Prothrombin Kringle-2 Activates Cultured Rat Brain Microglia

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Microglia, the major immune effector cells in the CNS, become activated when the brain suffers injury. In this study, we observed that prothrombin, a zymogen of thrombin, induced NO release and mRNA expression of inducible NO synthase, IL-1β, and TNF-α in rat brain microglia. The effect of prothrombin was independent of the protease activity of thrombin since hirudin, a specific inhibitor of thrombin, did not inhibit prothrombin-induced NO release. Furthermore, factor Xa enhanced the effect of prothrombin on microglial NO release. Kringle-2, a domain of prothrombin distinct from thrombin, mimicked the effect of prothrombin in inducing NO release and mRNA expression of inducible NO synthase, IL-1β, and TNF-α. Prothrombin and kringle-2 both triggered the same intracellular signaling pathways. They both activated mitogen-activated protein kinases and NF-κB in a similar pattern. NO release stimulated by either was similarly reduced by inhibitors of the extracellular signal-regulated kinase pathway (PD98059), p38 (SB203580), NF-κB (N-acetylcysteine), protein kinase C (Go6976, bisindolylmaleimide, and Ro31-8220), and phospholipase C (D609 and U73122). These results suggest that prothrombin can activate microglia, and that, in addition to thrombin, kringle-2 is a domain of prothrombin independently capable of activating microglia.

Microglia are the major immune effector cells in the CNS. Microglia become activated inflammatory cells in response to brain injury following trauma, ischemia, and neurodegenerative diseases (1–4). Activated microglia differ from inactive resting microglia morphologically and functionally. While inactive microglia are ramified in shape, activated microglia are round (5, 6) and produce inflammatory mediators such as NO, PGs, and proinflammatory cytokines (7–9). Many studies have reported that NO produced by activated microglia is toxic to neighboring cells. NO synthase (NOS) inhibitors profoundly reduce the neuronal toxicity of activated microglia (7), and microglia potentiate the toxicity of β-amyloid to neurons by producing NO (10). It has also been reported that acute high levels of NO produce necrosis, while chronic low levels of NO cause apoptosis in neuronal cells (11). Thus, these mediators influence both brain damage caused by ischemia (12) as well as the onset and progression of neurodegenerative diseases such as Alzheimer’s and Parkinson’s (13, 14). However, the mechanisms underlying microglial activation in the injured brain have not been clearly identified. Since microglial activation is accompanied by brain damage, a component(s) released from injured cells or infiltrated from blood could be involved in microglial activation.

Prothrombin is a zymogen of thrombin, and is converted to thrombin by factor Xa, resulting in blood coagulation following cleavage of fibrinogen into fibrin (15). Recently, it was reported that prothrombin can also inhibit blood vessel formation. Prothrombin inhibits basic fibroblast growth factor-stimulated capillary endothelial cell growth (16, 17). This function of prothrombin is independent of the thrombin moiety and the protease activity of thrombin. Instead, kringle-2, a domain of prothrombin distinct from thrombin, contains the antiproliferating effect of prothrombin (16, 17). In a previous study, we reported that thrombin can stimulate NO release and inducible NOS (iNOS) expression in microglia (18). In the current study, we provide evidence that prothrombin is also a microglial activator, and that in addition to thrombin, the kringle-2 domain can activate microglia.

Materials and Methods

Preparation of microglia

Microglia were cultured from the cerebral cortices of 1- to 3-day-old Sprague Dawley rats, as previously described (5, 19). Briefly, the cortices were triturated into single cells in MEM (Life Technologies, Grand Island, NY) containing 10% FBS (HyClone Laboratories, Logan, Utah) and plated in 75-cm² T-flasks (0.5 hemisphere/flask) for 2 wk. The microglia were then detached from the flasks by mild shaking and filtered through a nylon mesh to remove astrocytes and clumped cells. Cells were seeded in plates or dishes and washed 1 h later to remove unattached cells, and attached cells were used in experiments.

Determination of NO

Microglia were plated in 24-well plates (5 × 10⁴ cells/well) or 96-well plates (1.5 × 10⁵ cells/well) and treated with prothrombin (from human plasma; Sigma-Aldrich, St. Louis, MO, or Calbiochem, La Jolla, CA), recombinant kringle-2, or thrombin (from bovine plasma; Sigma-Aldrich, or ICN, Aurora, OH) for 48 h. Recombinant kringle-2 was obtained, as described previously (16). Kringle-2 used in this experiment contained less than 500 fg/ml endotoxin. The amount of nitrite formed from NO was
measured by mixing 50 μl culture medium with an equal volume of Griess reagent (0.1% naphthylethenyl diamine, 1% sulfanilamide, 2.5% H2PO4). The OD was measured at 540 nm (20). In some experiments, factor Xa (Sigma-Aldrich), inhibitors of protein kinase C (PKC; Go6976; Ro-31-8220, and bisindolylmaleimide; Calbiochem), inhibitors of phospholipase C (PLC; D609 and U-73122; Calbiochem), or an inhibitor of NF-κB (N-acetylcyesteine (NAC); Sigma-Aldrich) were added with prothrombin or kringle-2.

RT-PCR

Total RNA was isolated using RNAzol B (Tel-Test, Friendswood, TX), and cDNA was prepared using reverse transcriptase that originated from avian myeloblastosis virus (Takara, Otsu, Japan), according to the manufacturer’s instructions. The PCR primers for the iNOS, IL-1β, and TNF-α genes were as follows: iNOS, 5’-GAGAGGTGCTGATGACCCGTTG-3’ (sense primer) and 5’-CTGAGATGGTGGGGA-3’ (antisense primer); IL-1β, 5’-GATGTACCAGTT-3’ (sense primer) and 5’-GGGAGTTGAGGGGAGAC-3’ (antisense primer). PCR products were separated by electrophoresis in a 1.5% agarose gel and detected under UV light.

Measurement of mitogen-activated protein kinase (MAPK) activation

Microglia (7 × 10^5 cells) were incubated in serum-free medium overnight and treated with prothrombin or kringle-2. The cells were then washed with ice-cold PBS three times and lysed with 2X SDS-PAGE sample buffer, and the lysate was applied to an 8% SDS-polyacrylamide gel. After electrophoresis, the proteins were blotted onto a polyvinylidene difluoride membrane. Activation of MAPKs was determined by immunoblot analysis using Abs specific for the phosphorylated forms of extracellular signal-regulated kinase (ERK), p38, and c-Jun N-terminal kinase (JNK; New England Biolabs, Hertfordshire, U.K.). In addition, unphosphorylated forms of ERK or p38 were detected by immunoblotting using appropriate Abs to determine that equivalent amounts of protein were loaded in each lane.

Measurement of NF-κB activation by EMSA

EMSA was performed as previously described (18, 21). Microglia (2 × 10^6 cells) were harvested and suspended on ice for 15 min in 900 μl hypotonic solution (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF) containing 0.5% Nonidet P-40. The suspension was then centrifuged at 5000 × g for 10 min at 4°C, and the pellet (nuclear fraction) was collected. The nuclear fraction was resuspended in a buffer containing 20 mM HEPES, pH 7.9, 20% glycerol, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, and 1 mM PMSF, incubated on ice for 60 min with occasional gentle shaking, and centrifuged at 12,000 × g for 15 min. The crude nuclear protein in the supernatant was collected and stored at −70°C for use in EMSA. Two synthetic oligonucleotides (Genosys, Woodlands, TX) containing the NF-κB-binding sequence of the murine Ig light chain gene (5’-GGGAGTTGAGGGGA-3’) and its complementary sequence were end labeled using Klenow fragment and [α-32P]dCTP. The labeled DNA probe (−0.2 ng) was incubated for 30 min with 1 μg nuclear protein in a reaction buffer containing 8.5 mM EDTA, 8.5 mM EGTA, 8% glycerol, 0.1 mM ZnSO4, 50 μg/ml poly(dI-dC), 1 mM DTT, 0.3 mg/ml BSA, and 6 mM MgCl2. The reaction mixture was applied to an 8% polyacrylamide gel, and after electrophoresis, the gel was dried and an autoradiogram was obtained. For supershift assays, the nuclear extract was preincubated for 30 min with 1 μg anti-p50 or anti-p65 Ab (Santa Cruz Biotechnology, Santa Cruz, CA). The reaction mixture was subjected to electrophoresis through a 6% polyacrylamide gel.

Results

Prothrombin induced microglial activation

Microglia were treated with prothrombin at 0.1–1 U/ml for 48 h to determine whether prothrombin induced activation. The concentration of nitrite in the media formed from NO increased in a dose-dependent manner; 2 ± 0.4, 6.3 ± 0.7, 12.3 ± 0.6, 16.2 ± 0.9, and 14.5 ± 0.5 μM nitrite (mean ± SEM of three samples, unless indicated otherwise) was released from 5 × 10^5 cells treated with 0.1, 0.3, 0.5, 0.7, and 1 U/ml prothrombin, respectively, while 3.5 ± 1 μM nitrate was released from untreated cells (Fig. 1A). As we have previously reported, the basal level of nitrite observed was not likely to be due to NOS activity (18). The effect of prothrombin on mRNA expression of iNOS, IL-1β, and TNF-α was also determined using RT-PCR. The mRNA levels of iNOS and IL-1β, relative to those of GAPDH, increased within 1 h of 0.5 U/ml prothrombin treatment, with further increases observed for up to 6 h (Fig. 1B). The mRNA level of TNF-α increased within 1 h, sustained at 3 h, but decreased at 6 h (Fig. 1B). Prothrombin-induced microglial activation was not due to contamination of endotoxin since polymyxin B, a ligand for the lipid A region of endotoxin (22), did not reduce the effect of prothrombin on microglial activation (data not shown). The results suggest that prothrombin induced expression of proinflammatory mediators, such as iNOS, IL-1β, and TNF-α, and could be a microglial activator.

Previously, we reported that thrombin can induce iNOS expression in microglia (18). Therefore, we next examined whether prothrombin-induced microglial nitrite production/iNOS expression occurs as a result of conversion of prothrombin to thrombin. Hirudin is a specific inhibitor of thrombin (23). While hirudin significantly inhibited thrombin-induced NO release, it did not reduce prothrombin-induced nitrite production. In the presence of 10 U/ml hirudin, thrombin-induced nitrite production was reduced to 17.4 ± 8.8% of that in the absence of hirudin, but prothrombin-induced nitrate was not affected (Fig. 2). Consistent with these results, we were unable to detect factor Xa (which cleaves prothrombin to thrombin) in microglia treated with prothrombin (data not shown). These data suggest that induction of microglial activation by prothrombin occurs independently of thrombin activity.

Kringle-2 induced microglial activation

The kringle-2 domain of prothrombin is known to regulate biological processes such as angiogenesis (16). We examined whether
Factor Xa enhanced the effect of prothrombin on microglial NO release

Factor X is converted into factor Xa by the blood coagulation cascade system during tissue damage (15). Factor Xa cleaves prothrombin into both thrombin- and kringle-containing regions (15). Since both thrombin and kringle-2 induce microglial activation (Figs. 2 and 3), we examined whether factor Xa enhanced the effect of prothrombin. In the presence of 50 and 100 μM factor Xa, prothrombin-induced nitrite was increased to 189 ± 22.3% and 190 ± 27.7%, respectively, of that observed using prothrombin alone, while factor Xa alone had little effect on nitrite production (Fig. 4). These results suggest that factor Xa enhanced the effect of prothrombin by cleaving prothrombin into two active components, kringle-2 and thrombin.

Inhibitors of PKC and PLC reduced NO release and TNF-α mRNA expression from prothrombin- and kringle-2-treated microglia

Since PKC has been reported to be an important mediator of iNOS expression in microglia (18, 24, 25), we examined whether PKC was involved in prothrombin-induced nitrite production. In the presence of PKC inhibitors Go6976 (Go, 0.5 μM), Ro31-8220 (Ro, 0.5 μM), and bisindolylmaleimide (BIM, 10 μM), nitrite production was reduced to 26.9 ± 5.6%, 0.9 ± 12%, and 15.8 ± 9.8%, respectively, of that induced by prothrombin alone (Fig. 5A). These inhibitors similarly reduced kringle-2-induced nitrite production; Go6976 (Go, 0.5 μM), Ro31-8220 (Ro, 0.5 μM), and BIM (10 μM) reduced production to 29.2 ± 4.6%, 14.7 ± 7.2%, and 11.7 ± 13.7%, respectively, of that stimulated by kringle-2 alone. PKC inhibitors had little effect on nitrite production in untreated cells (data not shown). The reduced nitrite production was not caused by toxicity of these reagents, as determined by exclusion of trypan blue observed by light microscopy (data not shown).

The PLC pathway plays an important role in iNOS expression in macrophages (26, 27). Therefore, we examined whether PLC was involved in the prothrombin- and kringle-2-induced NO release from microglia by using the PLC inhibitors D609 (D), a phosphatidylycerine-PLC-specific inhibitor, and U-73122 (U), an inhibitor of PLCβ and γ. In the presence of 10 μM D609 or 20 μM U-73122, nitrite production induced by 0.5 U/ml prothrombin was reduced to 51 ± 4.8% and 33.2 ± 12.7%, respectively, of that observed in the absence of any inhibitor. D609 (10 μM) and U-73122 (20 μM) also reduced nitrite production induced by kringle-2 (10 μg/ml) to 36 ± 1.6% and 29.7 ± 7.2%, respectively, of that induced by kringle-2 alone (Fig. 5B). The absence of any

FIGURE 2. Hirudin does not inhibit prothrombin-induced NO release. Microglia were treated with thrombin (10 U/ml) or prothrombin (0.5 U/ml) preincubated with or without hirudin (10 U/ml) at 37°C for 30 min. Media were collected and assayed for nitrite concentration as in Fig. 1. Values are mean ± SEM of three samples. Data shown here are representative of three independent experiments.

FIGURE 3. The kringle-2 domain of prothrombin activates microglia. A. Microglia were incubated with the indicated amounts of kringle-2 for 48 h, and media were collected and assayed for nitrite concentration. Values are mean ± SEM of three samples. B. Cells were treated with 5 μg/ml kringle-2 for the indicated time periods. mRNA expression of iNOS, IL-1β, and TNF-α was measured by RT-PCR, as described in Materials and Methods. Data shown here are representative of three independent experiments.
Microglia were treated with 0.5 U/ml prothrombin or 10 μM kringle-2 for 48 h in the absence or presence of PKC inhibitors, 0.5 μM Go6976 (Go), or 0.5 μM M Ro31-8220 (Ro), 0.5 μM M BIM. Media were assayed for nitrite concentration. Values are mean ± SEM of three samples. Microglia were treated with 0.5 U/ml prothrombin for 3 h in the absence or presence of 10 μM D609 or 0.5 μM Go6976 (Go). TNF-α mRNA expression was measured by RT-PCR, as described in Materials and Methods. Data shown here are representative of three independent experiments.

**FIGURE 5.** Inhibitors of PKC and PLC reduce microglial activation. A, Microglia were treated with 0.5 U/ml prothrombin or 10 μg/ml kringle-2 for 48 h in the absence or presence of PKC inhibitors, 0.5 μM Go6976 (Go), 0.5 μM M Ro31-8220 (Ro), or 10 μM BIM. Media were assayed for nitrite concentration. Values are mean ± SEM of three samples. B, Microglia were treated with 0.5 μM prothrombin or 10 μg/ml kringle-2 for 48 h in the absence or presence of PLC inhibitors, 10 μM D609 (D) or 20 μM U-73122 (U). Media were assayed for nitrite concentration. Values are mean ± SEM of three samples. C, Microglia were treated with 0.5 U/ml prothrombin for 3 h in the absence or presence of 10 μM D609 or 0.5 μM Go6976 (Go). TNF-α mRNA expression was measured by RT-PCR, as described in Materials and Methods. Data shown here are representative of three independent experiments.

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Toxicity of these inhibitors was confirmed by the exclusion of trypan blue observed using light microscopy (data not shown). These data suggest prothrombin- and kringle-2-induced microglial activation is regulated by both PKC and PLC.

TNF-α and IL-1β released from prothrombin-treated microglia could contribute to iNOS expression and NO release (10, 28). We also found that iNOS mRNA expression was partially reduced in the presence of Abs blocking the function of TNF-α and IL-1β (data not shown). Thus, we examined whether PKC and PLC were involved in the prothrombin-induced TNF-α mRNA expression. Both D609 (20 μM) and Go6976 (Go, 0.5 μM) significantly reduced TNF-α mRNA expression from prothrombin-treated microglia (Fig. 5C). These results strongly suggest that prothrombin-induced microglial activation is regulated by both PKC and PLC.

**Effect of prothrombin and kringle-2 on activation of MAPKs**

MAPKs are known to mediate microglial activation by β-amyloid, LPS, gangliosides, and thrombin (18, 19, 29). We examined whether prothrombin and kringle-2 activated MAPKs by examining phosphorylation of MAPK tyrosine residues in immunoblot analyses. Phosphorylation of ERK was increased within 20 min of prothrombin (0.5 U/ml) treatment, and the activity remained elevated for 60 min (Fig. 6A). Phosphorylation of JNK/stress-activated protein kinase also slightly increased between 40 and 60 min (Fig. 6A). Phosphorylation of p38 increased within 5 min, and remained for 60 min (Fig. 6A). Kringle-2 activated MAPK as did prothrombin; within 20 min of kringle-2 addition (10 μg/ml), phosphorylation of ERK was increased and activation was sustained for up to 60 min (Fig. 6A). Phosphorylation of JNK/stress-activated protein kinase and p38 also increased during the experimental period (Fig. 6A). To test whether activation of MAPKs was involved in prothrombin-induced nitrite production, we treated microglia with prothrombin in the presence of PD98059 and SB203580, inhibitors of the ERK pathway and p38, respectively. In the presence of 5 and 10 μM PD98059, nitrite production was decreased to 75.7 ± 6.5% and 61.8 ± 8%, respectively, of that induced by prothrombin alone (Fig. 6B). In the presence of 10 and 20 μM SB203580, nitrite production was decreased to 43.9 ± 4.3% and 13.4 ± 1%, respectively. Kringle-2-induced nitrite production was similarly reduced by PD98059 and SB203580, with 5 and 10 μM PD98059 decreasing nitrite production to 77.6 ± 6.3%, 58.2 ± 5%, respectively, of that induced by kringle-2 alone (Fig. 6B), while 5, 10, and 20 μM SB203580 decreased nitrite production to 70.4 ± 4%, 51.9 ± 3.8%, and 17.4 ± 0.7%, respectively (Fig. 6B). Thus, the data suggest MAPK activity regulates prothrombin- and kringle-2-stimulated NO release from microglia.

**Prothrombin and kringle-2 activate NF-κB**

Since NF-κB binding sites are present in the promoter region of the genes encoding iNOS, IL-1β, and TNF-α (28, 30, 31), we examined whether prothrombin and kringle-2 activated NF-κB. The EMSA showed that both prothrombin and kringle-2 activated NF-κB within 15 min, with activity returning to basal level within 60 min (arrowheads in Fig. 7A, upper panel). To investigate which subtypes of NF-κB were activated by prothrombin and kringle-2, we performed supershift assays using Abs against p50 and p65 (Fig. 7A, lower panel). In the presence of p50 Ab, the intensity of the shifted bands was decreased and a supershifted band appeared (arrow in Fig. 7A, lower panel), but this was not observed when using p65 Ab, indicating that p50 may be activated by prothrombin and kringle-2. NAC is a known NF-κB inhibitor. In the presence of 5 mM NAC, prothrombin- and kringle-2-induced nitrite production was reduced to 3.5 ± 11.1% and 0.18 ± 19.3% of control levels, respectively (Fig. 7B). Thus, the results suggest that prothrombin- and kringle-2-induced microglial activation is regulated by NF-κB.

**Discussion**

The results of this study suggest that the kringle-2 domain of prothrombin can activate microglia. Kringle-2 mimics the effect of prothrombin in inducing microglial mRNA expression of iNOS, IL-1β, TNF-α, and MAPK and NF-κB activation. In addition, Kringle-2 and prothrombin appear to stimulate the same PKC and PLC pathways to activate microglia.

LPS, IFN-γ, β-amyloid, gangliosides, and thrombin have all been shown to be microglial activators, and several studies have examined the intracellular signaling mechanisms that mediate microglial activation. PKC may be a common mediator of microglial activation since PKC inhibitors reduce NO release/iNOS expression in microglia treated with thrombin (18), IFN-γ (24), and LPS (25). Recently, it has been reported for microglia that PKC isoforms such as α and ε are activated by β-amyloid and IFN-γ (24, 32).
In this study, PKC inhibitors also reduced prothrombin- and kringle-2-induced NO release and TNF-α mRNA expression (Fig. 5C).

MAPKs are also activated by microglial activators. However, the effect of PD98059 and SB203580 depends on the activator, since while both PD98059 and SB203580 suppressed LPS and thrombin-induced NO release, SB203580 had less effect on ganglioside-induced NO release than PD98059 (18, 19, 29). In the present study, prothrombin- and kringle-2-induced NO release was more strongly suppressed by SB203580 than by PD98059 (Fig. 6). NF-κB is also a common mediator of microglial activation. β-amyloid, IFN-γ, gangliosides, and thrombin all activated NF-κB (18, 29, 33), while inhibition of NF-κB reduced NO release (18, 29). Prothrombin and kringle-2 also activate NF-κB, and NAC reduced NO release from both prothrombin- and kringle-2-treated microglia (Fig. 7, A and B).

Kringle, a structure with three characteristic intradisulfide bonds, is found in a number of proteins, including prothrombin (34), plasminogen (35), hepatocyte growth factor (HGF) (36), macrophage-stimulating protein (MSP) (37), and apolipoprotein (38). Kringles of each protein have distinct functions in many cell types. Angiostatin, an internal fragment of plasminogen, has kringles that function to inhibit endothelial cell proliferation (39). HGF induces macrophage morphology changes (40), and MSP inhibits macrophage iNOS expression (41). Kringle domains of prothrombin function as suppressors of angiogenesis (16, 17). Kringles also have receptor specificity, with the MSP receptor, RON, being activated by MSP kringles, but not by HGF kringles (42), and HGF receptor being activated by HGF kringles, but not by MSP kringles (42). We also found that neither HGF nor angiostatin induced microglial NO release (data not shown). Thus, microglial NO release induced by prothrombin kringle-2 may be a specific function of prothrombin kringle-2 resulting from binding receptors specific for prothrombin kringle-2.

It has been reported that prothrombin mRNA is detected in most regions of the human and rat brain, and its expression changes during development (43). However, prothrombin in healthy human cerebrospinal fluid originates from the blood, and its level is less...
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