Protease-activated receptor-1 regulates cytokine production and induces the suppressor of cytokine signaling-3 in microglia

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Abstract. Protease-activated receptors (PARs) are cleaved and activated by thrombin and other extracellular proteases which are released during tissue trauma and inflammation. PAR-1 is the prototypic member of the PAR family and has been shown to be upregulated in several brain pathologies being expressed by neurons and glial cells. The present experiments show that the administration of the PAR-1 activating peptides (TRAP6 and TFLLR) inhibits the production of the pro-inflammatory cytokines TNF-α and IL-6 in microglial cells treated with lipopolysaccharide (LPS) while promoting the release of the anti-inflammatory cytokine IL-10. Conversely, the addition of the specific PAR-2 agonist SLIGRL had no effect on the amount of cytokines released following LPS treatment. Consistent with these data PAR-1, but not PAR-2, stimulation upregulates the expression of the suppressor of cytokine signaling-3 (SOCS-3). The present data support the hypothesis that in microglia PAR-1 may be involved in the regulation of inflammatory reactions modulating the balance between pro- and anti-inflammatory cytokines possibly through SOCS induction.

Introduction

Protease-activated receptors (PARs) are G-protein coupled receptors that signal in response to extracellular proteases. The proteolytic nature of PAR activation results in an irreversible activated receptor which is rapidly sorted to lysosomes for degradation. Four members of the PAR family have been described so far. PAR1, PAR3 and PAR4 are activated by thrombin whereas PAR2 is a receptor for trypsin (1). In addition to thrombin, PAR-1 is also cleaved and activated by plasmin, activated protein C (APC), factor Xa, factor VIIa and the matrix metalloprotease MMP-1 (2). Once

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activated PARs couple to heterotrimeric G-proteins. PAR1, in particular, couples to the $G\alpha_q$, $G\alpha_i$ and $G\alpha_{12/13}$ subtypes and induces activation of MAP kinases, mobilization of intracellular calcium, Rho and Rac signalling (1). Since PARs play a central role in hemostasis and thrombosis, as well as in inflammation and vascular development, their expression and activation must be tightly controlled.

PAR-1 activation has been suggested to mediate several pathological effects including cell death of spinal motoneuron cultures (3) and potentiation of N-methyl-D-aspartate receptor responses in hippocampal neurons (4). Acute and traumatic ischemic brain injury induces proliferation of glial cells, most of which are microglial cells (5). As recently reported, the activation of PAR-1 by thrombin or synthetic thrombin receptor agonist peptides induces micro-gliosis in hippocampal slice cultures (6). PAR-1 expression in microglia has been reported to occur *in vivo* after ischemic injury while restricted to neurons in untreated control animals (7). In mice lacking PAR-1 a reduction in infarct volume after transient focal cerebral ischemia was observed (8). On the contrary, PAR-1 activation by thrombin or PAR-1 activating peptides was also reported to protect rat hippo-campal neurons and astrocytes from cell death due to oxidative stress or treatments with glutamate or ß-amyloid (9-11).

While the effect of PAR-1 activation on cell survival and proliferation has been demonstrated in different cell culture system, more controversial is its role in modulating inflammatory responses. A number of previous studies reported that thrombin is able to induce release of cytokines, chemokines and nitric oxide from microglial cells both *in vivo* and *in vitro* (12-16). These results, however, were later reinterpreted because proved to be ascribed to a minor fraction of proteins contaminating thrombin preparations, leading to the conclusion that neither PAR activation nor proteolytic activity of thrombin were involved in inducing the release of pro-inflammatory mediators from microglia (17,18). In order to shed some light on this controversial matter, in the present study instead of purified thrombin we used certain PAR synthetic agonist peptides in combination with lipopolysaccharide (LPS) to study the role, if any, of PAR-1, the prototypic member of PAR family, in modifying the pro-inflammatory response of microglial cells.

Materials and methods

The murine microglial cell line BV-2 was grown in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Italy) supplemented with 10% fetal calf serum (FCS; Sigma, Italy) in 5% CO_2. Primary microglial cell cultures were derived from postnatal day 3-4 rat cortex as previously described (19). Briefly, free-floating microglia were collected from shaken astrocyte flasks and maintained in DMEM supplemented with 10% FCS in 5% CO_2. The purity of microglial cultures was assessed by a positive staining for *Griffonia simplicifolia* isolectin B4 (ILB4; Sigma) a selective marker of both resting and activated microglia.

For immunocytochemistry, microglial cells ($50x10^3$ cells/well) were seeded onto 8-well Permanox chamber slides and fixed in 4% paraformaldehyde for 20 min. Cells were quenched with 0.1 M glycine/HCl, pH 7.4, and treated with 3% H_2O_2 in methanol to inhibit endogenous peroxidase. After extensive washes in phosphate-buffered saline (PBS), cells were preincubated for 1 h with 5% non-fat milk and incubated with a polyclonal anti-PAR-1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA; H-111) overnight at 4˚C. The reaction was visualized by a standard avidin-biotin-peroxidase method. Briefly, after washes with PBS, slides were incubated for 1 h with a biotinylated secondary antibody (Vector Laboratories, Burlingame, CA), washed again, and incubated for 30 min with an avidin-biotin-peroxidase complex (Vectastain Elite ABC kit, Vector). Finally, cells were washed and treated with 0.05% 3-3 diaminobenzidine and 0.015% H_2O_2. Negative control experiments were done substituting Igs against PAR-1 with equivalent amounts of non-specific rabbit Igs.

Microglial cells ($3x10^5$ cells/well) were plated onto 24-well plates and exposed to *E. coli* lipopolysaccharide (LPS; 100 ng/ml) (serotype 0127:B8; Sigma) alone or in combination with 50 μM TRAP6 (Sigma) for 1, 3 and 24 h. The release of tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), interleukin-10 (IL-10) into culture supernatants was determined by standard ELISA techniques according to the manufacturer's instructions (R&D Systems, MN, USA). Statistical analyses were conducted using GraphPad Prism version 4.00 software. Data are expressed as means ± SEM. Comparisons were analysed using ANOVA with Bonferroni-corrected t-test.

Cell viability was measured by MTT reduction essentially as described (20). Following 48 h treatments, 10 μl of MTT solution (5 mg/ml) was added to each well and the incubation was continued for 3 h. Lysis buffer was prepared by dissolving 40% (w/v) sodium dodecyl sulphate (SDS) in deionised water, after adding an equal volume of N,N-dimethylformamide, the pH was adjusted to 4.7. After 3 h incubation with MTT, 100 μl of the lysis buffer was added to each well and the absorbance read at 570 nm on a microplate reader.

Lactate dehydrogenase (LDH) release in the culture medium was measured by Cytotoxicity Detection Kit (Roche, Mannheim, Germany) according to manufacturer's protocols.

For RT-PCR experiments, microglia and BV-2 cells were seeded onto 6-well plates and treated with thrombin (40 U/ml; Sigma), TRAP6 (50 μM; Sigma), TFLLR (50 μM; Bachem UK Ltd.) and SLIGRL (50 μM; Bachem UK Ltd.). After 3 h
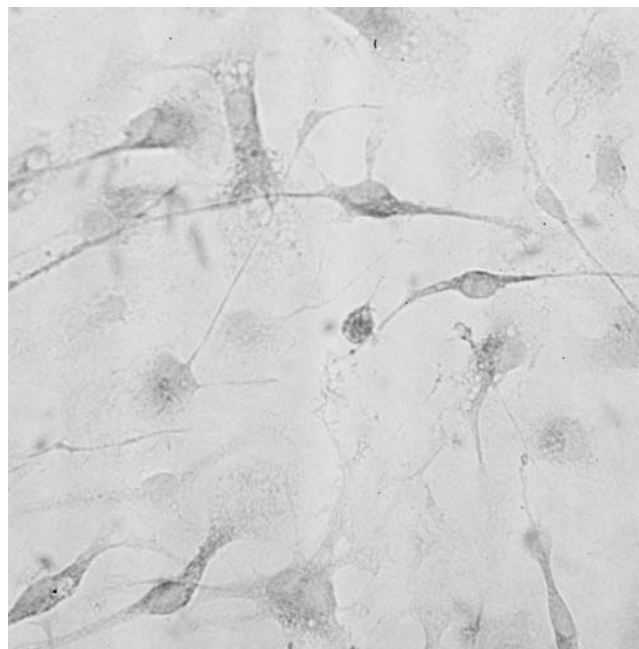

Figure 1. Expression of PAR-1 in microglial primary cell cultures. The anti-PAR-1 antibody labels strongly the perinuclear area and more faintly the cellular processes.

of treatment total RNA was isolated using TRIzol reagent (Invitrogen, CA, USA) according to manufacturer's instructions. One microgram of total RNA was reverse-transcribed by using Superscript III reverse-transcriptase (Invitrogen, CA). Briefly, a mixture of RNA, oligo(dT) and 4-dNTP mix was incubated at 65˚C for 5 min; then Superscript III reverse-transcriptase (200 U), RNase Ribonuclease Inhibitor, DTT and buffer (250 mM Tris, pH 8.3, 375 mM KCl and 15 mM $MgCl_2$) were added to the mixture and reaction was continued for 45 min at 50˚C. Finally, Superscript III was inactivated by heating at 70˚C for 15 min. The final volume was 20 μl. All reagents were from Invitrogen, Italy. Three microliters of the obtained cDNA were subjected to PCR by using specific primers for rat suppressor of the cytokine signaling-3 (SOCS-3) (forward ACCAGCGCCACTTCTTCA, reverse GTGGAGCATCA TACTGATCC). The PCR products were analysed by performing 1.5% TBE agarose gel electrophoresis (Submarine Agarose Gel Unit, Hoefer, CA, USA). Gels were prestained with ethidium bromide (0.5 ng/ml). A PC-assisted CCD camera (GelDoc 2000 System/Quantity One Software; Bio-Rad) was used for gel documentation and quantification. The data were normalized against GAPDH mRNA level.

Results

In primary microglial cell cultures we detected PAR-1 expression by immunocytochemistry (Fig. 1) and Western blot analysis (data not shown) consistent with previously reported data (14). This receptor is expressed in the BV-2 microglial cell line as well (data not shown).

The role of PAR-1 in modulating inflammation is controversial since it was reported to mediate the induction of both pro- and anti-inflammatory molecules (21,22). Here,
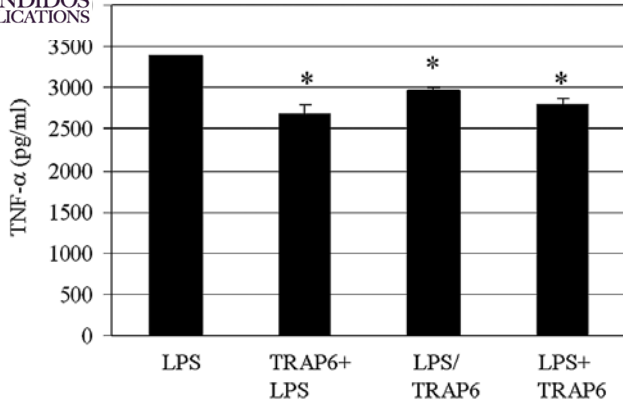
Figure 2. TRAP6 inhibits TNF-α production in the BV-2 microglial cell line stimulated with LPS. BV-2 cells were treated with 1 μg/ml LPS alone or in combination with 50 μM TRAP6 added 10 min before (TRAP6+LPS), simultaneously (TRAP6/LPS) or 10 min after LPS (LPS+TRAP6). Twenty-four hours later TNF-α released in the supernatants was measured by ELISA. *p<0.01 vs LPS alone.
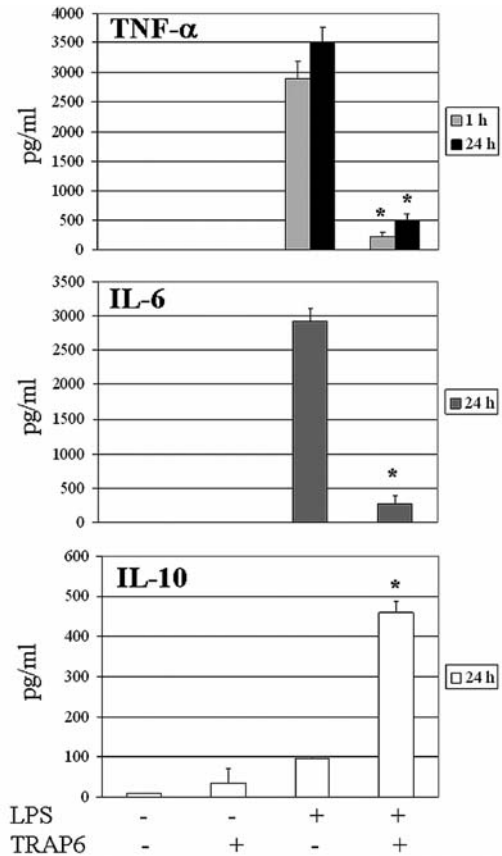

Figure 3. TRAP6 modifies LPS-induced cytokine production in primary microglial cells. Primary rat microglia was pre-treated for 10 min with TRAP6 (50 μM) before the addition of 100 ng/ml LPS; TNF-α, IL-6 and IL-10 were measured by ELISA after 1 and 24 h of treatment. *p<0.01 vs LPS alone.
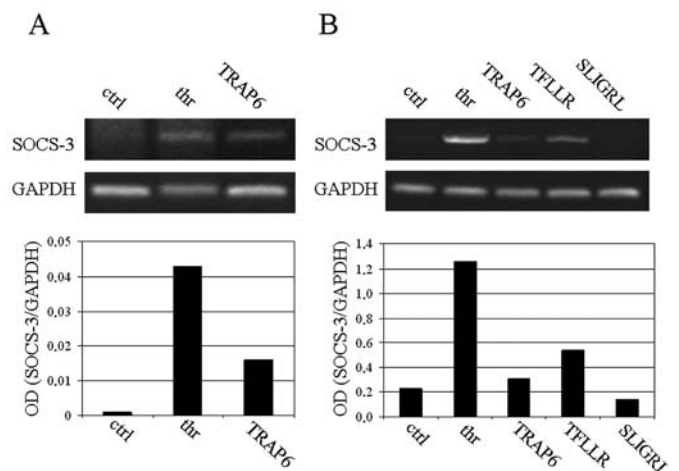

Figure 4. PAR-1 agonists upregulates SOCS-3 mRNA. (A) Primary microglial cells were treated with thrombin (thr; 40 U/ml) or TRAP6 (50 μM) and SOCS-3 mRNA was detected by RT-PCR after 3 h of treatment. (B) BV-2 microglial cells were treated with thrombin (thr; 40 U/ml), TRAP6 (50 μM), TFLLR (50 μM) or SLIGRL (50 μM) and SOCS-3 mRNA detected after 3 h of culture. Data are representative of three independent experiments.

we performed experiments in order to understand if PAR-1 activation could modify the production of pro- and/or anti-inflammatory cytokines by microglial cells. The BV-2 microglial cell line was treated with LPS in combination with the PAR-1 agonist peptide TRAP6 (50 μM) and TNF-α in the supernatant was measured by ELISA. Under these experimental conditions, we observed a reduction by about 20% of TNF-α release in cells treated with TRAP6 when the peptide was added 10 min before, simultaneously or 10 min after LPS. This low but significant reduction of LPS-induced TNF-α levels was observed from 3 (data not shown) to 24 h of treatment (Fig. 2). This result was confirmed in primary microglial cell cultures where TRAP6 administration more drammatically reduced LPS-induced TNF-α release (82% reduction with respect to LPS alone), similarly affecting the production of IL-6 (92% reduction with respect to LPS alone) (Fig. 3). TRAP6 was demonstrated to be a potent agonist of both PAR-1 and PAR-2 (23). Thus, we also tested the specific PAR-2 agonist SLIGRL which did not modify TNF-α release in microglia treated with LPS and the PAR-1 specific agonist TFLLR (24) which reduced LPS-induced TNF-α production similarly to TRAP6 (not shown). Furthermore, while reducing the production of pro-inflammatory cytokines in LPS-stimulated microglia, TRAP6 also determined an increased release of the anti-inflammatory cytokine IL-10 (Fig. 3). As already observed in BV-2 cells, also in primary microglia the reduction in LPS-induced TNF-α production by TRAP6 remained stable from early time points (1-3 h) up to 24 h. Vitality of the cultures was checked during these experiments by measuring LDH release and by the MTT test; no toxic effects of the peptides nor any increase in cell proliferation was observed under our experimental conditions (data not shown).

In microglia thrombin is known to induce SOCS-3 mRNA expression (25). Actually, SOCS family members represent the most potent and broadly acting suppressors of cytokine signaling and SOCS-3 plays crucial roles in negatively regulating the response of cells to growth factors and cytokines by inhibiting JAK/STAT signaling (26). In the present experiments, we analysed by RT-PCR the SOCS-3 mRNA levels in microglial cells treated with PAR-1 agonists. When primary microglia was treated with thrombin

(used as positive control) or TRAP6, SOCS-3 mRNA, which was barely detectable in untreated cells, was induced after 3 h as shown in Fig. 4A. Similarly, in BV-2 cells thrombin, TRAP6 and TFLLR upregulated SOCS-3 mRNA while the PAR-2 activating peptide SLIGRL had no effect (Fig. 4B).

Discussion

Several pathologic conditions of the central nervous system (CNS) are known to determine an upregulation of PAR-1 expression both in neurons and glia (7,27-30). In particular, the expression of this receptor in microglia seems to be restricted to the very early phase of the activation process of these cells (7). Microglial cells are the first line of defence during brain injury or disease responding with a morphological transition from resting into activated phenotype. In general, while acute microglial activation is considered part of the CNS tissue response to injury and an essential component of wound healing, chronic inflammation is thought to contribute to the development of neurodegenerative diseases (31). Actually, PAR-1 is considered a protective or a harmful factor in different model systems. For istance, PAR-1 -/- mice are protected from transient focal ischemia (8) and show an attenuated microglial response with reduced dopaminergic terminal damage in a model of Parkinson's disease (32). Conversely, activated protein C (APC) reduces damage in a mouse model of ischemic stroke by a mechanism involving PAR-1 (33). Moreover, PAR-1 was reported to switch from being harmful to protective during the progression of sepsis in mice while PAR-2 was shown to be required for PAR-1 protective effects (34). Recently, it has been demonstrated that in dendritic cells PAR-1 signalling sustains a lethal inflammatory response that can be interrupted by inhibition of either thrombin or PAR-1 signalling (35).

Our present data show that thrombin and PAR-1 agonists (TRAP6 and TFLLR) induce SOCS-3 expression in microglial cells. When administered alone TRAP6 is unable to induce the release of pro- or anti-inflammatory cytokines but under inflammatory conditions (LPS treatment) it does potentiate the production of the anti-inflammatory cytokine IL-10 while reducing the synthesis of the pro-inflammatory cytokines TNF-α and IL-6.

SOCS family members negatively regulates the JAK/STAT pathways (36) and the constitutive expression of SOCS-3 diminishes the amount of TNF-α and nitric oxide produced in response to LPS stimulation in macrophages (37). Moreover, IL-10-induced phosphorylation of SOCS-3 has been reported to be essential for the inhibition of TNF-α synthesis by IL-10 (38).

In human mononuclear cells thrombin and PAR-1 activating peptides were demonstrated to mediate the upregulation of the anti-inflammatory cytokine IL-10 (22). Our results are in line with data obtained in cells of the monocytic/macrophage lineage where TRAP6 potentiates IL-10 production induced by LPS or phytohemagglutin and suggest that in microglia PAR-1 may participate to the regulation of inflammatory responses.

Furthermore, in previous studies thrombin action in promoting pro-inflammatory cytokines by microglial cells was found to be mediated through PAR-4, while PAR-1 was considered to play an indirect pro-inflammatory role by upregulating CD-40 expression and thus potentiating CD-40 ligand-induced TNF-α production (21). In the present experiments, however, we demonstrate that PAR-1 plays also an important anti-inflammatory role by inducing an anti-inflammatory cytokine (IL-10) as well as SOCS-3, a negative regulator of the cytokine signal (26). Our finding extends a previous observation of a thrombin-induced expression of SOCS-3 in microglial cells (25), representing, to our knowledge, the first observation about the involvement of a member of the PAR family in SOCS regulation. This confirms the perspective of exploiting PAR agonists and antagonists as potential therapeutic agents in inflammatory conditions.

Acknowledgements

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