each from individual animals.

RESULTS

Previous studies have shown that trout thrombocytes aggregate in the presence of human fibrinogen (400 μg/ml) and the TXA_2 mimetic U-46619 (0.03–10 μM) in a dose-dependent manner (9). They also aggregate on stimulation with AA, but not EPA or DHA (10).

The proaggregatory response of thrombocytes in the presence of AA could have been due to its conversion to eicosanoids such as PGG_2, PGH_2, or TXA_2 rather than a direct effect. To ascertain if this was the case, the effect of three NSAIDs (which inhibit cyclooxygenase, the enzyme central to prostanoid synthesis), namely aspirin, ibuprofen, and indomethacin, on thrombocyte aggregation was studied. In these experiments, aspirin, ibuprofen (10–100 μM), or indomethacin (20–40 μM) were preincubated with thrombocyte suspensions before the addition of U-46619 (0.5 μM) or AA (10 μM) in the presence of human fibrinogen (400 μg/ml). Aspirin inhibited U-46619-induced aggregation, although this was only statistically significant at a concentration of 100 μM (Fig. 1*A*). Similarly, ibuprofen caused a significant decrease in U-46619-induced aggregation at a

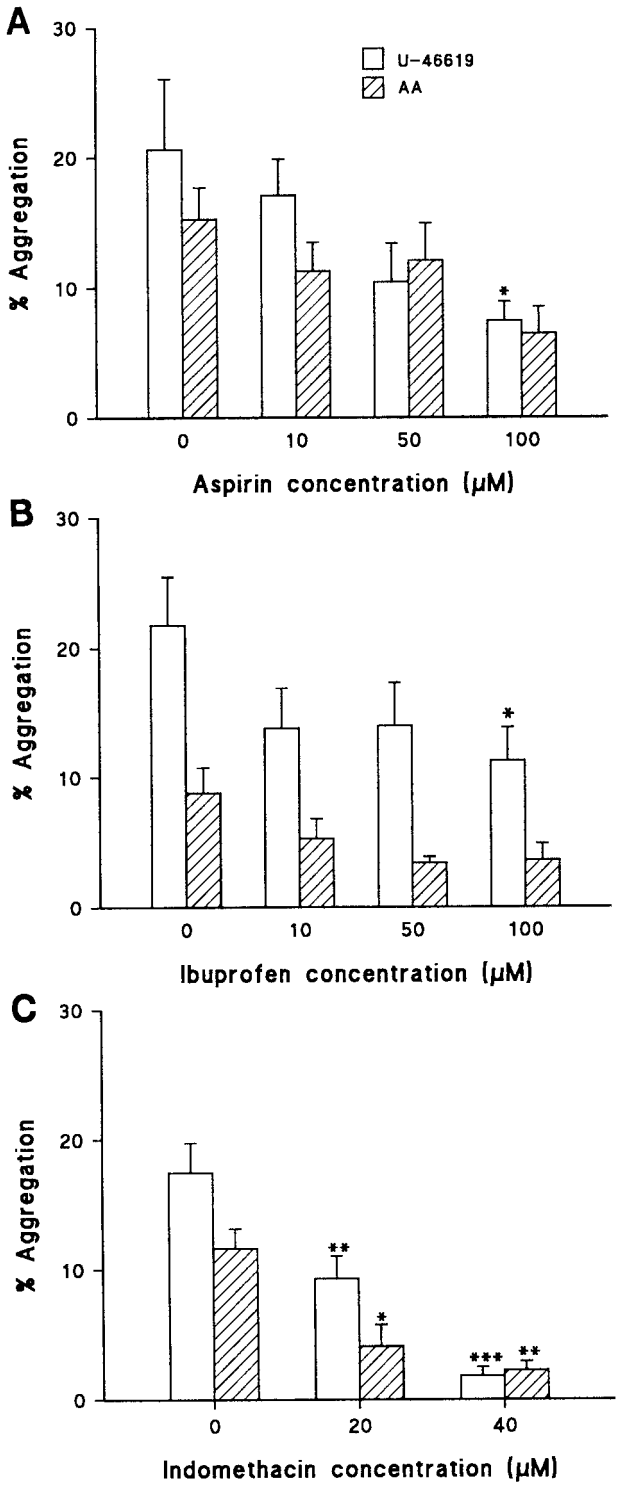

Fig. 1. Effect of aspirin (*A*), ibuprofen (*B*), and indomethacin (*C*) on thrombocyte aggregation induced by U-46619 (0.5 μM) or arachidonic acid (AA; 10 μM) in presence of human fibrinogen (400 μg/ml). $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ compared with appropriate control. Mean values ± SE, $n = 3–6$.

concentration of 100 μM (Fig. 1*B*). The most inhibitory NSAID was indomethacin (Fig. 1*C*), with significant inhibition of U-46619- and AA-induced aggregation at 20 and 40 μM.

The effects of the three NSAIDs tested in inhibiting thrombocyte aggregation induced by both AA and U-46619 suggested the involvement of prostanoid formation in the mechanism of action of both agonists. To test this, the levels of immunoreactive TXB (stable breakdown product of TXA) produced by thrombocytes in response to U-46619 (0.5 μM) or AA (10 μM) were determined (Fig. 2). The addition of exogenous AA alone resulted in the generation of a significant amount of immunoreactive TXB, whereas the presence of each inhibitor significantly decreased the amount of this product released by thrombocytes (Fig. 2). Indomethacin exhibited a significantly greater inhibition of TXB produced by the addition of AA than seen with aspirin or ibuprofen ($P < 0.05$). The addition of U-46619 (0.5 μM) to thrombocyte suspensions resulted in minimal (not above background) generation of immunoreactive TXB that was not significantly affected by the NSAIDs tested.

Inhibition of calcium uptake has been implicated in the action of indomethacin on purified human platelets, and this has been suggested as a mechanism of its antiaggregatory activity (6). To ascertain if this inhibitor affected calcium ion movements in trout thrombocytes, cytosolic free Ca^{2+} ion concentration measurements were performed. After incorporation of fura 2, thrombocytes were challenged with U-46619 and an increase in cytosolic free Ca^{2+} ion concentration was observed (Fig. 3) that was largely dose dependent. However, there was no significant effect of indomethacin (40 μM) on the U-46619-induced Ca^{2+} signal (data not shown).

AA has been shown to induce the aggregation of thrombocytes in the presence of human fibrinogen (9). However, because the prominent PUFAs in trout are DHA and EPA (20), the effect of these fatty acids (0.03–10 μM) on trout thrombocyte preparations in the presence of fibrinogen was studied. Neither induced aggregation (data not shown). However, the addition of DHA to thrombocyte preparations immediately before the addition of U-46619 resulted in a significant dose-

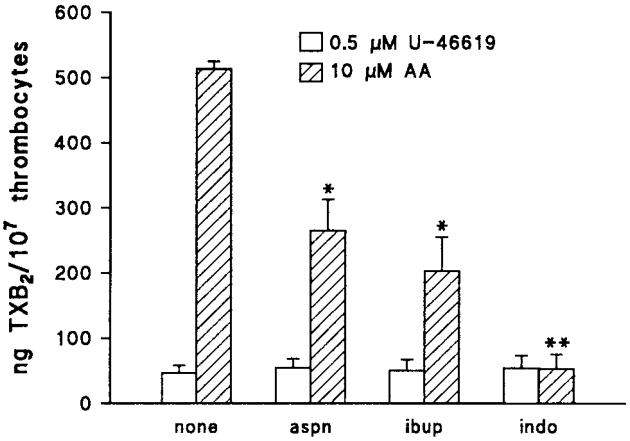

Fig. 2. Effect of aspirin (aspn), ibuprofen (ibup), and indomethacin (indo) on thromboxane (TX) B immunoreactive material production by thrombocytes stimulated by AA (10 μM) or U-46619 (0.5 μM). $*P < 0.05$, $**P < 0.01$ compared with control. Mean values ± SE, $n = 3$.

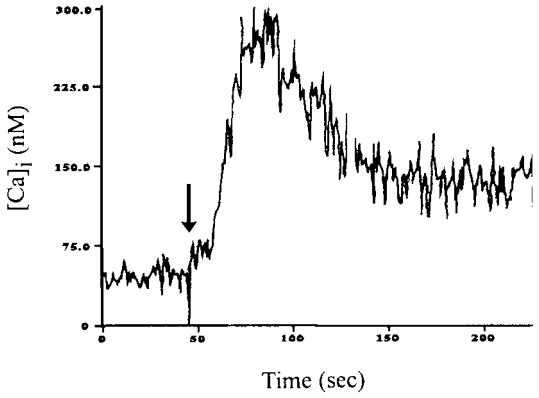

Fig. 3. Representative trace to show effect of U-46619 (10 μM) on cytosolic free Ca^{2+} ion concentration ($[Ca^{2+}]_i$) in fura 2-loaded thrombocytes in presence of 1.8 mM extracellular $CaCl_2$. Agonist was added at arrow.

dependent decrease in the amount of aggregation observed that was saturable at concentrations above 0.3 μM (Fig. 4). Experiments with EPA also indicated a similar inhibitory action on U-46619-induced thrombocyte aggregation (data not shown).

As well as TXB_2 production by trout thrombocytes, another cyclooxygenase product, PGE_2, is produced in significant amounts by both whole trout blood and purified thrombocytes (14, 22). Exogenous PGE_2 (0.001–1 μM) exhibited no proaggregatory affect on trout thrombocytes in the presence of fibrinogen. The addition of PGE_2 immediately before the addition of U-46619 resulted in a dose-dependent decrease in the resulting thrombocyte aggregation that was significant above concentrations of 0.01 μM (Fig. 5). As in mammals, PGI_2 is the most potent inhibitor of platelet function (18); therefore, the effect of PGI_2 on trout thrombocyte aggregation was studied. The addition of PGI_2 (5 and 10 μM) immediately before stimulation with U-46619 (0.5 μM) in the presence of human fibrinogen resulted in a significant decrease of thrombocyte aggregation (Fig. 6). No significant effect was observed at the other concentrations tested.

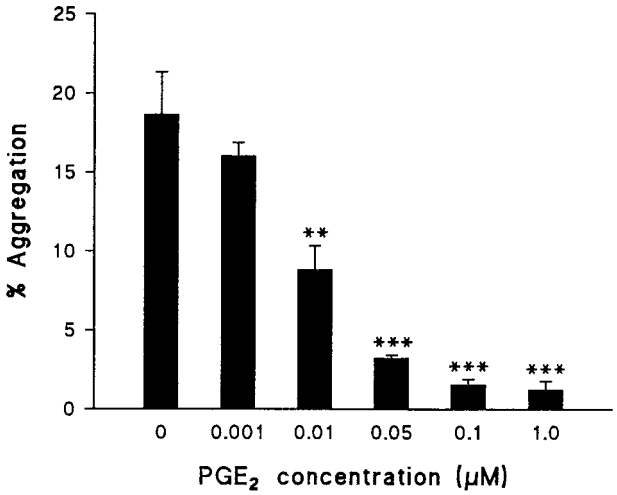

Fig. 5. Effect of prostaglandin (PG) E_2 thrombocyte aggregation induced by U-46619 (0.3 μM) in presence of trout fibrinogen (200 μg/ml). $^{**}P < 0.01$, $^{***}P < 0.001$ compared with control. Mean values ± SE, $n = 4$.

DISCUSSION

Both TXA_2 and its precursor fatty acid, AA, have been shown to induce human platelet aggregation (11). Similarly, trout thrombocytes aggregate in response to AA in the presence of fibrinogen. In human platelets, inhibition of cyclooxygenase, the central enzyme responsible for the initial conversion of AA to PGs, abolishes platelet responses to AA. This suggests that AA has no direct effect on human platelet hemostatic responses. In the present study, the effect of three NSAIDs, namely aspirin, ibuprofen, and indomethacin, on trout thrombocyte aggregation varied. Both aspirin and ibuprofen significantly inhibited U-46619-induced aggregation at the highest concentration tested (100 μM). Conversely, these inhibitors were without significant effect on AA-induced aggregation. Finally, indometha-

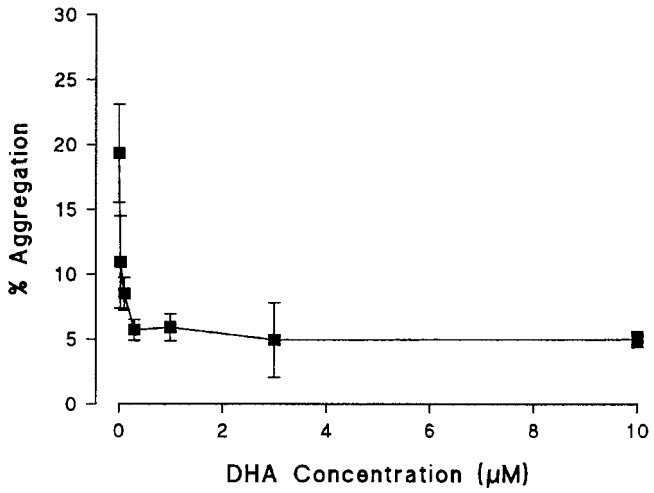

Fig. 4. Effect of docosahexanenoic acid (DHA) on U-46619-induced thrombocyte aggregation in presence of trout fibrinogen (200 μg/ml). Mean values ± SE, $n = 3$.

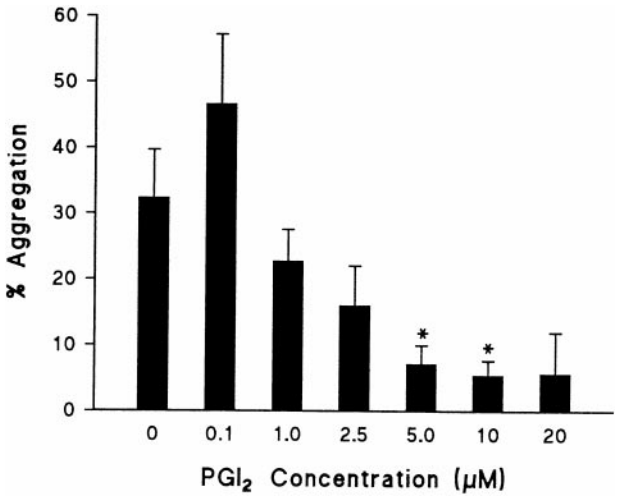

Fig. 6. Effect of PGI_2 on thrombocyte aggregation induced by U-46619 (0.5 μM) in presence of human fibrinogen (400 μg/ml). $^{*}P < 0.05$ compared with appropriate control. Mean values ± SE, $n = 4$.

cin significantly inhibited both AA and U-46619-induced aggregation at all concentrations used. The inhibition of U-46619-induced aggregation by the NSAIDs tested is of interest because as a stable mimetic of TXA_2, inhibition of cyclooxygenase activity would not be expected to affect its action. It is possible, therefore, that U-46619 elicits its proaggregatory effect on trout thrombocytes by stimulating further eicosanoid production rather than by a direct action, although in the present study this agent at 0.5 μM failed to induce significant generation of TXB immunoreactive material. Previous studies using higher concentrations of U-46619 (10 μM) have, however, been shown to cause the generation of TXB and other eicosanoids by trout thrombocytes (14). A further alternative is that the NSAIDs used in the present study may inhibit U-46619-induced aggregation by a route other than the inhibition of cyclooxygenase. Both indomethacin and ibuprofen have been shown to inhibit the uptake of calcium ions in ionophore- or epinephrine-stimulated human platelets by a mechanism other than the inhibition of cyclooxgenase (6). In the present study, however, indomethacin had no effect on calcium ion flux, hence implying that this is not an explanation for the effect of this inhibitor on thrombocytes.

Although AA produced a proaggregatory response in trout thrombocytes, other prominent PUFAs in fish, EPA and DHA, failed to induce aggregation. These findings correlate with the case in human platelets, in which EPA is converted to TXA_3, which is biologically inactive (12). DHA has been shown to inhibit the activity of cyclooxygenase on AA in human platelets (3). In the present study, U-46619-induced thrombocyte aggregation was inhibited by exogenous DHA; however, the mechanism of inhibition was not further investigated. These results are unexpected due to the high levels of DHA and EPA present in fish. Release of these fatty acids by enzymes such as phospholipase A_2 would lead to the generation of products with antiaggregatory activity. Therefore, a mechanism may exist to control the release of pro- and antiaggregatory PUFAs.

PGE_2 showed a marked inhibition of thrombocyte aggregation induced by U-46619 in the present study. In human platelets, however, PGE_2 has been shown to exert a dual effect on aggregation, being inhibitory at high concentrations (>50 μM) and stimulatory at low concentrations (5–500 nM) (34). The stimulatory effect of PGE_2 on human platelets is due to an inhibition of adenylate cyclase and the priming of protein kinase C to activation by other agonists such as TXA_2 (17, 34), whereas the inhibitory effect of PGE_2 has been attributed to its nonspecific binding to PGI_2 receptors on the surface of platelets (34). The inhibition of trout thrombocyte aggregation observed in the present study by PGE_2 occurs within a concentration range that would be produced by these cells under normal physiological conditions (14), suggesting that under such conditions it would be antiaggregatory in vivo as well as in vitro. PGI_2 is the most potent inhibitor of platelet aggregation, with an IC_{50} of ~1 nM for human platelet-rich plasma and 10 nM for sheep platelet-rich plasma (35). In the present study, trout thrombocyte aggregation was significantly inhibited by PGI_2, but only at concentrations of 5 and 10 μM. Although we cannot totally discount the possibility of some breakdown of PGI_2 to 6-keto $PGF_{1\alpha}$ (biologically inactive breakdown product), this is unlikely to have happened to any great extent due to the precautions used in our studies. Hence we conclude that, unlike the observed case with mammalian platelets, PGI_2 is not the most potent inhibitor of thrombocyte aggregation in trout and that PGE_2 has a more prominent role in the regulation of thrombocyte aggregation. This is also the situation in chickens, in which PGE_2 is a more potent inhibitor of aggregation than PGI_2 (2).

In summary, the present study identified both similarities and differences between the responses of trout thrombocytes and mammalian platelets to various prostanoids and their precursors. These and other studies (2, 10, 24) do, however, show that at a relatively early stage in the evolution of vertebrates, a mechanism of platelet-thrombocyte aggregation evolved that was dependent on eicosanoids for its induction and regulation. From the present study, the mechanism of action for the inhibition of thrombocyte aggregation by DHA, EPA, PGE_2, PGI_2, and the three NSAIDs tested remains to be elucidated. Ultimately, such studies should lead to a greater knowledge of hemostasis in stress-related disorders of commercially important fish and provide a better understanding of the origins of the mammalian hemostatic mechanism.

Perspectives

This paper is part of a wider study on the evolution of hemostatic mechanisms. Lower animals, such as invertebrates, have a range of hemostatic mechanisms, including plasma gelation and blood cell aggregation. Initial studies in this laboratory have investigated if eicosanoids are involved in the aggregatory response of coelomocytes (blood cells) in echinoderms (C. E. Ray and A. F. Rowley, unpublished observations). These studies showed that the TX mimetic U-46619 had no effect on the aggregatory response of these cells, suggesting that in echinoderms, at least, the mechanism of aggregation is independent of TX and potentially of eicosanoids in general. Perhaps this may be the case in all invertebrates, but clearly studies with one species are unlikely to be representative of the diverse range of invertebrate phyla. Furthermore, to our knowledge, no one has shown that invertebrates generate $TXA_{2/3}$ in significant amounts, and this may explain the lack of effect of U-46619 on blood cell aggregation. The evolution of the first vertebrates was accompanied by the appearance of a hemostatic mechanism involving thrombocytes in which aggregation is initiated by TX generation. The present study showed striking similarities in the role of eicosanoids in initiating and controlling the aggregation of both fish thrombocytes and mammalian platelets. This implies that this is a relatively ancient mechanism that was established with the evolution of teleost fish. Clearly, it would be of interest to see if similar mechanisms also operate in cartilaginous fish

such as sharks and rays as well as the evolutionarily more primitive agnathan fish (lampreys and hagfishes). Finally, although we know the basis by which eicosanoids are involved in the aggregatory response of trout thrombocytes, we still have little idea of the underlying mechanisms at the molecular level.

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