

The Microglia-activating Potential of Thrombin

THE PROTEASE IS NOT INVOLVED IN THE INDUCTION OF PROINFLAMMATORY CYTOKINES AND CHEMOKINES*

Received for publication, July 22, 2004, and in revised form, September 13, 2004
Published, JBC Papers in Press, September 27, 2004, DOI 10.1074/jbc.M408318200

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The serine protease thrombin is known as a blood coagulation factor. Through limited cleavage of proteinase-activated receptors it can also control growth and functions in various cell types, including neurons, astrocytes, and microglia (brain macrophages). A number of previous studies indicated that thrombin induces the release of proinflammatory cytokines and chemokines from microglial cells, suggesting another important role for the protease beyond hemostasis. In the present report, we provide evidence that this effect is not mediated by any proteolytic or non-proteolytic mechanism involving thrombin proper. Inhibition of the enzymatic thrombin activity did not affect the microglial release response. Instead the cyto/chemokine-inducing activity solely resided in a high molecular weight protein fraction that could be isolated in trace amounts even from apparently homogenous α - and γ -thrombin preparations. High molecular weight material contained thrombin-derived peptides as revealed by mass spectrometry but was devoid of thrombin-like enzymatic activity. Separated from the high molecular weight fraction by fast protein liquid chromatography, enzymatically intact α - and γ -thrombin failed to trigger any release. Our findings may force a revision of the notion that thrombin itself is a direct proinflammatory release signal for microglia. In addition, they could be relevant for the study of other cellular activities and their assignment to this protease.

Thrombin (EC 3.4.21.5, factor IIa) is a serine protease catalyzing the cleavage of fibrinogen and the activation of several other components of the blood coagulation cascade (1–3). The proteolytically active Arg-specific enzyme of about 39 kDa derives from its 72-kDa zymogen (prothrombin, factor II) via cleavage by the factor Xa-containing prothrombinase complex, while certain inhibitors can serve in its inactivation. The mo-

lecular mechanisms and the physiological consequences of thrombin activity have been intensively studied (3, 4). Nevertheless physiological substrates are not restricted to soluble proteins.

Proteinase-activated receptors (PARs)¹ serve in the direct control of cellular functions by proteases, including thrombin (4–6). By a very unusual mechanism of limited proteolysis, the N-terminal portion of a PAR can be cleaved off, resulting in the unmasking of a new N terminus, which serves as an intramolecular ligand. The autostimulation of PARs by their tethered ligand is coupled to cytosolic signaling events including G protein families, phospholipase C, or several protein kinases such as Src, p38, and p44/42 mitogen-activated protein kinase (1, 3–5, 7, 8). Thrombin thereby affects the growth, adhesion, chemotaxis, and release functions of several cell types such as endothelial and epithelial cells, vascular smooth muscle cells, fibroblasts, platelets, granulocytes, lymphocytes, and macrophages/monocytes (1, 4, 5, 9). Consequently thrombin is considered not only a clotting but a growth and wound-healing factor exhibiting a complex spectrum of restorative activities.

On the other hand, thrombin and other serum proteases are suspected to cause severe damage to CNS tissue upon disruption of the blood-brain barrier (BBB) (1, 7, 10–13). The BBB and blood-cerebrospinal fluid barrier ensure an organized exchange of molecules and limit the passage of cells between neural and extraneural compartments. Most serum proteins are normally denied CNS entry (5, 14). However, when the BBB integrity is compromised due to trauma, stroke, viral and bacterial infection, autoimmune diseases, or neurodegenerative processes, blood content can more or less inundate the tissue (1, 5, 12, 13, 15). Proteases, protease inhibitors or their complexes, lipid-loaded albumin, complement factors, immunoglobulins, or cytokines may then gain access to neurons and glial cells (1, 3, 5, 14, 16–22). Blood-borne proteases seem to have a tremendous impact on the tissue. Brain cell injury

* This work was supported by German Research Foundation Grant DFG/SFB507 (to U.-K. H. and H. K.) and NINDS, National Institutes of Health Grant NS44337 (to T. M.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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¹ The abbreviations used are: PAR, proteinase-activated receptor; BBB, blood-brain barrier; IL, interleukin; KC, mouse equivalent of GRO α (CXCL1); MCP-1, monocyte chemoattractant protein 1 (CCL2); MIP-1 α /1 β , macrophage inflammatory protein 1 α /1 β (CCL3/CCL4); PPACK, D-Phe-Pro-Arg-chloromethyl ketone; RANTES, regulated on activation, normal T-cell expressed and secreted (CCL5); TNF α , tumor necrosis factor α (TNFSF1A); CNS, central nervous system; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; LPS, lipopolysaccharide; Z, benzyloxycarbonyl; FPLC, fast protein liquid chromatography; MS, mass spectrometry.

associated with hemorrhage differs from pure ischemia, and the extent of CNS damage, for example upon stroke, is drastically reduced when certain protease activities are eliminated (13, 23, 24).

Microglial cells are major sensors and response elements in neuropathological scenarios of most heterogeneous etiology (25, 26). Microglia represent a CNS-intrinsic population of macrophage-like cells safeguarding innate defense. Inducible synthesis of chemoattractive and immunoregulatory factors and the ability to present antigen help the recruitment of leukocytes and the engagement of adaptive immune responses. While support for neuronal functions and protection of tissue integrity are beneficial contributions, deregulated or dysfunctional microglia can be critical or at least instrumental in a harmful way. Excessive or chronic reactions fuel destructive cascades upon trauma or during inflammatory and neurodegenerative processes (25, 26).

Both the measures of defense as well as the detrimental consequences require the normally "resting" microglia to transform into alerted and finally reactive states. Challenges by foreign material or disturbances in the CNS homeostasis deliver the required signals (14, 25). Molecularly these signals derive from bacterial cell walls and DNA, viral envelopes, or CNS endogenous factors normally not seen by microglia or usually not found at such concentration (14, 25). However, only a few factors are identified thus far as to their (bio)chemical structure and mode of action.

In principle, serum components could immediately inform microglia about disturbed BBB function or tissue injury (14). Indeed several serum proteins fulfil accessory functions in the cell surface interaction of bacterial toxins or themselves carry some microglia-activating potential. We previously showed *in vitro* that microglia are, indeed, a target of thrombin and that these cells express PARs (27, 28). By a PAR-dependent mechanism, as indicated by hirudin sensitivity and kinetics of desensitization, thrombin stimulation resulted in intracellular calcium signals. Moreover the cells responded with proliferation and the release of cytokines, a major executive feature of activated microglia. Induction of cytokines by thrombin would agree with an assumed profile as a proinflammatory signal (5–8, 10).

However, studying the kinetics and pharmacological and biochemical features of microglial cyto- and chemokine induction by α - and γ -thrombin preparations, we obtained evidence against a "typical" recruitment of PARs. Further experiments rapidly led us to conclude that neither PAR activation nor proteolytic or ligand-like activities of thrombin proper were essential. In the present report, we provide evidence that the release-inducing capacity of thrombin preparations resided in a minor fraction of associated protein.

EXPERIMENTAL PROCEDURES

Cell Culture Preparation and Treatment—Animals were kept and treated according to the Guidelines for Animal Care at the Max Delbrück Center for Molecular Medicine, Berlin, Germany. Primary microglial cultures were prepared from newborn mice (NMRI, purchased from Tierzucht Schönwalde GmbH, Schönwalde, Germany) and cultured in Dulbecco's modified Eagle's medium (DMEM) as described previously (29). After 10–14 days of primary cultivation, microglial cells were separated from other cell types by shaking and placed in 96- or 24-well plates or Petri dishes at densities of 10^4 , 5×10^4 , or 2×10^6 cells/cavity. After a 30-min attachment period, cells were extensively washed with DMEM containing 10% fetal calf serum (FCS) and kept in culture for 1–3 days before being used. Cultures routinely consisted of ~98% microglial cells as determined by staining with *Griffonia simplicifolia* isolectin B4 (Vector Laboratories, Burlingame, CA). Experiments were either carried out in FCS-containing DMEM or under serum-free conditions using macrophage serum-free medium supplemented with astrocyte-conditioned medium (30).

Primary cultures were stimulated for up to 24 h by addition of thrombin preparations (bovine, mouse, and human thrombin (catalog nos. T-3399, T-4648, T-7513, T-8397, and T-6884); human prothrombin (catalog no. F-5132); and bovine factor Xa (catalog no. F-2027) from Sigma and bovine and human α -thrombin, human γ -thrombin, bovine prothrombin, and bovine factor Xa from Enzyme Research Laboratories, Swansea, UK) or lipopolysaccharide (LPS, *Escherichia coli* K-235, Sigma). Thrombin activity is given in NIH units (except for γ -thrombin). In experiments on heat-inactivated thrombin, concentrated solutions of the protease (bovine α -thrombin) were incubated at 70 or 100 °C for various periods of time or at given temperature for 10 min, diluted to the final concentration, and then used to stimulate cells. Stimulations with thrombin preparations were also carried out in the presence of recombinant hirudin HV1 (*Hirudo medicinalis*, Calbiochem Merck Biosciences) and HV2 variants (Sigma), Z-D-Phe-Pro-methoxypropyl-boroglycinepinanediol ester, or D-Phe-Pro-Arg-chloromethyl ketone (PPACK) (Calbiochem).

Enzymatic Assay for Thrombin Activity—H-D-Phenylalanyl-L-pipecoyl-L-arginine-*p*-nitroaniline ($2 \times$ HCl, 500 μ M in 50 mM Tris/HCl, pH 7.0, 140 mM NaCl) was used as a chromogenic substrate (Chromogenix, Milano, Italy and Hemochrom Diagnostica, Essen, Germany). Absorbance at 405 nm was measured in a microplate reader (1420 Victor, Wallac Oy). All tests were run in triplicate using substrate solution as a blank. Inhibitors were mixed with thrombin 15 min before the assay. Note that γ -thrombin, although it cannot properly bind fibrinogen, is able to cleave small synthetic substrates as used in this assay.

Cyto- and Chemokine Measurements—Following microglial stimulations, culture supernatants were collected and stored at -70 °C for cyto- and chemokine measurements. Interleukin-6 (IL-6), total IL-12 (collecting the IL-12 forms p75, p40, and p40₂), KC (mouse equivalent of growth-related oncogene GRO α (CXCL1)), monocyte chemoattractant protein 1 (MCP-1 (CCL2)), macrophage inflammatory protein 1 α (MIP-1 α (CCL3)), MIP-1 β (CCL4), MIP-2, RANTES (regulated on activation, normal T cell expressed and secreted (CCL5)), tumor necrosis factor α (TNF α (TNFSF1A)), and soluble TNF receptor II were measured by enzyme-linked immunosorbent assay based on mouse- and factor-specific antibody pairs and standards or complete enzyme-linked immunosorbent assay kits following the instructions of the manufacturer (R&D Systems, Wiesbaden, Germany) (29, 31). The color reaction was analyzed in a microplate reader (SLT, Spectra, LabInstruments Deutschland GmbH, Crailsheim, Germany). Total protein was determined using the MicroBCA protein assay (Pierce).

Viability Assays—Metabolic activity was assayed using WST-1 reagent (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolol]-1,3-benzene disulfonate) based on the enzymatic cleavage of WST tetrazolium salt to formazan by the succinate-tetrazolium reductase system of the respiratory chain of intact mitochondria. The assay was performed according to the instructions of the manufacturer (Roche Diagnostics), and the color reaction was measured in a microplate reader (1420 Victor, Wallac Oy) at 540 nm wavelength (29, 31).

Reverse Transcription-PCR/PCR—For detection of cytokine mRNA induction, microglial cells (2×10^6 /Petri dish, 6-cm diameter, DMEM/FCS) were treated with LPS (100 ng/ml) or thrombin (10 units/ml) for 1 h. RNA was isolated using TRIzol reagent (Invitrogen). Briefly 2 ml of TRIzol reagent were used for the homogenization of the cells with the lysate pipetted several times to facilitate disruption. RNA was extracted by adding chloroform (0.2 volume eq), precipitated using 1 volume each of aqueous phase and isopropanol, and washed twice in 75% ethanol. Up to 5 μ g of the isolated RNA were then used for cDNA synthesis. RNA was incubated with 1 μ l of random primers (200–400 ng/ μ l) at 70 °C for 10 min. The mixture was then put on ice for 1 min. The reaction mixture consisting of 1 μ l of 10 mM dNTP mixture, 4 μ l of 5 \times Reverse transcriptase reverse transcription buffer, 0.5 μ l of RNasin (10 units), and 2 μ l of 100 mM dithiothreitol (all purchased from Invitrogen) was added to the RNA/random primer mixture and left for 5 min at room temperature. The reaction was started by addition of 1 μ l of Super-Script reverse transcriptase (200 units, Invitrogen). The final mixture was incubated for 50 min at 42 °C followed by 15 min at 70 °C to stop the reaction. To degrade the RNA of DNA-RNA hybrids, the mixture was treated with 1 μ l of 2 units/ μ l *E. coli* RNase H (Invitrogen) at 37 °C for 20 min. PCR for cytokines was carried out with a CytoXpress Mouse Cytokine Sepsis Set 2 kit following the instructions of the manufacturer (BioSource, Camarillo, CA). PCR products were analyzed by 2% agarose gel electrophoresis.

Microfiltration—Thrombin preparations were dissolved in 50 mM Na₂HPO₄/NaH₂PO₄, 150 mM NaCl, pH 7.0 (≤ 1 mg/ml). 500 μ l of the solution were loaded on a Microcon YM-100 filter unit with a 100-kDa molecular mass cut-off (Millipore) and centrifuged at $10,000 \times g$ until

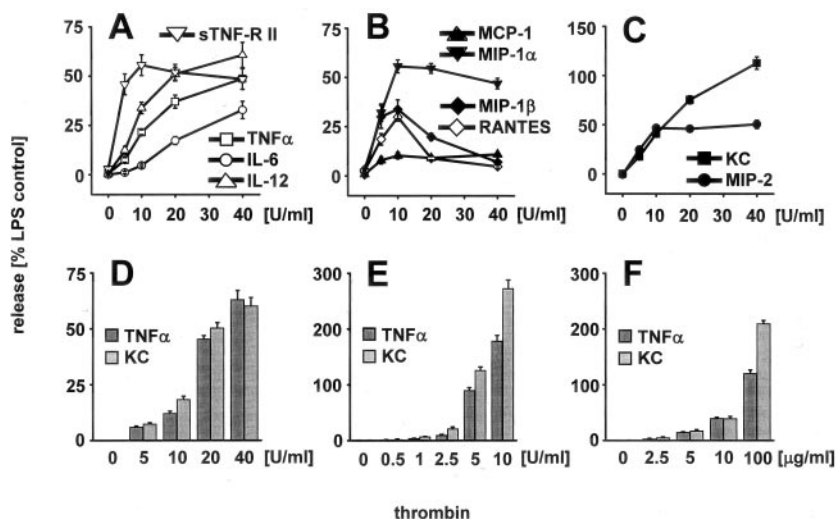


FIG. 1. Induction of microglial cytokines and chemokines by thrombin preparations. Microglial cells (10^4 /well) were incubated with various concentrations (given as units/ml) of bovine thrombin for 18 h, and accumulated cytokines (A), α -chemokines (CXCL) (B), and β -chemokines (CCL) (C) were determined in the culture supernatants. Release of soluble TNF receptor II (*sTNF-R II*, soluble TNFRSF2, or p75TNFR), a potential antagonist of TNF α , is also shown. TNF α and KC subsequently served as representative examples to illustrate microglial cyto- and chemokine production. D, cyto/chemokine induction was also determined in serum-free culture medium. Cells kept in macrophage serum-free medium/astrocyte-conditioned medium rather than FCS-supplemented medium were otherwise stimulated as described in A–C. E, mouse thrombin had a similar effect as shown here for TNF α and KC. F, besides α -thrombin preparations, human γ -thrombin proved effective as well. Note that concentrations are given here as μ g/ml as unit definition does not apply. In all cases, release was expressed as percentage of the respective value obtained from standard stimulations with LPS (100 ng/ml) always run in parallel. Data are mean \pm S.E. summarized from two to four (independent) experiments with (an average of) $n = 22$ /group (A–C), three experiments with $n = 54$ (D), five experiments with $n = 41$ (E), and four experiments with $n = 35$ (F), respectively.

the complete volume had been passed through. The filtrate was stored, the filter unit was filled with 500 μ l of buffer, shaken, and transferred onto a new test tube for another centrifugation (10,000 $\times g$, 15 min). The washing cycle was repeated several times. At the end, the filter was placed upside down on a new tube, and the retentate was collected by centrifugation (1000 $\times g$, 3 min). Aliquots of the original solution, the various filtrates, and the retentate were subsequently analyzed for enzymatic and release-inducing activity.

SDS-PAGE—Thrombin preparations were analyzed by SDS-PAGE under non-reducing conditions using 12.5% gels and in-gel protein staining as described previously (31).

FPLC—Thrombin preparations were separated by gel filtration on a Superose 12 HR 10/30 column using a FPLC system (Amersham Biosciences). Samples were prepared in elution buffer (50 mM Na₂HPO₄/NaH₂PO₄, 150 mM NaCl, pH 7.0), centrifuged, and filtered (0.2 μ m). Aliquots were separated using elution buffer at a flow rate of 0.5 ml/min. Protein was detected by absorbance at 280 nm, and fractions were collected and stored for further analyses. A mixture of dextran blue (2000 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), cytochrome *c* (12.5 kDa), and aprotinin (6.5 kDa) was used for calibration. For studies on proteolytic stability, aliquots of the same sample were repeatedly separated under the same conditions.

Mass Spectrometry—Microfiltration retentate from thrombin preparations was digested overnight at 37 $^{\circ}$ C in a solution of 25 mM ammonium hydrogen carbonate, pH 8, containing 0.1 μ g/ μ l trypsin (Promega, Madison, WI). Resulting tryptic peptides were concentrated on a vacuum centrifuge and submitted to mass spectroscopy. The tryptic peptides were separated using the Ultimate/Famos capillary liquid chromatography system (LC Packings, Amsterdam, Netherlands). The sample was loaded onto a 300- μ m-inner diameter \times 1-mm C₁₈ PepMap precolumn with a flow rate of 10 μ l/min of 0.1% acetic acid using a Rheos 4000 liquid chromatography pump (Flux Instruments, Danderyd, Sweden). After 7 min of preconcentration and clean-up, the precolumn was automatically switched in-line with the 75- μ m-inner diameter \times 50-mm analytical column, and the peptides were separated with a gradient of 2–40% acetonitrile in 40 min (0.1% HCOOH) at a flow rate of 200 nl/min. The liquid chromatography system was connected to a mass spectrometer with a Protana platform (Protana, Odense, Denmark) using a 30- μ m PicoTip from New Objective (Woburn, MA). Mass spectra were recorded using an LCQ quadrupole ion trap mass spectrometer (Thermoquest, San Jose, CA) using Triple-Play function. A first full scan mass spectrum was measured for a m/z 615–2000 range. A second scan was used to measure more precisely the molecular weight of the most abundant peptide signal in the first scan.

A third scan was used to measure the collision-induced mass spectrometry (MS)/MS spectrum of the selected peptide. The spray needle was set to 2.4–3 kV in the positive ion mode. The inlet capillary temperature was 200 $^{\circ}$ C. Other source parameters and the spray position were optimized for a myoglobin tryptic digestion. The peptides were identified with Excalibur and Sequest programs (Thermoquest, San Jose, CA).

RESULTS

Thrombin Preparations Induce Cyto- and Chemokine Release in Microglia—Incubation of mouse microglia with preparations of bovine, mouse, and human thrombin resulted in a dose-dependent release of multiple cytokines and chemokines (Fig. 1, A–E, and Table I). Bacterial LPS, a commonly used agent to activate macrophages/microglia and to mimic Gram-negative infection (29, 31), was used as a reference. Release induced by thrombin preparations was normalized to the LPS-inducible release and expressed as percentage. Absolute values are given in Table II. Exposure to thrombin did not result in impaired viability of the cells as determined in a WST-1 assay system (data not shown).

The release profile obtained with bovine α -thrombin is illustrated in Fig. 1, A–C. For clarity, TNF α and KC (GRO α) were chosen as representatives to illustrate subsequent findings, although other factors were measured as well. Extending the dose range for bovine thrombin up to 175 units/ml, their maximal release was obtained with 50 units/ml (not shown).

Cultures were routinely prepared in DMEM supplemented with FCS. To exclude effects relating to FCS, experiments were also carried out under serum-free conditions (30). Thrombin still triggered a dose-dependent release (Fig. 1D). One should bear in mind, however, that direct quantitative comparisons to the situation in the presence of serum either by relative (compared with LPS) or absolute means (pg/ml or pg/ μ g of protein) are not entirely suitable. LPS itself depends on serum factors (*i.e.* LPS-binding protein) (30), and microglia may behave differently under both culture conditions (25).

Based on enzymatic activity units, mouse thrombin caused stronger release induction than the bovine version (Fig. 1E).

TABLE I
Cyto- and chemokine-inducing activity of thrombin preparations

Preparation	Supplier/product code	Specific thrombin activity	Release induction activity
		<i>units/mg</i>	
Bovine α -thrombin	Supplier A/product 1	95	Yes
Bovine α -thrombin	Supplier A/product 2	40–165	Yes
Bovine α -thrombin	Supplier A/product 3	2000	No
Bovine α -thrombin	Supplier B/product 1	2031–2082	No
Mouse α -thrombin	Supplier A/product 4	1000	Yes
Human α -thrombin	Supplier A/product 5	2000	Yes
Human α -thrombin	Supplier B/product 2	3100	Yes/no ^a
Human γ -thrombin	Supplier B/product 3	Homogeneous by SDS-PAGE	Yes
Human prothrombin	Supplier A/product 6		No
Bovine prothrombin	Supplier B/product 4		No
Bovine factor Xa	Supplier A/product 7		No
Bovine factor Xa	Supplier B/product 5		Yes

^a Some release induction was seen for certain but not for other cyto-/chemokines.

TABLE II
Release of microglial cyto- and chemokines as induced by thrombin preparations

Cells (10^4 /well) were incubated with bovine thrombin (10 units/ml) for 18 h, and accumulated factors were determined in the culture supernatants. Release was calculated as pg/ μ g of total cell protein. Supernatants of untreated cultures were devoid of measurable quantities. MIP-1 α and MIP-1 β were the only exceptions as both chemokines showed some basal release (see also Ref. 29). Data are mean \pm S.E. summarized from two to four independent experiments with an average of $n = 27$. Other factors tested, such as soluble TNF receptor I (sTNF-RI), eotaxin, IL-10, or IL-18, were not induced.

Cyto-/chemokine	Release
	<i>pg/μg</i>
TNF α	76.0 \pm 5.4
sTNF-R II	122.7 \pm 15.7
IL-6	42.3 \pm 7.4
IL-12 ^a	1459.8 \pm 197.9
KC	168.3 \pm 9.6
MIP-2	568.4 \pm 51.3
MCP-1	38.6 \pm 4.8
MIP-1 α	1846.2 \pm 122.1
MIP-1 β	929.5 \pm 88.7
RANTES	783.1 \pm 57.8

^a Including IL-12p70, IL-12p40, and IL-12p40₂.

Mouse thrombin at 5 units/ml (equivalent to about 0.15 μ M) was apparently as effective as 10^{-7} g/ml of LPS, suggesting a rather potent induction activity. On the other hand, we could not detect any release-stimulating activity for the zymogen prothrombin (bovine and human sequences tested at ≤ 10 units/ml, data not shown). Together these findings demonstrated that thrombin preparations developed a robust release induction with some species preference and a profile distinct from that of LPS.

Exosite I Is Not Required for the Release Induced by Thrombin Preparations—Microglial cyto-/chemokine induction by thrombin is believed to be mediated through PARs. Indeed based on PCR and flow cytometry we previously showed that primary microglial cells express PAR1 and PAR3 mRNA and protein (27), functionally supported by the observation that hirudin was able to block calcium signaling of thrombin (28). Hirudin, a 7-kDa anticoagulant protein from leech, is a potent thrombin inhibitor. It forms 1:1 complexes with the protease and competes not only with fibrinogen but also with PAR1/PAR3 for binding to the thrombin exosite I, thereby interfering with PAR activation (1, 4, 5).

However, γ -thrombin readily induced the release of cyto-/chemokines (Fig. 1F). In this thrombin variant, the exosite is not functional due to cleavage (32). As a consequence, γ -thrombin cannot properly bind larger protein substrates, including PARs. Moreover we were also unable to block release inductions with recombinant hirudin variants (HV1

devoid of sulfate at Tyr⁶³ and [Lys⁴⁷]rHV2 (Refs. 33 and 34 and data not shown)). Together these observations and the fact that the required protease concentrations were somewhat high considering EC₅₀ values for PAR activation (1) questioned a PAR1/PAR3 involvement.

Cyto-/Chemokine Induction by Thrombin Preparations Reveals Kinetics Atypical for PAR—Challenged by activating agents such as LPS, microglia can rapidly respond with cyto- and chemokine production (29). Microglial cultures treated with thrombin preparations exhibited a similar time course with mRNA being induced within 60 min (Fig. 2A) and release being detectable between 2 and 4 h after the onset of stimulation (Fig. 2B, inset).

In addition to the time span between the onset of stimulation and the appearance of cytokines in the supernatant, the induction process *per se* can be fast, *i.e.* the period of stimulus presence necessary to organize for an effective response. We showed previously that stimulating agents can differ substantially in the efficacy of triggering release activities in microglia (31).

We determined the outcome of transient thrombin exposure to estimate the minimal stimulation period for an effective signaling. In this kiss-and-run study, cells were incubated with thrombin for 15 min to 3 h, the protease was removed, and cells were allowed to secrete cyto- and chemokines into the supernatant (Fig. 2B). Accumulated amounts were compared after a total of 18 h. To our surprise, even 3 h of thrombin exposure were still not sufficient to cause effective release. Since limited PAR proteolysis and subsequent signaling by the activated tethered ligand were believed to be rapid processes, these findings argued against a typical PAR recruitment. Obviously full execution of the cyto-/chemokine synthesis and release program required prolonged signal transduction periods.

Thrombin Does Not Act as a Protease in the Induction of Cyto-/Chemokines—We next directly addressed the question of whether any proteolytic mechanism would be involved in the induction of cyto-/chemokines. Two synthetic inhibitors of enzyme activity were tested. The specific and irreversible inhibitor PPACK, at a concentration of 50 nM, completely blocked the cleavage of a synthetic thrombin substrate (Fig. 3A). In contrast, treatment of microglia with PPACK-inactivated bovine, mouse, and human α - and γ -thrombin failed to abolish release induction (Fig. 3, B and C). Another inhibitor of thrombin enzymatic activity, Z-D-Phe-Pro-methoxypropylboroglycinepinanediol ester, gave virtually the same results. The compound is thought to form a tetrahedral adduct with the Ser or His of the active site and has a reported K_i of 7 nM. Tested for up to 1 μ M, no release inhibition was found (data not shown).

Thrombin Preparations Contain a High Molecular Weight Protein Fraction—Based on the findings thus far, we excluded a proteolytic mechanism and PAR activation. Alternatively we

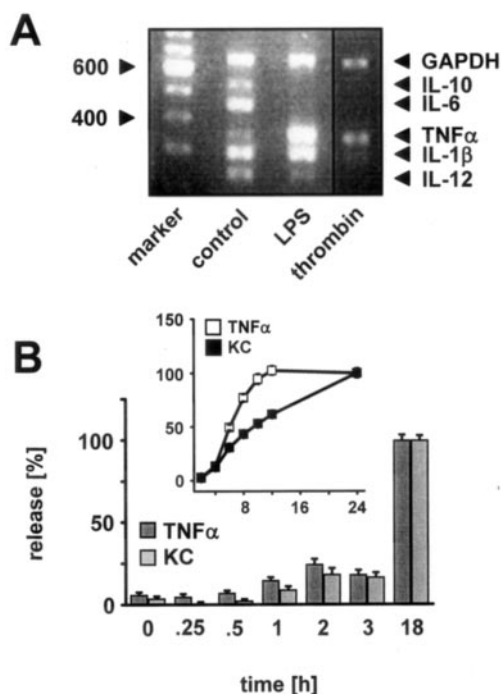


FIG. 2. Kinetics of microglial cyto-/chemokine induction by thrombin preparations. *A*, induction of cytokine mRNA by thrombin. Cells were incubated with thrombin (10 units/ml) or LPS (100 ng/ml) for 1 h, and mRNA for the five cytokines was amplified and detected by reverse transcription-PCR using a CytoXPress system. Untreated cells were used as control, and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) served as reference. Markers on the left indicated the size (bp) of the products, *i.e.* 351 (TNF), 294 (IL-1 β), 237 (IL-12p40), 453 (IL-6), 538 (IL-10), and 658 (glyceraldehyde-3-phosphate dehydrogenase, serving as housekeeping gene). *B*, induction of microglial TNF α and KC release upon transient exposure to thrombin. Cells were incubated with bovine thrombin (10 units/ml) for varying periods between 15 min and 3 h. Subsequently the thrombin-containing supernatant was removed to interrupt the protease-receptor interaction, and cells were rinsed and given fresh medium. The resulting release was determined after a total of 18 h and expressed as percentage of the maximal release induced by a continuous presence of thrombin for 18 h. Data are mean \pm S.E., summarized from three independent experiments with an average of $n = 36$. The inset reveals the time course of release. Accumulating cyto-/chemokines were measured in the supernatant at each given time point during a continuous stimulation and expressed as percentage of the release reached at 24 h. Data are mean \pm S.E. ($n = 24$). The apparent termination of TNF release at 12 h is unlikely to be due to soluble TNF receptor II interfering with TNF α detection by the enzyme-linked immunosorbent assay, although such a phenomenon has been observed with rat cultures and detection systems.

raised the hypothesis that thrombin preparations contain another factor with release-triggering activity, rendering thrombin itself dispensable. Experiments with heat-inactivated thrombin preparations suggested a thermostable compound, presumably a protein, that rapidly inactivated at 70 $^{\circ}$ C (data not shown). However, since even highly purified thrombin preparations, as indicated by high specific activity, had induction capacity any accompanying protein suspected to be its carrier could be present only in very low amounts. In fact, for some release-inducing preparations, protein impurities were hardly detectable by SDS-PAGE (data not shown). In contrast, some preparations were found to be devoid of any release effect (Table I). Therefore, we analyzed several thrombin preparations by FPLC. Proteins were separated by gel filtration, and eluted fractions underwent a systematic testing for enzymatic and cellular activities (Fig. 4, A–C).

The results substantiated the doubts about thrombin as the cyto-/chemokine release-controlling factor. The enzyme activity-confirmed bovine and mouse α -thrombin peaks (\sim 38 kDa)

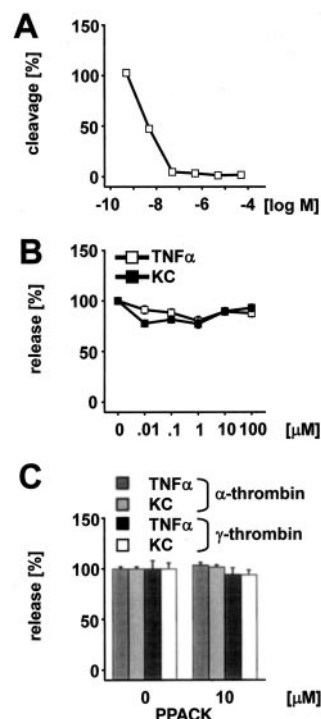


FIG. 3. Inhibition of the proteolytic activity of thrombin and its effect on microglial cyto-/chemokine release induction. *A*, inhibition of the thrombin-catalyzed cleavage of the chromogenic substrate *H*-D-phenylalanyl-L-pipecolyl-L-arginine-*p*-nitroaniline, revealing that PPACK at 50 nM completely blocks the enzymatic activity of bovine thrombin at 1 unit/ml. *B*, release induction by bovine α -thrombin (40 units/ml) in the presence of various concentrations of PPACK. PPACK alone had no effect on the cells. Data are mean \pm S.E., summarized from three experiments with $n = 36$ /group. *C*, release induction by mouse α -thrombin (5 units/ml) and human γ -thrombin (10 μ g/ml) in the presence of PPACK. Data are mean \pm S.E., from three and four experiments with $n = 48$ and $n = 46$ per group/treatment, respectively. Thrombin preparations were allowed to interact with the inhibitor for 15–30 min before being used in substrate cleavage assays or cultures. Microglial cells were then stimulated for 18 h with the respective thrombin in the presence of the inhibitor. Released cyto- and chemokine amounts were measured in the supernatants and expressed as percentage of the release obtained with thrombin in the absence of the inhibitor.

were devoid of release-triggering activity (Fig. 4, A and B, fractions 28–30). In contrast, cellular effects solely associated with a considerably higher molecular weight range (fractions 17–19). Even in such a heterogeneous preparation as given in Fig. 4A, there was only one fractional range for the proteolytic and one for the cellular activities. Their separation was also confirmed for γ -thrombin (Fig. 4C). The high molecular weight material was thus detected in thrombin preparations of different species, suppliers, and purity. As most obvious from the analysis of rather homogenous preparations, the release induction activity localized to a minor fraction of the total protein. For example, high molecular weight material in the mouse α -thrombin of Fig. 4B accounted for \leq 1% of the total protein while containing all of the induction activity.

The High Molecular Weight Material Contains Thrombin-related Peptides—While thrombin proper had no induction capacity, thrombin-derived peptides were identified within high molecular weight material by MS. All identified fragments resided in the sequences of the thrombin light and heavy chains, sparing the N-terminal portion of prothrombin that is cleaved off upon maturation (Fig. 4D). Accordingly peptides comprising the high molecular weight material most likely originated from mature thrombin.

Several lines of evidence thereby pointed to an aggregated

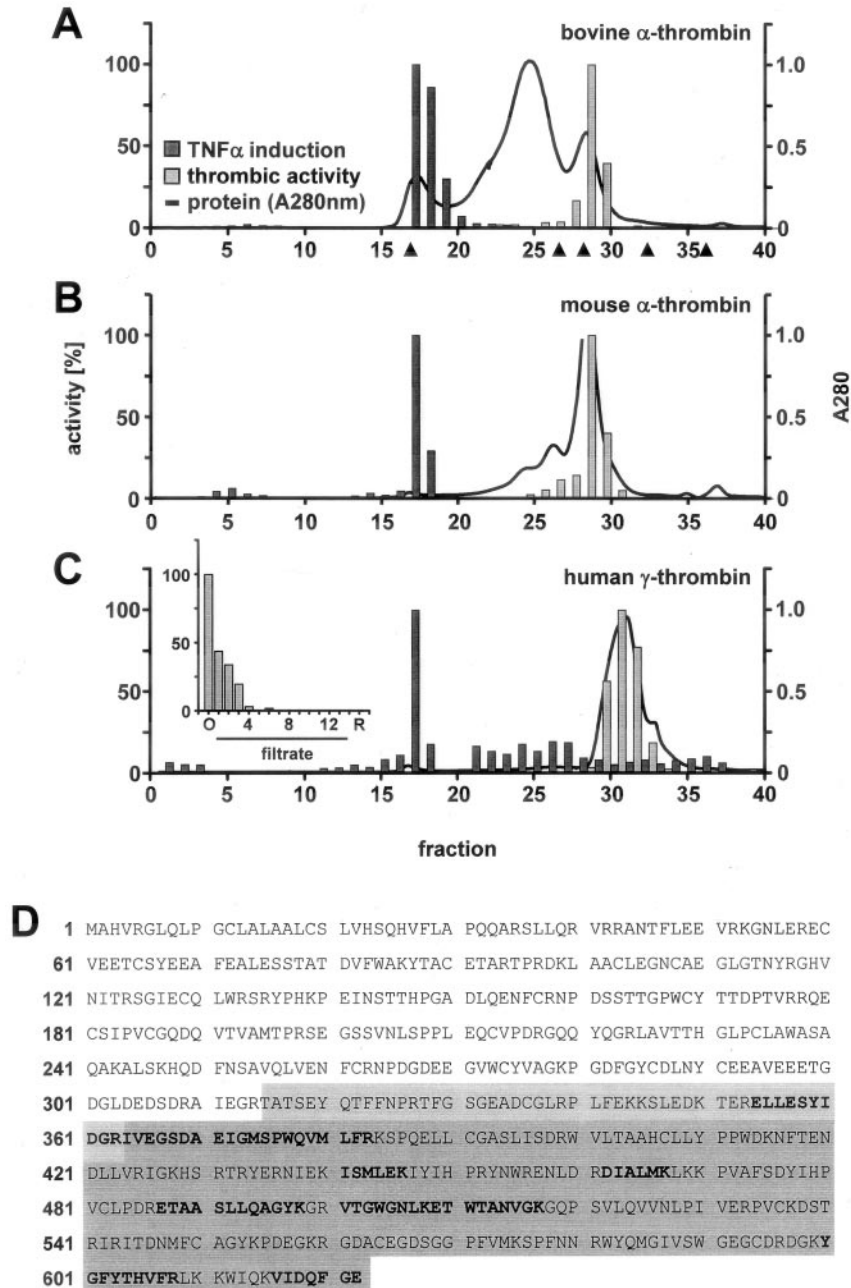


FIG. 4. FPLC and mass spectrometry analyses identifying high molecular weight protein material as the carrier of the cyto-/chemokine release induction activity in α - and γ -thrombin preparations. *A*, FPLC-based gel chromatographic separation of a bovine α -thrombin preparation. Protein was detected by absorbance at 280 nm (A_{280} , line). Light gray bars (fractions 28–30) and dark gray bars (fractions 17–19) indicate enzymatic thrombin activity and the cyto-/chemokine-inducing activity (illustrated for TNF α), respectively. For clarity, the distributions of enzymatic and cellular activities are shown for each fraction as percentage of the respective maximal value. Arrowheads indicate the elution positions for dextran blue (2000 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), cytochrome *c* (12.5 kDa), and aprotinin (6.5 kDa) used for calibration. *B*, FPLC gel filtration chromatogram and fractional activity distributions for a mouse α -thrombin preparation (details as in *A*). Note that the A_{280} peak of mouse thrombin is not shown by its full amplitude to make the small peak of the high molecular weight material visible. *C*, chromatographic and functional analysis of a human γ -thrombin preparation (details as in *A*). The inset shows the results of a differential microfiltration experiment on human γ -thrombin. The original preparation (*O*) was filtered (molecular mass cut-off of 100 kDa), the high molecular weight retentate (*R*) was rinsed 13 times with buffer, and thrombin activity was measured. The graph illustrates that after six rinses no additional enzyme activity could be detected in the filtrate. The recovery of the total thrombin activity was 102.6%. In addition, the high molecular weight material retained on the filter was also devoid of thrombin activity. On the other hand, release-inducing activity for microglia was detected only in the original solution and the retentate. *D*, several peptide sequences of thrombin origin were identified within the high molecular weight material by mass spectrometry. The respective sequences exclusively derived from the mature protease, rendering prothrombin as the parent structure unlikely. The identified peptides are shown in bold within the sequence of human prothrombin (Swiss-Prot: locus THRB_HUMAN, accession no. P00734). The light and heavy chains of thrombin are indicated by light and dark gray shadows (according to Ref. 3).

complex of inactive thrombin or its fragments rather than an intact protease. First, the FPLC fractions containing high molecular weight material did not exhibit enzymatic activity (Fig. 4, A–C). We could also not “wash off” further thrombin activity from high molecular weight material using a microfil-

tration approach (Fig. 4C, inset). Second, α -thrombin can readily undergo autolysis, although the breakdown varied with the species. Incubation of thrombin solutions under varying conditions produced degradation fragments as indicated by FPLC and SDS-PAGE (data not shown). *In vivo*, tryptic activ-

ities of the CNS tissue may contribute to thrombin cleavage. Indeed we observed substantial fragmentation when using agarose-immobilized trypsin for proteolysis of PPACK-inactivated thrombin. Yet the non-aggregated thrombin fragments were not found to cause release induction (data not shown), and the conditions leading to potential aggregates responsible for the effects remain to be defined.

Taken together, regardless of the biochemical formation of the high molecular weight material as the activity carrier, the body of evidence collected clearly rules out that thrombin proper alone triggers the cyto-/chemokine induction in microglia. Despite the expression of PARs in these cells the intact protease is *per se* unable to mount a proinflammatory release response by any proteolytic or non-proteolytic mechanism.

DISCUSSION

Thrombin as an Assumed Activator of Microglial Release Functions—The release of an array of cyto- and chemokines is essential for both the beneficial as well as the harmful consequences of microglial activation. A role of thrombin as a release stimulus has been suggested by the observations that preparations of the protease were able to induce microglial cyto-/chemokines and NO *in vitro* and *in vivo* (8, 10, 28, 35, 36). Induction of proinflammatory factors would fit into the general picture portraying multiple facets of thrombin beyond the role as a coagulation factor (1, 5, 10, 19, 37–39). Thrombin could link tissue injury to hemostatic reactions and inflammatory responses (4). Moreover, while having blood-clotting, wound-healing and, at low dose, neuroprotective properties (40), thrombin was also reported to have a profound neurotoxic potential (1, 5). Several studies revealed impairment of neuronal populations upon thrombin exposure (10, 13, 19, 35, 40). Cytokines could play a critical role in these *in vivo* scenarios.

Thrombin Is Devoid of Cyto-/Chemokine Release Induction Activity in Microglia—The present study, however, rendered a direct cyto-/chemokine induction by α - and γ -thrombin and a contribution of thrombin-mediated PAR activation unlikely. The ultimate proof derived from the biochemical separation of thrombin protein and enzyme activity from the release-inducing activity (Fig. 4). The release-triggering activity solely resided in a protein fraction of much higher molecule size (designated high molecular weight material), which was itself devoid of thrombin-like enzyme activity. LPS as an “entrapped impurity” can be excluded from consideration. The release profile was different, and the inducing activity could not be rinsed off the high molecular weight material and, most importantly, vanished with heat treatment. By size, high molecular weight material is also unlikely to be identical to (lipid-loaded) albumin or a recently described factor with a similar thermal stability profile (22).

Several thrombin preparations failed in stimulating microglial cyto- and chemokine production. Others were effective, including those of apparent homogeneity (Table I). Indeed high molecular weight material is easily overlooked. Protocols designed for thrombin preparation on a larger scale may not necessarily include size exclusion chromatography steps. The isolated enzyme can reach sufficient specific activity to suggest homogeneity. In SDS-PAGE, high molecular weight material could escape detection due to the large size and low abundance. Assuming a single polypeptide chain or an otherwise stable protein (aggregate) resisting SDS treatment, its size would simply impair gel penetration, and conventional protein staining would not visualize tiny amounts among a dominating thrombin.

Consequences of High Molecular Weight Material for the Assignment of Cellular Effects—In experiments intended to determine microglial responses to the protease, high molecular

weight material may simply obscure cyto-/chemokine release profiles (8, 41). Such “impurities” could also account for variable findings on other serum proteins (see Table I for factor Xa). We were also unable to demonstrate microglial effects for prothrombin, which was recently reported to be a factor for microglial NO release and cytokine mRNA induction (42). Attributing a function to the “dispensable” N-terminal prothrombin portion, in particular the Kringle-2 region, is as intriguing as it has been for the primary N terminus of PAR (43). Still a ready-to-go stimulus property for prothrombin without conversion is probably unlikely (3). Dissection of causal relations between protease administration, microglial activation, and induction of cyto-/chemokines would thereby be even more difficult *in vivo* (35). However, the discovery of high molecular weight material still leaves room for thrombin contributions. It may serve as a parent structure of microglia-active compounds or complexes.

Mass spectrometric analyses of high molecular weight material identified peptides of thrombin origin. Apparently, the material consisted, at least partially, of thrombin fragments or otherwise enzymatically inactive protein in a presumably aggregated state. Non-aggregated thrombin fragments (separated by FPLC from autoproteolytic digests) were inactive. Although the formation is not yet understood, functional aggregates may not only associate with the purification of blood-borne thrombin. Microglial release promotion was reported for recombinant protein as well (7), inspiring the assumption that thrombin-derived high molecular weight components may assemble even *in vivo*.

Conceivably the MS analysis missed out on additional high molecular weight constituents. Larger molecules would be present in low copy number only. Respective MS signals would be relatively weak. In contrast, multiple copies of (aggregated) smaller protein(s) give stronger signals that are detected more easily. Therefore, we currently are further investigating the release-inducing potential of high molecular weight fractions as well as of serum factors with suspicious molecular size or suspected interaction/aggregation behavior in highly purified preparations and in concert with thrombin. Dissection of the respective activity spectrum based on vigorous purification, analyses, and confirmation, via synthetic (recombinant) approaches, will thus warrant assignment of microglial cyto-/chemokine induction capacity to the true signals and their receptors.

Thrombin as a Potent(ial) CNS Maturation, Plasticity, and Emergency Factor—Proteolytic recruitment of PAR signaling is obviously dispensable for the microglial release stimulation by thrombin preparations. PAR contributions can differ among cells and species (4, 44), but our experiments on γ -thrombin, PPACK, and the induction kinetics ruled out an involvement. Similarly, testing a panel of PAR agonists, *i.e.* short peptides derived from the tethered ligands that directly activate the receptors (1, 5), we could not trigger release responses, although sequences and concentrations were proven effective in mouse cells (6, 44) (data not shown). Microglial PARs must thus serve other thrombin effects, and their expression has also been established for astrocytes and neurons (1, 5, 16–18, 45–47).

It is thus important to state that our work is not in conflict with the general concept that thrombin exhibits important CNS functions. Still thrombin could take the role of an immediate trauma signal upon BBB impairment (1, 10). Prothrombin circulates in the plasma at about 1 μ M, and nanomolar thrombin concentrations can persist for days in cerebrospinal fluid of brains with hematomas, sufficient amounts considering the picomolar EC₅₀ for PAR1 (1).

Like other components of the coagulation/fibrinolysis system, prothrombin and factor X(a), which is required for its processing, are expressed or detected within the CNS (1, 5, 48, 49). Glia and neurons even carry a membrane-associated prothrombinase complex (50). Although implications of this coagulation factor sequence as mirrored within the CNS are enigmatic, first hypotheses link thrombin activities to a shaping of synaptic connectivity during development (3, 24, 51, 52), the microanatomical alterations underlying plasticity, and the regulation of neuronal excitability (1, 3, 24, 46, 51, 53).

With regard to microglia, these cells are "designed" to recognize a broad variety of signals for instant alert and effective response (14, 26). We can reasonably assume that plasma contains an important pool of such latent signals that become functional upon injury. For thrombin proper, however, the proinflammatory release component did not appear to be a microglial response. This finding has practical relevance not only for the use of thrombin-related material in neurosurgery. Yet thrombin remains a microglia-modulating protease candidate because of its truly PAR-mediated effects. Clear separation of the blood-clotting, growth factor, and wound-healing actions from inflammatory contributions may thereby (re-)shape our understanding of how one of the key coagulation factors is involved with the control of CNS cell functions.

Acknowledgments—We thank Silke Fleischhauer, Bärbel Girresch, and Irene Haupt for excellent technical assistance; Sanja Pavlovic, Dr. Anja Hoffmann (all at the Max Delbrück Center for Molecular Medicine, Berlin, Germany) and Toni Ahtoniemi (Virtanen Institute, Kuopio, Finland) for experimental collaboration; and Christine Crozier and Drs. Marco Prinz, Wolfgang Brück (University of Göttingen), and Jonathan R. Weinstein (University of Washington, Seattle, WA) for reading the manuscript and critical discussion.

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