Persistent Protease-activated Receptor 4 Signaling Mediates Thrombin-induced Microglial Activation*

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We have previously reported that thrombin, the ultimate serine protease in the coagulation cascade, is a proinflammatory agent that causes proliferation and activation of brain microglial cells. However, participation of its principal receptor, the protease-activated receptor 1 (PAR1), appears to be limited to promoting microglial proliferation and not induction of inflammatory mediators. In the present study, we now report that thrombin action in promoting inflammatory mediators from brain microglia is mediated through another thrombin receptor, PAR4. Here we show that the PAR4 agonist peptide (PAR4AP, GYPGKP), but not the PAR1AP (TRAP, SFLLRN), induced tumor necrosis factor-α (TNF-α) production not only in cultured murine microglial cells in vitro but also in rat cortex in vivo. Down-regulation of PAR4 expression in microglial cultures by a specific antisense, but not a sense, oligonucleotide reduced PAR4AP-induced TNF-α production. Mechanistic studies indicated that, in comparison with PAR1 signaling, prolonged increase of [Ca²⁺]ᵢ, and phosphorylation of p44/42 mitogen-activated protein kinases, as well as NFκB activation may be responsible for PAR4AP-induced TNF-α production in microglia. Taken together, these results demonstrate that PAR4 activation mediates the potentially detrimental effects of thrombin on microglia, implying that perspectives of exploiting PAR1 as a potential anti-inflammatory target should be shifted toward PAR4 as a much more specific therapeutic target in brain inflammatory conditions associated with neurotrauma and neurodegenerations.

The inflammatory response is part of the normal defensive processes that may produce both beneficial and detrimental effects in tissues. In the central nervous system (CNS) microglia are the major immune effector cells and, reactive microglia has been implicated in the pathogenesis of a broad range of CNS disorders. These include not only infectious CNS diseases but also acute CNS injuries such as traumatic brain injury (TBI) (1), spinal cord injury (2), stroke, and brain ischemia (3). In addition, microgliosis is also intimately involved in several chronic neurodegenerative disorders such as Alzheimer’s disease (AD), and amyotrophic lateral sclerosis (ALS) (4, 5). Accompanying the focal accumulation of activated microglia are elevated inflammatory mediators in the brain, such as tumor necrosis factor-α (TNF-α) and several classes of pro- teases, which are believed to contribute to the delayed or extended neuronal degeneration (1–5). Since neurons are largely irreplaceable, microglial-based inflammatory damage in the CNS has attracted considerable attention recently. Therefore, efforts to understand the fundamental mechanisms of microglial activation, and unique proinflammatory mediators, may identify novel therapeutic strategies to eliminate microglial deleterious effects.

Among a myriad of microglial regulatory factors, the multifunctional serine protease, thrombin, is a recently discovered microglial activator. Prothrombin is highly concentrated in blood, circulating at micromolar levels (6). Prothrombin activation and extravasation of active thrombin into CNS parenchyma have been implicated in a number of CNS disorders such as TBI, stroke, ischemia, AD, and ALS (7–12). It has long been known that thrombin is a proinflammatory agent in other tissues (13) but it also can induce infiltration of inflammatory cells, edema, and reactive gliosis in the CNS in vivo (10). However, specific demonstration of direct activation of microglial cells by thrombin was only reported recently (14–16). From the therapeutic point of view, systemic inhibition of the thrombin proteolytic activity is certain to induce severe anti-coagulation side effects, possibly even fatal bleeding in brain and elsewhere (17). Therefore, identification of specific cellular/molecular target(s) mediating thrombin effect holds the key for developing safe and effective alternative therapeutic strategies.

Thrombin-induced cellular effects are primarily mediated by means of G-protein-coupled receptors (GPCRs) known as protease-activated receptors (PARs) (18). Among four PARs (PAR1–4) identified so far, thrombin activates PAR1, PAR3, and PAR4, but not PAR2 (19–22). Regarding molecular mechanisms underlying thrombin-induced microglial activation, others have suggested that PAR1 is not involved in activation of microglia, using parameters such as inducible nitric-acid synthase (15). Our recent study demonstrated that although the prototypic PAR1 participates in thrombin-induced microglial proliferation, it does not mediate release of thrombin-

linked immunosorbent assay; HE, hematoxylin and eosin; mAb, monoclonal antibody; EMSA, electrophoretic mobility shift assay.
induced potentially detrimental cytokines, such as TNF-α. This has cast in doubt the perspectives of PAR1 as the therapeutic target to exploit and suggested that more specific therapeutic target(s) remained to be identified. Since PAR3 has been suggested to function primarily as a chaperone for PAR4 rather than transducing signals on its own (18, 23), we have concentrated on determining just how PAR4 might participate in thrombin-induced microglial activation. The data provided here indicates that persistent PAR4 intracellular signaling is largely responsible for thrombin-induced TNF-α release in microglia. The mechanisms involved include prolonged phosphorylation of p44/p42 mitogen-activated protein kinases (MAPKs) and subsequent NFκB activation.

EXPERIMENTAL PROCEDURES

Materials—Recombinant human α-thrombin was a gift from John Fenton, II, Ph.D. (Wadsworth Public Health Laboratories, Albany, NY). Synthetic PAR1AP (SFFLEk) and PAR1AP (GYPGKF) were purchased from Bachem (Torrance, CA). PAR4 antisense (N185, 5′-TACAG-CAGCGGCCGACGACAT-3′-c6 amine) and sense (N186, 5′-ATGTTGCTGC-GCCGCTGCTGTA-3′-c6 amine) oligonucleotides were synthesized by ResGen, Invitrogen Corp. (Carlsbad, CA). Goat polyclonal antibody (pAb) to PAR4 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Phosphospecific and total p44/42 MAPKs pAbs were purchased from Cell Signaling (Beverly, MA). Griffonia simplicifolia isoelectin B4 (GSI-B4)-FITC and poly-L-lysine were from Sigma. A mouse anti-rat primary antibody was a gift from Dr. Robert E. Palazzo (University of Kansas, Lawrence, KS).

Fluorescent-conjugated secondary antibodies and dyes were bought from Molecular Probes (Eugene, OR). Alkaline phosphatase-conjugated antibodies were from Promega (Madison, WI). Polyclonal anti-thrombin rabbit antibody was fromDako (Glostrup, Denmark). Rabbit anti-tubulin (1:1000), total and phospho-p44/p42 MAPK, and β-actin (1:1000) were from Cell Signaling.

Cell culture—The mouse microglial cell line, N9, was a gift from Drs. Bottani and Ricciardi-Castagnoli (Universita ` Degli Studi Di Milano-Bicocca, Milan, Italy). Primary microglial cultures from the brains of neonatal C57BL6 mice were prepared and characterized as we previously described (16). One night before treatment, we replaced serum-containing medium in primary and N9 microglial cells with serum-free DMEM and IMDM (Gly/Ser-free) media, respectively. All treatments were performed under sterile conditions. Twelve male Sprague-Dawley rats (3–6 months old) were anesthetized with 2% xylazine plus 10% ketamine and immobilized in a rat stereotaxic apparatus. After incising the skin and removing the skull connective tissues, a small hole was drilled followed by placing a 30-gauge cannula into the cortical region using stereotactic coordinates of bregma: −4.80 mm, right lateral: 3.00 mm and ventral: 1.60 mm according to Paxinos’ Rat Brain Stereotactic Coordinates (36). The pre-filled ALZET osmotic minipump connected to the cannula was placed under the dorsal skin. Ten days following surgery, animals were re-anesthetized and perfused with saline followed by 4% paraformaldehyde before removing the brains for immunohistochemistry.

Immunohistochemistry—Rat brains were routinely paraffin embedded, formalin fixed and sectioned with a thickness of 20 μm. After routine de-paraffinization, rehydration and blocking, sections were stained with mAb to TNF-α (1:250) followed by goat anti-mouse-Cy3 conjugates (1:500) and GSI-B4-FITC (1:500) staining. The slides were then washed with Fluorescent washing buffer and mounted with Fluoromount and observed under a Nikon Bio-Radiance confocal microscope. Hematoxylin and eosin (HE) staining was performed as previously described (24).

Western Blot (WB) Analysis—WB for PAR4 (1:500 dilution of the PAR4 pAb), γ-tubulin (1:1,000), total and phospho-p44/42 MAPK, and semi-quantitative analysis of protein band density were performed routinely as previously described (16).

Cell Culture—The mouse microglial cell line, N9, was a gift from Drs. Bottani and Ricciardi-Castagnoli (Universita ` Degli Studi Di Milano-Bicocca, Milan, Italy). Primary microglial cultures from the brains of neonatal C57BL6 mice were prepared and characterized as we previously described (16). Cells were cultured in normal growth media, and the cell lysates were analyzed by WB with PAR4-specific pAb. The results were determined by the BCA assay.

Results

Mouse Microglia Express PAR4—To determine whether or not PAR4 is involved in thrombin-induced microglial activation, we first determined whether PAR4 was expressed in microglia. To accomplish this we used both primary microglial cultures derived from C57BL6 mouse cortices with a purity of 96–98%, as we previously reported (16), as well as a clonal mouse microglial cell line, N9. Both primary and clonal microglia were cultured in normal growth media, and the cell lysates were analyzed by WB with PAR4-specific pAb. The results

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shown in Fig. 1 indicated that both primary and clonal mouse microglial cells constitutively express equivalent levels of PAR4 protein. In addition, we have also found PAR4 mRNA expression in the clonal N9 microglial cells (100% purity without contamination of other cell types, Fig. 1). Moreover, others have recently reported in abstract form that N9 and other clonal and primary microglial cells expressed PAR4 mRNA (27). Therefore, these studies clearly show that murine microglial cells express PAR4 at both mRNA and protein levels.

PAR4AP Mimics Thrombin-induced Microglial TNF-α Production in Vitro—Having established that murine microglia express PAR4, we then assessed the effects of PAR4AP on microglial TNF-α production. For this purpose, primary microglial cultures were treated with increasing doses of either human α-thrombin, PAR1AP, or PAR4AP for 24 h as indicated (Fig. 2). TNF-α production in the culture media measured by ELISA showed that both thrombin (p < 0.001, dose versus thrombin) and PAR4AP (p < 0.01, dose versus PAR4AP), but not PAR1AP, dose-dependently induced TNF-α release from the microglial cultures. A peptide containing the same amino acids as PAR4AP, but in a scrambled sequence (KPGFYG), did not induce significant increase of TNF-α production, even at 100 μM, in a parallel experiment (data not shown). These results indicated that PAR4AP, but not PAR1AP, was able to mimic the thrombin-induced microglial TNF-α production in vitro.

Thrombin and PAR4AP Activate Microglia in Vivo—To confirm this in vitro finding and extend it in vivo, we infused either saline, α-thrombin (11 units/100 μl), PAR1AP (100 μM/100 μl) or PAR4AP (100 μM/100 μl) separately into rat cerebral cortices with the aid of a pre-set osmotic pump (at 100 μl/72 h) to control the infusion rate. Ten days after the initial stereotactic surgery, brain sections were obtained for histological staining with a microglial marker, GSI-B4, along with a mAb to TNF-α (Fig. 3). We found that thrombin infusion induced significant microgliosis compared with saline infusion (restricted to the needle track). A significant proportion of the reactive microglial cells stained positively for TNF-α. In contrast, although PAR1AP induced a mild microglial proliferation, we were unable to detect TNF-α-positive cells with this peptide. On the other hand, we were surprised to find that PAR4AP induced a massive TNF-α-positive microgliosis, even greater than that observed with α-thrombin. HE staining indicated that PAR1AP induced a mild increase of dark blue-stained small nuclei (assumed microglial cells) without significant lesion, while both thrombin and PAR4AP induced significant increase of microglial cells along with apparent cortical lesions. These results indicate that both thrombin and PAR4AP, but not PAR1AP, were able to induce TNF-α-positive microgliosis in vivo. As such, they generally agree with our in vitro findings. Therefore, these novel findings indicate that PAR4AP can mimic thrombin-induced microglial TNF-α production both in vitro and in vivo.

Down-regulation of PAR4 Reduces Thrombin- and PAR4AP-induced TNF-α—Although PAR4AP has been shown to specifically activate PAR4 in other cell types (28), to further confirm that PAR4AP-induced TNF-α production in microglial cells was indeed mediated via activation of PAR4, we attempted to downregulate PAR4 and measured TNF-α production. For this purpose, we synthesized an antisense oligonucleotide (N185) that spanned the initial coding sequence of the murine PAR4 gene as well as a sense oligonucleotide (N186) with a complementary sequence to N185 (“Experimental Procedures”). Following 24-hour treatment of N9 microglial cells, WB of PAR4 revealed a dose-dependent (p < 0.01) reduction of PAR4 expression induced by the antisense N185. In contrast, the sense N186, at a concentration similar to the highest (1 μM) used for N185, failed to show significant effect (Fig. 4). When we increased the concentration of the antisense N185 further to 5 μM, no additional effect on PAR4 down-regulation (~20% PAR4 remained after 1 μM N185 treatment, data not shown) was found. In addition, after stripping, re-probing with anti-γ-tubulin, a cytoskeletal marker, showed equivalent amount of γ-tubulin expression among different lanes. The results confirmed that the loss of PAR4 expression is not related to cell death or an anti-proliferative response to the oligonucleotides. In parallel with the PAR4 WB analysis, the same PAR4 oligonucleotide-treated microglial cells were further treated with a single dose of either thrombin (100 nM) or PAR4AP (100 μM) for an additional 24 h. TNF-α production in the culture media was quantified by ELISA (Fig. 4). We found that the PAR4 antisense N185 induced a dose-dependent decrease of TNF-α secretion induced by thrombin (p < 0.001, N185 dose versus thrombin) and PAR4AP (p < 0.001, N185 dose versus PAR4AP). In contrast, the PAR4 sense N186 showed no effects on TNF-α secretion induced either by thrombin or PAR4AP. These results indicate that activation of PAR4 specifically mediated effects of thrombin and PAR4AP on microglial TNF-α induction.

PAR4 Activation Induces Prolonged [Ca^{2+}]_i Increase and pp42/44 MAPK Activation—Since both PAR1 and PAR4 appear to be expressed and fully functional in microglia, we went further to investigate why these two thrombin receptors might mediate distinct functions, particularly regarding TNF-α induction. Previous data from fibroblasts indicated that soon after activation, PAR1 was immediately desensitized while PAR4 was shut off much more slowly than PAR1 (29). We have recently found that, once activated, microglial PAR1 is rapidly desensitized by G-protein-coupled receptor kinase-5 and -2, while a relatively nonspecific (unrelated to agonist stimulation), sluggish but constitutive PAR4 desensitization may take
Fig. 3. TNF-α-positive microgliosis induced by thrombin and PAR4AP but not by PAR1AP in rat brain. Indicated reagents were infused into cerebral cortex of rats with the aid of an osmotic pump to control the infusion rate (100 μl/72 h). One week after the treatments, the brain sections were obtained for histological staining with a microglial marker, GSI-B4 (green) and TNF-α (red). a-d, confocal images; e-f, HE staining; a and e, Control; b and f, thrombin (100 nM/100 μl); c and g, PAR1AP (100 μM/100 μl); d and h, PAR4AP (100 μM/100 μl). As shown, thrombin (~11 units) induced significant microgliosis with a portion of the microglial cells positively stained for TNF-α. PAR1AP induced a mild microglial proliferation but no TNF-α-positive cells were observed. By contrast, PAR4AP induced massive TNF-α-positive microgliosis. HE staining indicated that PAR1AP induced a mild increase of dark-blue stained small nuclei (assumed microglial cells) without significant lesion, while both thrombin and PAR4AP induced significant increase of microglial cells along with apparent cortical lesions.

To determine if the differential desensitization of these receptors might be responsible for their distinct functions, we compared PAR1 and PAR4 downstream signaling in parallel. Increase of [Ca^{2+}]_i is one such downstream signal following PAR activation. When microglial cells were treated with 100 μM PAR1AP, a sharp increase of [Ca^{2+}]_i was followed by an immediate and complete decrease of [Ca^{2+}]_i, to baseline (Fig. 5). Compared with PAR1AP, 100 μM PAR4AP induced a similar steep, although smaller, increase of [Ca^{2+}]_i, followed by a significantly slower decline curve to ~300 nm. It then remained at 200 nm above baseline until the end of the record (at least 4 min). The similar upwardly rising curves induced by PAR1AP and PAR4AP indicated that the ability for the two peptides to activate their corresponding receptors was not significantly different. However, the distinct downward curves implied that these two receptors were shut off differently. Changes of [Ca^{2+}]_i induced by 100 nM thrombin, which

Fig. 4. Down-regulation of PAR4 expression reduced thrombin- and PAR4AP-induced TNF-α release in microglial cells. N9 clonal microglial cells were treated with either sense (N185) or antisense (N185) oligonucleotides for PAR4 at indicated concentrations for 24 h. Changes of PAR4 expression levels were analyzed by WB (top panel). After stripping, the same blots were re-probed with anti-γ-tubulin (γ-Tub, middle panel), a centrosome marker. In parallel, additional PAR4 oligo-treated cells were further treated with either thrombin (100 nM) or PAR4AP (100 μM) for 24 h. TNF-α secretion into the culture media was quantified by ELISA (bottom panel). As seen, the PAR4 sense oligonucleotide showed no effects on either PAR4 expression or TNF-α secretion induced by thrombin and PAR4AP. By contrast, the PAR4 antisense oligonucleotide induced a dose-dependent decrease for both PAR4 expression and TNF-α secretion induced by thrombin (p < 0.001, N185 dose versus thrombin) and PAR4AP (p < 0.001, N185 dose versus PAR4AP).

Fig. 5. Prolonged [Ca^{2+}]_i increase induced by PAR4 activation in microglial cells. Primary cultures of microglial cells were preloaded with Fluo-4 (2 μM) in serum-free media for 45 min at 25 °C. After washing with media, basal [Ca^{2+}]_i, for regions of interest was recorded using a laser scanning confocal microscope, and cells were then treated with 100 nM thrombin, 100 μM PAR1AP, or 100 μM PAR4AP, respectively. The relative [Ca^{2+}]_i changes were calculated based on a standard curve generated with the same dye diluted in the media containing different concentrations of calcium. The images were collected at 5-s intervals in time series and analyzed by NIH Image software. The data represent average [Ca^{2+}]_i changes of 20–30 individual cells for each treatment.

are sufficient to activate both PAR1 and PAR4, showed a curve that appeared to reflect a combination of effects of both PAR1 and PAR4 activation: a sharp upwardly rising curve and a downward curve, comprised of a rapid declining phase followed

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by a sustained phase. Together, these data suggested that PAR4 activation induced a prolonged \([Ca^{2+}]\), increase as opposed to PAR1 activation, supporting the concept that PAR4 desensitization is considerably slower than PAR1.

In addition to \([Ca^{2+}]\), increase, we have previously shown that PAR1 activation in microglial cells induced a transient p44/42 MAPK activation with the peak occurring at 30 min (16). To further define differences between PAR1 and PAR4 signaling, we analyzed levels of phosphospecific and total p44/42 MAPKs in microglial cells at different time points (Fig. 6). We found that thrombin (100 nM) and PAR1AP (100 μM), as well as PAR4AP (100 μM), induced significant elevation of phosphospecific p44/42 MAPKs at 30 min. However, PAR1AP-induced p44/42 MAPK phosphorylation returned to control level while thrombin- and PAR4AP-induced p44/42 MAPK phosphorylation remained elevated at least 6–24 h after treatments. These results suggested that as compared with PAR1 signaling, PAR4 activation not only induced a prolonged \([Ca^{2+}]\), increase but also a prolongation of p44/42 MAPK activation, which further supports the observation of “sluggish” PAR4 desensitization in contrast to PAR1 in microglial cells.

**Persistent PAR4 Signaling via p44/42 MAPKs Induces NFκB Activation—**NFκB activation has previously been implicated in thrombin-induced rat microglial nitric oxide production (15). To determine the potential impact of differential PAR1 and PAR4 signaling on NFκB activation, N9 microglial cells were treated with control, thrombin (100 nM), PAR1AP (100 μM), and PAR4AP (100 μM) for 4 h. NFκB activation was assessed by EMSA (Fig. 7). We found that both thrombin and PAR4AP, but not PAR1AP, activated NFκB. To further assess the roles of p44/42 MAPKs in the NFκB activation induced by thrombin or PAR4AP, microglial cells treated with thrombin or PAR4AP were post-treated with the p44/42 MAPK inhibitor, PD98059 (10 μM), from 1 min to 2 hours of delay for the post-treatment. We found that early inhibition of p44/42 MAPK by PD98059 was sufficient to prevent NFκB activation induced by either thrombin or PAR4AP. These results implicated that thrombin and PAR4AP activated NFκB at least in part via the prolonged p44/42 MAPK activation.

**Inhibition of p44/42 MAPK or NFκB Reduces Thrombin- and PAR4AP-induced TNF-α Release in Microglial Cells—**In parallel with the NFκB activation assay, the roles of p44/42 MAPKs and NFκB in thrombin-induced microglial activation were further evaluated at the functional level with TNF-α secretion as the outcome. For this purpose, N9 microglial cells treated with thrombin (100 nM) or PAR4AP (100 μM) were either treated simultaneously with the NFκB inhibitor, parthenolide (20 μM), or post-treated with PD98059 from 1 min to 2 hours of delay. TNF-α secretion into the culture media at 24 h was quantified by ELISA (Fig. 8). We found that inhibition of NFκB by parthenolide completely abolished TNF-α secretion induced by thrombin or PAR4AP. In addition, inhibition of p44/42 MAPKs by PD98059 at earlier time points (1 min delay, \(p < 0.001\); 10 min, \(p < 0.01\)), but not at later time points (1 and 2 h, \(p > 0.05\)), significantly reduced TNF-α secretion induced by either thrombin or PAR4AP. These results suggested that PAR4 signaling via prolonged p44/42 MAPK activation and subsequent NFκB activation significantly contributed to the thrombin-induced microglial TNF-α production.

**DISCUSSION**

In this study, we demonstrate that murine microglial cells constitutively express PAR4 at both protein and mRNA levels. Functionally, a PAR4-specific agonist peptide mimics the thrombin-induced TNF-α production both in vitro and in vivo. The ineffectiveness of the scrambled PAR4AP on TNF-α induction confirmed the specificity of the PAR4AP effect. The inhibition of the effects of both thrombin and PAR4AP on TNF-α induction following down-regulation of PAR4 with PAR4-specific antisense oligonucleotide further demonstrated the critical role of PAR4 in thrombin- and PAR4AP-induced TNF-α production. Therefore, we conclude that PAR4 is at least one of the receptors that mediates the thrombin-induced TNF-α production in both mouse and rat microglia. An 80% down-regulation of PAR4 with the PAR4 antisense oligonucleotide at 1 μM accounted for ~70% reduction of the thrombin-induced TNF-α increase (Fig. 4). This suggests that PAR4 is likely to be the principal, if not the only, receptor that mediates the thrombin-induced microglial TNF-α production. Nevertheless, to conclude an exclusive role for PAR4 in this regard will require additional experimental approaches, such as studying microglial cells from PAR4-null (“knockout”) mice (30).

From our previous report on PAR1 in microglia (16), and the current results with PAR4, we have now demonstrated that both PARs are fully expressed and functional in murine microglial cells. Our preliminary analysis indicates that PAR3 mRNA is also present in murine microglial cells as well (data not shown). Recently, others have reported mRNAs for all four PARs in murine microglia (27), and our results support this conclusion. Since thrombin is known to activate three of these PARs (18), the functional consequences of thrombin in microglial cells may depend on both available thrombin concentrations (proteolytic activities), that might derive from a bridged blood-brain barrier or endogenously produced by brain astrocytes (31, 32), and coordination between PAR1, PAR3 and PAR4. Since the binding affinities between thrombin and different PARs are distinct, low concentrations (subnanomolar) of thrombin may only activate PAR1 and PAR3 while high nanomolar thrombin may activate all three PARs (18, 33). Because activation of PAR3 itself does not appear to transduce intracellular signals, but rather facilitates thrombin-PAR4 binding (18, 23, 30), the functional consequences of thrombin in microglial cells may be primarily determined by PAR1 and PAR4 signaling pathways. As we previously demonstrated, PAR1 appears to primarily participate in thrombin-induced microglial proliferation (16). In this study, infusion of PAR1AP into rat brain only induced a mild increase of microglial cells that were negative for TNF-α staining (Fig. 3). Therefore, we conclude that activation of microglial PAR1 alone may not produce significant inflammatory damage to the brain. On the contrary, the equivalent concentration of PAR4AP induced a massive TNF-α-positive microgliosis and significant neuronal damage, allowing us to conclude that PAR4 is the principal mediator for potentially detrimental effects of thrombin.

These current results may have profound therapeutic implications, given our previous assertion that for cellular intervention in thrombin-mediated events, systemic inhibition of the thrombin proteolytic activity would trade dangerous, even fatal, bleeding for any potential therapeutic effect (17). Although PAR1 is the ubiquitous PAR responding to thrombin, our data point to PAR4 as the paramount and the most specific proin-
flamatory receptor to target, using selective PAR4 antagonists. In addition, since PAR3 antagonists may reduce thrombin binding to PAR4 it may also have a potential therapeutic value. As a precaution, although our opinion is that it will not change ultimate results, species-specific expression patterns for different PARs have been shown for human and mouse platelets (18, 33). Therefore, potential species-specific differences for microglial distribution of PARs from current murine results need to be determined before application to human microglia.

One additional cautionary note must be mentioned. Our in vitro results show that 100 nM thrombin consistently induced more TNF-α production than 100 μM PAR4AP (Figs. 2, 4, and 8). However, 10-fold greater thrombin concentration (1 μM/11 units/100 μl) than that used in vitro induced a very modest TNF-α-positive microgliosis, which was much less than the effect produced by 100 μM/100 μl PAR4AP (Fig. 3). This apparent discordance implies that the effects of thrombin in vivo may be significantly inhibited. This inhibition of the thrombin effect may be attributed to several possible factors. First, the presence of endogenous protease inhibitors, such as protease nexin and other anti-thrombin factors, may be rapid and degrade a significant fraction of thrombin. Third, other potential thrombin substrates or receptors that are absent in serum-free microglial cultures may compete with PARs for binding to thrombin in vivo. One such receptor is the membrane proteoglycan, thrombomodulin, that binds thrombin with a 100–1000-fold greater affinity that we found active on brain astrocytes (34) and which may be neuroprotective for neurons (35). Consequently, there may be much fewer thrombin molecules available to actually bind and activate PAR4. By comparison, most of these factors would not have significant impact on PAR4AP, which may explain why PAR4AP induced more dramatic microgliosis than thrombin in vivo in this study.

As a minor point, although PAR4AP induced significantly prolonged [Ca2+] increase than did PAR1AP in microglial cells (Fig. 5), we also noticed that previously reported PAR4AP responses in other cell types, such as fibroblasts, appeared to be more delayed and prolonged (29). Such differential responsiveness might result from cell species differences. For example, PAR4 expression levels in microglial and fibroblastic cells might be different; and/or the mechanisms of PAR4 desensitization in these cells might also differ. For the latter, the previous study indicated that PAR4 activation in fibroblasts resulted in an absence of PAR4 C-terminal phosphorylation (assumed to be by GRKs or other desensitizing kinases). However, our results show that PAR4 activation in microglial cells indeed induced a 4- and 2-fold, rather than zero, increase of PAR4 binding to GRK5 and GRK2, respectively. This indicates that microglial PAR4, unlike fibroblastic PAR4, may be under loose control by GRKs.2

Besides pinpointing the critically involved cellular thrombin receptors, our mechanistic study also revealed that, compared with PAR1, the PAR4 signaling pathway in microglia is featured with prolonged [Ca2+] increase and p44/42 MAPK activation as well as subsequent NFκB activation. This may explain why PAR1 contributes primarily to promoting microglial proliferation while PAR4 is fully engaged in activating microglia and inducing cytokine (i.e. TNF-α) production. These results are consistent with previously reported thrombin signal-
ing in microglia (14–16), while adding more detailed features and clearer identification of upstream mediators. Together with our previous study for PAR1 (16), we now provide a clearer picture for how thrombin induces microglial proliferation and activation. Since thrombin is apparently involved in certain degenerative disorders such as Alzheimer’s disease, these findings not only illustrate the underlying mechanisms of thrombin-induced microglial activation, but will also manifest their guiding value in future therapeutic studies.

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