

**ROLE OF PROTEASE-ACTIVATED RECEPTORS  
IN PLATELET ACTIVATION**

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**By  
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## **ABSTRACT**

### **Role of Protease-activated Receptors in Platelet Activation**

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Platelets act as a fundamental component of the hemostatic process and their activation leads to the formation of a stable clot at the injured endothelium surface. Thrombin, as the important physiological agonist, activates platelets through protease-activated receptors (PARs). Protease-activated receptors are one of the major receptors in platelets and belong to the seven-transmembrane G-protein couple receptor family. Four protease-activated receptors are found, named as PAR1, PAR2, PAR3 and PAR4. Human platelets express PAR1 and PAR4 and murine platelets express PAR4 and PAR3 instead of PAR1. Thrombin activates PARs through a unique mechanism, involving the cleavage of N-terminus of PAR receptors and the newly exposed N-terminus acts as its own tethered ligand to bind and activate the receptor. In this study, we characterized a new PAR1 specific activating peptide (TFRRRLSRATR), generated from the c-terminus of human platelet P2Y<sub>1</sub> receptor, and evaluated its biological function. This peptide activated platelets in a concentration-dependent manner, causing shape change, aggregation, secretion and calcium mobilization. Its activation is completely inhibited by using BMS200261, a

PAR-1 specific antagonist. Its specificity to PAR1 receptor is further confirmed by using TFRRR-peptide-pretreated washed platelets and murine platelets. The shape change induced by 10  $\mu$ M peptide was totally abolished by Y-27632, an inhibitor of p160<sup>ROCK</sup> which is the downstream signal of G<sub>12/13</sub> pathways. The TFRRR-peptide, YFLLRNP, and the physiological agonist thrombin selectively activated G<sub>12/13</sub> pathways at low concentrations and began to activate both Gq and G<sub>12/13</sub> pathways with increased concentrations. Similar to SFLLRN, the TFRRR-peptide caused phosphorylation of Akt and Erk in a P2Y<sub>12</sub> receptor-dependent manner, and p-38 MAP kinase activation in a P2Y<sub>12</sub>-independent manner. The effects of this peptide are elicited by the first six amino acids (TFRRRL) whereas the remaining peptide (LSRATR), TFERRN, or TFEERN had no effects on platelets.

Beside thrombin, PARs also can be activated by other proteases. Previous studies in our lab show that plasmin, a major extracellular protease, activates both human and murine platelets through prototypical cleavage of PAR4 (Quinton *et al.*, 2004). In this study, we continue our study and investigate the molecular basis for the differential activation of murine and human platelets by plasmin. Plasmin-induced full aggregation is achieved at lower concentrations (0.1 U/mL) in murine platelets as compared to human platelets (1 U/mL). In COS7 cells expressing the murine PAR4 (mPAR4) receptor, 1 U/mL plasmin caused a higher intracellular calcium mobilization than in cells expressing the human PAR4 (hPAR4) receptor. This

difference was reversed when the tethered ligand sequences of mPAR4 and hPAR4 were interchanged through site-directed mutagenesis. This difference between human and murine PAR4 is not because of the cofactor effect of PAR3 in murine platelets by showing that in both transfected cell lines and platelet system, PAR3 inhibits plasmin-induced PAR4 stimulation. All of the data suggest that murine platelets are more sensitive to activation by plasmin than human platelets due to differences in the primary sequence of PAR4. In contrast to thrombin-dependent activation of platelets, wherein PAR3 acts as a co-receptor, mPAR3 inhibits plasmin-induced PAR4 activation.

Abnormal platelet activation causes thrombus formation and induces pathological conditions including stroke and atherosclerosis. Antithrombotic therapy is a widely used therapeutic method for stroke. However, currently used agents based on the irreversible inhibition of the platelet cyclooxygenases 1 and 2 or inhibition of P2Y<sub>12</sub> receptors can cause unexpected bleeding or resistant side effects. Antithrombotic therapy targeting thrombin signaling is one of the new treatments under investigation and PAR1 antagonists are now in clinical trials. In this study, we investigate the effect of one of thrombin receptors, protease-activated receptor 4 (PAR4) in mice transient middle cerebral artery occlusion/ reperfusion (tMCAO/R) model. Our data show that PAR4<sup>-/-</sup> mice have more than 80% reduction in infarct volume and significant improved neurological and motor function after 1 h MCAO followed by 23 h reperfusion. Examination of cellular responses to tMCAO/R indicates that PAR4<sup>-/-</sup>

mice have less cellular death. Platelet/endothelial and leukocyte/endothelial interactions have been shown to play a critical role in the inflammatory responses during cerebral ischemic/reperfusion injury. Comparing wild-type with PAR4<sup>-/-</sup> mice platelets/endothelial and leukocyte/endothelial interactions, deficiency of PAR4 causes a significant decrease in both platelet/endothelial and leukocyte/endothelial interactions. In addition, PAR4<sup>-/-</sup> mice attenuate blood-brain barrier (BBB) disruption during tMCAO/R. All the data suggest that deficiency of PAR4 will protect against brain ischemic injury through attenuation of cerebral inflammatory responses including inflammatory cells extravasation and BBB disruption. Protease-activated receptor 4 (PAR4) is the only thrombin receptor existing in both human and murine platelets. The data we get in this study also have a beneficial effect for human study and inhibition of PAR4 may provide a novel potential therapeutic strategy for ischemic injury.

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## **CHAPTER 1**

### **INTRODUCTION**

#### **Overview of Hemostasis and Thrombosis**

Hemostasis is the body's ability to maintain blood in a fluid state under physiologic conditions and arrest of flowing blood out of a vessel wall when vascular injury is occurred. Thrombosis will occur if the hemostatic system is unbalanced. The endothelium, platelets and coagulation system are the three important components in regulating the balance of human hemostatic system.

One of the key roles of endothelium is to maintain blood fluidity. It generates inhibitors to modulate vascular tone and permeability, inhibit platelet aggregation and coagulation system and promote fibrinolysis under physiologic conditions. When vascular injury occurs, endothelial cells will be damaged and lose their protective function. Subendothelial matrix will be exposed and the collagen on the surface of subendothelial matrix will bind to von Willebrand factor (VWF) to activate platelets and cause platelet rolling and adhesion to form a thrombus. The endothelial cells around the injury site will secrete inhibitors to inhibit platelet aggregation and hence direct platelets only to deposit on the injury site to heal the injury.

Platelets are among the fundamental components in maintaining the homeostasis. Platelets are produced from bone marrow megakaryocytes. Under physiologic condition, platelets do not adhere to normal vascular endothelial cells. Once platelets are activated, they will bind fibrinogen and aggregate, secrete granules and eventually lead to thrombotic events. In addition, at the site of vascular injury, activated platelets also express P-selectin on their surface and recruit leukocytes to deposit by interacting with leukocytes counter receptor P-selectin GP ligand-1. This interaction will further induce an inflammatory response under pathophysiological conditions, contributing to phenomenon such as ischemic stroke.

The coagulation system is also important to balance the hemostatic system. Under homeostatic conditions, the body is maintained in a balance of coagulation and fibrinolysis. The coagulation system includes intrinsic and extrinsic pathways. The activation of the coagulation cascade will produce thrombin that converts fibrinogen to fibrin. As the major initiating pathway *in vivo*, the extrinsic system is initiated by tissue factor, whereas the intrinsic cascade (which has less *in vivo* significance in normal physiological circumstances than the extrinsic cascade) is initiated when direct contact is made between blood and exposed negatively charged vascular surfaces. Fibrinolysis is the opposite process as coagulation. Plasmin, a serine protease that circulates as the inactive proenzyme (plasminogen), is the major activator for degradation of fibrin clots. The unbalance between fibrinolysis and coagulation will cause hemorrhage or thrombosis.

## **G-protein coupled receptor**

G protein-coupled receptors (GPCR), also known as seven transmembrane domain receptors, comprise a large family of transmembrane receptors that are activated by binding to ligand and initiate intracellular signal cascade pathways. G-protein coupled receptors are the major agonist receptor family in cells and are involved in a variety of physiological processes. They are also well represented on platelets and critical for platelet function. Platelets are activated through G protein-coupled receptors involving three major G protein-mediated signaling pathways:  $G_q$ ,  $G_{12/13}$ , and  $G_i$ .

## **Protease-activated receptor**

### **Protease-activated receptors family**

The protease-activated receptors are a subfamily of related G protein-coupled receptors and the major representatives of G-protein coupled receptors in platelets. The first protease-activated receptor was identified in 1991 by Coughlin and colleagues when searching for a receptor that confers thrombin signaling on human platelets and other cell types (Rasmussen *et al.*, 1991; Vu *et al.*, 1991a). PAR2 was the second identified PAR receptor by screening a mouse genomic library screen using probes homologous to the transmembrane regions of the substance K receptor (Nystedt *et al.*, 1994). Other PARs were found when studying PAR1-knockout mice.

Fibroblasts from PAR1-knockout mice did not respond to thrombin, whereas PAR1-null platelets responded normally to thrombin (Connolly *et al.*, 1996). All these findings led to the identification of PAR3 and PAR4 and to the discovery of the species-specific differences in PAR expression in platelets (Ishihara *et al.*, 1997) (Kahn *et al.*, 1998; Xu *et al.*, 1998). PAR3 and PAR4 are expressed and mediate thrombin signaling in mouse platelets, whereas PAR1 and PAR4 are responsible for thrombin-induced activation in human platelets.

Among these 4 members, PAR1, 3 and 4 are found to be activated by thrombin, which are called thrombin receptors. PAR2 does not respond to thrombin, instead it is activated by trypsin (Nystedt *et al.*, 1994), mast cell tryptase (Molino *et al.*, 1997b) and coagulation factor (F) VIIa (Camerer *et al.*, 2000) and FXa (Riewald *et al.*, 2001). Compared to human PAR1, 2 and 3, PAR4 is about 33% homologous to other human PAR receptors and has some distinct differences in the N- and C-terminal domains, which could be caused by the location of PAR4 in chromosome 19, while human PAR1, 2 and 3 locate in chromosome 5 and these differences may also explain the different activation from PAR1 when stimulated by thrombin, which will be discussed below.

### **Activation of protease-activated receptors**

Thrombin activates PAR1 and PAR3 differently from PAR4. PAR1 and PAR3 can be activated by a two-steps mechanism: the protease binds to receptor first and then

cleaves the receptor from N-terminal sequences. The extracellular amino terminus of human PAR1 contains a hirudin-like sequence of charged residues (D<sup>51</sup>KYE<sup>56</sup>PF<sup>56</sup>), which is distal to the thrombin cleavage site. This domain binds to an anion binding site on thrombin and concentrates the protease at the surface of the receptor. Deleting the hirudin-like domain is demonstrated to dramatically decrease the capacity of thrombin to activate PAR1. Furthermore,  $\gamma$ -thrombin, which lacks this hirudin-like domain, is 100-fold less potent than  $\alpha$ -thrombin, which has this site (Bouton *et al.*, 1995). Similar to PAR1, PAR3 also contains a hirudin-like site (FEEFP) distal to the thrombin cleavage site (Ishihara *et al.*, 1997), which will explain the cofactor effect of PAR3 to PAR4 activation in murine platelets. In contrast to the other thrombin receptors, PAR4 lacks a hirudin-like binding site for thrombin (Kahn *et al.*, 1998; Xu *et al.*, 1998). Because of lacking this binding site, PAR4 activation needs higher concentration of thrombin compared with PAR1 receptor. Both  $\gamma$ -thrombin and  $\alpha$ -thrombin have a similar potency for PAR4 activation (Xu *et al.*, 1998).

Thrombin cleaves PAR1 at R<sup>41</sup> ↓S<sup>42</sup>FLLRN. Similarly, trypsin cleaves PAR2 at R<sup>34</sup> ↓S<sup>35</sup>LIGKV to reveal the N-terminal tethered ligand SLIGKV in humans. PAR3 can be cleaved at K<sup>38</sup> ↓T<sup>39</sup>FRGAP and PAR4 can be cleaved at R<sup>47</sup> ↓G<sup>48</sup>YPGQV in human. Mutation of the cleavage site of these receptors will prevent agonist cleavage and signaling. In addition to the proteases, PARs also can be activated by peptides, which mimic the newly exposed tethered ligand, without cleaving the receptor. However, among these synthesized peptides, synthesized PAR4 peptide-AYPGKF is

more effective than natural tethered ligands for human PAR4—GYPGQV or murine PAR4---GYPGKF. In contrast to PAR1, 2 and 4, synthetic peptide corresponding to PAR3 tethered ligand does not activate PAR3.

The studies of the activation of PARs show a unique mechanism, which involves proteolytic cleavage of the amino terminus of the receptor and exposing a new amino terminus. The newly formed amino terminus will serve as a tethered ligand domain and bind to receptor's second extracellular loop to cause the receptor conformation change and initiate the intracellular signaling. Although the typical proteolytic cleavage causes activation of the same receptor, there are studies showing crosstalk between different PARs. The intermolecular signaling was first demonstrated by studying PAR1 receptor in a reconstituted system (Chen *et al.*, 1994). In human endothelial cells, PAR3 was also shown to form a dimer with PAR1 and regulated PAR1 signaling (McLaughlin *et al.*, 2007). One of the most important discoveries is that in murine platelets, although thrombin binds to PAR3, it can not induce any downstream signaling. On the other hand, PAR3 acts as a cofactor and localizes thrombin to activate PAR4. Another crosstalk was also reported in endothelial cells: the tethered ligand domain of signaling-defective cleaved PAR1 can transactivate PAR2 (O'Brien *et al.*, 2000).

The proteolytic activation of PARs is irreversible. Rapid phosphorylation and arrestin binding will desensitize activated GPCRs and hence promote uncoupling of the

receptor from G protein signaling in seconds. Unlike other GPCRs, activated PARs are unable to be recycled. Once they are activated, they will be directly internalized, sorted to lysosomes and rapidly degraded. Once PAR1 is activated, it will then be internalized by removing it from signaling effectors. Lysosomal sorting will prevent it from returning to the cell surface to continue signaling, terminating signaling (Trejo *et al.*, 1998). Internalization is also one of the signal termination mechanisms for activated PAR4; however, phosphorylation of the activated PAR4 was not detected (Shapiro *et al.*, 2000). Compared with PAR1, PAR4 has a slower internalization rate. This slower rate and lack of phosphorylation of activated PAR4 may explain the sustained signaling of PAR4, which is important for thrombin-stimulated platelet aggregation (Covic *et al.*, 2000; Shapiro *et al.*, 2000).

### **Protease-activated receptor-mediated platelet responses**

The effects of PARs in platelets were well investigated. Human platelets express PAR1 and PAR4. PAR1 acts as the major PAR receptor in human platelets. It is the high affinity thrombin receptor and can be activated by low concentration of thrombin. On the other hand, PAR4 acts as a low affinity thrombin receptor and need higher concentration of thrombin to be activated. Furthermore, PAR1 and PAR4 display different kinetics of calcium mobilization: PAR1 activating peptide SFLLRN causes a typical spike in calcium levels, on the other hand, AYPGKF activates PAR4 and induces a prolonged and sustained increase in calcium level (Covic *et al.*, 2000).

10 $\mu$ M SFLLRN, 500  $\mu$ M AYPGKF or 1 U/ml thrombin are enough to induce maximal calcium increase in platelets. PAR1 was not expressed in murine platelets, are demonstrated by the facts that PAR1<sup>-/-</sup> mice platelets did not respond to PAR1 activating peptide SFLLRN. In stead, PAR3 and PAR4 are expressed in murine platelets. By using the knock out approach, PAR4 was confirmed to be the major PAR receptor in murine platelet and thrombin could not activate PAR4 null mice platelets. However, PAR3 knock out mice show a delayed thrombin response and in transfected cells, PAR3 could potentiate thrombin-induced PAR4 activation at low concentration, which confirmed that in murine platelets, PAR3 acts as a cofactor to facilitate the response of PAR4 in the presence of low concentration of thrombin (Kahn *et al.*, 1998; Nakanishi-Matsui *et al.*, 2000).

Upon activating, PAR receptors will change their conformation and interact with heterotrimeric G proteins. The  $\alpha$  subunit and  $\beta\gamma$  heterodimer will activate different effectors to initiate downstream signaling cascades. The signal transduction of PARs in platelets was well investigated. Agonist stimulation of these receptors induces activation of G<sub>12/13</sub> and G<sub>q</sub> pathways. When coupled to G<sub>12/13</sub> pathways, it activates Rho and RhoA, p160ROCK pathways. G<sub>12/13</sub>- RhoA pathways are demonstrated to be important for platelet shape change (Paul *et al.*, 1999) and PAR-mediated dense granule release (Jin *et al.*, 2009). Inhibition of RhoA kinases by using exoenzyme C3 transferase significantly inhibited thrombin, SFLLRN or AYPGKF-induced platelet aggregation and secretion. Stimulation of G<sub>q</sub> pathway results in the activation of

phospholipase C $\beta$ 2 (PLC $\beta$ 2). Activated PLC $\beta$ 2 hydrolyzes membrane bound phosphatidylinositol-4, 5-trisphosphate (PIP $_2$ ) to inositol-1, 4, 5-trisphosphate (IP $_3$ ) and diacylglycerol (DAG). IP $_3$ , in turn, mobilizes calcium and DAG activates protein kinase C (PKC). Platelets, activated by thrombin, thus undergo shape change, aggregate and secrete their dense granular contents (Figure 1.1).

### **Protease-activated receptors in endothelial cells and central nervous system**

In addition to platelets, PARs also express in other systems, such as endothelial cells and central nerve system, to regulate cellular response when stimulated by thrombin under physiological and pathophysiological conditions. PAR1 and PAR4 mediate thrombin signaling in mouse endothelial cells (Kataoka *et al.*, 2003), however, in human endothelial cells, PAR1 and PAR3 instead of PAR4 respond to thrombin activation (O'Brien *et al.*, 2000). PAR1 and the coupled G $\alpha_{13}$  is the key mediator for the formation and maintenance of blood vessels in embryonic development. Both PAR1 deficiency and endothelial cell-specific G $\alpha_{13}$  knockouts displayed a similar phenotype (Ruppel *et al.*, 2005). In addition to thrombin, the anticoagulant-protease APC also can activate PAR1 on human endothelial cells. APC and thrombin both activate PAR1, but promote anti-inflammatory or pro-inflammatory responses respectively and furthermore, which can be reversed using low thrombin and high APC concentration (Riewald *et al.*, 2002). Endothelial cells also express PAR2, which

can be activated by coagulant factors VIIa and Xa (Camerer *et al.*, 2000; Riewald *et al.*, 2001).

Recently, studies show that PARs also express in central nerve system and play an important role in central nerve system diseases. PARs widely express in brain of rodent and human. Immunoreactivity corresponding to PAR1 was detected in normal human brain. The agonist of PARs, thrombin, also can enter the brain at high concentration after the disruption of blood-brain barrier under pathophysiological conditions. In addition, prothrombin is also detected in rat and human brains, which can form thrombin locally. Additionally, thrombin inhibitors, such as protease nexin-1 (PN-1) and antithrombin III, are expressed in the brain. Although the expression of prothrombin, thrombin inhibitors and thrombin receptor PARs suggest that thrombin can be generated and regulate brain function through PARs under physiological condition, Sinnreich *et al.* did not detect the increase of thrombin enzymatic activity without adding thrombin-activating factor ( such as blood-derived factors ) and over expression of prothrombin in neurons has no effect on increasing thrombin enzymatic activity (Sinnreich *et al.*, 2004). All these studies indicated that the effect of PAR activation was more important under pathological conditions when the blood-brain barrier is destroyed.

Thrombin has been demonstrated to modulate ischemic brain injury. High levels of thrombin are detected in ischemic rat brain which may be generated by increased

local prothrombin or infusion of bloodstream thrombin into brain parenchyma after disruption of blood-brain barrier. Studies also show that the expression levels of different PARs are different under experimental conditions in rat brain. PAR1 is found highly expressed in hippocampal slices after experimental ischemia. The inhibition of PAR1 by using PAR1<sup>-/-</sup> mice or PAR1 antagonist will protect brain injury from transient focal ischemia. It was shown that after transient focal ischemia in rat brain, PAR1 mRNA was found to be down regulated. However, PAR3 and PAR4 mRNA levels were increased (Rohatgi *et al.*, 2004) and there was enhanced expression of PAR1 and PAR3 on microglia and enhanced PAR4 labeling in the penumbra (Striggow *et al.*, 2001).

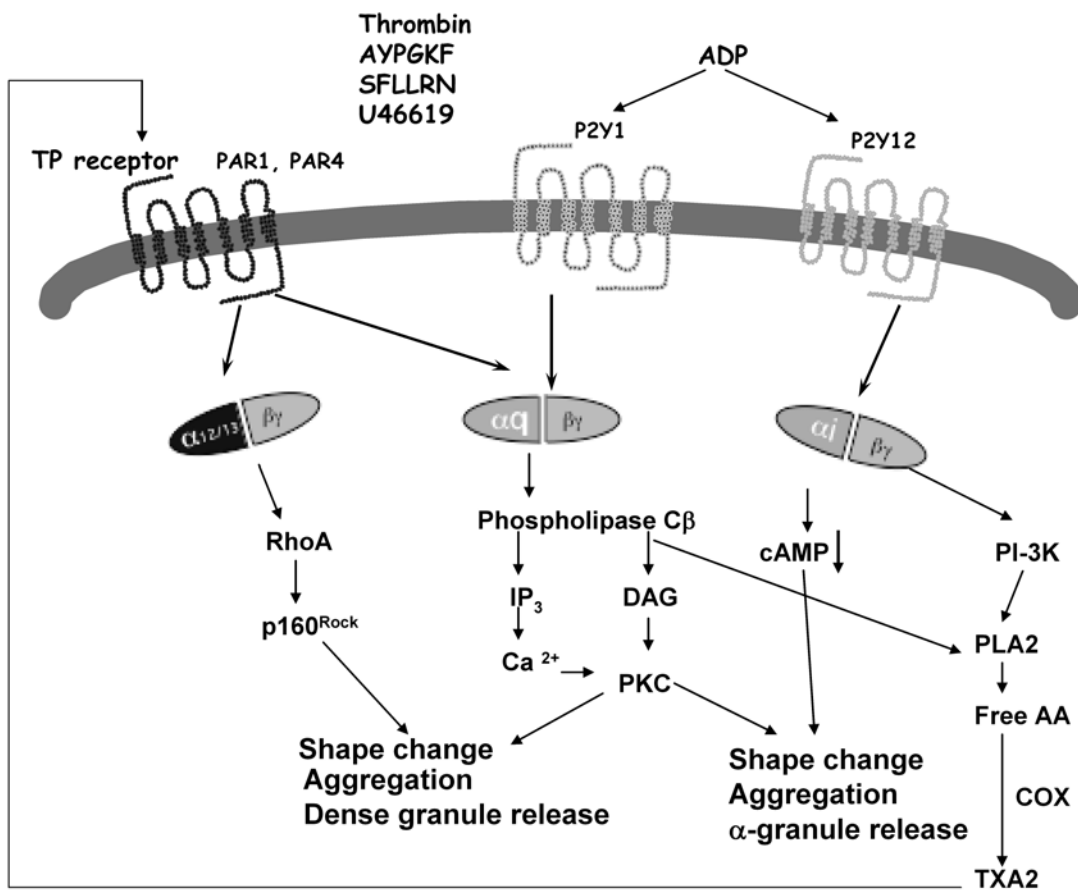


Figure 1.1. The schematic diagram of PAR and ADP receptors-induced platelet activation.

## ADP receptors

ADP is stored in platelet dense granule and is released when platelets are activated by thrombin, collagen or thromboxane A<sub>2</sub> (TXA<sub>2</sub>). The most important role of ADP is that it amplifies platelet activation initiated by other agonists (De Clerck *et al.*, 1990; Packham *et al.*, 1987). In addition, ADP is important for maximal platelet aggregation under high shear condition (Cattaneo *et al.*, 1994). Secreted ADP will interact with two G-protein coupled receptors: G<sub>q</sub>-coupled P<sub>2</sub>Y<sub>1</sub> receptor and G<sub>i</sub>-coupled P<sub>2</sub>Y<sub>12</sub> receptor. Both P<sub>2</sub>Y<sub>12</sub> and P<sub>2</sub>Y<sub>1</sub> receptors are required for ADP-induced platelet aggregation (Jin *et al.*, 1998). Through G<sub>q</sub>-coupled P<sub>2</sub>Y<sub>1</sub> receptors, ADP will cause calcium mobilization and PKC activation. P<sub>2</sub>Y<sub>1</sub> is responsible for calcium-mediated platelet shape change. Through G<sub>i</sub>-coupled P<sub>2</sub>Y<sub>12</sub> receptor, ADP will inhibit adenylyl cyclase and subsequently reduce cytosolic cAMP concentrations and the G $\beta\gamma$  dimers will activate downstream signals, such as phosphoinositide-3-kinases (PI3K) (Abrams *et al.*, 1996; Hirsch *et al.*, 2001; Jackson *et al.*, 2005), Akt/protein kinase B (Kim *et al.*, 2004), Rap1b (Woulfe *et al.*, 2002), Src family tyrosine kinases (Dorsam *et al.*, 2004), and G protein-gated inwardly rectifying potassium channels (GIRKs) (Shankar *et al.*, 2004). Both P<sub>2</sub>Y<sub>1</sub> null mouse platelet and platelets treated with P<sub>2</sub>Y<sub>1</sub> antagonist have no response to ADP. In addition, deficiency of P<sub>2</sub>Y<sub>1</sub> shows a prolonged bleeding time and has defect in vivo thrombus formation (Fabre *et al.*, 1999; Leon *et al.*, 1999) when compared with wild-type mice. The role of P<sub>2</sub>Y<sub>12</sub> receptor in platelet activation is investigated using both pharmacological antagonists

and knock out mouse models. After blocking the effect of P2Y<sub>12</sub> by using AR-C 69931MX, ADP can only induce platelet shape change, which is because of calcium mobilization downstream of G<sub>q</sub> pathways, which suggests that signaling events from P2Y<sub>12</sub> are essential for platelet aggregation. Similar results are shown in P2Y<sub>12</sub> (Foster *et al.*, 2001) or the G<sub>α<sub>i2</sub></sub> protein (Jantzen *et al.*, 2001) deficient mice, deleting either of them causes a significant reduction of ADP-induced platelet aggregation without affecting P2Y<sub>1</sub> receptor-mediated platelet shape change or intracellular calcium mobilization (Andre *et al.*, 2003; Foster *et al.*, 2001). Furthermore, patients with dysfunctional P2Y<sub>12</sub> receptor expression suffer from a severe defect in ADP-induced adenylyl cyclase inhibition and platelet aggregation, but retain a normal platelet shape change response (Cattaneo *et al.*, 1992).

### **Stroke and antithrombotic therapy**

Stroke is the third leading causes of death in the world and about 80% of strokes are caused by focal cerebral ischemia due to arterial occlusion (Caro *et al.*, 2000). Platelets play a critical role in cerebral ischemic events. Platelets deposit in ischemic basal ganglia during early reperfusion were detected by observing in vitro labeled platelets and aggregation of degranulated platelets with fibrin and leukocytes under the electron microscopic (Okada *et al.*, 1994). Antithrombotic therapy is one of the primary therapeutic methods used today to prevent stroke-induced injury. Currently,

the treatment options of ischemic stroke include thrombolytic therapy, anticoagulants and platelet inhibitors.

Thrombolytic therapy is used primary for acute thromboembolic stroke. Only less than 10% of patient can take this therapy because of the limitation of time after symptom onset. Later application could cause severe intracerebral hemorrhage (Adams *et al.*, 2005). Furthermore, the limitation of this therapy also includes the secondary arterial reocclusion which may still happen after a successful recanalization (Heo *et al.*, 2003) and the secondary injury which occurs mainly during reperfusion period. Anticoagulants are frequently used for acute stroke within 48 hours, however, anticoagulation carries a significant risk for intracerebral bleeding in the setting of acute stroke (Benatar, 2005; Gubitz *et al.*, 2004) and the recently updated American Heart Association guidelines (Adams *et al.*, 2007) state that “ urgent anticoagulation with the goal of preventing early recurrent stroke, halting neurologic worsening, or improving outcomes is not recommended.”

Numerous studies have been performed to improve stroke outcome by using of platelet aggregation inhibitors. Acetylsalicylic acid (ASA) has been used within 48 hours of stroke in 2 large trials (1997a; 1997b) based on its the irreversible inhibition of platelet cyclooxygenases 1 and 2 and it will lead to reduced prostaglandin and thromboxane A<sub>2</sub> synthesis. Studies show that ASA causes a moderate, but statistically significant benefit on stroke outcome. Two FDA proved P2Y<sub>12</sub> antagonists,

clopidogrel and ticlopidine, are also currently used for ischemic stroke treatment. Although the beneficial role of platelet aggregation inhibitors including ASA, ASA in combination with the platelet P2Y<sub>12</sub> receptor inhibitor clopidogrel in stroke prevention is well investigated (1996; Verro *et al.*, 2008), up to 33% of patients show resistance or partial responsiveness to these drugs and also the risk that unexpected bleeding will increase. To avoid these negative effects, the identification and development of new reagents against specific platelet signaling pathways is under investigation.

The thrombin receptor has become one of the new targets for antiplatelet drugs. Among thrombin receptor antagonists, SCH 530348 is the one currently under investigation. SCH 530348 is a nonpeptide antagonist and an oral reversible PAR1 antagonist derived from himbacine, a compound found in the bark of the Australian magnolia tree (Chackalamannil *et al.*, 2005) It blocks thrombin and PAR1 peptide-induced platelet aggregation without inhibiting the effect of ADP, collagen, U46619 or PAR4 agonist peptide on platelets (Chackalamannil *et al.*, 2008). It completely blocks platelet aggregation when stimulated with PAR1 agonist peptide TRAP within 1 hour of oral administration in monkeys. Clinical data show that a 40 mg dose of SCH 530348 inhibits more than 80% of PAR1 agonist peptide-induced platelet aggregation in 60 minutes among 68% of patients and this percentage will increase to 96% at the end of 120 min. Now two phase 3 trials are underway to test whether SCH350348 is useful for standard therapy to prevent ischemic events. In addition to

SCH 530348, E5555 is another PAR1 antagonist under 3 randomized, double-blind, placebo-controlled, Phase 2 trials.

## CHAPTER 2

### CHARACTERIZATION OF A NEW PEPTIDE AGONIST OF THE PROTEASE-ACTIVATED RECEPTOR-1

#### Introduction

Platelets are a fundamental component of the hemostatic process. Platelet activation leads to shape change, aggregation, secretion from granules, and finally in the formation of a stable clot at the damaged endothelium surface (Jurk *et al.*, 2005). Physiologically platelet activation occurs primarily by collagen in the subendothelium, but is rapidly amplified by recruitment of platelets to the site of the injury (Brass *et al.*, 1997; Kahner *et al.*, 2006; Shattil *et al.*, 1998). Adenosine diphosphate (ADP), released from the dense granules, thromboxane A<sub>2</sub>, produced in the activated platelets, and thrombin, generated on the activated platelet surface, are key players in the amplification response (De Clerck *et al.*, 1990; Offermanns, 2006; Packham *et al.*, 1987).

Thrombin activates platelets through cell surface receptors known as protease-activated receptors (PARs) (Coughlin, 1999). PARs belong to the class of seven transmembrane domain G protein-coupled receptors. Among the four members of

PARs characterized to date, PAR-1, PAR-3 and PAR-4 are activated by thrombin and considered primarily as thrombin receptors (Coughlin, 2000). Thrombin activates the PARs through a unique mechanism involving proteolytic cleavage of the amino terminal of the receptor and thus exposing a new amino terminus. The newly formed amino terminus binds to the extracellular domain of the receptor to cause intracellular signaling (Rasmussen *et al.*, 1991; Vu *et al.*, 1991a). Numerous studies on the structure-function analysis of PAR1 agonists have revealed a requirement of free amine group at the amino terminus, a short chain amino acid at position 1, aromatic amino acid at position 2 and arginine at position 5 for potent activation of PAR-1 (McComsey *et al.*, 1999; Scarborough *et al.*, 1992b).

Human platelets express PAR-1 and PAR-4, whereas the mouse platelets express PAR-3 and PAR-4 (Kahn *et al.*, 1999; Kahn *et al.*, 1998; Vu *et al.*, 1991a). In human platelets, PAR-1 is a high-affinity receptor that is activated at low concentrations of thrombin, and PAR-4 is a low-affinity receptor that mediates thrombin signaling at higher concentrations (Kahn *et al.*, 1999). In mouse platelets, PAR-3 serves as a co-receptor for PAR-4, wherein thrombin binds to PAR-3 and PAR-4, and cleaves the amino terminus of PAR-4 (Nakanishi-Matsui *et al.*, 2000).

In order to evaluate the physiological and pathophysiological functions of the protease-activated receptors, specific peptide agonists, which mimic the actions of the newly exposed N-terminal, were synthesized. These activation peptides are based on

the tethered ligand of the PARs, and hence are specific for the PARs. For example, SFLLRN, which mimics the new N-terminal amino terminal activates human PAR-1 and induces human platelet aggregation and degranulation without cleavage of PAR-1 receptor (Hung *et al.*, 1992; Vu *et al.*, 1991b). Resmussen et al find the peptide YFLLRNP works as a partial PAR-1 agonist that induces platelet shape change without calcium mobilization; however, full activation of platelets is achieved only at higher concentrations (Rasmussen *et al.*, 1993). The shape change caused by 60  $\mu$ M YFLLRNP appears to be mediated by the G<sub>12/13</sub> pathway through activation of RhoA and p160<sup>ROCK</sup>. Consistently, Y-27632, a p160<sup>ROCK</sup> inhibitor, is reported to totally block the stimulatory effects of YFLLRNP (Bauer *et al.*, 1999; Dorsam *et al.*, 2002).

In the current study, we evaluated the biological function of a synthetic peptide TFRRRLSRATR, which is derived from the carboxy terminus of P2Y<sub>1</sub> receptor that is important for Gq-coupling. Instead of interfering with the P2Y<sub>1</sub> receptor, this peptide is able to directly activate human platelet through PAR-1 receptor. Unlike the known PAR-1 peptide agonists, this peptide sequence is not derived from the amino terminus region of the receptor. Its structural diversity from the other peptide agonists of PAR-1 and its dose-dependent signaling effects make this peptide a distinct agonist that will help us in understanding the molecular mechanisms of ligand-receptor interactions.

## Materials and Methods

### *Materials*

Peptide (TFRRRLSRATR) was synthesized by New England Peptide, Inc. (Gardner, MA). Luciferin-luciferase reagent was purchased from Chrono-Log (Havertown, PA). Hexapeptides SFLLRN and AYPGKF were custom synthesized at Invitrogen (Carlsbad, CA). AR-C69931MX was a gift from Astra-Zeneca Research Laboratories (Charnwood, Loughborough, United Kingdom). The PAR 1 antagonist BMS-200261 was obtained as a generous gift from Dr. Steven Seiler (Bristol-Myers Squibb). Fura-2 AM was from Invitrogen (Eugene, OR). The p160<sup>ROCK</sup> inhibitor Y-27632 and Prostaglandin E<sub>1</sub> (PGE<sub>1</sub>) were from Biomol Research Laboratories (Plymouth Meeting, PA). Anti-phospho-Akt (Ser473), anti-phospho-Erk and anti-phospho-p38 MAPK were purchased from Cell Signaling Technology (Beverly, MA). Alkaline phosphatase-labeled secondary antibody was from Kirkegaard & Perry Laboratories (Gaithersburg, MD). CDP-Star chemiluminescent substrates were purchased from Applied Biosystems (Foster City, CA). PI-3 kinase inhibitors wortmannin and LY294002 were from Biomol Research Laboratories (Plymouth Meeting, PA). MRS-2179, apyrase grade VII, thrombin and acetylsalicylic acid were obtained from Sigma (St. Louis, MO). All the other reagents were of reagent grade, and de-ionized water was used throughout.

### *Washed human platelet preparation*

Washed human platelets were prepared as described previously, under the guidelines approved by the Institutional Review Board (Shankar *et al.*, 2006c). Whole blood from healthy, drug-free donors at the Sol Sherry Thrombosis Research Center of Temple University (Philadelphia, PA) was collected with informed consent, in tubes containing acid-citrate-dextrose (ACD: 2.5 g sodium citrate, 1.5 g citric acid, and 2 g glucose in 100 mL deionized water). Citrated blood was centrifuged to obtain platelet-rich plasma (PRP). The PRP was treated with 1 mM acetylsalicylic acid (aspirin) to block thromboxane A<sub>2</sub> production (Quinton *et al.*, 2002) and centrifuged to get the platelet pellet. Platelet pellet was resuspended in calcium-free Tyrode's buffer.

### *Preparation of washed mouse platelets*

Blood was collected from anesthetized mice, in accordance with the IACUC approved protocols, by cardiac puncture into syringes containing 3.8% sodium citrate as anticoagulant. The whole blood was centrifuged (IEC Micromax centrifuge; International Equipment, Needham Heights, MA) at 100 x relative centrifugal forces (RCF) for 10 minutes to isolate the PRP. Prostaglandin E<sub>1</sub> (PGE<sub>1</sub>, 1 μM) was added to PRP. The PRP were centrifuged at 400 RCF for 10 minutes and the pellet was resuspended in Tyrode buffer containing 0.01 unit/mL apyrase.

### *Platelet aggregation and secretion*

Aggregation and secretion of 0.5 mL washed platelets was analyzed using a P.I.C.A. lumiaggregometer (Chrono-log, Havertown, PA). Aggregation was measured by light transmission under stirring conditions (900 rpm) at 37°C. ATP secretion from platelet-dense granules was measured by using the Luciferin-Luciferase assay and the platelets were stimulated in a lumiaggregometer at 37°C with stirring at 900 rpm and the corresponding luminescence was measured. Agonists were added simultaneously for platelet stimulation and platelets were preincubated with each inhibitor at 37°C. The aggregation and secretion data are represented in the form of actual tracings (Dorsam *et al.*, 2002).

### *Platelet desensitization*

Platelet desensitization was performed as described earlier (Dubois *et al.*, 2003). Briefly, washed human platelets were incubated for 40 minutes at 37°C without stirring in the absence or presence of 300 µM peptide. Immediately after incubation, platelets were assayed for their responses to various agonists.

### *Intracellular Calcium Measurements*

For intracellular calcium mobilization studies, PRP was incubated with 2  $\mu$ M fura-2 with acetylsalicylic acid (aspirin) for 45 minutes and leave in room temperature for another 15 minutes. Platelets were then isolated and washed as described above. Changes in fluorescence were measured using Aminco-Bowman Series 2 luminescence spectrometer with a water-jacketed cuvette holder, equipped with a thermostat, at 37°C and set at constant stirring. 0.5 mL sample were analyzed with an excitation wavelength of 340 nm and an emission wavelength of 510 nm. Fluorescence measurements were converted to calcium concentrations using the equation reported by Grynkiewicz et al. (Grynkiewicz *et al.*, 1985), where  $F_{\min}$  and  $F_{\max}$  were determined with each respective platelet preparation (Shankar *et al.*, 2004).

### *Western blotting*

Washed human platelets (0.2 mL) were stimulated with agonists under non-stirring conditions for 3 minutes and lysed using 3 x sample loading buffer and boiled for 5 minutes. The platelet lysates were loaded on to a 10% Tris-glycine gel, subjected to SDS-PAGE, and transferred to polyvinylidene difluoride (PVDF) membrane. Nonspecific binding sites were blocked by incubation in blocking buffer (5% nonfat dry milk, 3% bovine serum albumin (BSA), 20 mM Tris, 140 mM NaCl) for 1 hour at room temperature (RT) under rocking conditions and then the membrane was

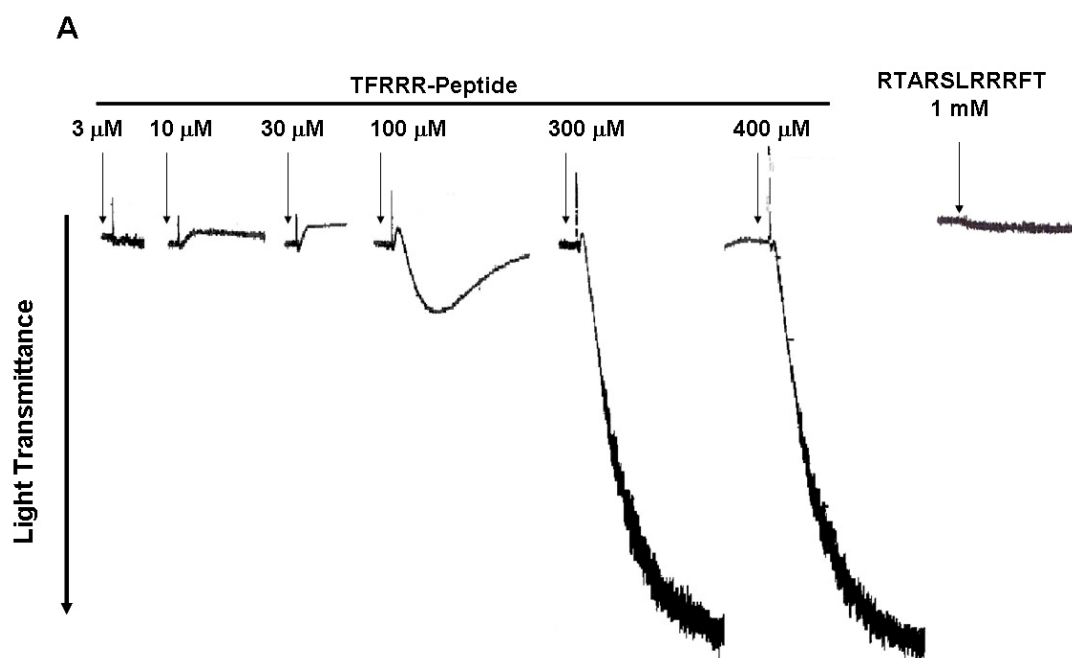
incubated with primary antibody (1:1000 dilution for phospho-Akt, phospho-Erk and phospho-p38MAP kinase in TBST, 2% BSA) overnight with gentle rocking. After three 5-minute washes with TBST, the membrane was probed with alkaline phosphatase-labeled goat anti-rabbit IgG (1:5000 dilution in TBST with 2% BSA) for 1 hour at RT. The membrane was washed 3 times for 5 minutes each time using the 1x TBST, once with deionized water and last with 1x tropix buffer (Tropix, Bedford, MA). Membrane was then incubated with CDP-Star chemiluminescent substrate for 15 minutes at RT and immunoreactivity was detected using Fujifilm Luminescent Image Analyzer (Fujifilm Medical Systems, Stamford, CT).

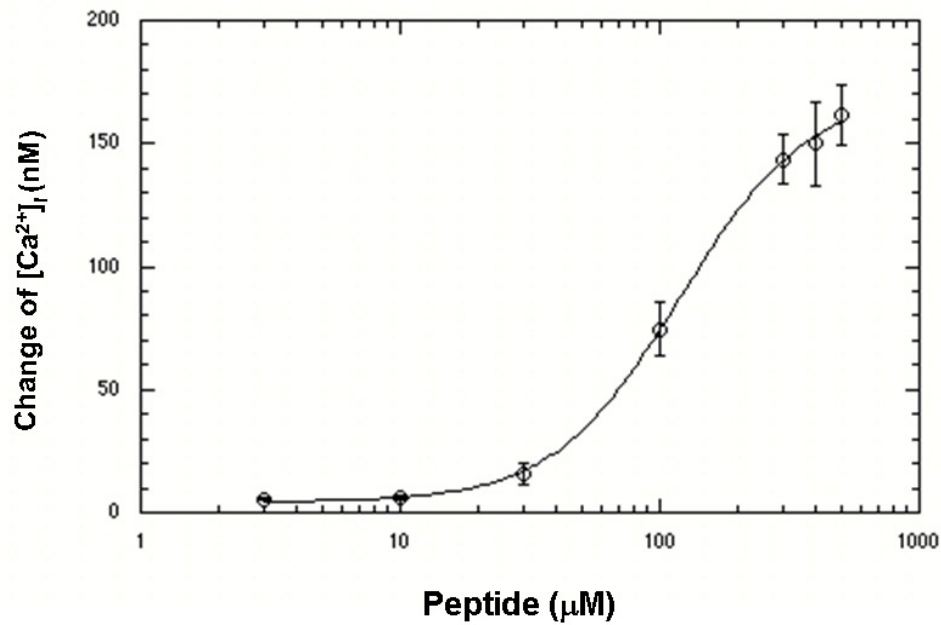
## Results

Recently we have identified a domain, TFRRLSRATR, in the carboxy terminus of the P2Y<sub>1</sub> receptor that is important for Gq coupling (Ding *et al.*, 2005). In efforts to develop intracellular antagonists to P2Y<sub>1</sub> receptor, we tested the ability of this peptide to competitively interfere with P2Y<sub>1</sub> receptor's ability to modulate Gq pathways using platelets as an experimental system. To facilitate the transport of the peptide across plasma membrane, an amino terminal palmitoylation was introduced. To our surprise, this palmitoylated peptide activated human platelets. Hence we began characterizing this peptide to understand the underlying mechanisms of platelet activation by this novel peptide.

## **TFRRR-peptide induced responses in human platelets**

We first evaluated whether the peptide without the lipid modification at the N-terminus would activate platelets. The non-palmitoylated TFRRR-peptide activated platelets in a concentration-dependent manner. At low concentrations (10  $\mu$ M) this peptide induced platelet shape change, and with increasing concentrations (100- 400  $\mu$ M), this peptide caused full platelet aggregation (Figure 2.1A). However, control peptide with scrambled sequence (RTARSLRRRFT) failed to cause either shape change or aggregation even at 1 mM (Fig. 2.1A). The TFRRR-peptide, however, did not cause any mobilization of calcium from intracellular stores at 10  $\mu$ M. This peptide caused calcium mobilization in human platelets starting at 30  $\mu$ M (Fig. 2.1B). The maximum increases in intracellular calcium occurred at 300  $\mu$ M, the concentration at which maximum platelet aggregation also occurred.



**B**

**Figure 2.1. TFRRR-peptide causes platelet aggregation and calcium mobilization.** Washed aspirin-treated human platelets were stimulated with unmodified TFRRR-peptide (3-400  $\mu\text{M}$ ) or 1 mM scrambled peptide (RTARSLRRRFT) under stirring conditions at 37  $^{\circ}\text{C}$ . In panel A, platelet aggregation was determined by measuring light transmittance. Tracings were representative of at least three separate experiments. In panel B, Calcium levels were analyzed. Each bar was the average of three experiments  $\pm$  SEM from three different donors.

## **Evaluation of the role of PARs in the TFRRR-peptide-induced platelet activation**

In order to investigate the mechanism of TFRRR peptide-induced platelet activation, we tested the effect of different platelet receptor specific antagonists on TFRRR-peptide-induced platelet aggregation. As this peptide caused mobilization of calcium from the intracellular stores, we evaluated the role of Gq coupled receptors in platelet aggregation induced by this peptide. Because peptide agonists activate PARs on platelets, we hypothesized that this TFRRR-peptide elicits its responses through PARs on platelets. Consistent with our hypothesis, SQ29548, a thromboxane receptor antagonist, or MRS2179, a P2Y<sub>1</sub> receptor antagonist did not have any effect on the TFRRR-peptide-induced platelet aggregation (Fig. 2.2A). Of the PAR specific antagonists, BMS200261, a PAR-1 specific antagonist, completely abolished the TFRRR-peptide-induced platelet aggregation, secretion and calcium mobilization (Fig. 2.2B). These data indicated that this peptide might activate platelets through the PAR-1 receptors. To further test our hypothesis, we desensitized the platelets by pre-treatment of washed human platelets with 300  $\mu$ M TFRRR-peptide for 40 minutes and then stimulated these platelets with either SFLLRN (a PAR-1 selective agonist) or AYPGKF (a PAR-4 selective agonist). The human platelets, pretreated with the peptide, failed to aggregate in response to SFLLRN (10  $\mu$ M) but aggregated normally in response to AYPGKF (500  $\mu$ M) (Fig. 2.3A).

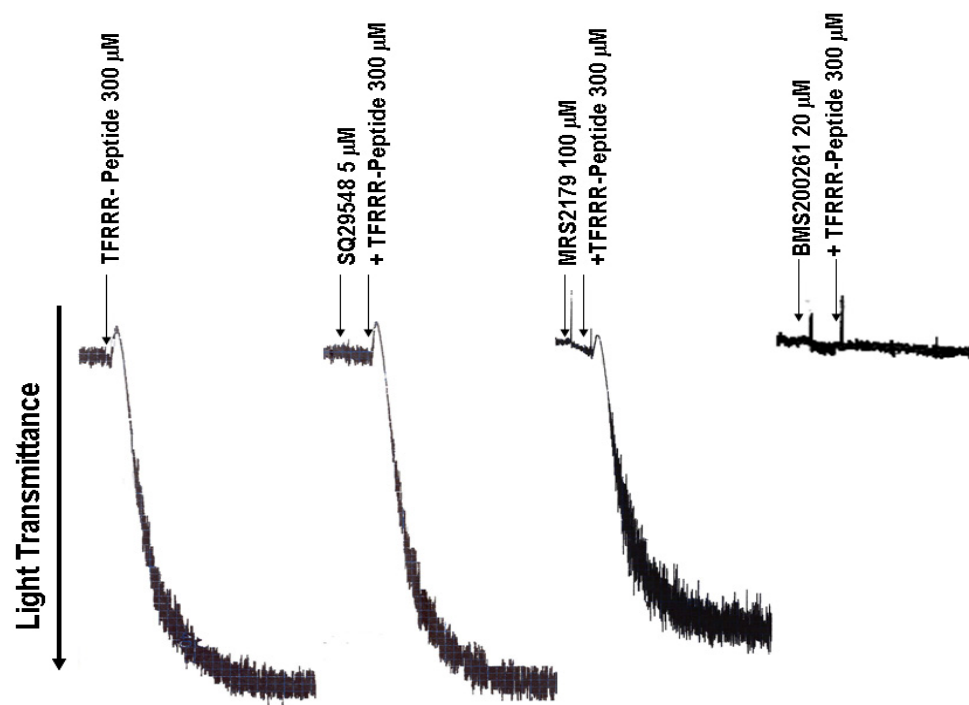
To further confirm these results, we used washed mouse platelets, which are devoid of the PAR-1 receptor. In mouse platelets, TFRRR-peptide failed to cause platelet aggregation, up to a concentration of 600  $\mu\text{M}$  (Fig. 2.3B). These results imply that TFRRR-peptide activates human platelets by activating PAR1. Since mouse platelets lack PAR1, this peptide fails to activate mouse platelets.

### **Differential activation of Gq and G<sub>12/13</sub> pathways by TFRRR-peptide and thrombin**

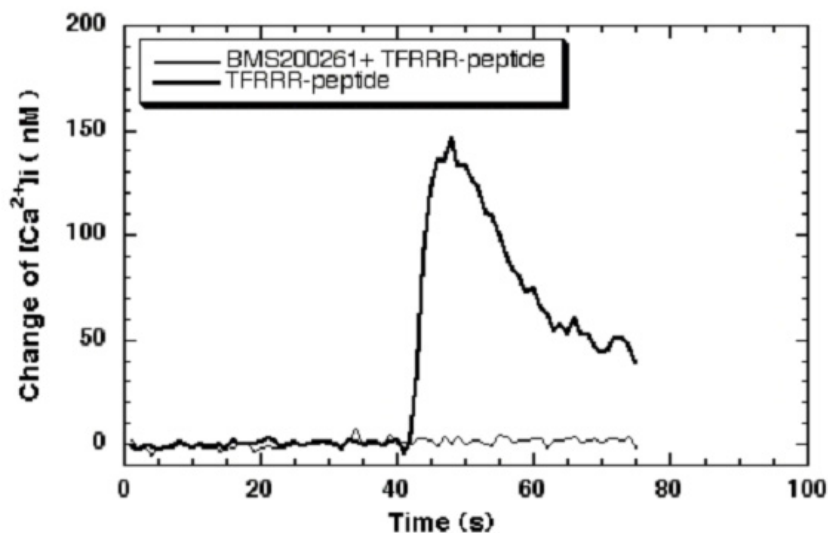
As the previous results showed that TFRRR-peptide lacking lipid modification activates PAR1 on the cell surface, we used the TFRRR-peptide without the N-terminal palmitoylation in the subsequent studies. Offermanns et al have shown that thrombin and thromboxane activate Gq and G<sub>12/13</sub> pathways (Offermanns *et al.*, 1994). In the Gq null mouse platelets, thrombin and thromboxane cause shape change through RhoA-p160<sup>ROCK</sup> pathway. As we have observed that TFRRR-peptide causes platelet shape change at 10  $\mu\text{M}$  concentration without causing increases in intracellular calcium levels, we evaluated the role the RhoA-p160<sup>ROCK</sup> pathway in this calcium-independent shape change. As shown in Fig. 2.4, 10  $\mu\text{M}$  TFRRR-peptide-induced platelet shape change was abolished by a p160<sup>ROCK</sup> inhibitor, Y27632. These data indicate that at 10  $\mu\text{M}$  concentrations the TFRRR-peptide selectively activates G<sub>12/13</sub> pathways, leading to the activation of RhoA-p160<sup>ROCK</sup> pathways. The results indicate that the TFRRR-peptide activates the G<sub>12/13</sub> and Gq pathways selectively in a

concentration-dependent manner. At low concentrations, the TFRRR-peptide activated only  $G_{12/13}$  pathways in platelets, but with the increasing concentrations, this peptide also activates  $G_q$  pathways and causes full platelet activation.

A



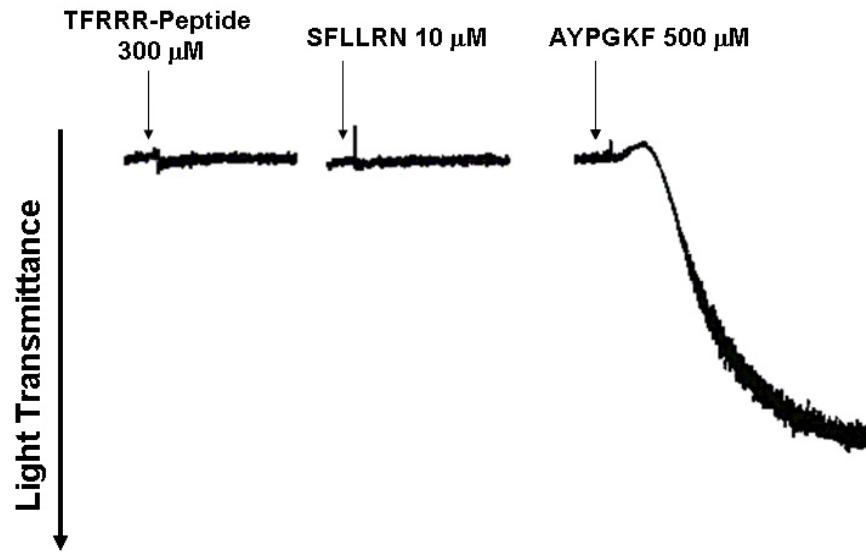
**B**



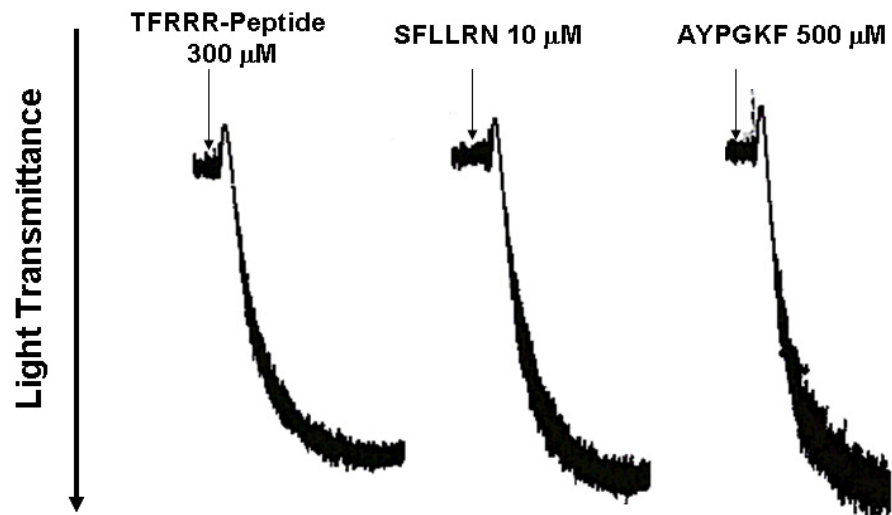
**Figure 2.2. The effect of receptor agonists on TFRRR-peptide induced platelet activation.** Washed aspirin-treated human platelets were stimulated with TFRRR-peptide (300  $\mu$ M), in the presence and absence of SQ29548 (5  $\mu$ M), MRS2179 (100  $\mu$ M) and BMS200261 (20  $\mu$ M) (panel A) under stirring conditions at 37°C. Panel B, Calcium mobilization was tested in human platelets stimulated with TFRRR-peptide (300  $\mu$ M) and with or without BMS200261 (20  $\mu$ M). Tracings were representative of at least three separate experiments.

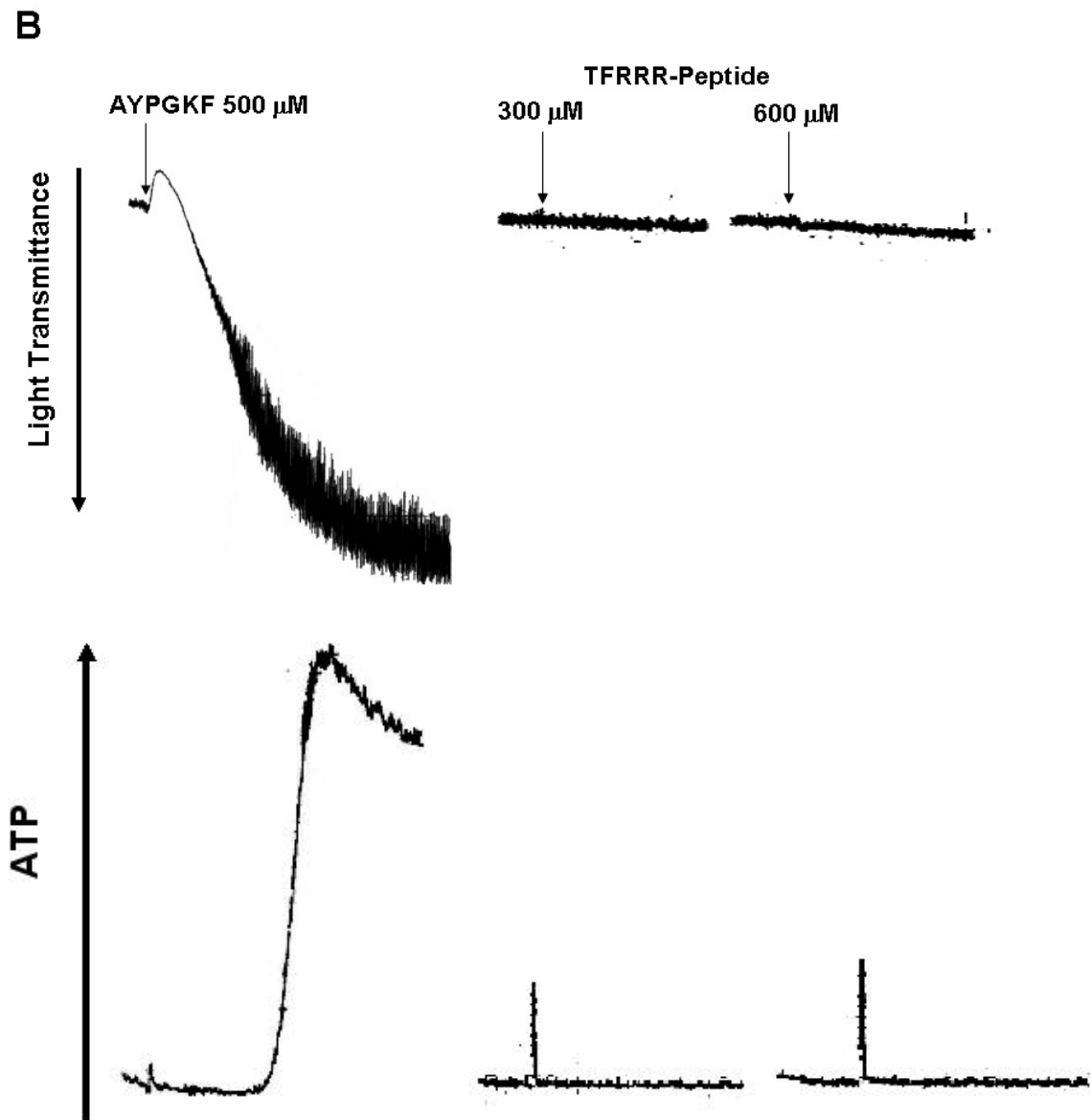
A

TFRRR-Peptide(300  $\mu$ M) preincubate 40 min



Control





**Figure 2.3. The role of PAR-1 in the TFRRR-peptide-induced platelet activation.**

Washed human platelets were incubated at 37 °C for 40 min in the absence or presence of 300  $\mu\text{M}$  peptide (panel A), followed by stimulation with TFRRR-peptide (300  $\mu\text{M}$ ), SFLLRN (10  $\mu\text{M}$ ) or AYPGKF (500  $\mu\text{M}$ ) to initiate aggregation Tracings

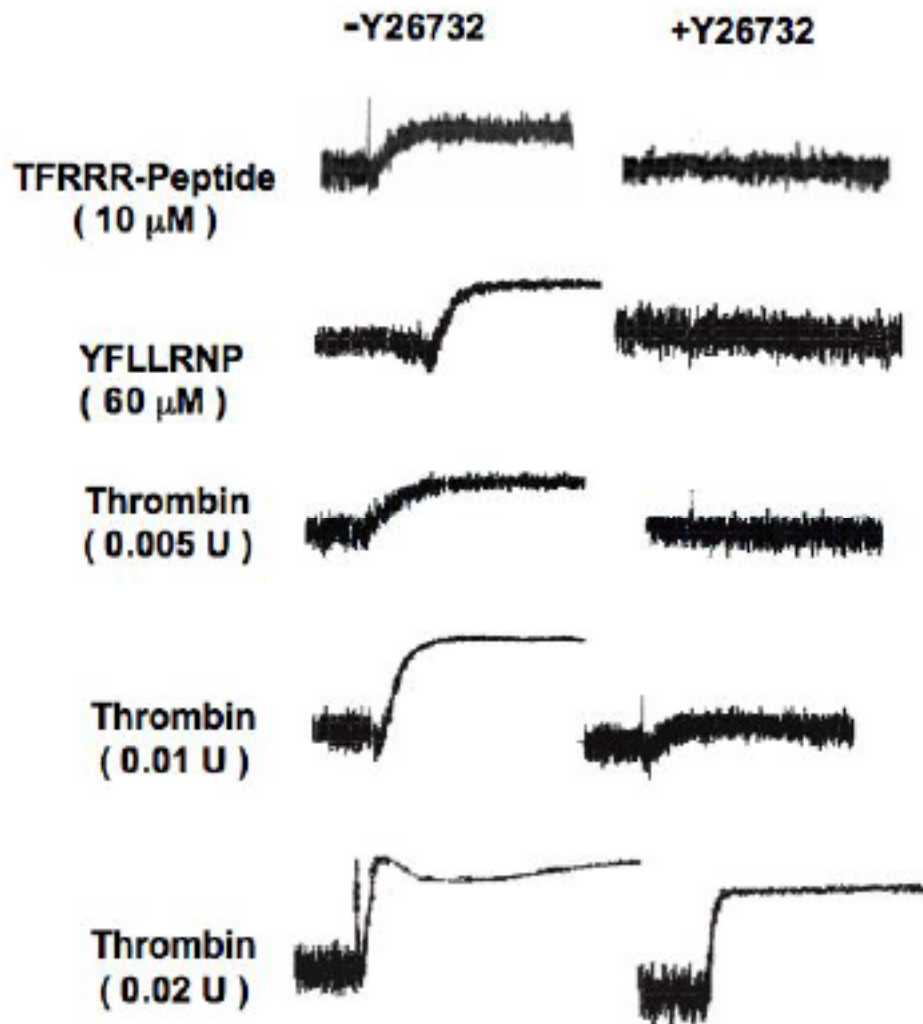
were representative of at least three separate experiments. In panel B, Effect of TFRRR-peptide on mouse platelet was tested. Washed mouse platelets were stimulated with AYPGKF (500  $\mu$ M) and TFRRR-peptide (300 and 600  $\mu$ M) under stirring conditions at 37°C. Platelet aggregation was determined by measuring light transmittance. ATP secretion from platelet dense granules was measured by using the Luciferin-Luciferase assay. Tracings were representative of at least three experiments.

We evaluated whether differential activation of G12/13 and Gq pathways occurs when platelets are activated by physiological agonist thrombin. As shown in Fig. 2.4, very low concentrations (0.005 U/ml) of thrombin, similar to YFLLRNP or TFRRR-peptide, cause platelet shape change that is totally blocked by p160<sup>ROCK</sup> inhibitor Y27632, indicating that such low concentrations of thrombin selectively activate only G12/13 pathways. As the concentration of thrombin increased to 0.01 and 0.02 U/ml, thrombin-induced platelet shape change is inhibited but not abolished by Y27632 (Fig. 2.4), indicating that Gq pathways are also activated by these concentrations of thrombin. Thus, thrombin also activates G12/13 and Gq pathways differentially based on the concentration.

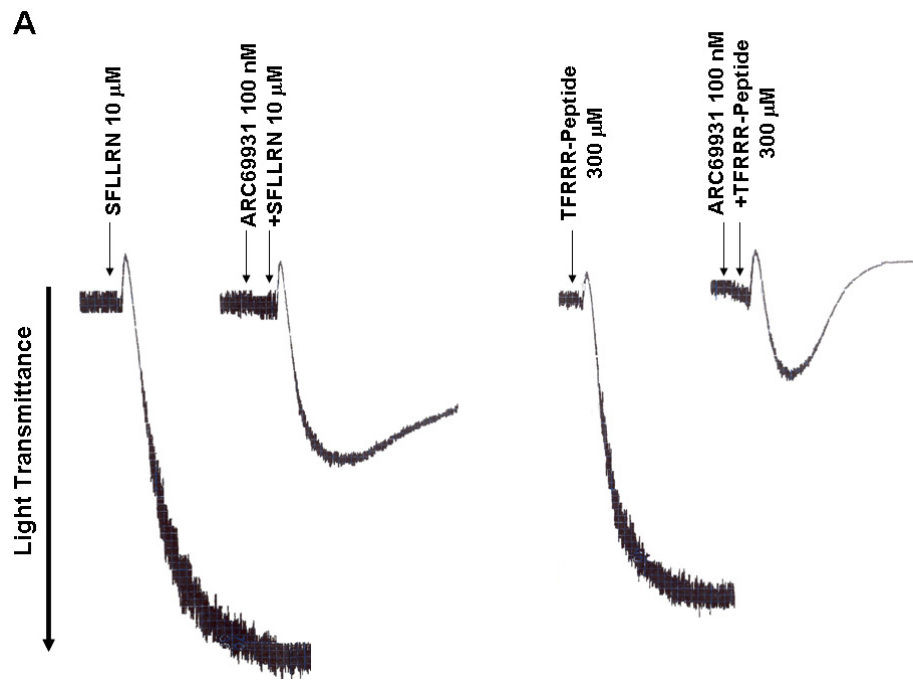
### **Role of secreted ADP in the PAR-1 activating peptide-induced platelet irreversible aggregation**

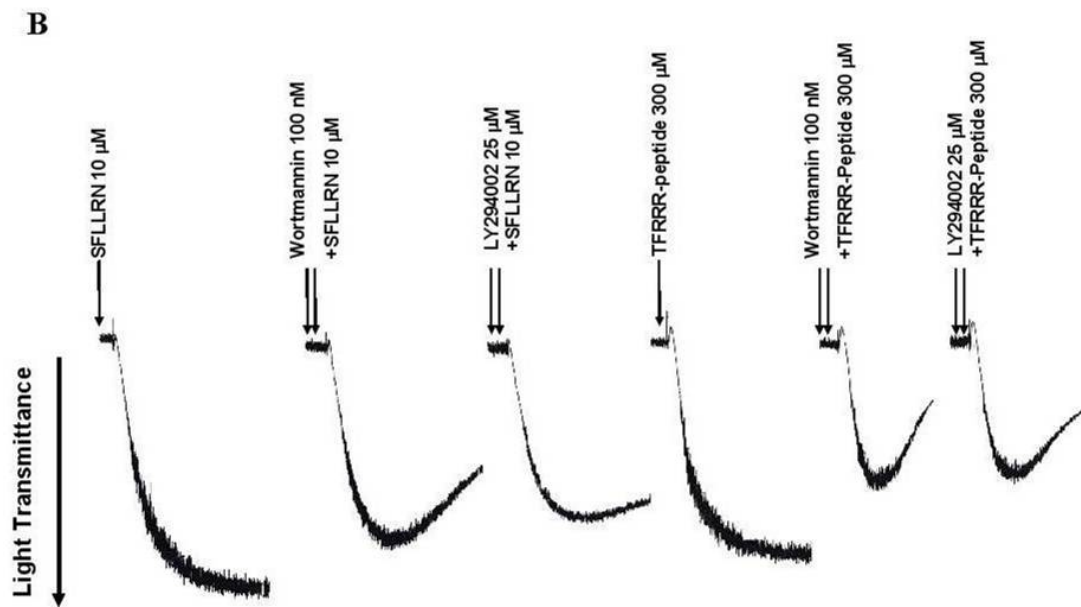
Trumel et al have shown that secreted ADP acting through the P2Y<sub>12</sub> receptor contributes to the irreversible aggregation by SFLLRN (Trumel *et al.*, 1999). We evaluated the role P2Y<sub>12</sub> receptor in TFRRR-induced platelet irreversible aggregation, using AR-C69931MX, a P2Y<sub>12</sub> receptor selective antagonist. In the absence of AR-C69931MX, TFRRR-peptide as well as SFLLRN caused irreversible aggregation. However, in the presence of AR-C69931MX, the aggregation induced by both SFLLRN and TFRRR-peptide became reversible (Fig. 2.5A). As PI-3 kinases are important for promoting irreversible aggregation, we evaluated whether they also

have a role in TFRRR-peptide-induced irreversible aggregation. As shown in Fig. 2.5B, wortmannin and LY294002 converted irreversible aggregation induced by TFRRR-peptide and SFLLRN to reversible aggregation. These results indicate that the TFRRR-peptide activates PAR-1 in a similar manner as SFLLRN.



**Figure 2.4. Low concentration of TFRRR-peptide or thrombin causes G12/13 pathway activation.** Washed aspirin-treated human platelets were stimulated with TFRRR-peptide (10  $\mu$ M), YFLLRNP (60  $\mu$ M), or different concentrations of thrombin in the presence and absence of Y27632 (10  $\mu$ M) under stirring conditions at 37°C. Tracings were representative of at least three separate experiments.

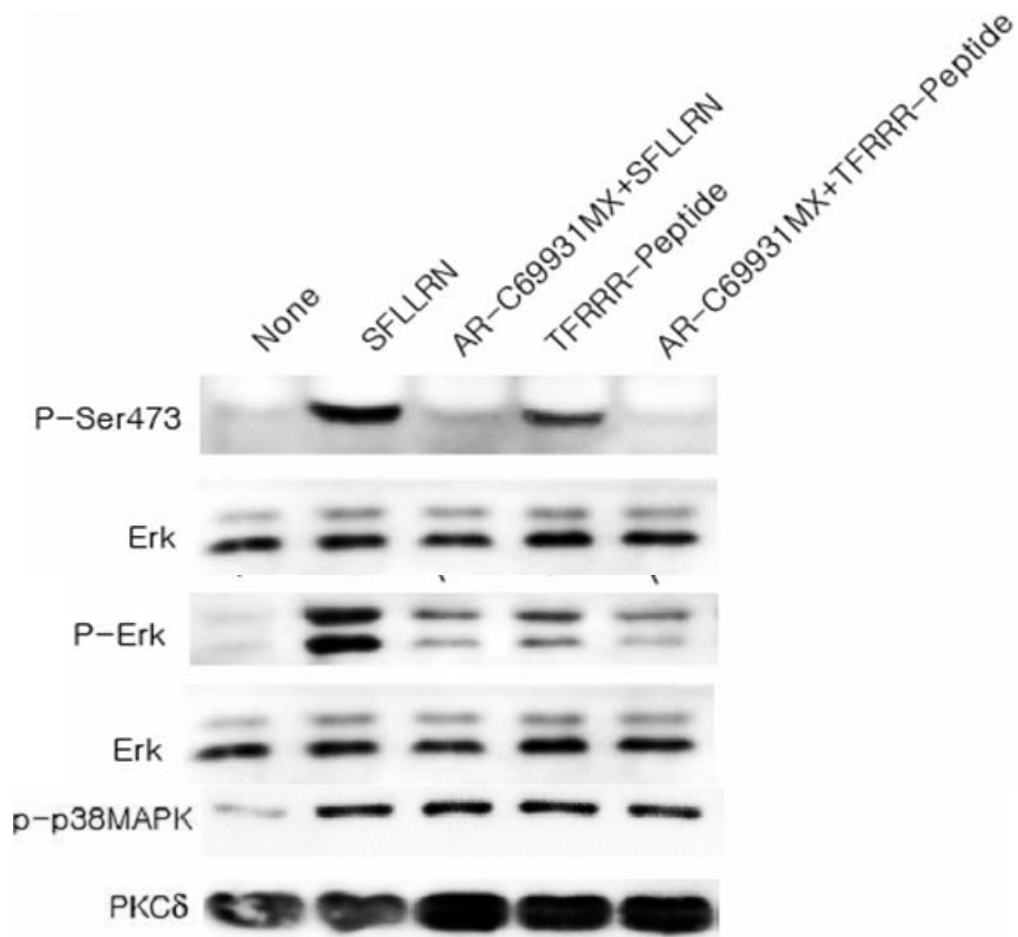




**Figure 2.5. Role of secreted ADP and PI3-K in the PAR-1 activating peptide induced platelet irreversible aggregation.** Washed aspirin-treated human platelets were stimulated with TFRRR-peptide (300  $\mu$ M) and SFLLRN (10  $\mu$ M), in the presence or absence of AR-C69931MX (100 nM) (panel A), wortmannin (100 nM) and LY294002 (25  $\mu$ M) (panel B) under stirring conditions at 37°C. Tracings were representative of at least three separate experiments.

### **Activation of Akt, Erk2 and p-38 MAPK by TFRRR-peptide in human platelets**

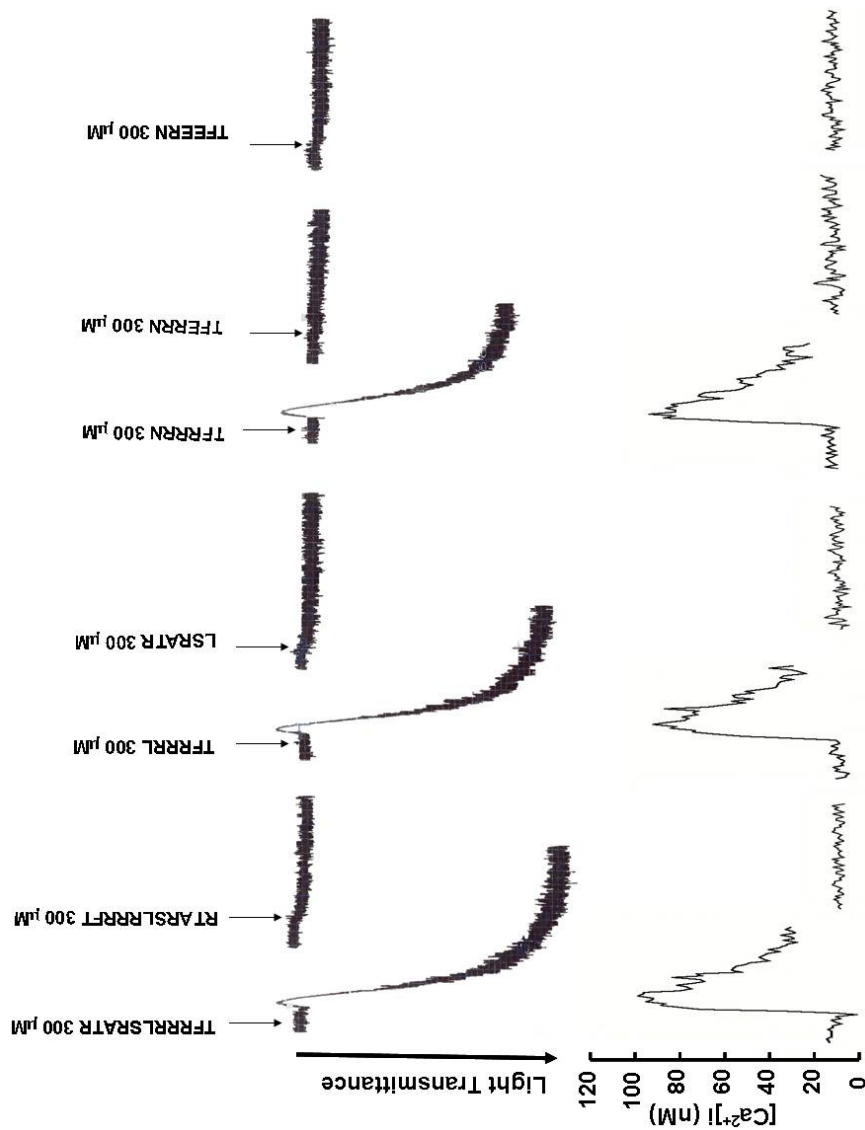
We have shown that the activation of P2Y<sub>12</sub> receptor and Gi pathways by secreted ADP is required for PAR-mediated Akt phosphorylation in platelets (Kim *et al.*, 2004; Kim *et al.*, 2006). Furthermore, we have shown that PAR-mediated Erk phosphorylation is potentiated by secreted ADP through the P2Y<sub>12</sub> receptor (Shankar *et al.*, 2006a). Hence we evaluated the role of P2Y<sub>12</sub> receptor in TFRRR-peptide-induced phosphorylation of Akt and Erk. As shown in Fig. 2.6, TFRRR-peptide or SFLLRN-induced Akt phosphorylation (Fig. 2.6A), and Erk phosphorylation (Fig. 2.6B) are inhibited by AR-C69931MX. However, p38 MAP kinase activation by SFLLRN or TFRRR-peptide was unaffected by AR-C69931MX, indicating that PAR-mediated p38 MAP kinase activation occurs independently of the P2Y<sub>12</sub> receptor (Figure 2.6C). These data indicate that TFRRR-peptide-induced platelet activation is due to PAR-1 activation.



**Figure 2.6. Role of P2Y<sub>12</sub> receptor in TFRRR-peptide induced phosphorylation of Akt, Erk and p38MAPK.** Washed aspirin-treated human platelets were stimulated with TFRRR-peptide (300  $\mu$ M) or SFLLRN (10  $\mu$ M) for 3 minutes, in the presence or absence of P2Y<sub>12</sub> antagonist AR-C69931MX (100 nM). Akt phosphorylation, Erk Phosphorylation and p-38 MAPK phosphorylation were measured by western blot analysis. The data are representative of experiments done using platelets from at least three different donors.

### **Structure-function studies on the TFRRR-peptide**

In order to evaluate the importance of specific amino acids in TFRRR-peptide, we also synthesized a series of peptides. Figure 2.7 shows that scrambled sequence RTARSLRRRFT failed to cause shape change or aggregation at 300  $\mu$ M, a concentration at which the TFRRR peptide elicits a range of responses. In addition, the first part of TFRRR-peptide (TFRRRL) has the similar effect as TFRRR-peptide, whereas the second part of TFRRR-peptide (LSRATR) failed to cause platelet aggregation and calcium mobilization (Fig. 2.7), which indicates that the first part of TFRRR-peptide is the key for the platelet activation. Interestingly, peptide TFRRRN, with a change in the last amino acid from Leu to Asn, also is able to activate the platelets. If the third or fourth Arg is altered to Glu, the peptide failed to elicit any platelet activation (Fig. 2.7). All of these data indicate that the effect of TFRRR-peptide depended on the first six amino acids and the third and fourth amino acids, rather than the sixth amino acid, are essential for this peptide-induced platelet activation.



**Figure 2.7. Structure-function studies on TFRRR-peptide:** Washed aspirin-treated human platelets were stimulated with different peptides derived from the TFRRR-peptide under stirring conditions at 37°C. In panel A, platelet aggregation and in panel B calcium release were analyzed. (TFRRR-peptide: TFRRRLSRATR; scrambled peptide: RTARSLRRRFT; first part of TFRRR-peptide: TFRRRL; second part of

TFRRR-peptide: LSRATR; point mutant peptide: TFRRRN, TFERRN, TFEERN).

Tracings were representative of at least three separate experiments.

## Discussion

Platelet activation plays a key role in the pathophysiology of thrombotic diseases (Coughlin, 2005; Jurk *et al.*, 2005; Ruggeri, 2002; Shankar *et al.*, 2006b). The major receptors on the platelet membrane include protease-activated receptors (PARs) and ADP receptors (Gachet, 2006; Macfarlane *et al.*, 2001). In platelets there are two ADP receptors: Gq-coupled P2Y<sub>1</sub> and Gi-coupled P2Y<sub>12</sub> receptors (Daniel *et al.*, 1998; Jin *et al.*, 1998). Coactivation of both receptors is required for ADP-mediated full activation of platelets as determined by shape change and aggregation (Jin *et al.*, 1998). ADP receptors belong to the seven-transmembrane domain G-protein-coupled receptor (GPCR) family. We have recently synthesized a ten amino acid domain in the carboxy terminus of the P2Y<sub>1</sub> receptor that is important for Gq stimulation (Ding *et al.*, 2005). The cell-penetrating peptides containing i3 loop peptides derived from protease-activated receptors PAR-1 and PAR-4 inhibit thrombin-mediated platelet aggregation (Covic *et al.*, 2002; Kuliopulos *et al.*, 2003). Based on these studies we investigated whether we could block P2Y<sub>1</sub>-Gq coupling using a cell penetrating peptide from this domain (TFRRRLSRATR). To our surprise, we saw that this peptide activated platelets. Although we initially thought that the peptide might be directly stimulating Gq pathways upon entering the cell, we ruled out the possibility by synthesizing the same peptide without the palmitoylation. The peptide thus made was also able to activate platelets suggesting that the peptide activates one of the cell

surface receptors. Hence we began characterizing the mechanism of platelet activation by this peptide (TFRRRLSRATR).

The TFRRR- peptide caused concentration-dependent platelets shape change, aggregation, secretion and calcium mobilization. In order to investigate the mechanism of this peptide, we used different platelet receptor specific antagonists and found that peptide-induced platelet activation was totally abolished by PAR-1 specific antagonist BMS200261. If we desensitize the platelet receptors by using 300  $\mu$ M of the peptide, platelets fail to respond to SFLLRN but not to AYPGKF. Furthermore, in mouse platelets, which are devoid of the PAR-1 receptor, peptide concentrations up to 600  $\mu$ M failed to cause platelet activation. In addition, blockade of PI-3 kinase rendered platelet aggregation induced by TFRRR-peptide reversible. These effects are similar to those observed with SFLLRN under similar conditions. Finally, the peptide caused activation of Akt, Erk, and p38 kinases in a similar manner as PAR-1 activating peptide, SFLLRN. These results clearly demonstrate that this new peptide specifically activates platelets through the PAR-1 receptor, and does not stimulate PAR-4 receptor.

Notwithstanding the similarities between SFLLRN and TFRRR peptide functions, the latter peptide at low concentration acts as a partial agonist similar to YFLLRN and activates G12/13 pathways (Kim et al., 2006). At higher concentrations, however, it mimics SFLLRN by activating G12/13 and Gq pathways together. In this respect, the

TFRRR-peptide resembles the YFLLRNP peptide, which also activates G12/13 and Gq pathways in a concentration-dependent manner (although TFRRR peptide is relatively more potent of the two). Thus, the TFRRR-peptide, like other PAR agonists (including thrombin), traffics signal through G12/13 and Gq pathways in a concentration-dependent fashion. However, physiologically these peptides do not exist and the PARs are activated by proteases. Upon proteolytic activation by thrombin, the tethered ligand intramolecularly activates the receptor (Rasmussen et al., 1991; Vu et al., 1991a). Thus, the concentration of the tethered ligand should be the same physiologically. A possible explanation for such a selective activation of G12/13 signaling pathways may be that PARs coupled to G12/13 proteins exist in a favorable conformation for binding and subsequent cleavage by thrombin relative to the Gq associated PARs. Thus at low concentrations of thrombin, these G12/13-coupled PARs are stimulated and transduce signal before Gq-coupled PARs become activated.

Another interesting observation is that the palmitoylated TFRRR- peptide activated platelets (data not shown). Previous studies have indicated that capping the amino terminus with acetyl moiety of peptide agonists completely abolished PAR1 activation (Scarborough *et al.*, 1992b). Thus it is not clear at this point whether the N-terminal lipid modified TFRRR-peptide and the non lipid modified TFRRR-peptide activate platelets through identical mechanisms.

PAR-1 is a high affinity thrombin receptor in human platelets and it is activated by serine protease, such as thrombin (Kahn *et al.*, 1999). After activation, it will cleave its extracellular N-terminus and expose a new N-terminal (S<sup>42</sup>FLLRNPNDK<sup>51</sup>) acting as a tethered ligand to bind the receptor surface C-terminal residues P<sup>85</sup>AFIS<sup>89</sup>, which is named as PAR-1 binding site-1 (LBS-1) (Seeley *et al.*, 2003). The intramolecular interaction of ligand residue Phe<sup>43</sup> and LBS-1 residue Ser<sup>89</sup>, and the hydrophobic contact of LBS-1 residue Ile<sup>88</sup> and ligand residue Leu<sup>44</sup> both are crucial for the ligand binding and receptor activation and mutation any of these residues will cause dramatically decrease of the receptor activation induced by peptide agonist, whereas the N-terminal of the ligand Ser<sup>42</sup> is not required for agonist activity and does not contact with binding site-1 (Bernatowicz *et al.*, 1996; Seeley *et al.*, 2003). Thus, the two PAR1 activating peptides SFLLRN and YFLLRNP are very similar in their sequence and have the crucial Phe and Leu residues. The difference between YFLLRNP and SFLLRN is that they have the different N-terminal residue, which suggests that a specific N-terminal is not necessary for agonist activation. The amino terminal may influence the agonist activation by binding to other residues of extracellular domains of the receptors. Thus, YFLLRNP is a partial agonist at the PAR-1 whereas SFLLRN is a full agonist.

The peptides used in our study have only Phe residue that presumably interacts with the Ser<sup>89</sup> residue in the LBS-1 of PAR-1. The Leu at fourth position was proposed to be important for activation (Ceruso *et al.*, 1999) with a preference for larger

hydrophobic residues (Seiler *et al.*, 2003). The TFRRR- peptide activates PAR-1, although it lacks the Leu residue that interacts with the Ile<sup>88</sup> in the PAR-1. It is interesting that changing of either of the two basic Arg residues to an acidic residue made the peptide inactive. Thus it appears that the Leu<sup>44</sup> could be substituted with Arg residue and hence is not essential for the activity of the peptide. It is interesting that the corresponding residue to Leu<sup>44</sup> in murine and hamster PAR1 is Phe (Connolly *et al.*, 1994; Hoogerwerf *et al.*, 2002; Rasmussen *et al.*, 1991; Vassallo *et al.*, 1992; Vu *et al.*, 1991b). In addition, mutation of this residue to a proline did not affect the activity of the peptide (Ceruso *et al.*, 1999). However, chemical modification of this residue (N-Me-L) or introduction of rigid spacers in this position resulted in the loss of activity (Ceruso *et al.*, 1999). Thus it appears that there is some flexibility for residues in the third position.

The consensus sequence among all the human PAR-1 activating peptides, including those used this study, is XFXXR, indicating the importance of the second residue (Phe) and fifth residue (Arg). Earlier work from Brass and co-workers has identified these two residues as the most important in the PAR-1 activating peptides (Vassallo *et al.*, 1992). Among various species, these two residues (Phe and Arg) are highly conserved in the PAR-1 (Connolly *et al.*, 1994; Hoogerwerf *et al.*, 2002; Rasmussen *et al.*, 1991; Vu *et al.*, 1991b; Zhong *et al.*, 1992). However, in the model proposed by Seeley *et al.*, the R<sup>46</sup> (fifth residue in the peptide) does not interact with the LBS-1 (Seeley *et al.*, 2003). These issue needs to be resolved in the future.

Thrombin receptors are the important molecular targets for anti-thrombotic drugs. In human platelets, PAR-1 is the predominant thrombin receptor (Kahn *et al.*, 1999). Several peptide or non-peptide agonists and antagonist based on its unique activating mechanism were synthesized to evaluate the function of PAR-1. The peptide we synthesized further confirmed that two residues (Phe and Arg) are critical for the agonist binding and activation. This work will enable us to find the smallest molecular PAR-1 ligand to initiate the PAR-1 activation. In summary, we report novel structural requirements for PAR 1 agonists and a signal trafficking downstream of PAR1 by a single agonist.

## CHAPTER 3

### REGULATION OF PLASMIN-INDUCED PROTEASE-ACTIVATED RECEPTOR 4 ACTIVATION IN PLATELETS

#### Introduction

Plasmin, generated from its precursor plasminogen, is an important enzyme present in blood that degrades fibrin clots. On the other hand, plasmin also activates platelets and causes additional thrombus formation (Ishii-Watabe *et al.*, 2000; Niewiarowski *et al.*, 1973). Earlier studies have demonstrated that plasmin activates a number of intracellular signaling events including calcium mobilization, protein phosphorylation, activation of phospholipase C, and protein kinase C in various cell systems (Chang *et al.*, 1993; Schafer *et al.*, 1985; Schafer *et al.*, 1986; Weide *et al.*, 1996). Studies with human platelets show that plasmin causes an increase of cytosolic  $Ca^{2+}$  and also inhibits thrombin binding (Kimura *et al.*, 1996; Nakamura *et al.*, 1995).

Platelets are involved in primary hemostasis and play a crucial role in the formation of the blood clot when they are activated. Thrombin is probably the most important protease that causes platelet activation. Thrombin is generated through coagulation cascades at sites of vascular injury and activates platelets through protease-activated

receptors (PARs). PARs are seven-transmembrane G-protein coupled receptors and are activated by cleavage of the amino-terminus by proteases. This new N-terminus acts as its own tethered ligand and causes platelet activation (Coughlin, 1999). To date, 4 PARs are identified (PAR1, PAR2, PAR3 and PAR4). Among these 4 members, PAR2 is not activated by thrombin (Nystedt *et al.*, 1994), and PAR3 binds thrombin but does not cause any downstream signaling in platelets. In fact, PAR3 is present on murine but not human platelets where it serves as a cofactor for PAR4 activation at low thrombin concentrations (Ishihara *et al.*, 1997; Nakanishi-Matsui *et al.*, 2000). PAR1 is the predominate thrombin receptor in human platelets due to its high affinity for thrombin but is not expressed on murine platelets (Kahn *et al.*, 1998). PAR4, also present on human platelets, is the major thrombin receptor in murine platelets and has a low affinity for thrombin compared to PAR1 (Kahn *et al.*, 1998; Xu *et al.*, 1998). In addition to thrombin, PARs also can be activated directly by binding with agonist peptides which mimic newly exposed N-terminal tethered ligands (Faruqi *et al.*, 2000; Hung *et al.*, 1992; Vu *et al.*, 1991b).

We have reported previously that plasmin is capable of proteolytically cleaving PAR4 with resultant activation of the receptor in both human and murine platelets (Quinton *et al.*, 2004). However, murine platelets are activated by lower concentrations of plasmin than human platelets (Nylander *et al.*, 2006). In this study, we investigated the molecular basis for this difference in potency and conclude that the primary sequences of the tethered ligand in the mouse and human PAR4 account for the

differential activation of murine and human platelets by plasmin. We also found that PAR3 is an inhibitory receptor for plasmin-induced activation of PAR4.

## **Materials and Methods**

### *Materials*

Plasminogen, streptokinase, and the chromogenic plasmin substrate S-2403 were purchased from DiaPharma (West Chester, OH). Homozygous PAR3-null mice have been previously characterized and were kindly provided by Dr. Shaun Coughlin (Cardiovascular Research Institute, University of California, San Francisco, CA). COS7 cells were purchased from the American Type Culture Collection (Manassas, VA). PCR amplification kit was purchased from Sigma Chemical Co. (St. Louis, MO). Hexapeptides SFLLRN and AYPGKF were custom synthesized at Invitrogen (Eugene, OR). The PAR1 antagonist BMS-200261 was obtained as a generous gift from Dr. Steven Seiler (Bristol-Myers Squibb). Fura-2 AM and LipofectAMINE 2000 reagent was purchased from Invitrogen (Eugene, OR). Unless specifically mentioned, all other reagents were purchased from Sigma Chemical Co. (St. Louis, MO).

### *Plasmin preparation*

The plasmin enzymatic activities were determined as described previously (Quinton *et al.*, 2004). Plasmin was prepared by incubating 5 mg/mL plasminogen with  $1.5 \times 10^4$  units/mL streptokinase for 3 min at 37°C.

### *Preparation of washed human and murine platelets*

Whole blood from healthy, drug-free donors was collected with informed consent, in tubes containing acid-citrate-dextrose (ACD: 2.5 g sodium citrate, 1.5 g citric acid, and 2 g glucose in 100 mL deionized water). Murine blood was collected from anesthetized mice by cardiac puncture into syringes containing 3.8% sodium citrate as anticoagulant. Human platelet rich plasma were treated with 1 mM acetylsalicylic acid (aspirin) and murine platelet rich plasma were treated with Prostaglandin E1 (PGE1, 1  $\mu$ M). The platelets were isolated and resuspended in Tyrode's buffer containing 0.01 unit/mL apyrase.

### *Platelet aggregation*

The platelet count was adjusted to  $2 \times 10^8$  cells/mL and aggregation of 0.5 mL washed platelets was analyzed using a P.I.C.A. Lumi-aggregometer (Chrono-log, Havertown, PA). Aggregation was measured by light transmission under stirring

conditions (900 rpm) at 37°C. The aggregation and secretion data are represented in the form of actual tracings.

### *Cytosolic Calcium Measurements*

Intracellular calcium mobilization was measured as described previously (Dorsam *et al.*, 2005). For human platelets, PRP was incubated with 2  $\mu$ M Fura-2 AM with acetylsalicylic acid (aspirin) for 45 minutes and left in room temperature for additional 15 minutes. For murine platelets, PRP was incubated with 2  $\mu$ M Fura-2 AM, 500 $\mu$ M EGTA, 10 $\mu$ M indomethacin and 1 $\mu$ M PGE1. Platelets were then isolated and washed as described above. For COS 7 cells, cells were incubate with DMEM medium containing 2  $\mu$ M Fura-2 AM and 0.02% pluronic F-127 for 60 min at 37°C with 5% CO<sub>2</sub>. Then cells were washed with 1X PBS and collected. Cells were then resuspended in 1X Tyrode's buffer containing 2 mM calcium and incubated 20 min at room temperature with gentle inversion every 5 min. After additional spin at 1,000 rpm for 3 min, cells were re-suspended in fresh 1X Tyrode's buffer without calcium and adjust to 10<sup>6</sup> cells/mL. 0.5 mL sample was analyzed and fluorescence measurements were converted to calcium concentrations using the equation reported by Grynkiewicz *et al* (Grynkiewicz *et al.*, 1985), where F<sub>min</sub> and F<sub>max</sub> were determined with each respective platelets or cells preparation.

*Construction of Wild-type and mutant human and murine PAR4 expression and transfection*

Human PAR4 (hPAR4) was generated as described previously (Quinton *et al.*, 2004). Murine PAR3 (mPAR3) is the gift from Dr. Shaun Coughlin. Murine PAR4 (mPAR4) coding sequence was amplified by using PCR with the forward and reverse primers specific for mPAR4 cDNA. The sense primer is 5'-CGCGAAGCTTATGTACCCATACGATGTTCCAGATTACGCTTGCTGGCCGCTGCTGTATCCTTTG-3', which contains a hemagglutinin epitope tag and HindIII restriction site. The antisense primer is 5'-CGCGGGATCCTCACAGAAGTG TAGAGGAGCAAATC-3', which contains a BamHI restriction site. Murine PAR4 DNA was then sub-cloned into the pcDNA3-zeocin (+) vector and the nucleotide sequence was confirmed by DNA sequence analysis.

Mutants of hPAR4 and mPAR4 were generated by using the QuickChange site-directed mutagenesis kit from Stratagene (La Jolla, CA). The changes in oligonucleotides and the corresponding amino acids are shown in table 1. Wild-type hPAR4 was used as a template of hPAR4-KF and wild-type mPAR4 was used as a template of mPAR4-QV. All mutations were confirmed by DNA sequencing.

COS7 cells were transient-transfected with pcDNA-based expression vectors or target DNAs by using LipofectAMINE 2000. Each transfection included 20  $\mu\text{g}$  DNA per 100 mm plate (10  $\mu\text{g}$  human PAR4, murine PAR4 and/or murine PAR3 or control expression vector).

### *Statistics*

Statistical analyses were made using student's *t* test. P values  $<0.05$  were considered statistically significant. All results are given as mean  $\pm$  SEM.

**Table 3.1. Primers for site-directed mutagenesis of the human and murine PAR4 receptors**

<b>TABLE 1. PRIMERS FOR SITE-DIRECTED MUTAGENESIS OF THE HUMAN AND MURINE PAR4 RECEPTORS</b>				
Name of mutant plasmid	Amino acid and position	Nucleotide change	Amino acid change	Primers used
HPAR4-KF	Gln52Val53	CAAGTC → AAAATTC	Gln → Lys Val → Phe	+5'-GGCTACCCAGGCAAAATTCTGTGGCCAATG-3' -5'-CATTTGGCACAGAAATTTGGCCCTGGGTAAGCC-3'
MPAR4-QV	Lys64Phe65	AAAATTC → CAAGTC	Lys → Gln Phe → Val	+5'-GGCTACCCGGGCGCCAAGTCTGTGCCCAACG-3' -5'-CGTTGGCACAGAGACTTGGCCCGGGTAGCC-3'
UNDERLINED NUCLEOTIDE INDICATES THE ALTERED NUCLEOTIDE FOR SITE-DIRECTED MUTAGENESIS				

## Results

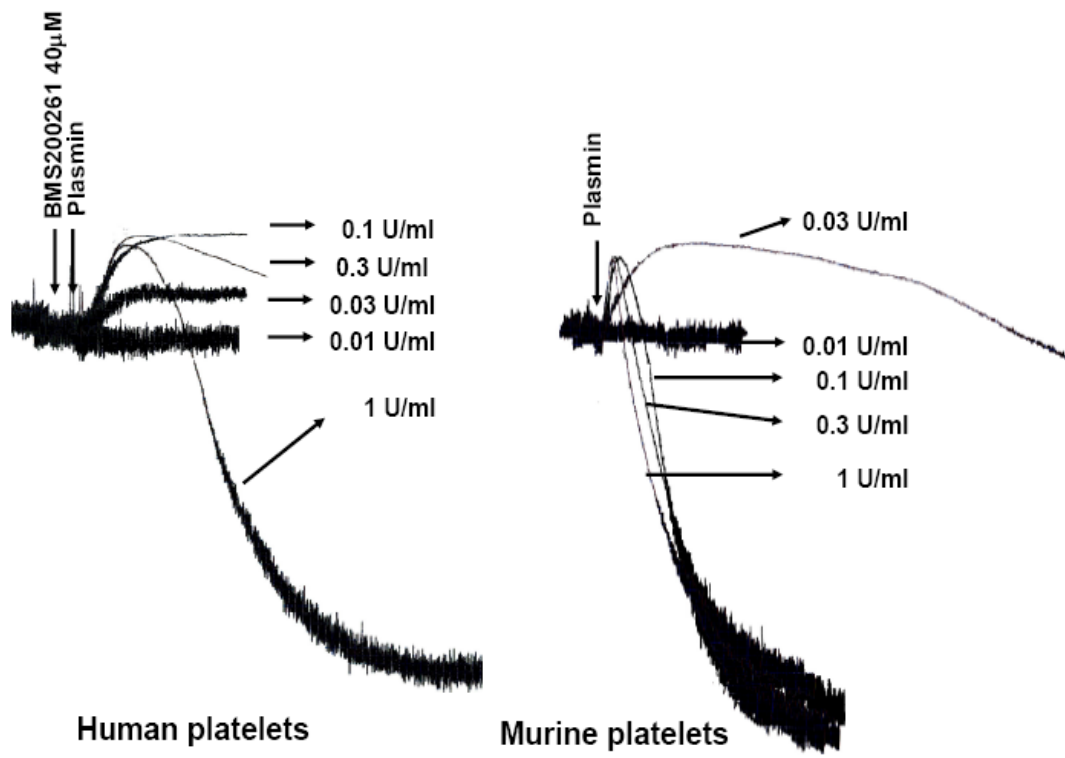
### *Plasmin-induced murine and human platelet activation.*

We have previously shown that plasmin-induced platelet activation primarily occurs through cleavage of PAR4 (Quinton *et al.*, 2004). The present work investigated the mechanisms underlying the differences in activation of murine versus human PAR4. We first compared the effect of plasmin on human and murine platelets. To rule out any possible contribution from human PAR1 receptors, PAR1 specific antagonist BMS-200261 was used to inhibit PAR1 activation without affecting PAR4 activation. The specificity of this antagonist was evaluated, wherein BMS-200261 completely inhibited platelet aggregation induced by PAR1 agonist SFLLRN but did not affect PAR4 agonist AYPGKF-induced aggregation (data not shown). The effect of BMS200261 in plasmin-induced murine platelet aggregation was also tested and there is no difference in the absence or presence of BMS200261 (data not shown). Figure 3.1A shows that plasmin causes shape change and aggregation of both murine and human platelets in a concentration-dependent manner, albeit with a different sensitivity. In murine platelets, 0.1 U/mL plasmin caused full platelet aggregation. However, the same concentration of plasmin caused only shape change in human platelets. To achieve complete activation of human platelets, a much higher concentration (1 U/mL) of plasmin was needed. We have stimulated human platelets with plasmin in the absence of BMS200261 and found that the aggregations were not

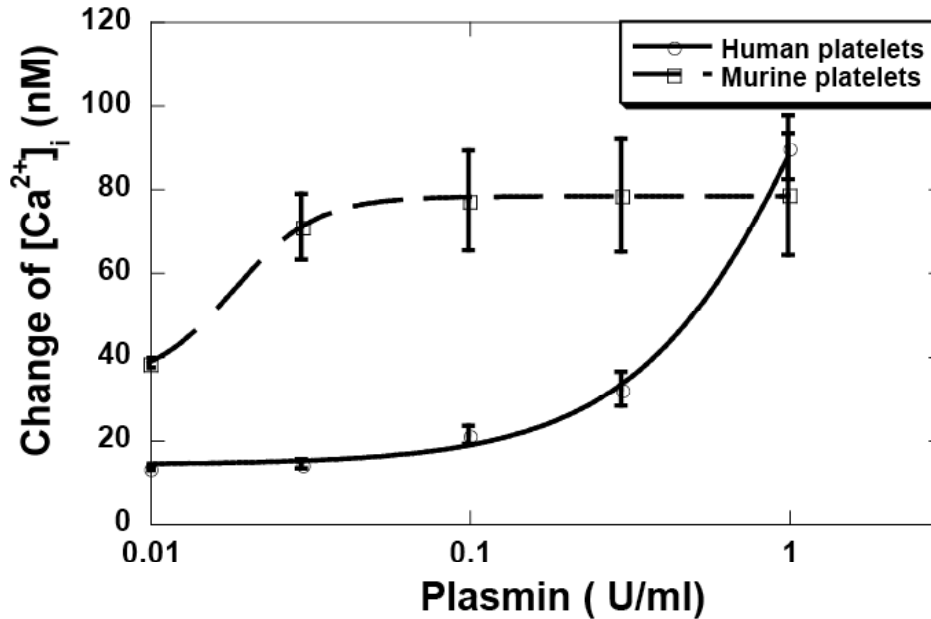
as robust as in murine platelets (data not shown). Figure .1B shows that, consistent with the aggregation data, in that over 10-fold higher concentrations of plasmin are required to give the same increase in intracellular calcium in human platelets compared to murine platelets. These data indicate that plasmin is more potent for murine platelet activation than human platelet activation.

We confirmed these results in COS7 cells that were transiently transfected with either hPAR4 or mPAR4. Transfection was monitored by stimulating cells, 48 h after transfection, with the PAR4 specific agonist AYPGKF. When stimulated with plasmin, cells expressing human or murine PAR4 showed an increase of calcium mobilization compared with control cells expressing the vector alone. Furthermore, there was a significantly higher increase of intracellular calcium in cells expressing mPAR4 compared to cells expressing the hPAR4 (Fig.3.2).

**A**



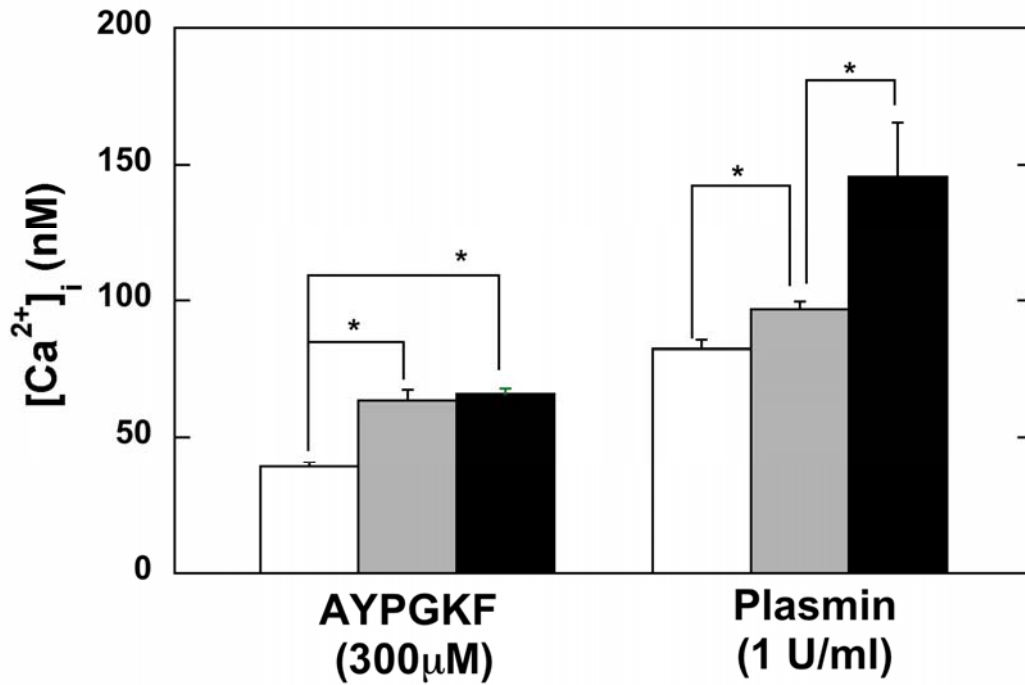
**B**



**Figure 3.1. Plasmin causes more activation in murine platelets than in human platelets.** Aspirin-treated, washed human or murine platelets were stimulated with different concentrations of plasmin (as indicated) under stirring conditions at 37° C and platelet aggregation (Panel A) and intracellular calcium mobilization (Panel B) were measured. BMS-200261 was used to block PAR1 on human platelets. Aggregation tracings are representative of at least three separate experiments. Calcium mobilization results are expressed as the mean ± SEM of the change of intracellular calcium release before and after adding agonist from at least three separate experiments.

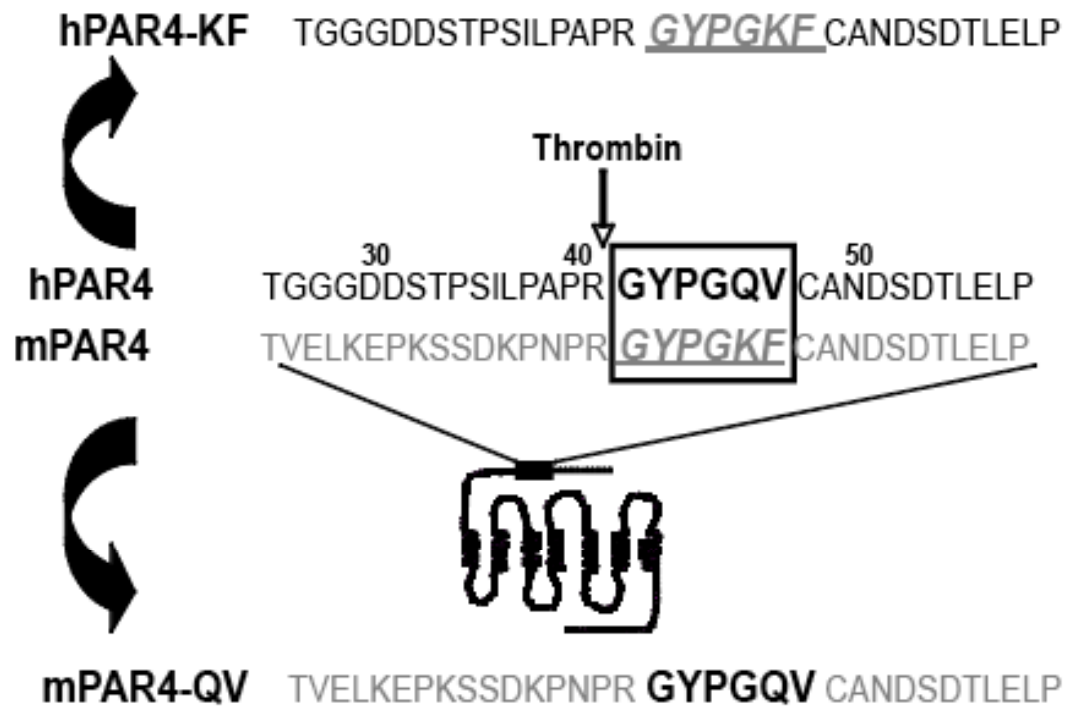
*Effect of different species PAR4 sequences on plasmin-induced PAR activation*

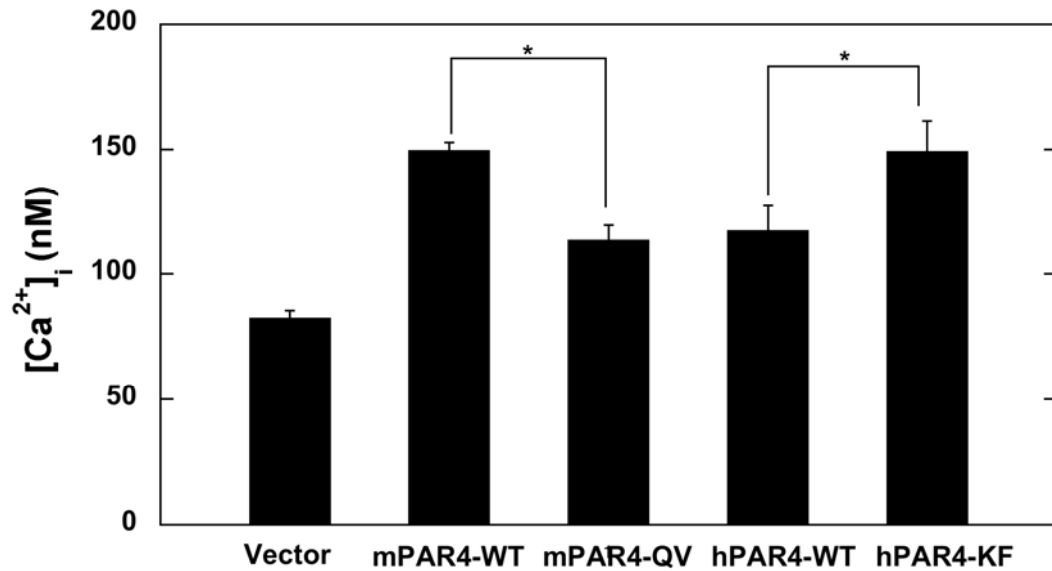
Human PAR4 shares more than 70% sequence homology to murine PAR4. However, their tethered ligand sequences, which are critical for receptor activation, are different. It is known that the PAR4 agonist AYPGKF, with greater similarity to mPAR4 tethered peptide, causes more activation of human platelets than GYPGQV, the hPAR4 tethered ligand (Faruqi *et al.*, 2000). Since we have shown previously that plasmin cleaves PAR4 at the same site as thrombin and generates an identical tethered ligand (Quinton *et al.*, 2004), it is possible that the observed difference in plasmin-induced activation is due to the difference between human and murine PAR4 primary tethered ligand sequences. To investigate this possibility, we made mutants of PAR4 receptors by exchanging the tethered ligand sequences of hPAR4 and mPAR4. Figure 3.3A shows the sequences of the mutant receptors. COS7 cells expressing mutant mPAR4 receptors with the hPAR4 tethered ligand sequences GYPGQV (mPAR4-QV) had less 1U/mL plasmin-stimulated intracellular calcium mobilization in COS7 cells than WT mPAR4 receptors (Fig. 3.3B). Consistently, mutant hPAR4 with mPAR4 tethered ligand sequences GYPGKF (hPAR4-KF) had increased plasmin-induced intracellular calcium mobilization as compared with cells expressing WT hPAR4 (Fig. 3.3B).



**Figure 3.2. Activation of transiently expressing human and murine PAR4 in COS7 cells by plasmin.** COS-7 cells were transiently expressing vector alone (open bars), human PAR4 (grey bars) or murine PAR4 (black bars) and stimulated with AYPGKF (300 μM) or plasmin (1 U/mL). Data are expressed as the mean ± SEM of the peak intracellular calcium release (n=3; \*, p<0.05).

A



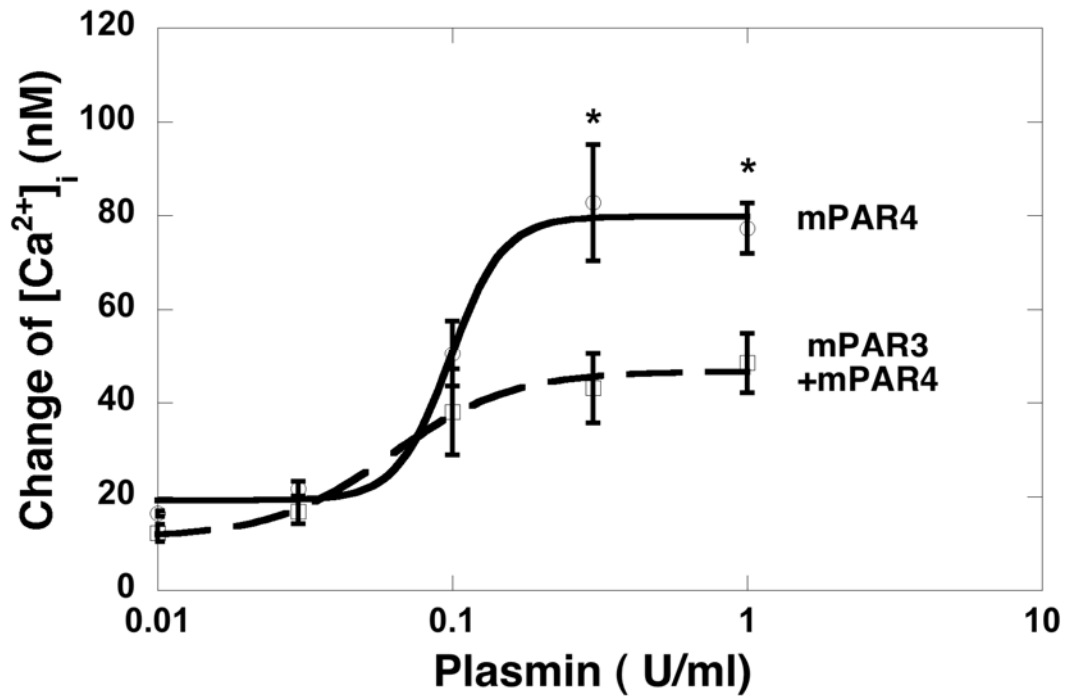
**B**

**Fig. 3.3. Effect of PAR4 mutants on Plasmin-mediated activation.** A) Depiction of the structures of the mutant mPAR4 and hPAR4 with interchanged tethered ligand sequences. B) Intracellular calcium increases were measured in COS7 cells transiently expressing human and murine wild type and mutant PAR4 upon stimulation with 1 U/mL plasmin. mPAR4-WT: wild type murine PAR4; mPAR4-QV: murine PAR4 with human PAR4 tethered ligand sequence; hPAR4-WT: wild type human PAR4; hPAR4-KF: human PAR4 with murine PAR4 tethered ligand sequence. Calcium mobilization results are expressed as the mean  $\pm$  SEM of the peak intracellular calcium release (n=3; \*, p<0.05).

### *Effect of murine PAR3 on plasmin-induced mPAR4 activation*

Since murine platelets express PAR3 in addition of PAR4 and mPAR3 acts as a co-receptor that promotes cleavage and activation of murine PAR4 at low concentrations of thrombin (Nakanishi-Matsui *et al.*, 2000), we investigated the effect of mPAR3 on plasmin-induced mPAR4 activation. We co-transfected mPAR3 and mPAR4 in COS7 cells. Co-transfection of mPAR3 and mPAR4 did not potentiate the plasmin-induced increase in intracellular calcium mobilization. Instead, presence of mPAR3 decreased the plasmin-induced intracellular calcium increases compared to cells that were transfected with only mPAR4 (Fig. 3.4).

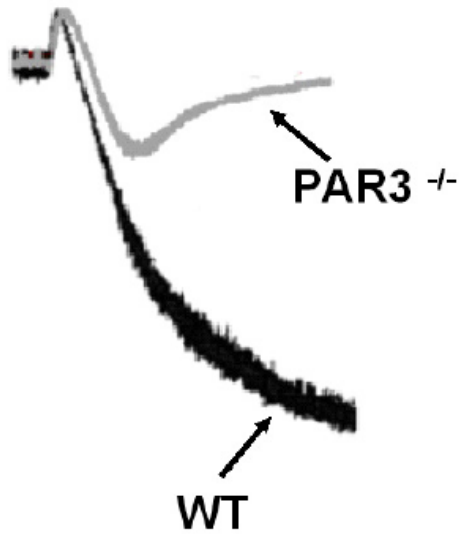
Platelets from PAR3<sup>-/-</sup> mice, expressing only PAR4, were used to confirm these results. As expected, PAR3 null mice platelets showed a diminished response when stimulated by thrombin (Nakanishi-Matsui *et al.*, 2000) (Figures 3.5A and 3.5B). However, plasmin (0.3 U/mL) caused a slightly greater extent of platelet aggregation in murine platelets lacking PAR3 receptors compared to platelets from wild type littermates (which express both PAR3 and PAR4). Furthermore, plasmin (0.3 U/mL) elicited a significantly greater increase in cytoplasmic calcium in PAR3<sup>-/-</sup> murine platelets compared to wild type platelets (Fig. 3.5B). These data indicate that PAR3 does not act as a co-receptor for plasmin, but actually represses plasmin-induced PAR4 activation.



**Fig. 3.4. mPAR3 inhibits plasmin-induced mPAR4 activation.** mPAR4 or mPAR3+mPAR4 transfected COS7 cells were stimulated with plasmin (0.01 -1 U/mL), and the change of calcium mobilization was measured. Vector transfected cells were used as control and 1 U/ml plasmin caused  $39.1 \pm 2.6$  nM calcium mobilization in vector group. Data are expressed as the mean  $\pm$  SEM (n=3; \*, p<0.05).

**A**

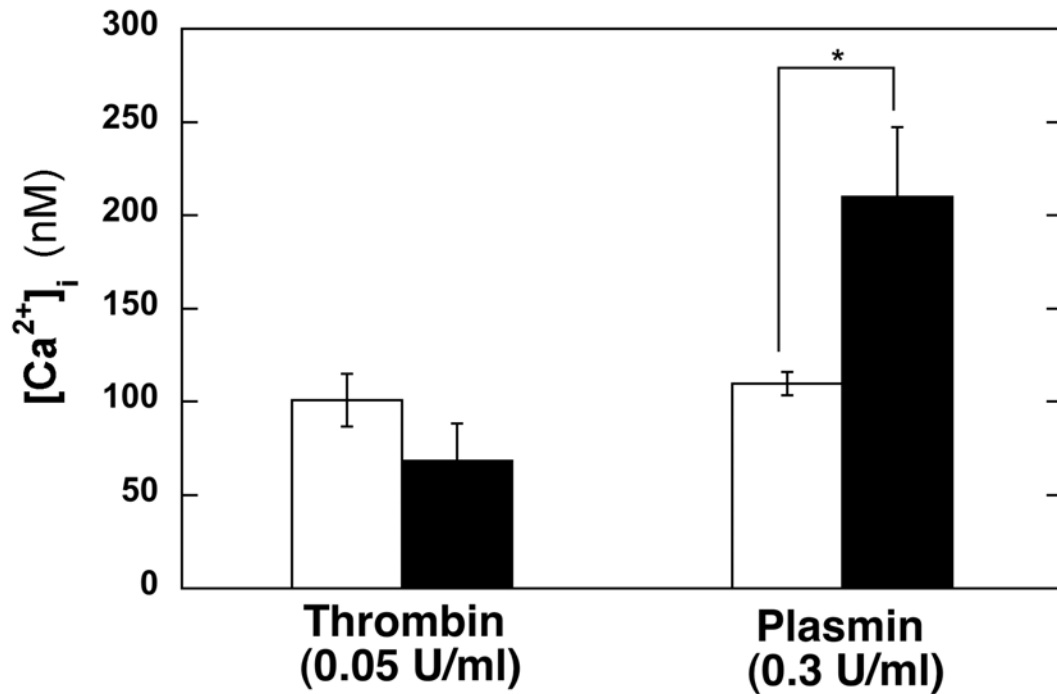
**Thrombin  
(0.05 U/ml)**



**Plasmin  
(0.3 U/ml)**



**B**



**Fig. 3.5. Plasmin causes increased platelet activation in  $PAR3^{-/-}$  mice.** Wild type or  $PAR3^{-/-}$  murine platelets were stimulated with thrombin (0.05 U/mL) or plasmin (0.3 U/mL) (panel A). In panel B, agonist-induced calcium mobilization was compared between wild type and  $PAR3^{-/-}$  murine platelets. The calcium mobilization of wild type murine platelets was taken as 100% (open bar) and agonist-induced calcium mobilization in  $PAR3^{-/-}$  murine platelets was expressed as a percentage of control platelets (black bar). Data are expressed as the mean  $\pm$  SEM ( $n=3$ ; \*,  $p<0.05$ ).

## Discussion

We have previously shown that plasmin-mediated platelet activation occurs primarily through slow cleavage of PAR4 (Quinton *et al.*, 2004). In addition, we found that less plasmin is needed to cause the same extent of aggregation in murine platelets as compared to human platelets. The present work investigated the mechanisms underlying the differences in plasmin-dependent activation of murine versus human PAR4. Our results suggest that mPAR4 is more sensitive than hPAR4 when assayed as plasmin-stimulated receptor-transfected COS7 cells measuring calcium mobilization and when assessed by plasmin-induced platelet aggregation and calcium mobilization.

In order to explain the difference in plasmin-mediated PAR4 activation between human and murine platelets, we considered the following possibilities: (a) human platelets only express PAR4 while murine platelets have both PAR3 and PAR4. (b) mPAR4 and hPAR4 have sequence divergence in the N-terminal tethered ligand. (c) structural differences in mPAR4 compared to hPAR4 may make the cleavage site more accessible.

PAR3, as a member of PAR family, has 27% amino acid sequence similarity to PAR1 (Hou *et al.*, 1998). In human platelets, thrombin activates both PAR1 and PAR4 receptors (Kahn *et al.*, 1999), whereas murine platelets express PAR3 and PAR4

(Kahn *et al.*, 1998) and mPAR3 acts as a co-receptor that promotes cleavage of PAR4 resulting in platelet activation at low concentration of thrombin (Nakanishi-Matsui *et al.*, 2000). However, our data shows that unlike the effect of mPAR3 on thrombin-induced murine PAR4 activation, mPAR3 did not act as a cofactor of mPAR4 for plasmin activation; on the contrary, expression of mPAR3 in COS7 decreases the ability of plasmin to activate mPAR4 receptors.

We believe that the difference in primary sequence between plasmin and thrombin may be responsible for a differential engagement of PAR3. It is possible that plasmin binds to mPAR3 at a different site than thrombin does and this binding might sequester some plasmin rendering it unavailable to activate at PAR4. However, despite the inhibitory effect of PAR3, murine platelets are still activated by lower concentrations of plasmin than human platelets. Thus, the primary structural differences lead to a more potent tethered ligand upon cleavage of mPAR4 with plasmin than that generated with the cleavage of hPAR4 with plasmin.

Previous study reported that platelets from the different species differed in their sensitivity to ADP and thrombin. Murine platelets were more sensitive to both ADP and human thrombin with significantly lower  $EC_{50}$  values compared to human platelets (Nylander *et al.*, 2006). In addition, studies on PAR4 tethered ligand-derived peptides showed that peptides based on either the tethered ligand sequence of human, rat or murine PAR4 cause different potency of PAR4 activation and AYPGKF, which

was generated according to murine PAR4 tethered ligand sequence has a highest potencies to activating PAR4 (Faruqi *et al.*, 2000; Hollenberg *et al.*, 2004). The fact that plasmin cleavage produces the same tethered ligand as thrombin may explain why mPAR4 has a higher affinity for plasmin activation than hPAR4 (Quinton *et al.*, 2004).

Another possibility is that the different sequences of mPAR4 compared to hPAR4 may have three dimensional protein structures which may be more accessible for plasmin binding and cleavage. In addition, according to the N-terminal extra-domain sequences, mPAR4 had more basic Arg/Lys residues than hPAR4. It is possible that besides thrombin cleavage site, there are more potential residues in mPAR4 could be cleaved by plasmin than in hPAR4.

Physiologically, plasmin acts as a fibrinolytic reagent that dissolves fibrin clots. Our results show that under our experimental conditions, plasmin activates PAR4 in both transfected cell lines and platelet system. We do not have any direct evidence, whether plasmin also activates platelets under physiological conditions. Even if activation of platelet PAR4 has little physiological relevance, it is possible plasmin-dependent activation of PAR4 on other cells may have an important physiological function.

In conclusion, this study finds that murine PAR4 is more sensitive for plasmin-mediated activation compared to human PAR4 because of the primary tethered ligand sequence differences between hPAR4 and mPAR4. In addition, unlike acting as a cofactor for thrombin, mPAR3 negatively regulates plasmin-mediated mPAR4 activation.

## CHAPTER 4

# THE CONTRIBUTION OF PROTEASE-ACTIVATED RECEPTOR 4 TO MICROVASCULAR INFLAMMATION DURING CEREBRAL ISCHEMIC/REPERFUSION INJURY

### Introduction

Protease-activated receptors (PARs) belong to the superfamily of seven-transmembrane domain G protein coupled receptors. Four subtypes of PAR members (PAR1-PAR4) are known to date. PAR1, PAR3 and PAR4 can be activated by thrombin, and therefore are also called thrombin receptors (Coughlin, 2000), whereas, PAR2 is activated by trypsin (Nystedt *et al.*, 1995). PAR receptors can be activated by the cleavage of the receptor at the extracellular N-terminus with the newly exposed N-terminus acting as a ligand to bind to the receptor to initiate intracellular signaling (Rasmussen *et al.*, 1991; Vu *et al.*, 1991a). The activation of PAR1, PAR3 and PAR4 has been intensively investigated in platelets (Coughlin, 2000; Ishihara *et al.*, 1997; Kahn *et al.*, 1998; Molino *et al.*, 1997a). In addition to platelets, PAR receptors are also found to be widely expressed in the brain (Strigow *et al.*, 2001; Wang *et al.*, 2002). PAR4 protein is found on dendrites of the hippocampus and all

cortical layers(Striggow *et al.*, 2001). Studies involving thrombin and PARs in the nervous system showed that these receptors play a critical part in maintaining a delicate balance between neuroprotection and neurodegeneration during inflammation, injury and disease states (Striggow *et al.*, 2000; Vaughan *et al.*, 1995).

The thrombin and PARs have been demonstrated to modulate ischemic, hemorrhagic and traumatic brain injury. It was shown that after transient focal ischemia in rat brain, PAR1 mRNA was found to be down regulated. However, PAR3 and PAR4 mRNA levels were increased (Rohatgi *et al.*, 2004) and there was enhanced PAR4 labeling in the penumbra (Striggow *et al.*, 2001). The effects of thrombin were also demonstrated in other experimental ischemic models. Studies with PAR1 null mice and a PAR1 antagonist showed that PAR1 increases infarct volume and causes neuronal damage after both transient focal cerebral ischemia and combined cerebral hypoxia/ischemia (Junge *et al.*, 2003; Olson *et al.*, 2004).

Antithrombotic therapy is the primary therapeutic method used today to prevent stroke-induced injury, however, agents currently used can cause unexpected bleeding and other unwanted side effects (Smyth *et al.*, 2009). Therapies targeting thrombin signaling have become one of the new directions to avoid the risk of bleeding. PAR1 acts as a major PAR receptor in human platelets and its antagonists, SCH530348 and E5555 have been evaluated in clinical investigations (TRA•CER; ClinicalTrials.gov identifier: NCT00527943 and TRA-2<sup>o</sup>P - TIMI 50; ClinicalTrials.gov identifier:

NCT00526474; ClinicalTrials.gov identifiers: NCT00619164, NCT00548587, and NCT00312052). However, PAR1 does not exist in murine platelets; it is difficult to investigate the antithrombotic role of PAR1 antagonists in mouse models of cerebral ischemic injury. In murine platelets, PAR3, instead of PAR1 and PAR4 are expressed. PAR4 is the major thrombin receptor in murine platelet and also the only PAR receptor that expressed in both human and murine platelets. Although both PAR4 and PAR1 are activated by thrombin, they respond differently. PAR4 requires a higher concentration of thrombin and causes a prolonged and sustained response, whereas PAR1 can be activated by low concentration of thrombin, which induces a rapid and transit calcium mobilization (Shapiro *et al.*, 2000). To fully understand the effect of thrombin in brain injury, it is important to investigate the role of PAR4 in ischemic injury. It has also been clearly demonstrated that microvascular inflammation, including leukocyte infiltration and blood brain barrier disruption play important roles in secondary injury following cerebral ischemia. To the best of our knowledge, we are the first to examine the effects of PAR4 in cerebral I/R injury and our data suggested that PAR4 is a critical contributor to the inflammatory responses in the cerebral microvasculature.

## Material and methods

### *Animals*

The cerebral ischemia/reperfusion studies were carried out on 8-10 week old male PAR4<sup>-/-</sup> C57BL/6 or C57BL/6 wild-type mice. PAR4<sup>-/-</sup> mice breeding pairs were a gift from Dr. Shaun Coughlin, University of California San Francisco and wild-type mice were purchase from Jackson Laboratories (Bar Harbor, ME). All experiments were conducted in accordance with the guidelines approved by Institutional Animal Care and Use Committee at Temple University.

### *Middle cerebral artery occlusion and reperfusion (MCAO/R)*

Middle cerebral artery occlusion and reperfusion (MCAO/R) was performed as described previously(Zhang *et al.*, 2007). The animals were anesthetized with an intraperitoneal injection of a Ketamine (100mg/ml) - Xylazine (20mg/kg) mixture (1:1) at a dose of 1ml/kg. The intraluminal filament method(Hata *et al.*, 1998) was used for middle cerebral artery occlusion. The middle cerebral artery was occluded for 60 minutes and reperfusion was confirmed when pulsations were again observed in the ICA. The same surgical procedures were performed on sham animals without occlusion of the middle cerebral artery. Body temperature was maintained at 37±5°C and regional cerebral blood flow (rCBF) was monitored by laserPro Blood Perfusion

Monitor (TSI Inc, Shoreview, MN, USA). The MCAO was considered adequate if rCBF showed a sharp drop to 25% of the baseline (pre-ischemia) level.

### *Cranial windows*

Two days before the MCAO/R experiments, cranial windows were implanted in those animals using for observing platelets and leukocytes rolling and adhesion as previously described (Zhang *et al.*, 2007). A 4mm diameter circular craniotomy was performed using a high speed drill (Champ-Air Dental Drill Benco Dental) over the right parietal cortex and a 5mm diameter coverglass was placed over the exposed brain and sealed with Nexaband Quick Gel. The coverglass provided adequate mechanical protection from infection or contamination.

### *Platelet preparation*

Approximately 0.9ml blood was harvested and collected in syringe with 0.1 ml acid citrate-dextrose buffer and centrifuged at 120g for 10 minutes. Platelet-rich plasma was transferred to a polypropylene tube. Carboxyfluorescein diacetate succinimidyl ester (CFDASE; Molecular Probe, Eugene, OR, U.S.A) was dissolved in DMSO to a concentration of 14.9 $\mu$ mol/L. PRP was diluted with Dulbecco's phosphate-buffered saline (DPBS) to 1500  $\mu$ L and incubated with 9  $\mu$ l CFDASE solution for 5 minutes at room temperature in the presence of PGE1 (1 $\mu$ M). After centrifugation for 10 minutes

at 450g, the platelet pellet was resuspended with 300  $\mu$ l of DPBS, stored on ice and protected from light. Platelet number was manually counted and leukocytes were controlled to be less than 0.05% in the platelet suspension. In each mouse,  $50 \times 10^6$  platelets were injected before performing MCAO/R. Platelet function was checked to make sure that the isolation procedure did not activate platelets.

*Intravital fluorescence microscopy and measurement of platelet, leukocyte/endothelial interactions*

An Olympus epi-illuminescence intravital microscopy (BX10, Olympus, Japan) with a digital Camera (Cooke 1600, Cooke Corporation, Romulus, Michigan) was used to observe the cerebral microcirculation at 24 hours after MCAO. Leukocytes were stained *in vivo* by injection of 0.05 ml of a 0.01% solution of the fluorescent dye Rhodamine 6G (Sigma Inc, St Louis, MO) through the facial vein (Levene *et al.*, 2007). Platelets were stained *in vitro* by CFDA5E. The image from the camera was displayed on a computer monitor, captured and recorded by Camfire software at a video frame rate of 25 frames/sec.

Three venules (with diameter 30-40  $\mu$ m) in each animal were assessed. Rolling was considered to be the total number of platelets or leukocytes moving along the endothelial cells at substantially slower velocity compared with the midstream blood cell velocity. They were counted when they passed an arbitrary line perpendicular to

the longitudinal axis of the vessel in 30 seconds. Adhering platelets and leukocytes were defined as the total number of the platelets and leukocytes firmly attached to the microvascular endothelium for more than 2 seconds and 30 seconds, respectively. Adhering platelets and leukocytes were scored as the number of cells per mm<sup>3</sup> of the vascular surface area, calculated from the diameter and standardized length (100 µm) of the vessel segment under investigation.

#### *Infarct volume and cerebral edema assessment*

The animals were euthanized with an overdose of pentobarbital (200 mg/kg *i.p.*) 24 hours after MCAO and the brains were removed. After chilling on ice for 10 minutes, five 2mm-coronal sections were cut using a mouse brain matrix (Zivic lab, Pittsburgh, PA, USA). The sections were immersed in a 2 % triphenyltetrazolium chloride (TTC) (Sigma Inc, St Louis, MO, USA) saline solution for 20 minutes at 37°C in the dark. The brain sections were then fixed in 4% paraformaldehyde at 4°C for 24 hours and the anterior and caudal face of each section was scanned by a flatbed color scanner (Microtek Inc, Carson, CA, USA). The resulting images were analyzed with Image G software. The infarct volumes were corrected for brain edema/swelling. For this purpose, the hemispheric infarct volume in each section was calculated by subtracting the area of normal, TTC stained tissue in the hemisphere ipsilateral to the ligation from the contralateral nonischemic area to generate the infarct fraction (%) as described by Swanson et al. and Lin et al (Lin *et al.*, 1993; Swanson *et al.*, 1990).

Cerebral edema was determined by the percent increase of the ipsilateral/contralateral hemisphere area (Vannucci *et al.*, 2001).

#### *Neurological deficit score and Rotarod test*

The severity of neurological deficits was evaluated 24 hours after ischemic injury using a five-point deficit score (0=normal motor function; 1=flexion of torso and of contralateral forelimb upon lifting of the animal by tail; 2=circling to the contralateral side but normal posture at rest; 3=leaning to contralateral side at rest; and 4=no spontaneous motor activity) (Hata *et al.*, 1998). A Rotarod was used to evaluate the motor function 24 hours after MCAO (Gupta *et al.*, 2002). Animals were trained on rotarod at speed of 10rpm before MCAO and tested 24 hours after MCAO at same speed. The running time on rotarod was recorded automatically.

#### *Quantitative evaluation of Evans blue extravasation*

Blood-brain barrier disruption was assessed by quantitatively measuring Evans blue (EB) extravasation as described previously with some modifications (Belayev *et al.*, 1996). Mice were intravenously injected with 0.1ml 2% Evans blue 24 hours after MCAO. After 60 minutes the animals were perfused transcardially with 0.9% saline to remove intravascular EB dye. Ischemic and non-ischemic hemispheres were dissected and weighed. The hemispheres were homogenized in 500  $\mu$ l 0.1M PBS and

500 µl of TCA (trichloroacetic acid). The samples were incubated at 4 °C for at least 1h and centrifuged at 10000 g for 30 min. The resulting supernatants were measured for absorbance of EB at 610 nm using a spectrophotometer.

### *Histology*

TTC stained brain sections were postfixed in 4% paraformaldehyde and dehydrated in 30% sucrose. After freezing, the 2-mm sections were cut into 40 µM slices on a cryostat and mounted on gelatincoated slides. Slides were stained with cresyl violet and microscopic images were collected.

### *Statistical analysis*

Students' t-test was used to evaluate the difference of cerebral infraction and neurological score, rotarod running time, and leukocyte/endothelial or platelet/endothelial interactions in PAR4<sup>-/-</sup> and wide type mice. One-way ANOVA was used to analyze Evans blue extravasation in PAR4<sup>-/-</sup> and wild type mice. Data were presented as means ± SEM. A statistically significant difference was assumed at  $P < 0.05$ .

## Results

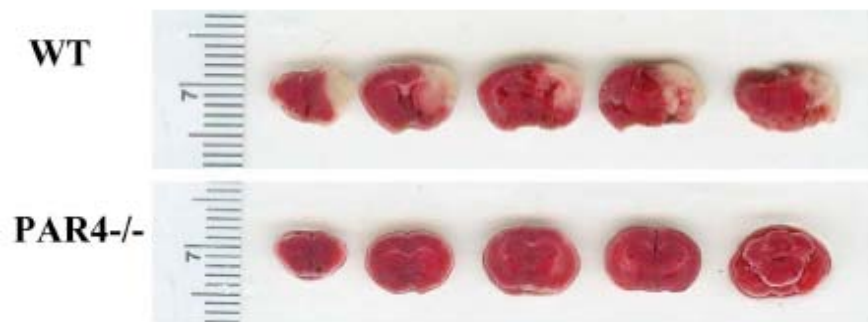
### *Evaluation of tissue damage after cerebral I/R injury in WT and PAR4<sup>-/-</sup> mice*

PAR4<sup>-/-</sup> and wild-type mice were subjected to MCAO for 1 hour and 23 hours of reperfusion. Cerebral infraction lesion area was determined by using the vital dye TTC. Figure 4.1 shows that compared to wild-type mice, deficiency of PAR4 caused a more than a 10-fold reduction of cerebral infarct fraction ( $23.86\% \pm 1.86$  vs  $2.16\% \pm 1.23$  respectively ( $p < 0.001$ )).

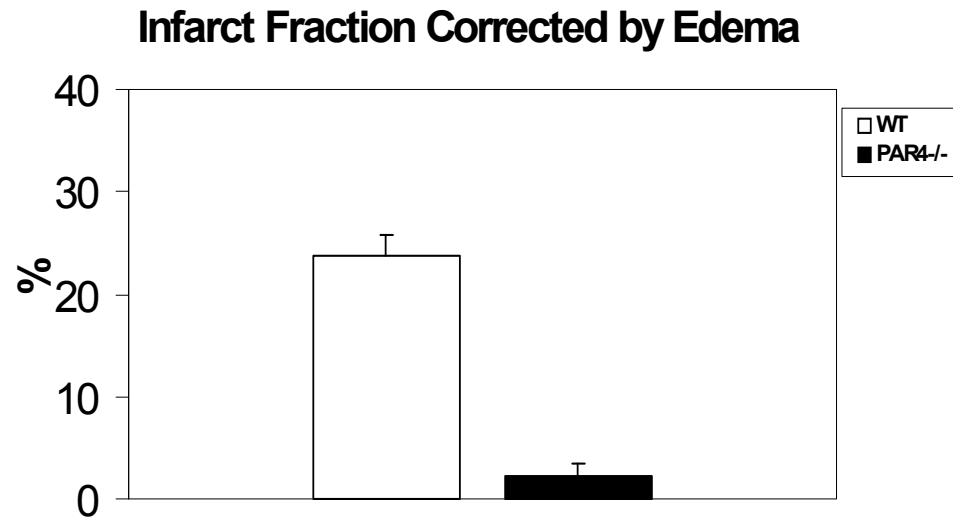
To further characterize the cellular response in wild-type and PAR4<sup>-/-</sup> mice after I/R injury, cresyl violet staining was used to evaluate neuronal death after stroke. More cell loss was observed in ipsilateral hippocampus (Fig. 4.2) in wild-type mice than PAR4<sup>-/-</sup> mice. Neurodegeneration was noticed in both CA1 pyromidal cells and the dentate gyrus of the wild-type ipsilateral hippocampus compared to the contralateral side (Fig. 4.2A and Fig. 4.2B). Loss of the hilar neurons in the dentate gyrus in wild-type ipsilateral side was also greater compared to the contralateral side (Fig. 4.2C and Fig. 4.2D). Similar cell loss in the hippocampal CA1 was also observed in wild-type ipsilateral side compared to the contralateral side (Fig. 4.2E and Fig. 4.2F). Ischemia and reperfusion did not cause significant cell loss in PAR4<sup>-/-</sup> mice (Fig. 4.2G-2L). There was no detectable cell loss in either the hippocampal CA1 or the dentate gyrus of the PAR4<sup>-/-</sup> mice. Similar results were observed in the cortex (data not shown).

These results indicate that deficiency of PAR4 decreased neuronal death after ischemic injury.

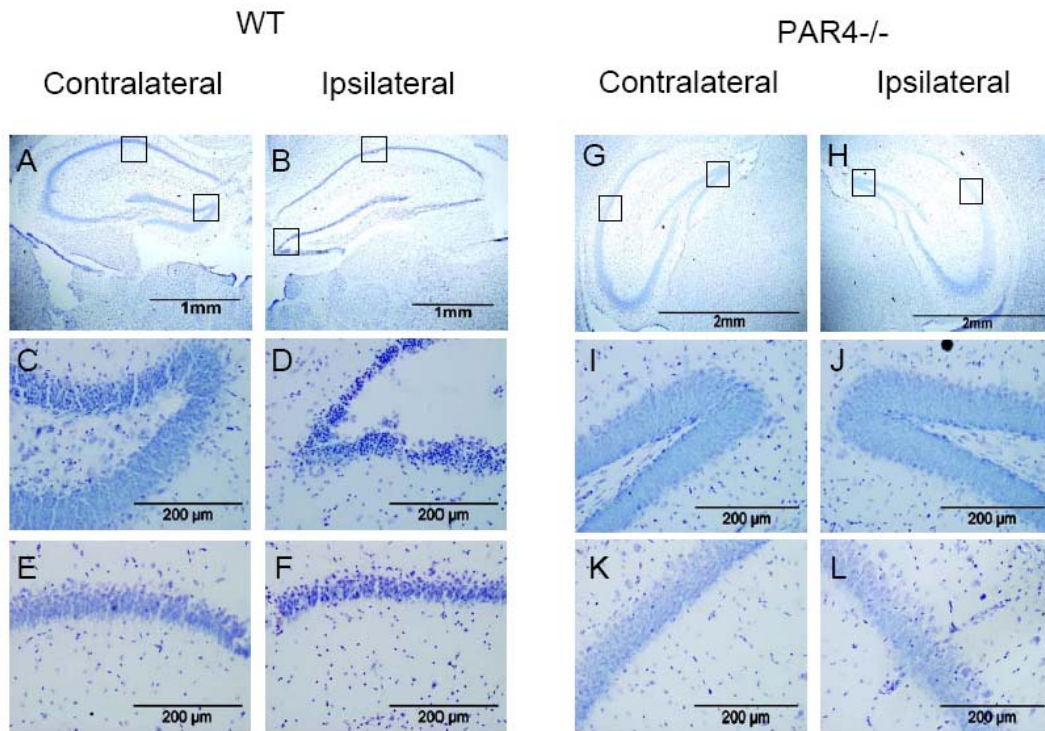
**A**



**B**



**Figure 4.1. Infarct volume is significantly reduced in PAR4<sup>-/-</sup> mice. (A).** Representative TTC-stained coronal sections of wild-type and PAR4<sup>-/-</sup> mice. The white area represents lesion area measured. (B) Average infarct volume was measured and adjusted for brain edema/swelling as infarct fraction:  $\text{Infarct fraction} = \frac{[\text{Contralateral area} - (\text{Ipsilateral area} - \text{Infarct area})]}{\text{Contralateral area}}$ . Wild type infarct size (23.725% ± 2.156, n=8; Mean ± SEM) was significantly larger (\*\*\*) P<0.001) than PAR4<sup>-/-</sup> infarct size (2.156% ± 1.22, n=10; Mean ± SEM).



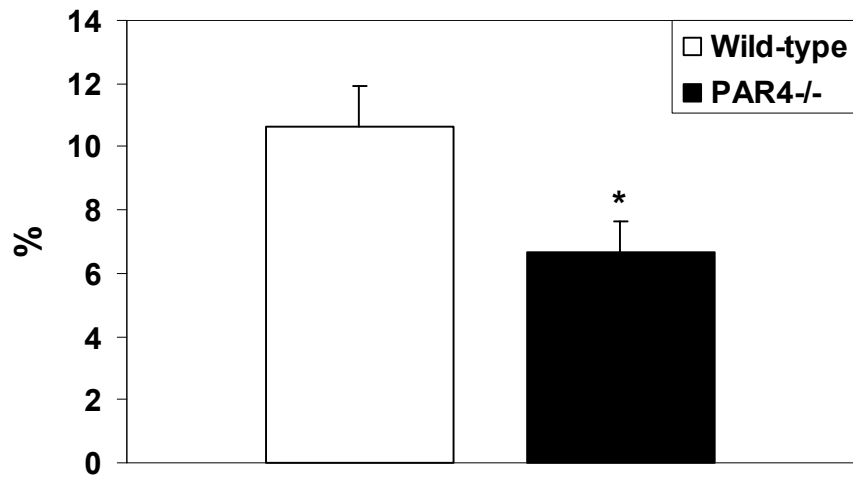
**Figure 4.2. Neurodegeneration in the ipsilateral, but not contralateral hippocampus of wild-type but not PAR4 null mice after I/R injury.** Representative photographs with Cresyl violet staining at low (A, B, G, H) and high (C, D, E, F, I, J, K, L) magnification of the wild-type ipsilateral hippocampus (B, D and F); wild-type contralateral hippocampus (A, C and E); PAR4<sup>-/-</sup> ipsilateral hippocampus (H, J and L) and PAR4<sup>-/-</sup> contralateral hippocampus (G, I and K). Wild-type (n= 5). PAR4<sup>-/-</sup> (n= 5).

*Effects of PAR4 on cerebral edema after I/R injury*

As determined from the percent increase of the ipsilateral/contralateral volume, cerebral I/R injury caused brain edema. There was a significantly reduction in the magnitude of edema in in PAR4<sup>-/-</sup> mice (6.67%±0.99) compared to wt mice (10.66%±1.23) (Fig. 4.3).

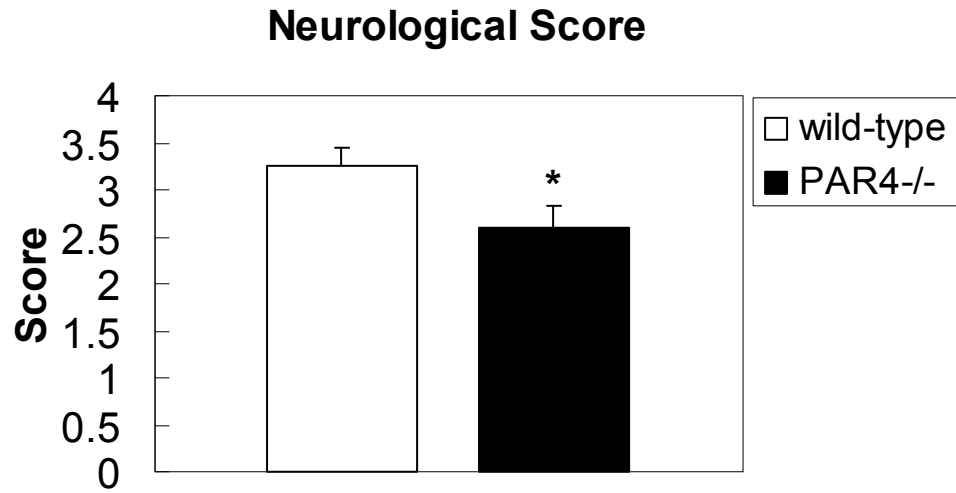
*Deficiency of PAR4 attenuated neurological deficits after cerebral I/R injury*

The neurological deficit score and Rotarod test were used to evaluate the neurological function after MCAO. PAR4<sup>-/-</sup> mice showed improved neurological function (2.6±0.233) and prolonged running time on the Rotarod (21.02±7.21) compare to wild-type mice (3.33±0.19) and (7±1.59). (Fig. 4.4)

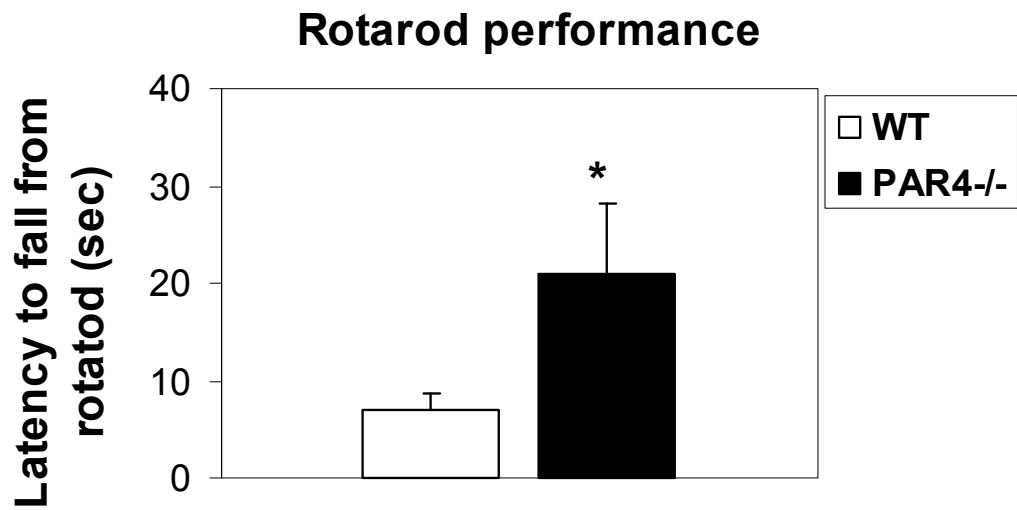


**Figure 4.3. Deficiency of PAR4 reduced edema after I/R injury.** Edema was assessed after 1-hour MCAO and 23-hours reperfusion in both wild-type (n=9) and PAR4-/-mice (n=10. \* p<0.05 is considered as significantly different compared to wild-type (Mean ± SEM).

**A**



**B**



**Figure 4.4. Effect of PAR4 on neurological function in mice subjected to I/R injury.** In panel A, neurological score was evaluated after 1h MCAO and 23 h reperfusion. In panel B, Rotarod testing of sensorimotor deficits in wild-type (n=8) and PAR4<sup>-/-</sup>(n=10) mice after 1 h MCAO and 23 h reperfusion was tested. Deficiency of PAR4 causes both significantly improved neurological score and sensorimotor function compare to wild-type. (\* P<0.05; Mean ± SEM)

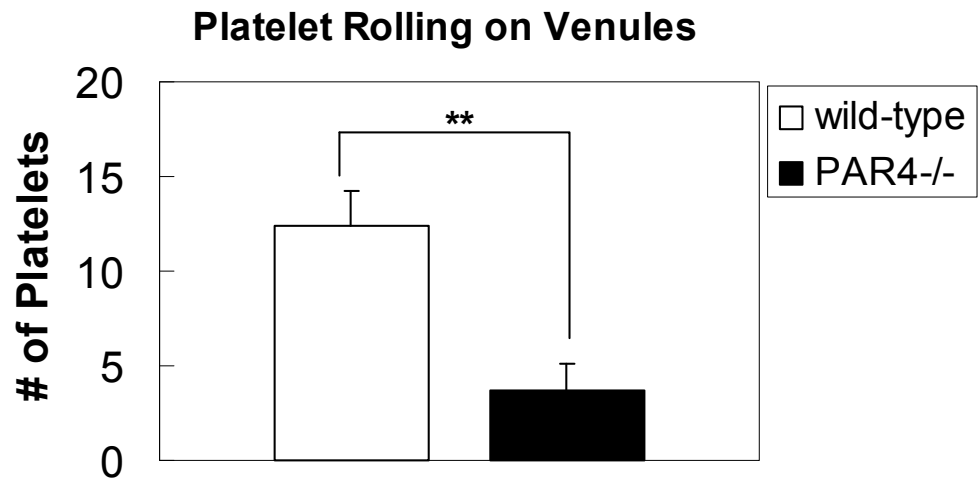
*Effects of PAR4 on platelet/endothelial and leukocyte/endothelial interactions during MCAO/R*

The level of platelet and leukocyte rolling and adhesion were observed in cerebral venules of both wild-type and PAR4  $-/-$  mice 24h after cerebral ischemia/reperfusion injury. PAR4  $-/-$  mice subjected to MCAO/R demonstrated a lower level of leukocyte rolling ( $10.25 \pm 1.25/30$  seconds) and adhesion ( $60 \pm 20/\text{mm}^2$ ) on venules compared to wild-type mice ( $19.75 \pm 1.75/30$  seconds and  $(118 \pm 13.61/\text{mm}^2)$ ) (Fig. 4.5). In addition, the number of platelets rolling ( $3.65 \pm 1.43/30$  seconds) and adhering ( $28.22 \pm 7.95/\text{mm}^2$ ) in PAR4  $-/-$  mice also significantly decreased compared to wild-type mice ( $12.38 \pm 1.84/30$  seconds) and ( $110.4 \pm 33.98/\text{mm}^2$ ) (Fig. 4.5).

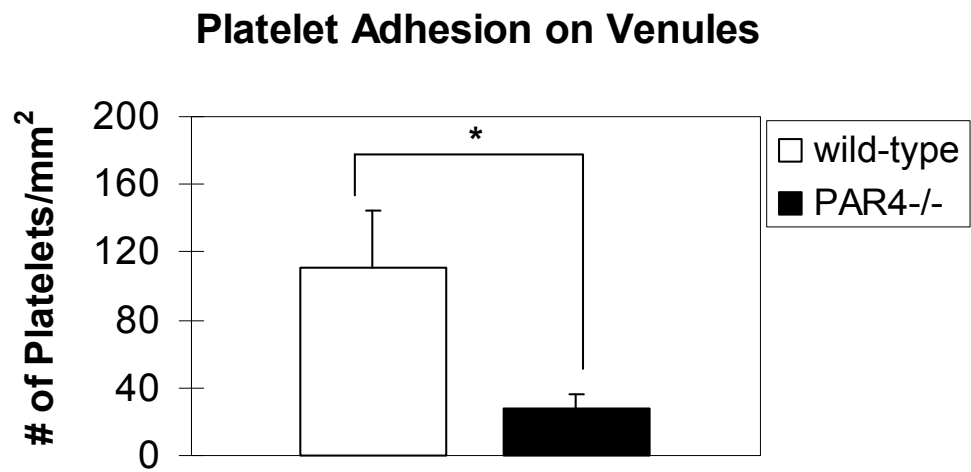
*Effects of PAR4 on blood brain barrier disruption after MCAO/R*

Focal cerebral ischemia significantly increased Evans blue extravasation in the ischemic hemisphere of wild-type mice ( $3876 \pm 393.7$  ng/g) compared to sham animals ( $1522 \pm 280.4$  ng/g). PAR4  $-/-$  mice showed significantly attenuated Evans blue extravasation in the ischemic hemisphere ( $2352 \pm 586.1$  ng/g) compared to wild-type mice ( $3876 \pm 393.7$  ng/g) following 1h of ischemia and 24h reperfusion. The results also indicated that the blood brain barrier was not altered in non-ischemic hemisphere in either wild-type mice ( $1113 \pm 215.6$  ng/g) or PAR4  $-/-$  mice ( $1218 \pm 129.2$  ng/g) when compared to sham mice ( $1475 \pm 168$  ng/g). (Fig. 4.6)

**A**

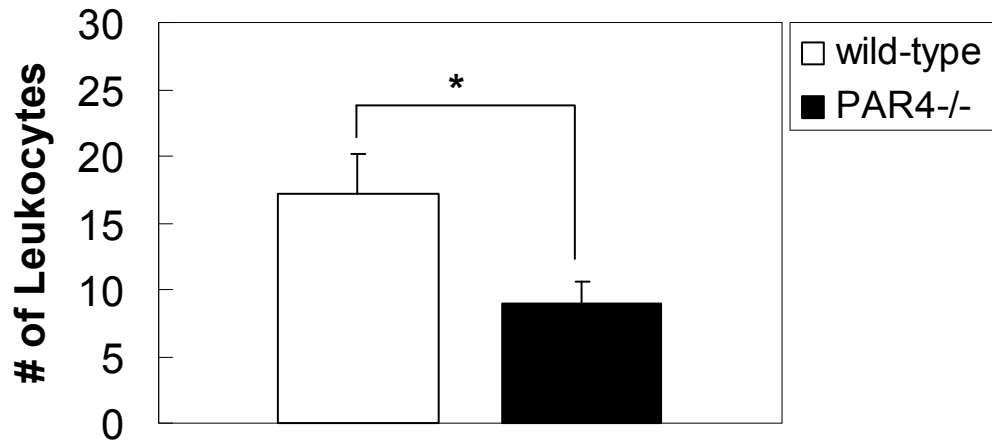


**B**



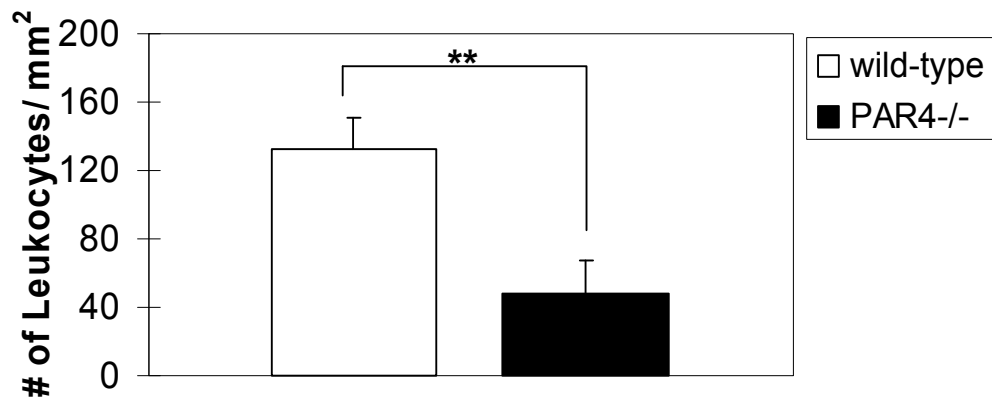
**C**

### Leukocyte Rolling on venules

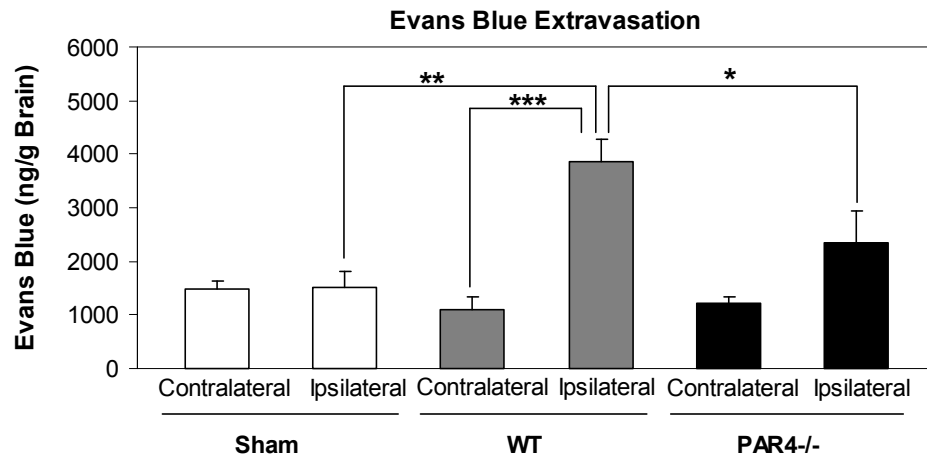


**D**

### Leukocyte Adhesion on Venules



**Figure 4.5. Effect of PAR4 on the rolling and adhesion of platelets and leukocytes.** The rolling and adhesion of platelets (A and B) and leukocytes (C and D) in murine brain microvascular venules after 1-hour MCAO and 23-hours reperfusion were evaluated. Five animals were evaluated in each group. \*P<0.05; \*\* P<0.01 relative to the corresponding wild-type group values.



**Figure 4.6. Effect of PAR4 on the changes in the BBB permeability to Evans blue.** The leakage of Evan's blue dye after 1-hour MCAO and 23-hours reperfusion in PAR4 null mice was significantly decreased than wild-type mice. (\*  $P < 0.05$ ,  $n = 10$ , Mean  $\pm$  SEM).

## Discussion

In this study, we investigated the role of PAR4 in a mouse model of cerebral ischemic/reperfusion injury. The most important finding is that deficiency of PAR4 protected the mouse brain from I/R-induced injury. We observed that the infarct volume of PAR4 deficiency mice decreased more than 10-fold compare to wild-type and as expected, motor function in PAR4 null mice was significantly improved. In addition cresyl violet staining showed a significant neuronal loss in ipsilateral cortex and hippocampus of wild-type but not PAR4<sup>-/-</sup> mice following MCAO/R. We found that PAR4 null mice showed less cerebral microvascular inflammation, including attenuated platelet and leukocytes/endothelial cells interactions, BBB disruptions and cerebral edema following I/R injury.

The mechanism of ischemia-induced brain injury is complicated and still under investigation. Lack of blood –derived oxygen causes direct neuronal death while the secondary injury, mainly due to the inflammatory responses following reperfusion, exacerbate tissue damage and neurological deficits. Inflammatory responses in cerebral microvessels lead to leukocyte and platelet accumulation on the surface of endothelial cells where they physically occlude microvessels, release inflammatory cytokines and break down the blood-brain barrier. There is subsequent extravasation of inflammatory cells and fluid into the brain parenchyma contributing to exacerbation of tissue damage and cerebral edema (Sandoval *et al.*, 2008). This study

was the first demonstration that deficiency of PAR4 attenuates BBB breakdown after ischemia injury. BBB function was significantly improved when deleting the PAR4 receptor.

PARs are widely expressed. Although PAR4 is mainly expressed in platelets, it also expressed on endothelial cells. On endothelial cells, PAR1 is the major PAR receptor and responsible for the down stream signaling in endothelial cells upon activation (Kataoka et al., 2003). Previous studies have shown that PAR1 is important for mediating pulmonary and intestinal microvascular permeability (Chin et al., 2003; Vogel et al., 2000). However, a recent study also showed that PAR4 and its agonist cathepsin G are involved in alterations of the colonic epithelial barrier in ulcerative colitis (Dabek et al., 2009). In addition, PAR1 deletion did not influence BBB disruption in the central nervous system after cerebral ischemic injury (Junge et al., 2003). Our study show that deficiency of PAR4 prevents BBB disruption after stroke, which indicated that in the central nervous system, PAR4, instead of PAR1, played a role in the regulation of BBB function following ischemic injury.

The neuroprotective effects in PAR4 deficient mice could involve at least two possible mechanisms. First, as indicated by diminished platelet and white cell interaction with endothelial cells and improved preservation of BBB function, PAR4 deficiency may reduce microvascular inflammation and diminish inflammatory cells infiltration,. Since PAR4 is also expressed on neurons, it is also possible that

inhibiting expression of PAR4 in the brain has a direct neuroprotective effect after brain ischemic injury. Consistent with a potential role in a protection following ischemia, PAR4 mRNA levels were found to be significantly increased 12 hr after ischemia in the rat injured side and this increased level was reduced only after 7 days (Rohatgi *et al.*, 2004). Since PAR4 receptors are widely distributed and deficiency of PAR4 showed multiple effects in cerebral I/R injury, it is still difficult to determine the exact mechanisms through which PAR4 acts.

PAR4 is the only PAR member that is expressed on both human and murine platelets and plays an important role in thrombin-induced platelet activation. Platelet activation plays a key role in vascular occlusion during ischemic stroke and is the target of anti-thrombotic therapy (Smyth *et al.*, 2009). Platelets are regarded as classic inflammatory cells, which contain and release adhesion proteins, interact with other inflammatory cells and produce numerous chemokines and express chemokine receptors (Gear *et al.*, 2003). Platelet-leukocyte-endothelial cells interactions have been implicated in the pathogenesis of ischemia/reperfusion injury in several vascular beds (Ishikawa *et al.*, 2004). P-selectin secreted from platelet- $\alpha$  granules and Weibel-palade bodies of endothelial cells is critical for leukocyte rolling. P-selectin from platelets is important for the formation of platelet-leukocyte aggregation and recruitment of leukocytes to the endothelial surface (Ishikawa *et al.*, 2004). Thrombin activates the PAR4 receptor and causes P-selectin secretion in platelets. In rat mesenteric venules, thrombin-induced leukocyte rolling and adhering is related to

PAR4 (Vergnolle *et al.*, 2002). Recently, the activation of PAR-4 but not other PARs on platelets was demonstrated to play a major role in soluble tissue factor-induced inflammation (Busso *et al.*, 2008). In our study, we found that both leukocytes and platelet rolling and adhesion after ischemic injury in PAR4 null mice were decreased compared to wild-type animals, which may contribute to the inhibition of inflammatory effect in PAR4 null mice due to the decreased P-selectin secretion through thrombin-induced PAR4 activation.

Furthermore, since PAR4 is the major thrombin receptor in murine platelets, deficiency of PAR4 will completely abolish the thrombin-induced murine platelet response. This will mimic a double inhibition of both PAR1 and PAR4 response in human platelets. Studies on guinea pigs already showed a prolonged occlusion time when blocking both PAR1 and PAR4 compared to blocking only PAR1 in a ferric chloride-induced injury model (Derian *et al.*, 2003). Our data further demonstrate that completely inhibition of the thrombin-induced platelet response will also dramatically prevent brain ischemic injury.

In conclusion, this study demonstrated that deficiency of PAR4 is neuroprotective in cerebral I/R injury, partially through the attenuation of cerebral microvascular inflammation. Hence, blockade of PAR4 might indicate a new therapeutic strategy for the treatment of stroke.

## **CHAPTER 5**

### **DISCUSSION**

Platelets play a critical role in regulating the balance of hemostasis although they are small, anuclear and have a short lifespan. By using pharmacological inhibitors, recombination system and genetically engineered mice, more and more receptors and their downstream signaling pathways, which regulate platelet activation under physiological and pathological condition, have been discovered and investigated. In platelets, glycoprotein, integrin and G protein-coupled receptors are three major receptors. Protease-activated receptors are among the G-protein-coupled receptors expressed in platelet and regulate platelet function by responding to the most important physiological agonist thrombin. The work in this thesis focuses on the three important aspect of PAR receptor activation:

- 1) Characterization of a new peptide agonist of the protease-activated receptor-1 by investigating the novel structural requirements for PAR1 agonists and the signal trafficking downstream of PAR1 induced by this new peptide.
- 2) Investigation of the other serine protease-plasmin induced PAR4 activation and species-dependent different activation

3) Investigation of the effect of PAR4 under pathological condition (cerebral ischemia/reperfusion injury) by comparing wild-type and PAR4 knock out mice.

### **The receptor structure and its activation**

Protease-activated receptors are special members of the G-protein coupled receptor families, with regard to their ability to be intramolecularly activated after cleavage by agonists. In addition to identifying the physiological agonists, the function of PAR receptors was further investigated by synthesizing peptides which mimic the tethered ligand domain and directly activate PARs without the requirement for hydrolysis by agonists.

SFLLRNPNDKYEPF (TRAP-14) is the first synthesized peptide, mimicking the 14 new amino terminus created by cleavage at Arg41 and is able to activate both wild-type and cleavage site mutant PAR1 receptors (Vu *et al.*, 1991a). Further study shows that the first 6 amino acids of this peptide are enough to fully activate PAR1 (Scarborough *et al.*, 1992a). The activation function of the peptide agonists suggests that besides N-terminal sequences, other sites in extracellular loops are also important for receptor activation. The human and *Xenopus* receptors chimeras or antibodies targeting different domain of thrombin receptors provide the evidence that the second extracellular loop is the target for SFLLRN binding and initiating receptor activation

(Bahou *et al.*, 1994). This was further determined by testing in PAR1/PAR2 chimeras (Lerner *et al.*, 1996) or in point mutated *Xenopus* receptors (Nanevicz *et al.*, 1995). Since then, a series of studies were undertaken to investigate the structure-function relationship for activation of the thrombin receptor by using substituted TRAP analogs representing the cleaved N terminus. SFLLRN was the minimal requirement for full activation and an extension of C-terminus will not affect its activity, whereas truncation from the N terminus will decrease the peptide potency (Chao *et al.*, 1992; Sabo *et al.*, 1992; Scarborough *et al.*, 1992a). Study shows that Ser<sup>1</sup> is important for peptide binding while changing to other amino acid could decrease potency but is still active once the free amino group is maintained. For example, YFLLRNP, which is a partial PAR-1 agonist, only activates G12/13 pathways at low concentration; however, full activation of platelets is achieved only at higher concentrations (Rasmussen *et al.*, 1993). Phe<sup>2</sup> was found the most essential amino acid for peptide activity, the agonist activity was completely lost when it changed to alanine (Scarborough *et al.*, 1992a) with the exception of tyrosine (Nose *et al.*, 1993). On the other hand, the third, fourth and fifth position are more tolerated for activity (Chao *et al.*, 1992; Natarajan *et al.*, 1995; Scarborough *et al.*, 1992a; Vassallo *et al.*, 1992).

My work in this thesis (explained in chapter 2) further investigates the synthesized peptide structure- receptor function relationship. My work shows that the peptide TFRRRLSRATR, which is derived from the carboxy terminus of P2Y<sub>1</sub> receptor, is able to activate platelet specifically through PAR1 receptors, which suggests that a

peptide could specifically activate certain kind of PAR receptors, even if not related to its tethered ligand domain if it is in accordance with the PAR receptor binding and activation mechanism. Similar to TRAP-14, our data also show that the first 6 residues are enough for PAR1 activation. In addition, my work point out the importance of the third and fourth residues for agonist –induced platelet activation, showing the completely lost of function upon mutating the third or fourth residues to Glu.

One of the interesting questions is why we started to investigate this peptide. The cell-penetrating peptides containing i3 loop peptides derived from protease-activated receptors PAR-1 and PAR-4 were generated by Covic, et al., which constitute a new molecular strategy for inhibiting the receptor function from inside of the cells (Covic *et al.*, 2002; Kuliopulos *et al.*, 2003). According to the previously study in our lab, the carboxy terminus of the P2Y<sub>1</sub> receptor is important for Gq coupling (Ding *et al.*, 2005). We started to investigate the possible new P2Y<sub>1</sub> antagonist according to the mechanism of pepducins by using P2Y<sub>1</sub> C-terminal sequences. However, this peptide activated platelets instead of inhibiting P2Y<sub>1</sub> and Gq interaction. Although we initially thought that the peptide might directly stimulate Gq pathways upon entering the cell, we ruled out the possibility by synthesizing the same peptide without the palmitoylation. The peptide thus made was also able to activate platelets suggesting that the peptide activates one of the cell surface receptors. Hence we began

characterizing the mechanism of platelet activation by this peptide (TFRRRLSRATR).

### **The opposite effect of plasmin in homeostasis and its effect on platelet activation**

Plasmin is a serine protease that is released as the zymogen plasminogen from the liver into the circulation. It is activated by tissue plasminogen activator (tPA), urokinase plasminogen activator (uPA), and factor XII (Hageman factor). It is inactivated by a-2-antiplasmin, a serine protease inhibitor. Early study showed that plasmin is the critical component in fibrinolysis by dissolving fibrin blood clots. Currently, tissue plasminogen activator is the only Food and Drug Administration-approved therapy for stroke in a narrow 3-hour therapeutic window. In 1970's, Niewiarowski, Gurewich, Senyi and Mustard reported that plasmin can cause aggregation of suspensions of washed human, pig and rabbit platelets and cause serotonin secretion. This finding is further confirmed by injection of streptokinase (SK) in vivo, which showed that plasmin generated after SK injection causes platelet release reaction in vivo. This opposite function of plasmin raises the interest of people to study the mechanism of plasmin-induced platelet activation. However, there are controversial results regarding plasmin-induced platelet activation. Early studies show that low concentrations of plasmin inhibit platelet aggregation in response to thrombin (Miller *et al.*, 1975), ionophore A23187, and collagen (Schafer *et al.*, 1985). Furthermore, pretreatment of 0.05 to 1.0 CU/mL plasmin reduces ristocetin-mediated

agglutination of washed platelets in the presence of von Willebrand factor (vWF) from 66% of control to 2% of control. Streptokinase, which activates the plasmin, also inhibits 2.9  $\mu\text{mol/L}$  adenosine diphosphate, 0.25 U/mL thrombin, and 0.025 mg/mL collagen-induced platelet aggregation in PRP (Adelman *et al.*, 1985). On the other hand, studies also show that plasmin could activate platelets instead of inhibiting agonist-induced platelet aggregation in human and rabbit platelets at high concentration (Guccione *et al.*, 1985; Schafer *et al.*, 1986), which indicates that the concentration difference is very likely to result in the controversial results. Plasmin will activate platelets only at concentration higher than 1.5 caseinolytic units [CU]/mL and will inhibit platelet activation induced by thrombin, collagen, or calcium ionophore A23187 at lower concentrations (0.1 to 1.0 CU/mL). In addition, another report claimed that in addition to concentration, incubation temperature is also important for plasmin-induced platelet activation, suggesting that the change of temperature may modulate the balance of activating and inhibitory processes in platelet (Lu *et al.*, 1991). Later on, the signaling pathways studies of plasmin-induced platelet activation show the involvement of thrombin receptor cleavage (Kimura *et al.*, 1996). Kuliopulos, A *et al.* reported that plasmin could desensitize PAR1 receptor and cleave the R41 thrombin-cleavage site in a soluble N-terminal exodomain (TR78) as a model for the full-length PAR1 receptor (Kuliopulos *et al.*, 1999). Furthermore, plasmin-induced signaling through PAR1 was also reported in response to cell migration (Majumdar *et al.*, 2004).

Previous work done in our lab, on the other hand, showed a different pathway for plasmin-induced platelet activation. We reported that plasmin-induced activation of platelets by cleavage of PAR 4 instead of PAR1 (Quinton et al., 2004). In order to explain the controversial results between our lab and other groups and rule out any other possible role of PAR1 receptor, three approaches were used: 1) plasmin-induced human platelet aggregation was blocked only when PAR4 receptors were desensitized or PAR4 activation was inhibited by PAR4 specific antagonist. 2) plasmin could cause murine platelet aggregation, which did not express PAR1 receptors. 3) the effect of plasmin was further confirmed in PAR4 recombination system.

In this study, one of the interesting findings is that lower concentration of plasmin is enough to cause the same extent of aggregation in murine platelets compared to human platelets. In this thesis (explained in chapter 3), my work continues the study of plasmin-induced platelet activation and investigates the mechanisms underlying the differences in plasmin-dependent activation of murine versus human PAR4. The possible explanations for this differential activation in different species are: first, different PARs are expressed in human and murine platelets; second, the N-terminal sequences of human and murine PAR4 are different; third, the structural differences existing in murine PAR4 and human PAR4 may make the cleavage site accessibility different. Our results show that the potency of plasmin-induced human and murine platelet activation are exchanged when exchanging the tethered ligand sequences of

hPAR4 and mPAR4 in recombination system, suggesting that the differences of sequences do play a role in differential activation.

The unexpected result in this study is the inhibition effect of PAR3 in plasmin-induced murine platelet activation. Although plasmin cleaves PAR4 at the same site as thrombin cleaves, it acts differently compared to thrombin, which activates mPAR3 and makes it as a cofactor for low concentration of thrombin-induced PAR4 activation in murine platelets. For plasmin, in both platelet system and recombination system, murine PAR3 inhibits plasmin-induced PAR4 activation.

The question raised in this study is whether plasmin also activates platelets under physiological conditions. To date, we do not have any direct evidence to show that this phenomenon also occurs in physiological conditions. However, it is also possible that it will happen under pathological conditions or plasmin-dependent activation of PAR4 on other cells may have an important physiological function. My work explained in chapter 4 may give a hint that plasmin-activated PAR4 in brain may play a role in ischemic injury. If this is the case, it will also on the other side explain that deficiency of PAR4 will protect brain from ischemic stroke.

## **Effect of protease-activated receptors in brain ischemic injury**

PARs, as one of most important receptors in platelets, are well investigated in hemostasis and thrombosis. In addition to platelets, PARs are found to be expressed in other tissues and systems, such as the central nervous system (CNS) and studies find that regulation of PARs are important under pathological conditions. Previously, a lot of studies investigated the role of different PARs in ischemia by using experimental ischemic models. Studies show that the level of thrombin is increased in the ischemic rat brain. On the other hand, the regulation of different PARs is different under experimental ischemic conditions. The expression level of PAR1 is increased in hippocampal slices in a oxygen-glucose deprivation (OGD) model and by using this model, low concentration of thrombin (10 pM-10 nM) decrease cell death in hippocampal neurons and astrocytes cells. These data demonstrate the evidence to support the hypothesis that low concentration of thrombin through PAR1 receptor is neuroprotective in ischemic brain. However, on the other hand, by using OGD model, other studies show that neuronal death is exacerbated with the increased thrombin concentration. That might indicate, different concentrations of thrombin play different roles through PAR receptors during cerebral ischemic injury. Since PAR4 is the low affinity thrombin receptor and can be activated only in high concentration of thrombin, it is possible thrombin-induced controversial role is due to the activation of different PARs. This hypothesis is supported by studies which show that PAR4 labeling in the penumbra is enhanced under focal ischemia model.

Furthermore, different from other PARs, PAR4 mRNA levels are found to be significantly increased 12 hr after ischemia in the rat injured side and this increased level was reduced only after 7 days (Rohatgi *et al.*, 2004).

The effect of PARs on ischemia brain also is investigated by using knock out mice. After transient focal cerebral ischemia, PAR1 knock out mice showed decreased infarct volume but without any effect on the blood-brain barrier (Junge *et al.*, 2003). This finding is further confirmed by using combined cerebral hypoxia/ischemia model and study shows that deficiency of PAR1 causes less neuronal death, decreases glial fibrillary acidic protein (GFAP) expression and improves the motor function compared with wild-type mice (Olson *et al.*, 2004). PAR2 is activated by trypsin and its function is mostly involved with inflammation. The effect of PAR2 on focal cerebral ischemic injury suggested a controversial effect compared to PAR1 knock out mice. PAR-2 has neuroprotective effects after ischemia as demonstrated by the increased infarct volume and the number of TUNEL positive cells at the 24 hours of reperfusion in PAR2 knock out mice (Jin *et al.*, 2005).

Considering this complex and unclear regulation mechanisms among different PARs in cerebral ischemic injury, my work (explained in chapter 4) studied the effect of one of PAR receptors, PAR4 using a brain ischemia injury model. The results in this study show that deficiency of PAR4 protects mice brain from transient focal ischemic injury. We observed that the infarct volume of PAR4 deficiency mice decreased more

than 10-fold compare to wild-type, which is even more better than PAR1 knock out mice as previously showed in other group. In mice platelets, there are PAR4 and PAR3 instead of PAR1. To date, all the studies show that PAR3 can not induce downstream signaling by itself, although it can bind to thrombin. Deficiency of PAR4 will block mice platelets to respond to thrombin, which is similar to human platelet inhibiting both PAR1 and PAR4. PAR1 antagonist as a new antithrombotic therapy is under clinical study now. Our study may give a new target for antithrombotic therapy: additional blockade of the PAR4 may confer a benefit beyond the achievement by blocking PAR1 signaling alone. Furthermore, as expected, the behavior function in PAR4 null mice was significantly improved. Cresyl violet staining showed a significant neuronal loss in ipsilateral cortex and hippocampus of wild-type but not PAR4<sup>-/-</sup> mice following MCAO/R.

The mechanism of ischemia-induced brain injury is complicated and still under investigation. The inflammatory response following reperfusion is one of the most critical responses after stroke. Lack of blood –derived oxygen causes direct neuronal death. The secondary injury mainly refers to post ischemic inflammatory responses which include leukocyte and platelet accumulation on the surface of endothelial cells where they physically occlude microvessels, release inflammatory cytokines and break down the blood-brain barrier. This study provides the first evidence that PAR4 play an important role in regulation of inflammation after brain ischemic injury by attenuating platelet and leukocytes/endothelial cells interactions, blood-brain barrier

disruption and cerebral edema following I/R injury in PAR4 knock out mice. However, the study of PAR1 knock out mice shows no benefit effect on blood-brain barrier disruption. The selectins, integrins and the immunoglobulin superfamily are the three classes of cell adhesion molecules that mediate leukocytes, platelets and endothelial cell interactions. Although the mechanism of regulation of the adhesion molecules after ischemia is still under investigation, tremendous progress has been made recently. There is evidence showing that PAR4 is not only expressed in platelets, but also expressed in endothelial cells and leukocytes. P-selectin secreted from platelet- $\alpha$  granules and Weibel-palade bodies of endothelial cells is critical for leukocyte rolling. P-selectin from platelets is more important for the formation of platelet-leukocyte aggregation and recruitment of leukocytes to the endothelial surface (Ishikawa *et al.*, 2004). P-selectin can be secreted when activating PAR4 with thrombin. Integrin  $\alpha$ IIb $\beta$ 3 is also a critical adhesion molecule and can join the endothelial cells, astrocytes and basal lamina to maintain the integrity of the cerebral microvasculature. Both of them can be activated by thrombin. Platelet endothelial cell adhesion molecule-1, which can be expressed upon thrombin stimulation, also plays additional roles in attaching endothelial cells and in negotiating leukocyte transmigration. Taking all of the studies together, our study may give a clue that PAR4 may be a key mediator to regulate the inflammatory response after brain ischemic injury.

## Summary

In summary, the work described in this thesis provides new evidences for the important role of PARs in platelet activation. The peptide we characterized in this study indicated that mimicking newly exposed tethered ligand domain was not necessary for generating PAR receptor activating peptide agonist. The peptide we investigated is from P2Y<sub>1</sub> receptor C-terminal sequences and it activates platelets through PAR1 receptor with highly specificity. PAR receptors can be activated by other proteases other than thrombin which are confirmed in our study of plasmin-induced PAR4 activation. Furthermore, our study also indicates that although plasmin cleaves PAR4 at the same site as thrombin does, it acts differently from thrombin. In murine platelets, plasmin-induced murine PAR4 activation does not depend on the cofactor effect of PAR3; instead, PAR3 negatively regulates plasmin-mediated mPAR4 activation. The importance of PARs is further investigated under in vivo conditions. Deficiency of PAR4 attenuated the disease outcomes in animal models of stroke, which is associated with decreased platelet/endothelial and leukocyte/endothelial interactions in CNS and protection of BBB function. This is the new evidence to support the development of anti-thrombotic drugs by targeting thrombin receptors.

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