Thrombin induces NO release from cultured rat microglia via protein kinase C, mitogen-activated protein kinase and NF-κB

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Running title: Thrombin induces microglial activation.

Key words: microglia, thrombin, NO, PKC, MAPK, NF-κB

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Summary

Microglia, brain resident macrophages, become activated in brains injured due to trauma, ischemia, or neurodegenerative diseases. In this study, we found that thrombin treatment of microglia induced nitric oxide (NO) release/inducible nitric oxide synthase (iNOS) expression, a prominent marker of activation. The effect of thrombin on NO release increased dose-dependently within the range of 5-20U/ml. In immunoblot analyses, iNOS expression was detected within nine hours after thrombin treatment. This effect of thrombin was significantly reduced by protein kinase C inhibitors, such as Go6976, bisindolylmaleimide, and Ro31-8220. Within 15min, thrombin activated three subtypes of mitogen activated protein kinases (MAPKs): extracellular signal-regulated kinase (ERK), p38, and c-jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK). Inhibition of the ERK pathway and p38 reduced the NO release of thrombin-treated microglia. Thrombin also activated NF-κB within 5 min, and N-acetyl cystein (NAC), an inhibitor of NF-κB, reduced NO release. However, thrombin receptor agonist peptide (TRAP, an agonist of protease activated receptor-1 (PAR-1)), could not mimic the effect of thrombin, and cathepsin G, a PAR-1 inhibitor, did not reduce the effect of thrombin. These results suggest that thrombin can activate microglia via PKC, MAPKs, and NF-κB, but that this occurs independently of PAR-1.
Introduction

Microglia are major immune effector cells in the central nervous system, and activation of microglia is a common phenomenon that appears when the brain suffers injury. Many studies have reported that activated microglia produce inflammatory mediators such as nitric oxide (NO), tumor necrosis factor-alpha, and prostaglandins, factors that affect the onset and progression of brain diseases (1-4). For example, microglial activation precedes neuronal cell death in ischemia, and profound microglial activation is commonly observed in seriously injured regions of the brain (5-6). Furthermore, neuronal cell death due to ischemia can be reduced by the inhibition of microglial activation (7). The risk and progression of Alzheimer’s disease and Parkinson’s disease can also be reduced by anti-inflammatory drugs that suppress microglial activation (8-9). However, it has not been clearly shown how the microglial activation occurs in the injured brain.

Thrombin is a well-known protease involved in blood coagulation and wound-healing. In addition, thrombin seems to have diverse functions in many different types of cells. Thrombin induces proliferation of fibroblasts (10) and smooth muscle cells (11). Thrombin induces neurite retraction and synapse reduction (12-13) and changes the morphology of astrocytes (14). Many studies have reported inflammatory functions of thrombin. Thrombin acts as a chemotactic agent for inflammatory cells such as monocytes, macrophages, and neutrophils, and it induces secretion of cytokines from these kinds of cells (15-17). More importantly, thrombin injected into a rat brain induces the infiltration of inflammatory cells, brain edema, and reactive gliosis (18). However, the effect of thrombin on microglial cells has not yet been studied, although microglia are the major immune effector cells in the brain.
The intracellular signaling mechanisms activated by thrombin are diverse. In endothelial cells, thrombin activates Src-family tyrosine kinases, phosphatidylinositol 3-kinase and mitogen-activated protein kinases (MAPKs) (19). In vascular smooth muscle cells and endothelial cells, thrombin produces reactive oxygen species (ROS) (20-21). In astrocytes, thrombin activates protein kinase C and small GTP-binding protein Rho (22-23, 14). Protease activated receptor-1 (PAR-1) has been reported to serve as a thrombin receptor in many types of cells including platelets, endothelial cells, and astrocytes (24-25). In activating PAR-1, thrombin cleaves the extracellular N-terminus of this receptor. The newly formed N-terminus then binds to the receptor and activates it (26). Non-PAR thrombin receptors have also been reported on fibroblasts (27). On these cells, the non-PAR has high affinity for thrombin, and activation of the non-PAR induces expression of genes that are not induced by the activation of PAR-1. However, the intracellular signaling mechanisms linked to non-PAR have not yet been clarified.

The results of this study revealed that thrombin induces NO release/iNOS expression in microglia via PKC, mitogen-activated protein kinase (MAPK), and NF-kB. However, PAR-1 seems not to be involved in this function of thrombin.

**Experimental procedures**

*Preparation of microglia* - Microglia were cultured from the cerebral cortices of 1-3day-old Sprague Dawley rats as previously described (1, 28). Briefly, the cortices were triturated into single cells in minimal essential medium (Gibco, Grand Island, NY) containing 10% fetal bovine serum (Hyclone, Logan, Utah) and plated in 75 cm²
T-flasks (0.5 hemisphere/flask) for 2-3 weeks. Then the microglia were detached from the flasks by mild shaking and filtered through a nylon mesh to remove astrocytes and clumped cells. Cells were plated in 24-well plates (5 x 10^4 cells/well) or 60 or 100mm dishes (0.1-5 x 10^6 cells/dish). One hour later, the cells were washed to remove unattached cells before being used in experiments.

**Determination of NO** - Microglia plated in 24-well plates were treated with thrombin from bovine plasma (Sigma, St. Louis, MO or ICN, Aurora, OH) for 48hrs. The amount of nitrite formed from NO was measured by mixing the culture medium (50µl) with an equal volume of Griess reagent (0.1% naphthylethylene diamine, 1% sulfanylamide, 2.5% H_3PO_4). The optical density was measured at 540nm (29). To examine the involvement of PAR-1, MAPKs, PKC, and NF-κB in the NO release, cathepsin G (inhibitor of PAR-1, Calbiochem, La Jolla, CA), TRAP (agonist of PAR-1, BACHEM, Switzerland), PD98059 (inhibitor of ERK kinase, Calbiochem), SB203580 (inhibitor of p38, Calbiochem), Go6976, Ro-31-8220, and bisindolylmaleimide (all three inhibitors of PKC, Calbiochem), or N-acetyl cystein (inhibitor of NF-κB, Sigma, St. Louis, MO) were included in the treatment with thrombin.

**Detection of expression of iNOS by immunoblot analysis** - Microglia (5 x10^5 cells) were treated with thrombin for 5-25 hours, washed with ice-cold phosphate-buffered saline (PBS), and lysed with modified radioimmune precipitation assay (RIPA) lysis buffer (150mM NaCl,10mM Na_2HPO_4, pH 7.2, 0.5% sodium deoxycholate, 1% nonidet P-40) containing protease inhibitors (2mM phenylmethylsulfonyl fluoride, 100µg/ml leupeptin, 10µg/ml...
pepstatin, 2mM EDTA). Proteins were separated in a 7.5% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane. The membrane was incubated with iNOS antibody (1:2000 dilution, UBI, Lake Placid, NY) for 1 hour. The membrane was then washed three times with Tris-buffered saline including 0.05% Tween-20 and incubated with peroxidase-conjugated secondary antibody (Vector Lab., Burlingame, CA). iNOS was visualized using an enhanced chemiluminescence system (Sigma).

**Measurement of MAPK activation** - Microglia (5 x 10^5) were incubated in serum-free medium overnight and treated with 10U/ml thrombin for 5, 15, 30, 60, and 120 min. The cells were then washed with ice-cold PBS three times, lysed with 2 x SDS PAGE sample buffer and the lysate applied to an 8% SDS-polyacrylamide gel. After electrophoresis the proteins were blotted onto a polyvinylidene difluoride membrane. Activation of MAPKs was examined by immunoblot analysis using antibodies specific for the phosphorylated forms of ERK, p38, and JNK (New England Biolab, UK).

**Measurement of NF-κB activation by electrophoretic mobility shift assay (EMSA)** - Electrophoresis mobility shift assays were carried out as previously described (30). Microglia (2 x 10^6 cells) were harvested and suspended in 900μl of a hypotonic solution (10mM HEPES, pH 7.9, 10mM KCl, 0.1mM EDTA, 0.1mM EGTA, 1mM dithiothreitol, 0.5mM phenylmethylsulfonyl fluoride) including 0.5% nonidet P-40 on ice for 5 min. The suspension was then centrifuged at 500xg for 10 min at 4°C, and the pellet (nuclear fraction) was saved. The nuclear fraction was resuspended in a buffer containing 20mM HEPES, pH 7.9, 20% glycerol, 0.4M NaCl, 1mM
EDTA, 1mM EGTA, 1mM dithiothreitol, and 1mM phenylmethylsulfonyl fluoride, incubated on ice for 60 min with occasional gentle shaking, and centrifuged at 12,000xg for 15 min. The crude nuclear protein in the supernatant was collected and stored at -70°C and later used for electrophoretic mobility shift assays. Two synthetic oligonucleotides (Genosys, Woodlands, TX) containing the NF-κB binding sequence of the murine immunoglobulin light chain gene (5'-GGGAGTTGAGGGACTTTCCGAGG-3') and its complementary sequence were end-labeled using Klenow fragment and [α-32P]-dCTP. The labeled DNA probe (approximately 0.2 ng) was incubated with 0.5 μg of nuclear proteins 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 dithiothreitol, 0.3 mg/ml bovine serum albumin, and 6 mM MgCl2 for 30 min. The reaction mixture was applied to an 8% polyacrylamide gel. After electrophoresis, the gel was dried and an autoradiogram was obtained. For super-shift assays, the nuclear extract was pre-incubated with 1 μg of anti-p50 or anti-p65 antibody (Santa Cruz Biotechnology) for 30 min. The reaction mixture was subjected to electrophoresis through a 6% polyacrylamide gel.

Reverse transcription polymerase chain reaction (RT-PCR) - Total RNA was isolated using RNAzol™ B (TEL-TEST, Inc., Friendwood, TX), and cDNA was prepared using reverse transcriptase that originated from Avian Myeloblastosis Virus (TaKaRa, Japan) according to the manufacturer’s instructions. The PCR primers for the inducible nitric oxide synthase (iNOS) gene were 5'-GCAGAATGTGACCATCATGG-3' (sense primer) and 5'-ACAACCTTGTGTGTTGAGGC-3' (antisense primer). PCR products were separated by electrophoresis in a 1.5% agarose gel and detected under UV light.
Results

Thrombin induces microglial NO release and inducible nitric oxide synthase (iNOS) expression. To determine whether thrombin induced microglial activation, we looked at nitrite formed from NO in the media and the expression of inducible nitric oxide synthase (iNOS), a prominent marker of microglial activation. Microglia were treated with thrombin at 5-20U/ml for 48 hours. Nitrite production increased in a dose-dependent manner: 6.9 ± 0.8 (mean ± SEM of three samples unless indicated otherwise), 10.2 ± 0.8, and 16.1 ± 0.3 μM nitrite was produced from 5 x 10^4 cells treated with 5, 10, and 20U/ml of thrombin, respectively, while 3.7 ± 1.3 μM nitrite was produced from untreated cells (Fig. 1A). Basal level of nitrite production was not likely from nitric oxide synthase (NOS) since it was not reduced by iNOS inhibitors (AMT and 1400W) and non-selective NOS inhibitors (L-NAME and L-NMMA) (data not shown). iNOS expression was also detected by immunoblot analysis within 9 hours with further increase for up to 21 hours and maintained to 25 hours (Fig. 1B). This suggested that the effect of thrombin on nitrite production was due to the expression of iNOS.

PKC inhibitors reduce NO release from thrombin-treated microglia. In several types of cells, PKC has been known to mediate the function of thrombin (19, 22, 31). Furthermore, PKC has been reported as an important mediator of iNOS expression in microglia and astrocytes (32-34). We therefore examined whether a PKC pathway could be involved in the thrombin-induced nitrite production. In the presence of PKC inhibitors, bisindolylmaleimide (BIM, 2 μM), Go6976 (Go, 0.1 μM), and Ro31-8220 (Ro, 0.5 μM), nitrite production was reduced to 32.8 ± 0.1, 27.6 ± 7.9, and 10.9 ± 4% of that induced by thrombin in the absence of any
inhibitor (Fig. 2A). PKC inhibitors had little effect on nitrite production from untreated cells (data not shown).

Thrombin-induced iNOS expression was also inhibited in the presence of BIM and Go (Fig. 2B). The reduced nitrite production and iNOS expression were not caused by any toxicity of these reagents, as was confirmed by the exclusion of trypan blue observed through a light microscope. These data, therefore, strongly suggest that a PKC pathway is involved in the thrombin effect.

*Thrombin stimulates mitogen-activated protein kinase.* Since MAPKs have been known to mediate microglial activation (28,35), we examined whether thrombin induced the activation of MAPKs. Within 15 min, thrombin activated ERK, p38, and JNK/SAPK, which was confirmed by increased phosphorylation of tyrosine residues of these kinases as determined by immunoblot analysis. The activity status of ERK and p38 remained sustained for 120 min, while that of JNK/SAPK lasted up to 60 min but fell back to the control level after 120 min (Fig. 3). To test whether activation of the MAPKs could be involved in the thrombin-induced nitrite production, we treated microglia with thrombin in the presence of PD98059 and SB203580, inhibitors of the ERK pathway and p38 pathway, respectively. PD98059 (5 µM) and SB203580 (5 µM) significantly reduced nitrite production to 20.9 ± 14 and 19.3 ± 4.9% of that induced by thrombin in the absence of any inhibitor (Fig. 4). These results suggest that MAPKs, particularly ERK and p38, mediate NO release from microglia treated with thrombin.

*Thrombin activates NF-κB.* Since NF-κB binding sites are present in the promoter region of the iNOS gene, we examined whether thrombin activated NF-κB. The electrophoretic mobility shift assay showed that thrombin
activated NF-κB within 5 min. The activity was sustained for 15 min but was significantly reduced after 30 min (arrows in Fig. 5A). To investigate which subtypes of NF-κB was activated by thrombin, we carried out super-shift assays using antibodies against p50 and p65 (Fig. 5B). The intensity of the shifted bands was decreased and a super-shifted band appeared in the presence of antibody against p50 (arrow head in Fig. 5B) but not in the presence of antibody against p65, indicating that p50 could be activated by thrombin.

We further examined whether the activation of NF-κB was directly related to the induction of NO release. For this, NAC, a known NF-κB inhibitor, was applied together with thrombin. NAC dose-dependently reduced the thrombin-induced nitrite production: in the presence of 5, 10, and 20 mM NAC, nitrite production was reduced to 71.5 ± 12.7, 7.7 ± 6.4, and 12.6 ± 5.4%, respectively (Fig. 6). Thus, the results suggest that NF-κB could be a mediator of the thrombin effect in the induction of microglial NO release.

Thrombin-induced NO release is independent of PAR-1. We also examined whether PAR-1 mediated the effect of thrombin to induce microglial NO release, since PAR-1 has been suggested to act as a thrombin receptor in many types of cells. For this, we looked at the effects of the agonist of PAR-1, thrombin receptor agonist peptide (TRAP). TRAP did not mimic the effect of thrombin even at a 500 µM concentration: 50, 100, 200, and 500 µM TRAP induced 1.5 ± 0.5, 2.3 ± 0.9, 1.5 ± 0.7, and 1.1 ± 0.6 µM of nitrite, respectively, whereas 10 U/ml of thrombin induced 14.9 ± 1.1 µM nitrite (Fig. 7A). We then used RT-PCR to detect iNOS expression, since the RT-PCR method could be more sensitive than immunoblot analysis or measuring nitrite. Even with RT-PCR, the result was the same: iNOS was not detected in 500 µM TRAP-treated cells while it was detected within three
hours in thrombin-treated cells. (Fig. 7B)

We also tested the effect of cathepsin G, an inhibitor of PAR-1. As we expected based on the results of the experiments using TRAP, cathepsin G (40mU/ml) did not reduce nitrite production from thrombin-treated microglia. Microglia pretreated with cathepsin G for 30min produced $12.6 \pm 0.5 \mu M$ nitrite in response to 10U/ml of thrombin compared to $12.6 \pm 0.9 \mu M$ of nitrite produced by microglia treated with thrombin alone. Cathepsin G (40mU/ml) alone had no discernible effect (Fig. 8).

Discussion

The results in this study indicate that thrombin could be a mediator of brain inflammation, since thrombin induces NO release and iNOS expression in microglia, which are major immune effector cells in the brain. The intracellular signaling mechanisms that mediate the function of thrombin in microglia can be compared to those of other microglial activators. LPS and beta-amyloid peptide have been known to be microglial activators. Recently we reported that gangliosides, components of the plasma membrane, could induce microglial activation (35). Thrombin’s microglial activation could be achieved via similar intracellular signaling pathways. PKC may thus be the common mediator of microglial activation. PKC inhibitors reduce NO release by microglia treated with LPS (33-34) and gangliosides (data not shown) as they inhibited thrombin-induced NO release. (Fig. 2). Recently, it has been reported that in microglia PKC isoforms such as $\alpha$, $\varepsilon$, and $\delta$, are activated by beta-amyloid (36). MAPKs are other common mediators of microglial activation. ERK, p38, and JNK/SAPK are activated by thrombin, LPS, beta-amyloid, and gangliosides (28, 35). However,
some discrepancies still exist with regard to the effect of inhibitors of the ERK pathway (PD98059) and p38 (SB203580) on the action of these activators. LPS- and thrombin-induced nitrite production was significantly reduced by PD98059 and SB203580, whereas ganglioside-induced nitrite production was inhibited by PD98059, but not by SB203580 (28, 35). NF-κB is also a common mediator of microglial activation. Microglial activators, such as beta-amyloid, interferon-gamma, and gangliosides activate NF-κB (35, 37), and inhibition of NF-κB reduced NO release (35).

In addition to PKC, MAPK, and NF-κB, Src-family tyrosine kinases, PI3-K, and reactive oxygen species (ROS) have also been reported to mediate the function of thrombin in many kinds of cells. Thrombin has been known to activate a Src-family tyrosine kinase in astrocytes (22) and endothelial cells (19). In microglia, however, selective inhibitors of Src-family tyrosine kinases, PP2 and lavendustin A, did not inhibit the action of thrombin (data not shown). PI3-K has been known to mediate thrombin-induced proliferation of smooth muscle cells (38), and ROS have been known to induce the expression of chemokines and cell adhesion molecules in endothelial cells, epithelial cells, and lymphoma cells (39-40). However, PI3-K may not be involved in the thrombin effect on microglia, since PI3-K inhibitors, such as wortmannin and LY294002, had little effect on thrombin-induced NO release (data not shown). Although NAC that increases reduced form of glutathione level dose-dependently reduced thrombin-induced NO release (Fig. 6), ROS scavengers, such as catalase, superoxide dismutase, and trolox, did not inhibit thrombin-induced nitrite production (data not shown). Thus, ROSs that could be removed by these scavengers might not mediate the action of thrombin to induce NO release.

PAR-1 has been suggested as a thrombin receptor. In astrocytes and C6 glioma cells, thrombin-induced
Ca\textsuperscript{2+} mobilization is mimicked by TRAP, and blocked by cathepsin G (41-42). Activation of platelets could be achieved by both thrombin and TRAP (43). However, in microglia TRAP did not induce NO release and iNOS mRNA expression (Fig. 7). Furthermore, cathepsin G had no effect on the thrombin-induced NO release (Fig. 8). In addition, the effect of thrombin was not reduced by hirudin that inhibits protease function of thrombin and binding of thrombin to PAR (44-46) (data not shown). We also tested the effect of prothrombin in the absence of factor VII and X that change prothrombin into thrombin. Prothrombin dose-dependently induced microglial NO release (data not shown). These results suggest that, in microglia, thrombin induces nitrite production via a PAR-1-independent pathway. Recent studies in other laboratories could support this conclusion. In myoblasts, thrombin protected myoblasts from apoptosis, while TRAP did not (47). In fibroblasts, TRAP and TP508 (a peptide fragment of thrombin different from TRAP) induced differential patterns of gene expression, suggesting additional non-proteolytic action of thrombin (27). However, until recently, information regarding thrombin receptors other than PAR-1 has been limited although PAR-3 and 4 also have been suggested. Therefore, further studies are needed to characterize thrombin receptors in microglia.

**Acknowledgement**

This work was supported by HMP-98-N-6-0010 of the 98 Good Health R&D Program, Ministry of Health and Welfare, Korea, and Grant No. 1999-2-207-004-5 from the interdisciplinary research program of the KOSEF to E Joe.
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**Footnotes**

Abbreviations: AMT, 2-amino-5,6-dihydro-6-methyl-4H-1,3-thiazine; ERK, extracellular signal-regulated kinase; JNK/SAPK, c-jun N-terminal kinase/stress-activated protein kinase; LPS, lipopolysaccharide; L-NAME, N\(^G\)-nitro-L-arginine, methyl ester; L-NMMA, N\(^G\)-monomethyl-L-arginine; MAPK, mitogen-activated protein kinase; NAC, N-acetyl cystein; NF-κB, nuclear factor-kappa B; NO, nitric oxide; iNOS, inducible nitric oxide; PAR-1, protease activated receptor-1; PI3-K, phosphatidylinositol 3-kinase; PKC, protein kinase C; TRAP, thrombin receptor agonist peptide.
Figure legends

Figure 1. Thrombin induces NO release (A) and iNOS expression (B) in cultured rat microglia. (A) Microglia were incubated with the indicated amounts of thrombin for 48 hours. The amount of nitrite formed from NO was determined as described under Experimental Procedures. Values are means ± SEM of three samples. (B) iNOS expression was detected by immunoblot analysis. Cells were treated with 10U/ml of thrombin for the indicated time periods.

Figure 2. Protein kinase C inhibitors, bisindolylmaleimide, Go6976, and Ro31-8220 significantly reduce NO release (A) and iNOS expression (B). (A) Microglia were treated with 10U/ml of thrombin for 24 hours in the absence or presence of 2µM bisindolylmaleimide (BIM), 0.1µM Go6976 (Go), or 0.5µM Ro31-8220 (Ro). The amount of nitrite formed from NO was determined as in Fig. 1. Values are means ± SEM of three samples. (B) For immunoblot analysis, cells were treated with 10U/ml of thrombin for 24 hours in the absence or presence of 10µM BIM or 0.1µM Go.

Figure 3. Thrombin activates ERK, p38, and JNK/SAPK. Microglia were treated with 10U/ml of thrombin for the indicated time periods. Activated ERK (P-ERK), p38 (P-p38), and JNK/SAPK (P-JNK) species were detected by immunoblot analysis with antibodies specific for the phosphorylated forms of each kinase. The amount of protein loaded in each lane was confirmed by measuring the amount of ERK using antibody against the unphosphorylated form of ERK (data not shown). The same results were obtained in more than three
independent experiments.

Figure 4. PD98059 and SB203580 reduce NO release. Microglia were treated with 10U/ml thrombin for 48 hours in the presence or absence of 5µM PD98059 or 5µM SB203580. Values are means ± SEM of three samples.

Figure 5. Thrombin activates NF-κB. Nuclear extracts were prepared from microglia treated with 10U/ml thrombin for the indicated time periods. (A) The NF-κB specific oligonucleotide-protein complexes are marked by arrows. (B) In the super-shift assay, antibodies against p50 and p65 were mixed with nuclear extracts obtained from microglia treated with thrombin for 30min. A super-shifted band was detected in the presence of p50 antibody and is marked by an arrow head. The electrophoresis mobility shift assay was carried out as described under Experimental Procedures.

Figure 6. N-Acetyl cystein (NAC) inhibits thrombin-induced NO release. Microglia were treated with 10U/ml of thrombin for 48 hours in the absence or presence of 5, 10, and 20mM of NAC. Values are means ± SEM of three samples.

Figure 7. TRAP does not mimic the effect of thrombin. (A) Microglia were treated with 10U/ml of thrombin or the indicated amounts of TRAP for 48hours. Values are means ± SEM of three samples. (B) mRNA for iNOS
is detected in thrombin-treated microglia but not in TRAP-treated ones. Cells were treated with 10U/ml of thrombin or 500µM TRAP for 12 hours. RT-PCR was performed as described in Experimental procedures.

Figure 8. Cathepsin G does not reduce NO release from thrombin-treated microglia. Microglia were pretreated with 40mU/ml of cathepsin G (CG) for 1 hours before thrombin was added. The amount of nitrite formed from NO was determined 48 hours after thrombin treatment. Values are means ± SEM of three samples.
Fig. 7

A

B

control thrombin TRAP

iNOS

GAPDH

Nirite (µM)
Fig. 8

- Thrombin
- + + -

- Cathepsin G
- - + +

Nitrite (μM)
Thrombin induces NO release from cultured rat microglia via protein kinase C, mitogen-activated protein kinase and NF-kappaB
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J. Biol. Chem. published online July 12, 2000

Access the most updated version of this article at doi: 10.1074/jbc.M001220200

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