Administration of a Potent Antagonist of Protease-Activated Receptor-1 (PAR-1) Attenuates Vascular Restenosis Following Balloon Angioplasty in Rats

PATRICIA ANDRADE-GORDON, CLAUDIA K. DERIAN, BRUCE E. MARYANOFF, HAN-CHENG ZHANG, MICHAEL F. ADDO, WAI-MAN CHEUNG, BRUCE P. DAMIANO, MICHAEL R. D'ANDREA, ANDREW L. DARROW, LAWRENCE DE GARAVILLA, ANNETTE J. ECKARDT, EDWARD C. GIARDINO, BARBARA J. HAERTLEIN, and DAVID F. MCCOMSEY

Drug Discovery, The R. W. Johnson Pharmaceutical Research Institute, Spring House, Pennsylvania

Received December 26, 2000; accepted March 14, 2001 This paper is available online at http://jpet.aspetjournals.org

ABSTRACT

Human platelets possess two distinct thrombin-activated receptors, PAR-1 (protease-activated receptor-1) and PAR-4, whereas human vascular smooth muscle cells possess only PAR-1. Although such thrombin receptors have been studied extensively in vitro, their physiological roles are still rather ill-defined. We have now employed a potent, selective PAR-1 antagonist, RWJ-58259, to probe the in vivo significance of PAR-1 in thrombosis and vascular injury. RWJ-58259 was examined in two thrombosis models in guinea pigs: the arterial (A-V) shunt assay (monitoring thrombus weight) and the Rose Bengal intravascular photoactivation assay (monitoring time to occlusion). Administration of RWJ-58259 (10 mg/kg, total i.v. dose) did not inhibit thrombus formation in either thrombosis model, although local, intrashunt delivery in the A-V shunt model did elicit a modest antithrombotic effect (thrombus weight reduction from 35 ± 2 to 24 ± 4 mg). These results are consistent with the presence of more than one thrombin-sensitive receptor on guinea pig platelets, in analogy with human platelets. Indeed, we were able to establish that guinea pig platelets express three thrombin receptors, PAR-1, PAR-3, and PAR-4. We also examined RWJ-58259 in a vascular restenosis model involving balloon angioplasty in rats. Perivascular administration of RWJ-58259 (10 mg) significantly reduced neointimal thickness (77 ± 5 μm to 45 ± 5 μm, P < 0.05), clearly demonstrating an important role for PAR-1 in vascular injury. From these results, it is evident that a PAR-1 antagonist is not especially effective for treating platelet-dependent thrombosis; however, it could well be beneficial for treating restenosis attendant to arterial injury.

α-Thrombin is a powerful agonist for a variety of cellular responses, and these actions are mediated by a special type of G protein-coupled transmembrane receptor known as a protease-activated receptor (PAR). Important biological effects of thrombin are mediated by such PARs in platelets, fibroblasts, monocytes, neutrophils, osteoblast-like cells, smooth muscle cells, nerve cells, and endothelial cells (Coughlin, 1994; Dennington and Berndt, 1994; Olegate et al., 1994; Van Obberghen-Schilling et al., 1995). Perhaps the best-characterized receptor function of thrombin is the activation of platelets, which is a crucial process in thrombosis and hemostasis. Thrombin is the most potent stimulator known of platelet aggregation and degranulation, and it may also be the most significant mediator of platelet recruitment during arterial thrombus formation. Thrombin-induced aggregation of human platelets is mediated by two PARs, PAR-1 and PAR-4 (Kahn et al., 1999), whereas PAR-1 is not relevant to thrombin-induced aggregation of rat or mouse platelets (Connolly et al., 1994; Derian et al., 1995). This species dependence makes it problematic to derive a good understanding of the in vivo physiology associated with different PARs.

The role of thrombin receptor activation in thrombosis and hemostasis could be demonstrated more clearly with specific pharmacological agents that can interrupt receptor function. Recently, we identified a series of potent, indole-based peptide-mimetic PAR-1 antagonists, represented by RWJ-56110, the biological function of which was characterized in vitro (Andrade-Gordon et al., 1999). This antagonist was very selective in blocking the actions of PAR-1 over the actions of PAR-2, PAR-3, or PAR-4. Interestingly, we found that the inhibitory effect of RWJ-56110 in thrombin-induced human platelet aggregation is attenuated at high enzyme levels (e.g., 8 nM), consistent with PAR-4 playing a role in thrombin signaling (at the elevated concentrations). This scenario raises a serious question about the ability of a PAR-1 antagonist to serve effectively as an antithrombotic agent, an issue of keen interest since the identification of PAR-1 in 1991 (Vu et al., 1991). We report herein the first in vivo investigation

ABBREVIATIONS: PAR, protease-activated receptor; RT, reverse transcriptase; PCR, polymerase chain reaction; bp, base pairs.
of the antithrombotic effects of a potent peptide-mimetic PAR-1 antagonist, RWJ-58259, by using two standard animal models. Since thrombin is implicated in the proliferative and inflammatory events associated with restenosis, we have also investigated the effects of RWJ-58259 in a rat model of vascular injury. Our results clearly suggest that a PAR-1 antagonist has the potential for therapeutic utility in restenosis following balloon angioplasty.

Experimental Procedures

Materials. RWJ-58259 was synthesized in our laboratories, purified by flash-column chromatography, and isolated as a dihydrochloride dihydrate (off-white powder). Details on the synthesis and isolation will be published separately. The structure of RWJ-58259 was confirmed by NMR spectroscopy and mass spectrometry; the purity was established by elemental microanalysis and reverse-phase high-pressure liquid chromatography.

Platelet Aggregation. Human platelet-rich plasma concentrate containing the anticoagulant acid-citrate dextrose (Biological Specialty Corp., Colmar, PA) was gel-filtered (Sepharose 2B, Amersham Pharmacia Biotech Inc., Piscataway, NJ) in Tyrode's buffer (140 mM NaCl, 2.7 mM KCl, 12 mM NaHCO₃, 0.76 mM Na₂HPO₄, 5.5 mM dextrose, 5.0 mM Heps, and 2 mg/ml bovine serum albumin, pH 7.4). Gel-filtered platelets were diluted with Tyrode's buffer (143,000 platelets/μl per well), compound solution in buffer, and 2 mM CaCl₂ in a 96-well microtiter plate. All fresh blood samples were obtained using sodium citrate (0.38% final concentration) as the anticoagulant. For platelet-rich plasma studies, human blood was obtained by venipuncture from healthy volunteers who were drug free for a minimum of 10 days. Guinea pigs (Hartley; Covance Inc., Denver, PA) or rats (Sprague-Dawley, Charles River, Raleigh, NC) were anesthetized and blood drawn via an intra-arterial catheter. Platelet-rich plasma was prepared by centrifugation at 200g for 10 min. Platelet-rich plasma aggregation was performed in the presence of 4 mM H-Gly-Pro-Arg-Pro-NH₂ to inhibit fibrin polymerization. Platelet aggregation was initiated by addition of an agonist shown to achieve 80% aggregation. The θ-thrombin concentrations for gel-filtered platelet and platelet-rich plasma aggregation studies were 0.15 and 7.5 mM, respectively. The SPLLLRN-NH₂ concentration used was 2 μM. The assay plate was gently mixed constantly. Aggregation was monitored at 0 and 5 min after agonist addition in a microplate reader by optical density at 650 nm (Molecular Devices, Sunnyvale, CA). Aggregation was calculated as the decrease in optical density between the two measurements. All samples were tested in duplicate wells on the same plate.

Cell Cultures. Human aortic smooth muscle cells and growth media were obtained from Cascade Biologics (Portland, OR). Rat aortic smooth muscle cells were obtained from Cell Applications (San Diego, CA) and were cultured as described (Owens et al., 1986).

Calcium Mobilization. Intracellular calcium mobilization was measured using a fluorescence technique. Rat aortic smooth muscle cells in 96-well microtiter plates were loaded with 5 μM fluo-3-AM (Molecular Probes, Eugene, OR) for 90 min. Plates were washed five times to remove unincorporated dye. Subsequent steps were performed using a fluorometric imaging plate reader (FLIPR, Molecular Devices, Sunnyvale, CA). Gel-filtered platelets on the same plate.

Drugs. Thrombin (2 nM) was added in fresh serum-free Media 231 (Cascade) and supernatants were collected after overnight incubation. Samples were analyzed by enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, MN).

PCR Analysis for PAR-1 and PAR-4. Total RNA was isolated from guinea pig washed platelets using Trizol Reagent (Life Technologies, Grand Island, NY). For conversion of RNA to first-strand cDNA, RNA samples were incubated with random primers in the presence or absence (minus RT for negative controls) of Superscript II reverse transcriptase (Life Technologies) according to the manufacturer's recommendations. PCR reactions were carried out on ca. 50 ng of cDNA, or equivalent amounts of RNA in the RT reactions, using the Advantage-GC cDNA polymerase mix (CLONTECH, Palo Alto, CA). Primers to generate and detect the respective guinea pig PAR amplifications were designed using the nucleic acid alignments of the known species for PAR-1 and PAR-3. However, numerous attempts to use this strategy to detect guinea pig PAR-4 were unsuccessful. Thus, the sequences used to amplify and detect the guinea pig PAR-4 PCR product were obtained from the partial sequence analysis of the guinea pig PAR-4 gene (manuscript in preparation). The sense and antisense primers used for the amplification of PAR sequences were: PNP1-U, 5'-CATAGACTGACGTGACATCAATCAG-3'; PNP1-L, 5'- CAAAGCAAGAAGTGAGTGCAGA-3'; PNP2-U, 5'-CAATGCGCA- AACATGGTATTTG-3'; PNP3-L, 5'-AAAATCAGGGATGAGG- GAG-3'; GPNP4-U, 5'-TGCCCGTGGGCGTCCGGCC-AATG-3'; and PNP4-L, 5'-GTCAACACGCCTGGTACGGTGCT-3'.

Reactions were conducted at a volume of 50 μl and at 25 cycles of 94°C for 30 s, 60°C for 30 s, and 68°C for 48 s for PAR-1; 20 cycles of 94°C for 30 s, 54°C for 30 s, 68°C for 56 s for PAR-2; and 28 cycles of 94°C for 30 s, 63.5°C for 30 s, and 68°C for 90 s for PAR-4. The products of each reaction (5.0 μl for PARs 1 and 3, and 50.0 μl for PAR-4) were electrophoresed through 2% agarose gels and transferred to Hybond N+ membranes (Amersham). The appropriate oligonucleotide primer probes, corresponding to nested sequences within the respective PAR PCR product, were digoxigenin-labeled, hybridized, and detected using the Genius nucleic acid detection system (Roche Molecular Biochemicals, Indianapolis, IN). The sequences used for these nested primer probes were: PNP1PP-L, 5'-CCAAGTGCTGGCAGAGGCTACCTGAGTACACC-3'; PNP3-L, 5'-TCTCATTGCGAGAATTAACCC-3'; GPNP4-U, 5'-TGGCCCGTGGGCGTCCGGCC-AATG-3'; and GPNP4-L, 5'-GTCAACACGCCTGGTACGGTGCT-3'.

Animal Models. All procedures involving the use of animals were performed in accordance with the Guide for the Care and Use of Laboratory Animals (1996) and the Animal Care and Use Committee, The R. W. Johnson Pharmaceutical Research Institute, Spring House, PA.

Ex Vivo Platelet Aggregation. RWJ-58259 was administered i.v. to anesthetized guinea pigs at the indicated doses as a 5 min infusion. Blood was withdrawn 5 min after dosing. Inhibition of thrombin or SPLLLRN-induced platelet aggregation was assessed using platelet-rich plasma.

Guinea Pig Arteriovenous Shunt Thrombosis Model. Adult male guinea pigs (Hartley, 600–750 g) were anesthetized with a ketamine hydrochloride/xylazine hydrochloride solution i.m. The left jugular vein was cannulated (PE-50) for drug administration. The left carotid artery and right jugular vein were cannulated with silicon treated (SigmaArote, Sigma Chemical, St Louis, MO), saline-filled polyethylene tubing (PE-60) and connected with a 6-cm section of silicon-treated tubing (PE-190) to form an extracorporeal arteriovenous shunt. Shunt patency was monitored using a Doppler flow.
system (model VF-1, Crystal Biotech Inc., Hopkinton, MA) and flow probe (1.0 mm, Titronics, Iowa City, IA) placed proximal to the shunt.

On completion of a 15-min postsurgical stabilization period, RWJ-58259 was administered intravenously as a loading-plus-maintenance infusion or directly into the shunt as a constant infusion. An occlusive thrombus was formed by the placement of a thrombogenic surface (#50 cotton thread, 6 cm in length) into the extracorporeal shunt. After 15 min exposure to flowing blood, the cotton thread was carefully removed and thrombus weight was calculated by subtracting the weight of the thread (3 mg) prior to placement from the total wet weight of the thread upon removal from the shunt. Arterial blood was withdrawn immediately at the conclusion of the study to assess ex vivo platelet function and coagulation.

Platelet count determinations were performed using a Sysmex K1000 differential cell counter (Sysmex Corporation, Kobe, Japan). Platelet-rich plasma aggregation induced by α-thrombin (35 nM) or SFLLRN-NH₂ (50 μM) was measured using an aggregation profiler (Bio/Data model PAP-4, Bio/Data Corp., Horsham, PA). Activated clotting time was determined using a whole-blood microcoagulation analyzer (Hemochron Jr., International Technidyne Corp., Edison, NJ). Template bleeding-time measurements were performed by the teналip-clip method, monitoring the time to clot formation.

RWJ-58259 was intravenously administered as a 5 mg/kg loading dose (over 5 or 10 min) with a subsequent 5 mg/kg maintenance infusion (over 20 min) for a total cumulative dose of 10 mg/kg. Inogatran (synthesized at the R. W. Johnson Pharmaceutical Research Institute) was administered as a 0.7 mg/kg loading dose (over 1 min) with a subsequent 0.3 mg/kg maintenance infusion (over 19 min) for a total cumulative dose of 1 mg/kg. Aspirin was administered at 100 mg/kg (over 2 min) and the shunt protocol was started 5 min later. This dose of aspirin was chosen based on previous studies whereby lower doses of aspirin had been ineffective in reducing thrombus weight. In a separate series of experiments, RWJ-58259 was administered directly into the shunt at a constant infusion of 0.1 or 0.3 mg/kg/min (over 20 min) for a total cumulative dose of 2 or 6 mg/kg, respectively. Inogatran was administered directly into the shunt at a constant infusion of 0.01 mg/kg/min (over 20 min) for a total cumulative dose of 0.2 mg/kg.

**Intravascular Photocactivation Model.** Male guinea pigs (Hartley, 375–700 g) were anesthetized with ketamine/xylazine (90/12 mg/kg, i.m.) and the right carotid artery gently isolated from the surrounding connective tissue. A 1-mm ultrasonic Doppler flow probe was secured around the artery proximal to the occlusion area and flow was continuously measured. Rose Bengal (Sigma), a photoprotective dye, was dissolved in saline and infused i.v. at 20 mg/kg over 10 min. A green, heat-filtered xenon light source, positioned 0.5 cm from the artery to illuminate a 1-cm length of the vessel, was turned on 5 min before Rose Bengal infusion and remained on for 15 min. Arterial flow was monitored for a total of 30 min following the start of the Rose Bengal infusion. RWJ-58259 was administered at a total dose of 10 mg/kg, i.v., split into a 5 mg/kg infusion for 10 min prior to Rose Bengal and 5 mg/kg infusion starting after the conclusion of the Rose Bengal infusion for the remaining 20 min of the 30-min observation period. Recombinant hirudin (Hoechst Marion Roussel, Kansas City, MO) was infused at either 1 or 3 mg/kg i.v. for 10 min prior to the Rose Bengal infusion. RWJ-58259 was dissolved in 5% dextrose and r-hirudin was dissolved in saline. In a separate group of RWJ-58259-treated guinea pigs, not exposed to Rose Bengal or light, the animals were exsanguinated, platelet-rich plasma was prepared, and ex vivo platelet aggregation to α-thrombin and SFLLRN-NH₂ was measured.

**Rat Restenosis Model.** Vascular injury was induced by balloon catheter inflation of the rat common carotid artery. A 2F embolectomy catheter was inserted via the external carotid into the left common carotid of male Sprague-Dawley rats (350–450 g) anesthetized with ketamine/xylazine (75/5 mg/kg, i.m.). The balloon tip was advanced to the aorta, inflated to 35 psi, and slowly withdrawn a total of three times. RWJ-58259 (1, 5, or 10 mg) was suspended in 150 μl of a polymer gel consisting of 50% caprylate and 50% glycolate and applied to the adventitia of the left common carotid. This polymer was shown not to affect the vascular injury response in this model. Perivascular treatment was used for these studies because RWJ-58259 is not orally active. Required intravenous infusion rates were not practical via minipump. This particular polymer has been successfully used for slow release of compounds. Since the material is absorbed slowly, we anticipated that RWJ-58259 would be released slowly over a period of time. Release kinetics were not performed for these studies. However, material tends to stay where placed and compound concentrations are expected to be high locally, likely resulting in significant levels reaching the luminal edge of the vessel. Fourteen days after injury, rats were anesthetized and perfusion-fixed with buffered formalin. Eight left carotid tissue sections (5 μm, 100 μm apart) were stained for elastin and used for morphometric analysis (Cheung et al., 1999). Medial and intimal area and thickness were measured using image analysis software. Percent stenosis was computed as intimal area as a percentage of the total area within the internal elastic lamina.

**Data Analysis.** All results are presented as mean ± S.E. Statistical analysis was performed either by the Student’s t test or one-way analysis of variance where indicated. Mean values were considered statistically significant when P < 0.05.

**Results**

**RWJ-58259 Is a Potent PAR-1 Antagonist.** We recently described a series of indole-based peptide mimetics represented by RWJ-56110, which inhibits thrombin-induced PAR-1 activation in human platelets and vascular cells (Andrade-Gordon et al., 1999). Replacement of the indole template with an indazole template afforded an improved chemical series, represented by RWJ-58259 (Fig. 1). We selected this PAR-1 antagonist for animal studies because of its good potency, PAR-1 selectivity, and particularly, in vivo safety profile.

RWJ-58259 inhibited 0.15 nM α-thrombin and 2 μM SFLLRN-induced aggregation of human gel-filtered platelets with IC₅₀, values of 0.37 ± 0.07 μM (n = 12) and 0.11 ± 0.01 μM (n = 9), respectively. The PAR-1 action of RWJ-58259 was verified by its failure to inhibit human gel-filtered platelet aggregation stimulated by either collagen or the thromboxane mimetic U46619. In addition, RWJ-58259 effectively inhibited human platelet-rich plasma aggregation induced by 7.5 nM α-thrombin (IC₅₀, 8.0 ± 2.0 μM, n = 3). The higher IC₅₀ observed for RWJ-58259 in platelet-rich plasma studies most likely reflects both the elevated thrombin concentration required to activate platelets in plasma due to endogenous thrombin inhibitors as well as increased binding of RWJ-58259 to plasma proteins. At elevated concentrations of

![Fig. 1. Chemical structure of indazole-based peptide-mimetic RWJ-58259.](image-url)
thrombin (e.g., 10–30 nM) with either human gel-filtered platelets or platelet-rich plasma, as observed previously for RWJ-56110 (Andrade-Gordon et al., 1999), RWJ-58259 became refractory in a thrombin dose-dependent manner, reflecting the dual PAR system on human platelets. The PAR-1 selectivity of RWJ-58259 was confirmed in the same, detailed fashion as described for RWJ-56110 (results not shown) (Andrade-Gordon et al., 1999).

In rat aortic smooth muscle cells, RWJ-58259 was found to inhibit α-thrombin-induced calcium mobilization (IC\textsubscript{50} = 0.07 ± 0.01 μM, n = 4) and proliferation (IC\textsubscript{50} = 2.3 ± 0.0 μM, n = 2). RWJ-58259 also blocked α-thrombin-induced interleukin-6 release from human aortic smooth muscle cells (IC\textsubscript{50} = 3.6 ± 2.3 μM, n = 2). By contrast to human platelets, full antagonism of thrombin's action was observed in these vascular cells at high thrombin concentrations (e.g., 200 nM; results not shown). The ability of RWJ-58259 to inhibit signaling and function in smooth muscle cells, independent of thrombin concentration, is reflective of PAR-1 being the only thrombin-sensitive receptor on these cells (Andrade-Gordon et al., 1999).

**Effects of RWJ-58259 on Guinea Pig Platelets.** Guinea pig platelets have been widely used to test for PAR-1 action in platelet aggregation because they are responsive to the PAR-1-activating peptide SFLLRN-NH\textsubscript{2} (Connolly et al., 1994; Derian et al., 1995), which indicates the presence of functional PAR-1 on the cell surface. Since guinea pig platelets have a lot in common functionally with human platelets, functional PAR-1 on the cell surface. Since guinea pig platelets have a lot in common functionally with human platelets, we chose this small animal to explore PAR-1 antagonism in vivo. Our previous findings with the PAR-1 antagonist RWJ-58259, which was ineffective at 6 mg/kg. This dose was the maximally tolerated intravenous dose for RWJ-58259.

**Guinea Pig Arteriovenous Shunt Thrombosis Model.** In this thrombosis model, a thrombus comprised of platelets, fibrin, and red blood cells forms on a section of cotton thread placed in an extracorporeal shunt between the carotid artery and jugular vein. Antithrombotic efficacy is indicated by decreases in the weight of thrombus accumulated during 15 min of exposure to flowing blood. Intravenous administration of RWJ-58259 (10 mg/kg) did not reduce thrombus weight (42 ± 4 mg, n = 2) when compared with a control group (43 ± 2 mg, n = 15) even though α-thrombin and SFLLRN-induced platelet-rich plasma aggregation were completely inhibited (Fig. 3A). The direct thrombin inhibitor inogatran (1 mg/kg, i.v.) or aspirin (100 mg/kg, i.v.) significantly decreased thrombus weight to 18 ± 3 mg (n = 6) and 16 ± 1 mg (n = 4), respectively. In a separate group of guinea pigs, RWJ-58259 was administered directly into the shunt just proximal to the thread in a protocol to maximize potential antithrombotic efficacy. An infusion rate of 0.1 mg/kg/min (2.0 mg/kg total dose) decreased thrombus weight slightly from a control of 35 ± 2 mg (n = 5) to 28 ± 4 mg (n = 4) (Fig. 3B). Increasing the infusion rate to 0.3 mg/kg/min (6.0 mg/kg total dose) further decreased thrombus weight to 24 ± 4 mg (n = 3). In these studies, the drug concentration (22 μM and 66 μM, respectively) was high enough to effectively inhibit α-thrombin and SFLLRN-induced platelet-rich plasma aggregation. Higher doses of RWJ-58259 could not be evaluated due to a combination of drug solubility and infusion volume. Bleeding times and activated clotting times were not changed. By comparison, administration of inogatran directly into the shunt at a rate of 0.01 mg/kg/min (0.2 mg/kg total dose) significantly decreased thrombus weight to 14 ± 2 mg (n = 3).

**Guinea Pig Photostimulation Thrombosis Model.** Intravascular photoactivation of the dye Rose Bengal with a green, heat-filtered xenon light results in endothelial damage that stimulates platelet adhesion to the vessel wall and generalized initiation of a platelet-rich thrombo-occlusive event. Antiplatelet agents and to a lesser extent, anticoagulants are

![Fig. 2. Effects on ex vivo platelet-rich plasma aggregation after i.v. administration of RWJ-58259. Platelet-rich plasma aggregation in response to increasing concentrations of thrombin was inhibited after 0.3, 1, and 3 mg/kg of RWJ-58259.](image-url)
effective in this model. Figure 4 (top panel) depicts carotid arterial perfusion and occlusion, as measured by Doppler flow, with each bar representing an individual animal. Initial occlusion times for the saline- and dextrose-treated animals averaged approximately 15 min. In all vehicle-treated animals except one, the arterial occlusion remained stable, whereas in the drug-treated groups the occlusion was unstable with intermittent flow observed over 30 min. At 1 mg/kg of the thrombin inhibitor r-hirudin, three of six treated animals were flowing at 30 min, and two of six did not experience occlusion. Two of eight RWJ-58259 treated animals were flowing at 30 min and one of eight did not experience occlusion. Total cumulative perfusion times (Fig. 4, middle panel) were significantly extended by r-hirudin at 1 and 3 mg/kg. RWJ-58259 at 10 mg/kg tended to increase perfusion times but this effect was not significant. RWJ-58259 significantly inhibited thrombin and SFLLRN-NH₂-induced platelet aggregation ex vivo (Fig. 4, bottom panel). Aggregation to low concentrations of α-thrombin (7–25 nM) was significantly inhibited, whereas aggregation at higher concentrations was much more variable and was determined not to be significantly different from that in the untreated animals. Aggregation to SFLLRN-NH₂ was completely inhibited by RWJ-58259 at all concentrations evaluated.

**Guinea Pig Platelet PAR Profile.** In vitro studies with our selective PAR-1 antagonist RWJ-58259 indicated the
presence of more than one thrombin receptor on both human and guinea pig platelets. Furthermore, results from the guinea pig in vivo thrombosis models suggested that another thrombin receptor, possibly PAR-4, plays a role in platelet-dependent thrombosis. Therefore, it was necessary to characterize the thrombin-receptor profile of guinea pig platelets. Although PAR-1 has been cloned from several species, only human and murine PAR-4 have been cloned and characterized (Kahn et al., 1998; Xu et al., 1998). Thus, we tested human PAR-1 (SFLLRN-NH₂)-, human PAR-4 (GYPGQV-NH₂)-, and murine PAR-4 (GYPGKF-NH₂)-activating peptides on human, rat, and guinea pig platelets. Whereas the human PAR-1 and PAR-4 peptides induced human platelet aggregation and the human and murine PAR-4 peptides induced rat platelet aggregation (no PAR-1 in rat platelets), only the PAR-1 peptide induced guinea pig platelet aggregation (not the PAR-4 peptides; results not shown). This outcome agrees with a recent communication by Nishikawa et al. (2000), in which washed guinea pig platelets do not respond to the murine PAR-4 peptide up to a concentration of 1 mM. To follow up on this observation, we examined the constitution of PARs in isolated guinea pig platelets by RT-PCR and were able to detect the mRNAs corresponding to PAR-1, PAR-3, and PAR-4 (Fig. 5). The apparent paradox of guinea pig platelets containing the message for PAR-4, but failing to respond to the human or murine PAR-4 peptides, was probed by isolating the guinea pig PAR-4 gene and characterizing the second exon. Like the genomic organization of other so-characterized PARs, exon 2 of the guinea pig PAR-4 gene contains the coding sequences of the entire receptor, without the initiation codon and signal sequence. Interestingly, sequence analysis revealed that guinea pig PAR-4 contains the activation motif SFPGQA, which diverges from the motifs in human (GYPGQV) or murine (GYPGKF) PAR-4. We synthesized SFPGQA-NH₂ and found that it does induce the aggregation of guinea pig platelets with an EC₅₀ of 131 μM. This result illustrates a notable flexibility in the evolution of the PAR-4 gene. In the final

A. Darrow, C. Derian, M. Addo, and P. Andrade-Gordon, manuscript in preparation.
analysis, guinea pig platelets possess two functional thrombin-responsive systems, PAR-1 and PAR-3/PAR-4.

Effects of RWJ-58259 in a Rat Restenosis Model. Since α-thrombin-mediated vascular smooth muscle cell responses associated with vascular injury (inflammatory cytokine release and cell proliferation) were inhibited by RWJ-58259, this agent would be a good candidate to assess the role of PAR-1 in a rat balloon angioplasty model of vascular injury. Furthermore, since rat platelet aggregation stimulated by α-thrombin was not inhibited by RWJ-58259, confirming the lack of PAR-1 on these cells as well as the PAR-1 selectivity of RWJ-58259, this in vivo model would reflect effects directly on the vasculature. Perivascular treatment (1, 5, 10 mg) with RWJ-58259 produced dose-related reductions in intimal area and thickness, and a decrease in percent stenosis (Table 1), which became statistically significant at the 10 mg dose. Medial area and thickness were not changed, resulting in a significant reduction in the intimal to medial ratio. There was no evidence of an effect on remodeling. There was a trend toward increased lumen area at the 10 mg dose, but this was not significant. There was no significant difference in the vessel size among the treatment groups. An example of the effect of RWJ-58259 on vascular injury is shown in Fig. 6. Thus, there is a clear reduction in neointimal thickness in the section from a rat treated with RWJ-58259 compared with a section from a rat treated with vehicle. These results indicate that inhibition of thrombin-induced activation of PAR-1 in vivo can reduce the vascular injury response.

Discussion

The thrombin receptor PAR-1 has been implicated in a variety of cellular events mediated by thrombin, including those associated with thrombosis and vascular injury. In this report, we have demonstrated that PAR-1 is involved in the restenotic events associated with balloon angioplasty in rats by using a potent, selective PAR-1 antagonist, RWJ-58259. Furthermore, results with RWJ-58259 in two different guinea pig thrombosis models reveal that PAR-1 may partially mediate platelet-dependent thrombus generation; however, there are serious concerns about the suitability of this, and other, species for such antithrombotic studies.

Antithrombotic Effect of RWJ-58259. The presence of divergent thrombin-receptor profiles for platelets of different species was first recognized in studies employing the PAR-1 agonist peptide SFLLRN (Connolly et al., 1994; Derian et al., 1995). Platelets isolated from the blood of humans, primates, and guinea pigs, but not rabbits, rodents, and dogs, were responsive to SFLLRN, although all of the species responded to thrombin. Based on the species studies, we reasoned that the guinea pig would provide an appropriate small-animal model to assess platelet PAR-1-dependent responses associated with thrombosis. In both models evaluated, inhibition of thrombin’s proteolytic activity resulted in significant antithrombotic effects, confirming a significant role for thrombin-mediated thrombus formation. Our results with RWJ-58259 revealed just a modest effect on thrombus formation in the two guinea pig models, raising the distinct possibility that PAR-1 is not a significant contributor to platelet thrombus formation. Our in vitro and in vivo platelet aggregation results with RWJ-58259 indicated that it is an effective antagonist of guinea pig PAR-1; however, its effectiveness was dependent on thrombin concentration. Complete antagonism of thrombin in vitro was achieved at low thrombin concentrations, but the effect diminished as the thrombin levels rose above 10 nM. Thus, we hypothesized that another thrombin-responsive receptor existed on guinea pig platelets.

Three thrombin receptors, PAR-1, PAR-3, and PAR-4, have been described and the PAR profiles of human and murine platelets have been reasonably well defined (Vu et al., 1991; Ishihara et al., 1997; Xu et al., 1998). On the basis of studies with PAR-3-deficient mice, it appears that a dual thrombin receptor system (PAR-3/PAR-4) exists on the platelets of wild-type mice (Kahn et al., 1998). However, human platelets do not express PAR-3 and thus PAR-1 was considered to be the only thrombin receptor on these cells. The discovery of human PAR-4 then suggested that human platelets do have a dual thrombin receptor system (PAR-1/PAR-4) (Xu et al., 1998). The presence of PAR-4 on human platelets is consistent with the loss of thrombin antagonist activity with our PAR-1 antagonists, RWJ-56110 and RWJ-58259 at elevated thrombin concentrations (Andrade-Gordon et al., 1999). Since the activity of RWJ-58259 was similar in isolated human and guinea pig platelets, we hypothesized that the results of our in vivo thrombosis models reflected a dual thrombin receptor system, PAR-1 and PAR-4, on guinea pig platelets.

Therefore, we sought to determine the PAR profile of guinea pig platelets, first by agonist peptide studies, then by RT-PCR. Surprisingly, our results indicate a triple PAR expression pattern with the presence of PAR-1, PAR-3, and PAR-4. This result raises important questions about the complex interactions of the different PARs during thrombus formation in different species and ultimately, the significance of PAR-4 activation in human thrombotic disease. The interaction of PAR-3 and PAR-4 was elegantly described by Nakanishi-Matsui et al. (2000), who demonstrated that PAR-3 serves as a cofactor for PAR-4, thereby increasing the thrombin sensitivity of PAR-4 by as much as 10-fold. The coordinated action of PAR-3/PAR-4 appears to mirror the action of

**TABLE 1**

<table>
<thead>
<tr>
<th>Group</th>
<th>No.</th>
<th>Intima Area, mm²</th>
<th>Media Area, mm²</th>
<th>Stenosis %</th>
<th>Intima Thickness, μM</th>
<th>Media Thickness, μM</th>
<th>I/M</th>
<th>Lumen Dimensions, mm²</th>
<th>Vessel Dimensions, mm²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>10</td>
<td>0.129 ± 0.005</td>
<td>0.123 ± 0.006</td>
<td>44 ± 3</td>
<td>77 ± 5</td>
<td>58 ± 2</td>
<td>1.35 ± 0.09</td>
<td>0.231 ± 0.026</td>
<td>0.480 ± 0.028</td>
</tr>
<tr>
<td>1 mg</td>
<td>9</td>
<td>0.118 ± 0.010</td>
<td>0.118 ± 0.007</td>
<td>38 ± 2</td>
<td>68 ± 5</td>
<td>55 ± 2</td>
<td>1.24 ± 0.09</td>
<td>0.258 ± 0.019</td>
<td>0.482 ± 0.026</td>
</tr>
<tr>
<td>5 mg</td>
<td>9</td>
<td>0.110 ± 0.008</td>
<td>0.113 ± 0.007</td>
<td>36 ± 3</td>
<td>63 ± 4</td>
<td>53 ± 2</td>
<td>1.19 ± 0.09</td>
<td>0.239 ± 0.025</td>
<td>0.447 ± 0.035</td>
</tr>
<tr>
<td>10 mg</td>
<td>8</td>
<td>0.084 ± 0.007*</td>
<td>0.121 ± 0.006</td>
<td>26 ± 3*</td>
<td>45 ± 5*</td>
<td>54 ± 2</td>
<td>0.83 ± 0.10*</td>
<td>0.329 ± 0.031</td>
<td>0.507 ± 0.034</td>
</tr>
</tbody>
</table>

I/M: intima/media

*P < 0.05, significantly different from vehicle by one-way analysis of variance.
PAR-1 with respect to thrombin sensitivity. The expression of all three PARs in guinea pig platelets suggests that two equally responsive thrombin systems, PAR-1 and PAR-3/PAR-4, exist there. The lack of significant antithrombotic activity for RWJ-58259 in the two guinea pig thrombosis models can be explained by the occurrence of thrombin-dependent platelet activation via PAR-3/PAR-4 during complete PAR-1 blockade. Our results indicate that guinea pigs may not be a suitable animal model for evaluating PAR-1 antagonists as potential antithrombotic drugs for humans.

The physiological role of this PAR redundancy may be a protective system to assure effective, rapid platelet aggregation during severe vascular injury, when concentrations of thrombin would be explosively elevated. Because of the complexity of multiple PARs, it has been difficult to dissect the contributions of individual PARs to the process of thrombosis, and this may have prevented the development of a PAR-1 antagonist as a potential therapeutic agent. The potential significance of PAR-4 activation in human clinical disease remains to be determined. Future studies in nonhuman primates, whose platelet PAR profile is similar to that of humans (unpublished observation), should provide a better means to evaluate the antithrombotic efficacy of selective PAR-1 antagonists.

**Antirestenotic Action of RWJ-58259.** Vascular injury associated with angioplasty procedures results from both thrombotic and restenotic components. While our results with RWJ-58259 in the thrombosist models did not conclusively determine the impact of PAR-1 antagonism on thrombotic processes, RWJ-58259 showed significant inhibition of neointimal thickening in the rat model of vascular injury, consistent with a direct effect on PAR-1-mediated vascular smooth muscle function. These results are highly significant, since rat platelets are fully responsive to thrombin through PAR-3/PAR-4 activation. Our results are consistent with a recent study that showed a reduced vascular injury response in rats treated with an antibody to PAR-1 (Takada et al., 1999). We have also found that the vascular injury response is reduced in mice deficient in PAR-1 compared with wild-type mice (Cheung et al., 1999).

PAR-1 is up-regulated in vascular smooth muscle cells in response to vascular injury in animal models (Wilcox et al., 1994; Cheung et al., 1999) and in human atherosclerotic coronary arteries (Nelken et al., 1992). This up-regulation is associated with proliferating cells. Thus, the effectiveness of PAR-1 antagonism in reducing vascular injury may be the result of inhibition of PAR-1-mediated vascular smooth muscle proliferation (McNamara et al., 1993). Consistent with this view, RWJ-58259 effectively inhibited thrombin-induced calcium mobilization and proliferation in rat aortic smooth muscle cells. Thrombin levels are also greatly increased at sites of vascular injury (Hatton et al., 1989; Harker et al., 1995). Although thrombin inhibitors have reduced vascular injury responses in several animal models (Heras et al., 1990; Barry et al., 1996; Gerdes et al., 1996), initial clinical trials have been unable to show the effectiveness of thrombin inhibition in vascular injury (Serruys et al., 1995; Burchenal et al., 1998). This observation may derive from inadequate treatment regimens. Alternatively, there may be some advantage to the specific blockade of PAR-1 as opposed to the inhibition of all of thrombin's many actions with a direct enzyme inhibitor.

In summary, we were unable to ascertain the antithrombotic potential of a PAR-1 antagonist in guinea pig models of thrombosis because of interference from the PAR-3/PAR-4 system present on guinea pig platelets. Thus, a determination of possible antithrombotic utility preclinically would require studies that surmount the species issue, such as through the use of primate models. However, our results with RWJ-58259 in rats indicate that selective antagonism of PAR-1 can significantly attenuate restenosis following balloon angioplasty. Accordingly, inhibition of PAR-1 may have therapeutic potential in human vascular injury.

**References**

Andrade-Gordon P, Maryanoff BE, Derian CK, Zhang H-C, Addo MF, Darrow AL, Eckardt AD, Hoekstra WJ, McComsey DF, Oksenberg D, Reynolds EE, Santulli...
Andrade-Gordon et al.


Address correspondence to: Dr. Patricia Andrade-Gordon, The RW Johnson Pharmaceutical Research Institute, R-348, Welsh and McKean Roads, Spring House, PA 19477-0776. E-mail: pandrade@prius.jnj.com