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THE *IN VITRO* EFFECT OF EPTIFIBATIDE, A GLYCOPROTEIN IIb/IIIa ANTAGONIST, ON VARIOUS RESPONSES OF PORCINE BLOOD PLATELETS

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Abstract: The current study systematically evaluates the *in vitro* effect of eptifibatide, a GPIIb/IIIa blocker, on various responses of porcine platelets evoked by principal physiological stimulators. Eptifibatide at concentrations up to 40 mg/mL did not affect the calcium signal produced by thrombin, partly reduced the procoagulant response evoked by collagen, and strongly inhibited (IC_{50} ~11 mg/mL) adhesion of these cells to fibrinogen coated surfaces. Eptifibatide in a concentration-dependent manner reduced ADP, collagen, and thrombin-induced platelet aggregation (IC_{50} = 16-27 mg/mL), dense granule secretion (IC_{50} = 22-31 mg/mL) and lysosome secretion (IC_{50} = 25-50 mg/mL). Substantial (up to 30-40%) collagen or thrombin-evoked platelet aggregation still occurred at high (52 mg/mL) eptifibatide concentrations. Direct comparison of the susceptibility of platelet aggregation and dense granule secretion to the inhibitory action of eptifibatide indicates that aggregation is appreciably more sensitive than secretion. Eptifibatide (8 mg/mL) added together with a low (70 ng/mL) concentration of bivalirudin (a direct thrombin inhibitor) effectively (~90%) reduced platelet aggregation induced by thrombin (0.2 U/mL). Based on these results, eptifibatide is not expected to reduce efficiently thrombus formation initiated by rapid local production of large amounts of thrombin. One practical consequence of our *in vitro* studies is the suggestion that the anti-thrombotic efficacy of eptifibatide, especially in preventing acute thrombotic events, may be largely improved by its combination with direct thrombin inhibitors.

Keywords: integrilin, aggregation, secretion, porcine platelets, GPIIb/IIIa receptors

Platelets, among others, play a crucial role in the initiation of occlusion of arteries at the sites of high-grade stenosis or ruptured atheromatous plaques, thereby contributing to cardiovascular, cerebrovascular, and peripheral vascular diseases (1). Platelets are also involved in acute restenosis after coronary angioplasty (2) and are the major cause of thrombi formation at the sites of implants, providing artificial surfaces (e.g. indwelling catheters) (3).

Final step in the process of activation of platelets by various physiological stimulators (e.g. thrombin, ADP, collagen) is the exposure of active GPIIb/IIIa ($\alpha_{IIb}\beta_3$) complexes on their surface (4, 5). The expression of GPIIb/IIIa complexes enables the formation of platelet aggregates (through the interaction of fibrinogen molecules with the GPIIb/IIIa exposed on the neighboring platelets) and the adhesion of platelets to uncovered collagen fibers in the subendothelium of the injured vessel wall (6, 7). It is now commonly accepted that the binding of plasma fibrinogen to the activated platelet GPIIb/IIIa receptors is a prerequisite and crucial event in platelet aggregation, and therefore, in thrombus formation, regardless of the type of platelet stimulus. This has led to the development of a new antiplatelet strategy which utilizes GPIIb/IIIa antagonists as drugs reducing thrombotic complications in human medicine. Several anti-GPIIb/IIIa agents – including monoclonal antibodies (abciximab – Reopro®), peptides containing Arg-Gly-Asp (RGD) or Lys-Gly-Asp (KGD) sequences (eptifibatide – Integrilin®), or non-peptide compounds (tirofiban – Aggrastat®) – have been developed in recent years (8-10).

Integrilin® (eptifibatide) is a cyclic heptapeptide with low affinity and high specificity for the GPIIb/IIIa complex (11). The eptifibatide molecule is derived from the structure of barbourin, an anticoagulant found in the venom of the southeastern pigmy rattlesnake. The eptifibatide molecule contains a KGD amino acid sequence, identical with that in the distal terminus of the γ-chains of fibrinogen (12-14). The interaction between fibrinogen γ-chains and the GPIIb/IIIa receptor is through the binding site situated in a IIIa subunit. Eptifibatide tight fit into GPIIb/IIIa's fibrinogen binding pocket (a KGD bind-

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ing motif) was proposed to be responsible for its ability to block integrin function with minimal receptor activation (12). Eptifibatide is one of the cheapest antiplatelet drugs with high specificity and affinity to the GPIIb/IIIa receptor and a relatively short biological half life (11, 15). Eptifibatide was approved for human use in 1998 for prevention of thrombotic complications during percutaneous coronary intervention (12).

Although it is not of great importance for veterinary practice, eptifibatide antiplatelet action is of great potential value in experimental medicine in which pig animal models are utilized. Pigs are used for testing antiplatelet drugs in *in vivo* as well as in *in vitro* studies (16, 17) and also are often used as an experimental model in research connected with cardiovascular system diseases (16, 18, 19). The anatomy of the cardiovascular system in swine is similar to that of humans. Both species have similar cardiac physiology, including the administration of the coronary artery blood supply and cardiac conduction system (20). The experimental cardiovascular models of swine are related to the testing of interventional catheter devices, myocardial infarction, cardiovascular surgery, or the implantation of biomechanical devices (21). Swine are also valuable because of the development of transgenic technology, which could provide an unlimited source of genetically modified cells and organs for transplantations in humans (22).

No information is available about the effect of eptifibatide on porcine platelet responses. Consequently, the purpose of the present study was to evaluate systematically the *in vitro* effect of eptifibatide on various responses of platelets, including aggregation, release reaction, adhesion, procoagulant response, and the calcium signal evoked by principal physiological stimulators.

EXPERIMENTAL

Chemicals

Integrilin® (eptifibatide) was from Glaxo Smith Kline (U.K.). Reptilase (Batroxobin®) was the product of Diagnostica Stago (France). Adenosine 5'-diphosphate (ADP), fibrinogen, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (Hepes), ethyleneglycol-bis (β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), prostaglandin E_1, Russel's viper venom (RVV), bovine serum albumin (BSA), apyrase, digitonin and Fura-2/AM, p-nitrophenyl-N-acetyl-D-glucosaminide, p-nitrophenylphosphate were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Chrono Lume® luciferase reagent was from Chrono-Log Corp. (Havertown, PA, USA). Collagen (fibrillar, from equine tendon) was from Hormon Chemie (Munich, Germany). Thrombin (human) was from La Roche (Basel, Switzerland).

Animals

A total of 50 domestic pigs (breed: Polish Large White) of both sexes, approximately 9 months old, with a mean weight of 90-100 kg were used in experiments. The animals were raised on local farms under normal agricultural husbandry conditions. Pigs were fed a grower chow diet consisting of grain (rye, barley, wheat, maize) and soybean. The animals for this study were selected at random from available litters. Prior to blood collection, pigs were starved for at least 12 hours. The study protocol and procedures were approved by the Ethics Committee at the Medical University of Białystok.

Blood collection

Blood collection was performed in a local slaughterhouse (PMB, Białystok, Poland). Forty milliliters of blood were withdrawn by a direct carotid catheterization and collected into 3.8% (w/v) sodium citrate, one volume per nine volumes of blood. To avoid contamination of blood by tissue factor, the initial 10 mL of blood was discarded. Platelet numbers in the blood of studied animals varied from 368×10^6/mL to 548×10^6/mL.

Platelet preparation

Platelet rich plasma (PRP) was obtained by centrifugation of whole blood at $200 \times g$ for 20 min. To prepare washed platelets, PRP suspension was acidified to pH 6.5 with 1 M citric acid; the sample was centrifuged at $1500 \times g$ for 20 min to obtain a pellet which was resuspended in a Ca^{2+}-free Tyrode-Hepes buffer [152 mM NaCl, 2.8 mM KCl, 8.9 mM $NaHCO_3$, 0.8 mM KH_2PO_4, 0.8 mM $MgCl_2$, 5.6 mM glucose, apyrase (2 U/mL), 10 μM EGTA, BSA (3.5 mg/mL) and 10 mM Hepes, pH 6.5]. The platelets were washed once with the above buffer and finally suspended in the same buffer with the exception that, in the final suspension medium, apyrase and EGTA were omitted and pH was adjusted to 7.4. The platelet concentration was standardized to 4×10^8 cells/mL by dilution with Tyrode-Hepes buffer. Platelet number was determined using a Coulter® Hematology Analyzer.

Assay of platelet aggregation

Platelet aggregation was followed turbidimetrically by recording the light transmission through a

stirred platelet suspension in the plastic cuvette of an aggregometer (Chrono-Log Corp., Havertown, PA, USA) (23).

Simultaneous measurement of platelet aggregation and ATP secretion

The platelet release reaction was monitored simultaneously with optical aggregation using a lumiaggregometer (Chrono-Log Corp., Havertown, PA, USA). All procedures were conducted as described previously (24). The results of the chemiluminescence light signal were compared with an ATP standard curve and converted to nanomoles per 10^8 cells.

Platelet lysosome secretion

Lysosomal granule release was measured by a quantitative β-hexosaminidase assay as described by Holmsen (25). Aliquots (0.5 mL) of washed platelets were incubated at 37°C with desired concentrations of eptifibatide in the cuvette of an aggregometer without stirring for 2 min. Then, threshold concentrations of stimulator were added and platelet suspension was incubated with stirring for the next 5 min. Incubation was stopped by centrifuging of the platelet suspensions for 1 min at 11000 × *g* in an Eppendorf centrifuge. Aliquots (50 µL) of supernatant were transfered into the wells of a microtiter plate containing 50 µL of citrate-phosphate buffer pH 4.5 with 3.3 mmol/L substrate (*p*-nitrophenyl-N-acetyl-β-D-glucosaminide). After incubation at 37°C for 3 h, 100 µL of 0.1 M NaOH was added to stop the hexosaminidase reaction. The absorbance of the released color product was read at 405 nm, using a Sunrise (Tecan, Austria) plate reader. The percentage release was calculated using the following equation:

[OD_{405} (release from sample with eptifibatide) – [OD_{405} (spontaneous release)] / [OD_{405} (release from no addition of inhibitor)] – [OD_{405} (spontaneous release)] × 100.

To measure the total amount of hexosaminidase, platelets were lysed with 2 % Triton X-100.

Assay of platelet adhesion

Platelet adhesion was quantified by measure the alkaline phosphatase activity of adherent cells, as described by Bellavite et al. (26). The wells of a microtiter plate were precoated by means of overnight incubation at 4°C with 0.2 mg/mL fibrinogen. The wells were blocked with 2% bovine serum albumin in phosphate buffered saline (PBS) at an ambient temperature for 60 min. Immediately before use, the wells were washed twice with 0.9 % NaCl in an automatic plate washer (Tecan, Austria). To explore the effects of eptifibatide on platelet adhesion, aliquots (0.5 mL) of washed platelets (8×10^7 cells/mL) were incubated in the cuvette of an aggregometer for 5 min at 37°C without (control) and with the desired concentrations of eptifibatide. Then, 50 µL aliquots of this suspension were transferred to the wells of the microtiter plates coated with fibrinogen and supplemented with 25 µL of Tyrode-Hepes buffer (pH 7.4) containing 2 mM $CaCl_2$, 2 mM $MgSO_4$ and stimulatory agent 3 × the final desired concentration. These platelet suspensions were incubated at 37°C for 45 min in a humidified thermostatic chamber (37°C, 5% CO_2). At the end of the incubation period, the microtiter plates were transferred to an automatic washer and subjected at room temperature to 2 washing cycles with PBS. Each well contained 4×10^6 platelets in a final volume of 75 µL/well. Platelet adhesion was evaluated by assaying the number of adhering cells, measured as the activity of platelet acid phosphatase (26). The percentage of adherent cells was calculated on the basis of a standard curve obtained with a defined number of platelets.

Measurement of cytosolic free Ca^{2+}

Cytosolic free Ca^{2+} ($[Ca^{2+}]_i$) was determined in Fura-2-loaded platelets. All procedures were conducted exactly as described previously (27).

Measurement of platelet procoagulant activity

The assay system used was similar to that described by Rota et al. (28). It is based on Russell's viper venom, which induces generation of thrombin by activation of factors V and X (29) and which, in the presence of Ca^{2+} ions, is dependent on the availability of phosphatidylserine (PS). Defibrinated plasma was prepared by adding one part of reptilase (20 BU of Batroxobin, Diagnostica Stago, France) to 200 parts of fresh pooled plasma obtained from 5 healthy donors. The clot was left to form for 10 min at 37°C and discarded after centrifugation at 2000 × g for 10 min. The defibrinated plasma was filtered through a 0.1 µm cellulose filter (Whatman International Ltd., Maidstone, England) to remove platelet microparticles, frozen in liquid nitrogen (-196°C), and then stored in refrigerator in 1 mL aliquots at -70°C. To measure thrombin generation, 40 µL aliquots of washed platelets (4×10^8 cells/mL) suspended in Tyrode-Hepes buffer were transferred to an aggregometer cuvette containing 0.2 mL of the defibrinated plasma preincubated at 37°C for 60 s with 10 mL of Russel's viper venom (0.1 mg/mL in

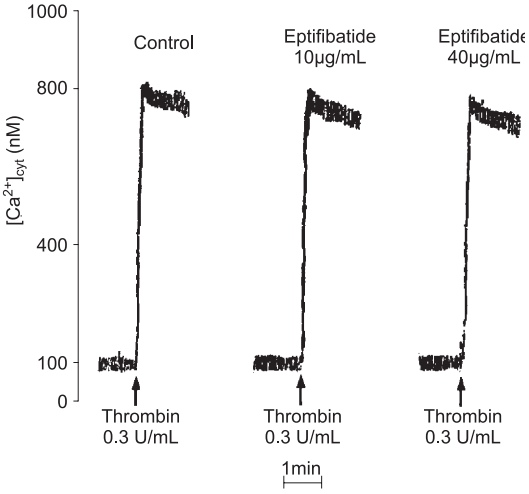

Figure 1. Effect of eptifibatide on thrombin induced $[Ca^{2+}]_i$ in porcine platelets.
Fura-2-loaded platelets suspended in normal Tyrode/Hepes buffer were treated at times indicated by arrows at 37°C with 0.3 U/mL of eptifibatide. As indicated, vehiculum or eptifibatide (20 or 40 mg/mL final conc.) were added 2 min before stimulus. $[Ca^{2+}]_i$ was calculated using the calibration procedure described in "Experimental". Each trace is representative of at least six determinations performed in three different platelet preparations.

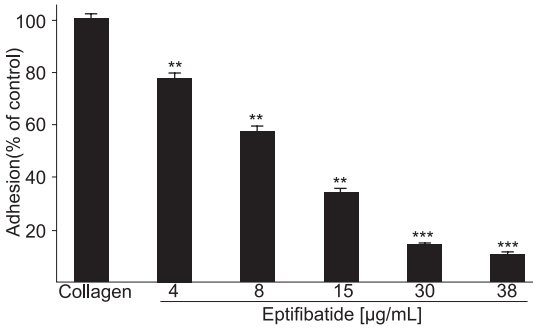

Figure 2. The effect of eptifibatide on platelet adhesion to fibrinogen coated surfaces.
Aliquots of washed platelets were incubated at 37°C in fibrinogen coated wells of a microtiter plate without (control) or with eptifibatide added to the final concentration as indicated. Adhesion was initiated by the addition of collagen threshold concentration (15 – 20 µg/mL). The extent of platelet adhesion was measured 60 min after the addition of the agonist and the maximum extent of adhesion evoked by collagen was taken as 100%. The data represent the mean values and standard deviations of seven experiments, each performed on a separate platelet preparation (n = 28), **p < 0.01, ***p < 0.005 vs. collagen.

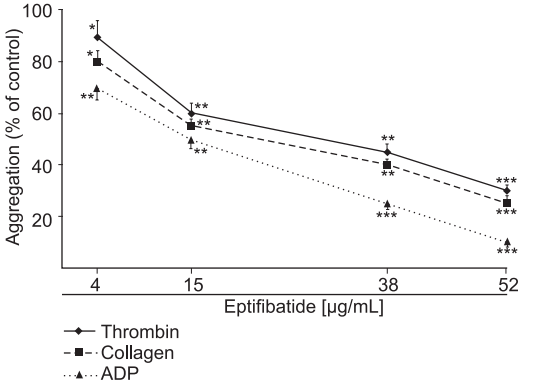

Figure 3. The effect of tirofiban on thrombin-, collagen- and ADP-induced platelet aggregation.
0.5 mL of PRP (collagen, ADP) or washed platelets (thrombin) were incubated at 37°C without (control) or with eptifibatide added to the final concentration as indicated. Aggregation was initiated by the addition of threshold concentrations of thrombin (0.2 – 0.25 U/mL), collagen (15 – 20 µg/mL) or ADP (8 – 12 µM). The extent of platelet aggregation was measured 3 min after addition of the agonist, and the maximum extent of aggregation for each stimulus was taken as 100%. The data represent the mean values and standard deviations of seven experiments, each performed on a separate platelet preparation (n = 28), *p < 0.05, **p < 0.01, ***p < 0.005 vs. control.

deionized water). After 60 s, thrombin generation was initiated by the addition of 40 µL of 0.1 M $CaCl_2$, in buffer (50 mM Tris-HCl, 0.1 M NaCl, 0.1 M $CaCl_2$, 0.5% BSA, pH 7.5). Immediately (for control) and 4 min after the addition of calcium chloride, thrombin generation was stopped by transferring 40 µL aliquots of the mixture into the wells of a microtiter plate containing 197.5 µL of buffer (50 mM Tris HCl, 0.1 M NaCl, 20 mM EDTA, 0.5% BSA, pH 7.5). To estimate thrombin activity the reaction mixture was supplemented with 12.5 µL of 4 mM S-2238 in (50 mM Tris HCl, 0.1 M NaCl, 0.5% BSA, pH 7.5) and incubated at 37°C. The absorbance of the released color product was recorded during 6 min incubation at a wavelength of 405 nm, using a Sunrise (Tecan, Austria) plate reader. The amidolytic activity of thrombin was expressed in international units (U) as the number of micromoles of *p*-nitroaniline liberated during 1 min and was calculated for 1 mL of platelet suspension.

Data analysis

Data reported in this paper are the mean (± S.D.) of the number of determinations indicated (n). Statistical analysis was performed by the Student's test and elaboration of experimental data by the use of Slide Write plus (Advanced Graphics Software, Inc. Carlsbad, CA, USA.)

RESULTS

Results presented in Figure 1 show that in platelets stimulated by thrombin, eptifibatide at a

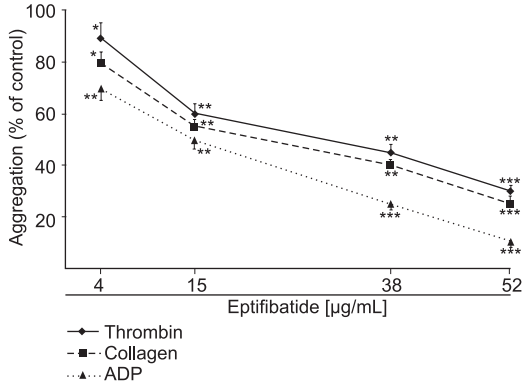

Figure 4. The effect of eptifibatide on platelet dense granule secretion.
0.5 mL of PRP (collagen, ADP) or washed platelets (thrombin) were incubated at 37°C without (control) or with eptifibatide added to the final concentration as indicated. Secretion was initiated by the addition of threshold concentrations of thrombin (0.2 – 0.25 U/mL), collagen (15 – 20 μg/mL) or ADP (8 – 12 μM). The extent of platelet secretion was measured 3 min after addition of the agonist, and the maximum extent of secretion for each agonist was taken as 100%. The data represent the mean values and standard deviations of seven experiments, each performed on a separate platelet preparation (n = 28), *p < 0.05; **p < 0.01; ***p < 0.005 vs. control.

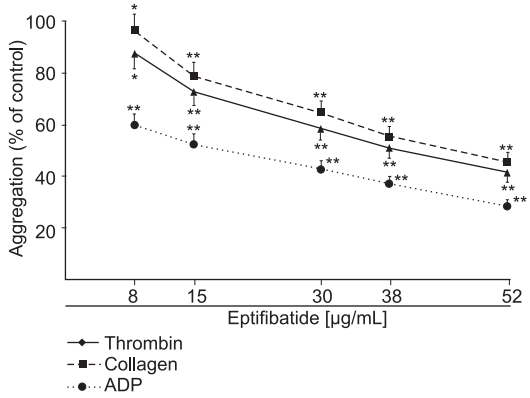

Figure 5. The effect of eptifibatide on platelet lysosome secretion. Lysosome release was measured by a quantitative β-hexosaminidase assay and the percentage release was calculated as described in "Experimental". The maximum extent of secretion for each agonist was taken as 100%. The data represent mean values and standard deviations of seven experiments, each performed on a separate platelet preparation (n = 28), *p < 0.05; **p < 0.01 vs. control.

relatively high concentration (40 μg/mL) failed to affect the generation of the calcium signal.

As can be seen from Figure 2, eptifibatide (4 – 38 μg/mL) reduced adhesion of collagen-activated platelets to a fibrinogen-coated surface, in a concentration dependent manner. The estimated IC_{50} value was ~11 μg/mL.

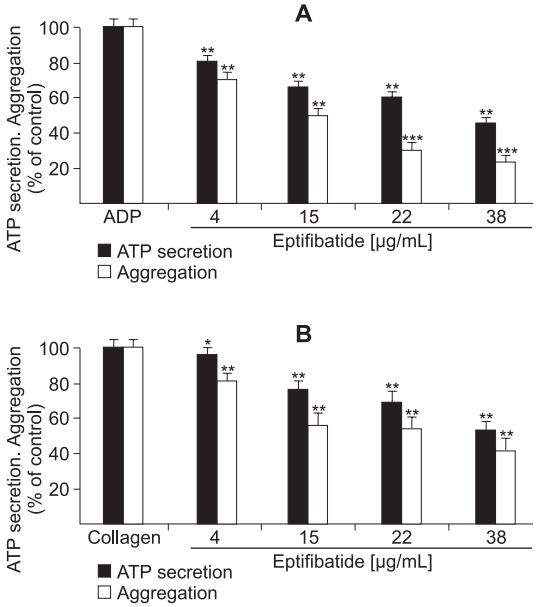

Figure 6. The effect of eptifibatide on collagen and ADP-induced platelet aggregation and secretion
0.45 mL of PRP supplemented with 50 mL of Chrono-lume (luciferin/luciferase reagent) was incubated without (control) or with eptifibatide added to the final concentrations as indicated. Aggregation and secretion were initiated by the addition of threshold concentrations of collagen (15 – 20 μg/mL) or ADP (8 – 12 μM). Optical aggregation and platelet release reaction were monitored simultaneously on a dual channel recorder. The extent of platelet aggregation and secretion was measured 3 min after addition of the agonist, and the maximum extent of aggregation and concentration of secreted ATP in control was taken as 100 %. The data represent the mean values and standard deviations of seven experiments, each performed on a separate platelet preparation (n = 28), *p < 0.05; **p < 0.01; ***p < 0.005 vs. control.

We subsequently studied the effect of eptifibatide on platelet optical aggregation induced by the threshold (TAC) concentrations of the most common physiological stimulators: thrombin, collagen, ADP. As shown in Figure 3, eptifibatide at the concentrations 4 – 52 μg/mL reduced thrombin, collagen, and ADP-induced platelet aggregation in a dose-dependent manner. The estimated IC_{50} values were ~16 μg/mL for ADP, ~25 μg/mL for collagen and ~27 μg/mL for thrombin. In the presence of 52 μg/mL eptifibatide, only aggregation induced by ADP was reduced by more than 80%. Substantial (up to 30 – 40%) aggregation still occurred in platelets stimulated by collagen or thrombin.

As seen from Figure 4, eptifibatide (4 – 52 μg/mL) dose-dependently reduced platelet dense granule secretion evoked by TAC concentration of the thrombin, collagen, and ADP. The estimated

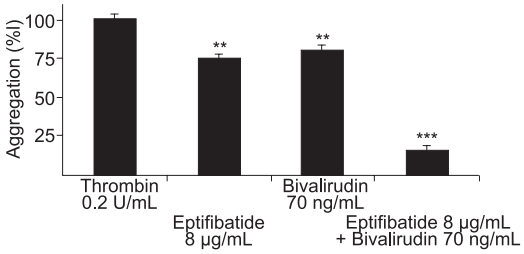

Figure 7. The effect of eptifibatide and bivalirudin on thrombin-induced platelet aggregation.
0.5 mL of washed platelets were incubated at 37°C without (control) or with eptifibatide or bivalirudin added to the final concentration as indicated. Aggregation was initiated by the addition of threshold concentrations of thrombin (0.2 –0.25 U/mL). The extent of platelet aggregation was measured 3 min after addition of the agonist, and the maximum extent of aggregation for each stimulus was taken as 100%. The data represent the mean values and standard deviations of seven experiments each performed on a separate platelet preparation (n = 28), **p < 0.01; ***p < 0.005 vs. thrombin.

Table 1. Platelet procoagulant response in the presence of eptifibatide.

Additions	**Thrombin generation (mU/mL)**
Collagen 15 µg/mL	85 ± 7
Collagen + Eptifibatide 5 µg/mL	74 ± 7*
Collagen + Eptifibatide 15 µg/mL	73 ± 6*
Collagen + Eptifibatide 22 µg/mL	51 ± 4**
Collagen + Eptifibatide 38 µg/mL	38 ± 3**
Collagen + Eptifibatide 52 µg/mL	28 ± 2***

Aliquots (300 µL) of washed platelets (4×10^8 cells/mL) were preincubated 2 min at 37°C in polypropylene cuvettes of an aggregometer without stirring. Tested substances were added to the final concentration as indicated, and after an initial mixing (30 s) incubation was continued without stirring for 10 min. In control (none) appropriate vehiculum was added instead. Phospholipid-dependent thrombin generation was evaluated as under "Experimental". The amidolytic activity of thrombin in international units (U) per 1 mL of platelet suspension is shown. The data represent the mean values and standard deviations of four experiments each performed on a separate platelet preparation (n = 16), *p < 0.05, **p < 0.01, ***p < 0.005 vs. control.

IC_{50} values were ~22 µg/mL for ADP, ~29 µg/mL for collagen, and ~31 µg/mL for thrombin. Partial (about 40%) platelet secretion still occurred even at a high (52 µg/mL) eptifibatide concentration.

As can be seen in Figure 5, eptifibatide (8 – 52 µg/mL) dose-dependently inhibited platelet lysosome secretion. A high eptifibatide concentration (52 µg/mL) reduced lysosome secretion induced by ADP, collagen, and thrombin by 70, 60, and 50 %, respectively.

Experiments shown in Figure 6 were performed to compare the susceptibility of ADP (panel A) and collagen (panel B) -induced platelet aggregation and secretion to the inhibitory effect of eptifibatide. Platelet responses were measured in PRP samples using a lumiaggregometer. Aggregation and secretion of ATP from platelet-dense granules were recorded simultaneously in the same platelet sample. As shown, treatment of platelets with eptifibatide (4 – 38 µg/mL) resulted in a distinctly stronger inhibition of aggregation than secretion.

Results presented in Figure 7 show that eptifibatide (8 µg/mL) or bivalirudin (70 ng/mL) added separately inhibited thrombin induced platelet aggregation by ~25 and ~20%, respectively. However, eptifibatide and bivalirudin added together reveal a synergistic effect, and inhibit aggregation by ~85 %.

As is shown in Table 1, eptifibatide (5 – 38 µg/mL) affects the procoagulant response in platelets induced by threshold concentrations of collagen in a dose-dependent manner. The estimated IC_{50} value was ~28 µg/mL. High eptifibatide concentrations (52 µg/mL) reduced collagen-induced procoagulant response by 70%.

DISCUSSION AND CONCLUSION

The results presented here reveal that in porcine platelets eptifibatide does not affect early stages of platelet activation since it has no effect on the generation of the calcium signal evoked by thrombin or the shape change triggered by collagen or thrombin (not shown here). Finding that eptifibatide inhibits adhesion of porcine platelets to fibrinogen coated surfaces, indicates that also in porcine platelets eptifibatide blocks GPIIb/IIIa complexes. This is because interaction between platelets and fibrinogen is believed to occur exclusively through the activated GPIIb/IIIa receptors (1, 4, 14). Eptifibatide inhibits optical aggregation of porcine platelets induced by all the principal (ADP, collagen and thrombin) physiological platelet stimulators. However, only aggregation induced by TAC concentrations of ADP was inhibited by more than 80%. In human medicine, the inhibition of aggregation by more than 80% is required in patients undergoing percutaneous coronary angioplasty (30, 31).

Ineffective reduction by eptifibatide of platelet aggregation is likely explained by the fact that GPIIb/IIIa receptors are not only constituents of the surface membrane of platelets but they are also present in internal membrane compartments like the canalicular system and alpha-granules (32-34). It has been proposed that an internal pool of GPIIb/IIIa receptors constitute about half of the total population of GPIIb/IIIa complexes present in platelets (33). In the process of platelet activation, centrally located GPIIb/IIIa receptors have been proposed to be translocated to the platelet surface (32, 35). It is hypothesized that an internal pool of GPIIb/IIIa complexes may be unavailable to the inhibitory action of eptifibatide and may interact with platelet fibrinogen (stored in the α-granules) in the course of translocation to the platelet surface (34). This may explain the low susceptibility of platelets to inhibitory action of eptifibatide, especially when cells are activated by thrombin, which is known to be the strongest inducer of platelet secretion (36).

Secretion is a very important platelet response (36). Substances released from platelet storage granules are known to potentiate aggregation and contribute to the development of platelet procoagulant response (37-39). The results presented here show that, in porcine platelets, eptifibatide is a rather weak inhibitor of dense granule and lysosome secretion, and that the inhibitory effect of eptifibatide on platelet secretion depends on the type of stimulus. Secretion induced by ADP was more strongly inhibited by eptifibatide than that evoked by collagen or thrombin. Direct comparison of the inhibitory effect of eptifibatide on platelet optical aggregation and dense granule secretion (induced by collagen or ADP) indicates that aggregation is more sensitive to the inhibitory effect of eptifibatide than secretion. These findings are similar to those of Dickfeld et al. (40) who have reported that in human platelets eptifibatide is a stronger inhibitor of platelet aggregation than secretion induced by ADP or TRAP.

It is commonly appreciated that activated platelets provide a catalytic surface (phosphatidylserine) necessary to the assembly of the tenase and prothrombinase complexes involved in the local generation of large amounts of thrombin (41, 42). It has been suggested, that the GPIIb/IIIa complexes are involved in platelet dependent thrombin generation (43, 44). It is therefore expected that the blocking of GPIIb/IIIa receptors may affect platelet procoagulant response. Our results indicate that eptifibatide is a weak inhibitor of procoagulant response since its high concentration (52 μg/mL) inhibited collagen-induced procoagulant response by ~60%.

The concentration of eptifibatide required to inhibit efficiently porcine platelet aggregation (~38 μg/mL) is higher than that used in human medicine (~2 μg/mL) (45, 46). The reason for this phenomenon is not entirely clear but it can be explained by some differences in the structure of GPIIb/IIIa receptors in humans and pigs (47).

Among three major physiological stimulators of platelet (thrombin, collagen, and ADP) eptifibatide is able to inhibit efficiently only platelet responses induced by ADP. Eptifibatide added alone is a relatively poor inhibitor of platelet responses evoked by thrombin. Since thrombin is one of the most often occurring physiological inducers of platelet activation, this may eliminate eptifibatide as an efficient antiplatelet agent in swine. The possible solution may be a combination of etifibatide with direct thrombin inhibitors. This approach may be fruitful since, as is reported here, low concentrations of eptifibatide and bivalirudin – a direct thrombin inhibitor (48), synergistically inhibit platelet aggregation induced by high thrombin concentrations.

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