PAR-1 upregulation by trimethyltin and lipopolysaccharide in cultured rat astrocytes

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Abstract. We have previously shown that various protease-activated receptor (PAR) isoforms, mainly PAR-1, are up-regulated in reactive astrocytes of rat hippocampus following i.p. administration of trimethyltin (TMT), a neurotoxicant which is known to cause neuronal death and reactive gliosis. In the present paper, we demonstrate that this PAR-1 up-regulation was also mimicked in primary cultures of neonatal rat cortex astrocytes after exposure (24 and 48 h) to TMT (10-100 μM). This result suggests that the PAR-1 increase we have observed *in vivo* may represent a direct effect of TMT on astrocytes rather than a consequence of a complex astrocytic reaction following neuronal death. Furthermore, an evident upregulation of PAR-1 in cultured primary astrocytes also occurred following exposure to lipopolysaccharide (LPS) (a well-known inductor of glial cell activation) whereas other neurotoxic agents (such as staurosporine, hydrogen peroxide and sodium azide), which are known to induce cell death, were unable to determine any PAR-1 variation. Similarly to astrocytes, both TMT and LPS induced an upregulation of PAR-1 in the rat astrocytoma cell line, C6, thus indicating that this phenomenon was independent from microglial cells eventually contaminating astrocyte primary cultures. Furthermore, after exposure to TMT and LPS, the levels of tumor necrosis factor-α and interleukin-1ß were also increased in astrocyte cultures, suggesting that the PAR-1 upregulation we have detected may be involved in glial inflammatory response rather than in cell death.

Introduction

Protease-activated receptors (PARs) are transmembrane receptors that are coupled to G-proteins. These receptors are activated by a unique mechanism whereby serine proteases hydrolyse, at a specific cleavage site, the extracellular N-terminus of the receptor. This cleavage exposes a new N-terminus that acts as a tethered ligand, which binds intramolecularly to the receptor to initiate cellular signals. Four members of this family have been cloned so far: PAR-1, PAR-3 and PAR-4, which are activated by thrombin, and PAR-2, which is cleaved by trypsin or mast cell tryptase. Furthermore, to a lesser extent, other proteinases such as Factor Xa, cathepsin G, plasmin and granzyme A are also able to activate PARs (1,2). PARs are expressed widely in a variety of tissues, including that of the nervous system. Although various PAR subtypes have been detected at both the neuronal (3) and the glial level (4-6), their precise biological role is not fully understood at present. Numerous studies suggest that these receptors can affect neural cell proliferation, morphology and physiology (7,8). These various cellular activities probably involve different signalling pathways triggered through binding of PARs to a series of G-proteins (G12/13, Go/i and Gq) (9-11).

PARs seem to be also involved in neurodegenerative processes. For example, an upregulation of PARs has been detected following optic nerve crush (12), spinal cord injury (13) and sciatic nerve lesion (14). Furthermore, changes in PAR expression have been reported in several models of experimental cerebral ischaemia (3,15). More recently, an increase of PAR-1 and prothrombin was found to occur in astrocytes of brains from patients affected by HIV encephalitis (HIVE) (16). As a matter of fact, brain inflammation is also known to occur in other neurodegenerative diseases, including Alzheimer's disease (17,18) and stroke (19). In this connection, numerous studies have demonstrated that thrombin may participate in inflammatory processes in CNS disorders by activating astrocytes and microglia to propagate focal inflammation and to produce potential neurotoxic factors (reviewed in ref. 20). In CNS, active thrombin derives primarly from the bloodstream following increased permeability or breakdown of the blood-brain barrier (21). On the other hand, neural cells themselves synthetize prothrombin (22-24).

Recently, our group has undertaken a study to clarify whether the expression of the thrombin system is altered in rat hippocampus following administration of trimethyltin (TMT), a neurotoxicant that produces neuronal loss associated with astrogliosis and microglia response (25-29). In particular, we were able to show the appearance of an evident specific immunoreactivity for various PAR isoforms, mainly PAR-1, in reactive astrocytes of CA3 and hylus regions (30), i.e. hippocampal areas that represent the main target of TMT injury

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(26,29). The question now arises as to whether this PAR induction may represent a direct effect of TMT on astrocytes or, alternatively, may be due to a complex indirect astrocytic reaction following neuronal damage.

In order to gain insights into this matter in the present study, we investigated the expression of PAR-1, the main astrocytic PAR isoform (30,20), in primary cultures of rat astrocytes treated with TMT and other neurotoxic agents.

Materials and methods

Materials. TMT was obtained from Heraeus (Karlsruhe, Germany). Escherichia coli lipopolysaccharide (LPS) (serotype 0127:B8), hydrogen peroxide, staurosporine, sodium azide, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and protease inhibitors AEBSF [4-2(2-aminomethyl)-benzenesulfonylfluoride hydrochloride], aprotinin, leupeptin, bestatin, pepstatin A, and E-64 [trans-epoxysuccinyl-l-leucylamido-(4-guanidino)-butane] were from Sigma (Sigma, St. Louis, MO, USA). Rabbit and goat polyclonal antibodies against PAR-1, goat polyclonal antibody against actin and donkey anti-goat IgG conjugated to horseradish peroxidase (HRP) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Donkey anti-rabbit IgG-HRP linked was purchased from Amersham (Amersham Biosciences, Buckinghamshire, UK).

Cell culture. Astroglial cultures were prepared from mixed primary glial cultures obtained from neonatal rat cortex as described previously (31). Glial cells were seeded onto 24-well plates, and mantained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) (Invitrogen, Carlsbad, CA, USA) in 5% CO_2. Astroglial cultures consisted of >95% glial fibrillary acidic protein-positive cells.

Rat astrocytoma cell line C6 (ATCC, Manassas, VA, USA) was cultured in DMEM supplemented with 10% FCS (Invitrogen).

Cell viability. Lactate dehydrogenase (LDH) release in the culture medium was measured using a cytotoxicity detection kit (Roche, Mannheim, Germany) according to the manufacturer's protocols. Cell viability was also assayed by MTT reduction, essentially as described (32).

Immunocytochemistry. Astrocytes ($3x10^5$) were plated onto 24-well plates. Cells were exposed to TMT (10, 50 or 100 μM) for 24 and 48 h, washed in PBS 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 PBS, cells were preincubated for 1 h with 5% non-fat milk and incubated with rabbit polyclonal IgG against PAR-1 (Santa Cruz Biotechnology) at a final concentration of 2-4 μg/ml (overnight at 4˚C). These experiments were also performed with goat PAR-1 polyclonal antibody (Santa Cruz Biotechnology). Negative control experiments were performed substituting Igs against PAR-1 with equivalent amounts of non-specific Igs (2-4 μg/ml) or by preincubating the primary antibody with saturating amounts of the specific blocking peptide (Santa Cruz Biotechnology) (antigen/antibody weight ratio = 5 according to the manufacturer's instructions) (30). After washes with PBS, slides were incubated (1 h at RT) with a biotinylated secondary antibody (Vector Laboratories, Burlingame, CA), washed again, and incubated (30 min at RT) 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.

Western blot analysis. Cells were collected at 24 and 48 h after treatment with various neurotoxic agents: TMT (1, 10 μM), LPS (0.1, 1 μg/ml), staurosporine (0.5 μM), hydrogen peroxide (100 μM) and sodium azide (500 μM). Cells were then lysed in the following lysis buffer: 10 mM Tris/HCl, pH 7.4, 1% SDS, 2 mM AEBSF [4-2(2-aminomethyl)benzenesulfonyl-fluoride hydrochloride], 2 μM aprotinin, 40 μM leupeptin, 70 μM bestatin, 30 μM pepstatin A, and 30 μM E-64 [trans-epoxysuccinyl-l-leucylamido-(4-guanidino)-butane]. Samples were clarified by centrifugation at 1000 x g for 5 min. Equivalent amounts of protein (30 μg) were subjected to electrophoresis on 12.5% precast SDS-polyacrylamide gels (ExcelGel; Pharmacia Biotech, Uppsala, Sweden) using horizontal apparatus (Pharmacia Biotech). Separated proteins were then electro-transferred to nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany) by a semi-dry system (Novablot; Pharmacia Biotech) using a continuous buffer (39 mM glycine, 48 mM Tris) containing SDS (0.037% w/v) and methanol (20% v/v). To reduce the background, the blotted membranes were treated with 3% non-fat milk (Sigma) for 1 h at RT. The membranes were then incubated (overnight at 4˚C) with anti-PAR-1 Igs (Santa Cruz) at a final concentration of 0.1-0.2 μg/ml in 3% BSA. Controls were performed by substituting specific Igs with equivalent amounts of non-specific Igs or preabsorbing the primary PAR-1 antibody with the specific blocking peptide. To obtain an internal protein control, membranes were incubated with anti-actin antibody (Santa Cruz Biotechnology). The membranes were then washed with PBS-Tween 0.1% and treated with a secondary antibody conjugated to horseradish peroxidase (HRP). After a further wash, proteins were visualized using a chemiluminescence protein detection kit from Amersham Biosciences according to the manufacturer's instructions. Band intensity was measured using a PC-assisted CCD camera (GelDoc 2000 System/Quantity One software, Bio-Rad Laboratories, Hercules, CA, USA).

RT-PCR. Total RNA was isolated from rat astrocytes and C6 glioma cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The electrophoresis of 1 μg RNA in a 1% agarose/3% formaldehyde gel was used to check the quality of the total RNA. Only RNA revealing equally intense fluorescence bands of 18S and 28S rRNA after ethidium bromide staining was taken for further use. Then, 1 μg of total RNA was reverse-transcribed by using Superscript III reverse-transcriptase (Invitrogen). 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, 15 mM $MgCl_2$) were added to the mixture and the reaction was continued for 45 min

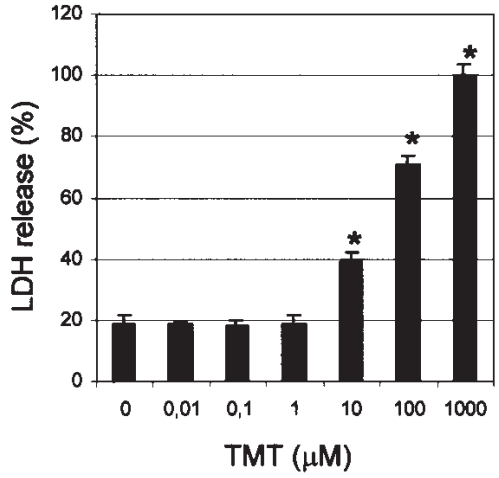

Figure 1. Cytotoxic effect of TMT on rat primary astrocytes revealed by LDH release at 24 h. Astrocytic death occurs in a concentration-dependent manner, becoming statistically significant at 10 μM TMT. Cell death is massive with doses above 100 μM TMT. n=3 experiments; $^{*}p<0.001$ versus controls (untreated cultures); ANOVA with Bonferroni-corrected t-test.

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. Three μl of the obtained cDNA were subjected to PCR by using specific primers for rat PAR-1 as previously described (30). The PCR products were analysed by performing 1.5% TBE agarose gel electrophoresis (Submarine Agarose Gel Unit, Hoefer, San Francisco, CA, USA). Gels were prestained with ethidium bromide (50 ng/100 ml). A PC-assisted CCD camera (GelDoc 2000 System/Quantity One Software; Bio-Rad) was used for gel documentation and quantification. All data were normalized against GAPDH mRNA level.

Cytokine assays. Interleukin-1ß (IL-1ß) and tumor necrosis factor-α (TNF-α) in culture supernatants and in cell lysates were assessed by specific ELISA kits purchased from R&D Systems (Abingdon, UK). All ELISA kits were used according to the manufacturer's instructions.

Nitrite levels. Nitrite (NO_2^-) levels were determined by using Griess reagent (1 mM sulfanilamide, 1 mM naphthylene-diamine dihydrochloride, and 100 mM HCl) in culture supernatants. Absorbance was measured at 540 nm, and NO_2^- concentration was determined using sodium nitrite as a standard.

Statistical analysis. All values in the figures are the arithmetic means ± SE of n determinations. Statistical analysis was performed by using Student's t-test and ANOVA followed by multiple comparison Bonferroni test, where appropriate.

Results

Cytotoxic effect of TMT. The cytotoxic effect of trimethyltin (TMT) was determined by LDH release (Fig. 1) and confirmed by MTT assay (not shown). Fig. 1 shows that TMT exerted its cytotoxic effect in a concentration-dependent manner.

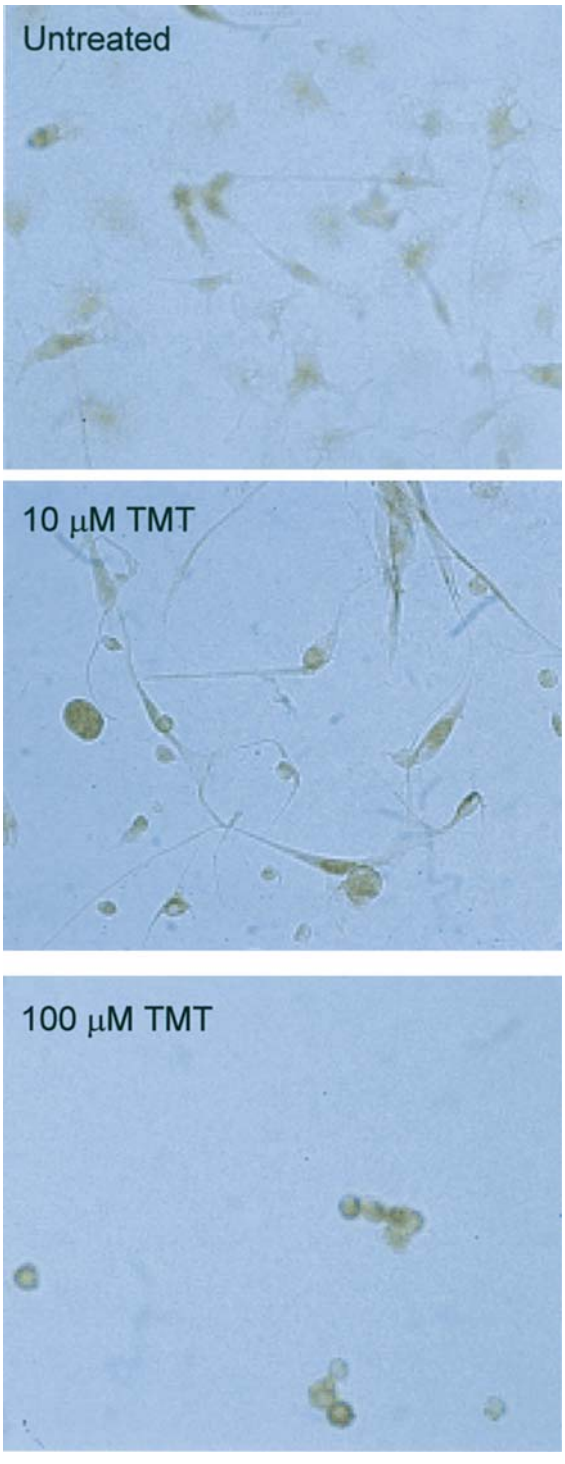

Figure 2. PAR-1-related immunoreactivity in rat primary astrocytes. Untreated astroglial cells show a specific diffuse immunostaining. After exposure to 10 μM TMT for 24 h, some cells undergo morphological changes; in particular, a rounding up of the cell body and a retraction of cell processes. These cells show strong PAR-1-related immunoreactivity. Following exposure to 100 μM TMT, all surviving astrocytes are rounded and intensely positive for PAR-1. Photographs show representative fields from four independent experiments.

Under these experimental conditions, TMT cytotoxicity became statistically significant at 10 μM and doses above 100 μM induced massive cell death. Thus, the following experiments were performed in a range of TMT concentrations between 10 and 100 μM.

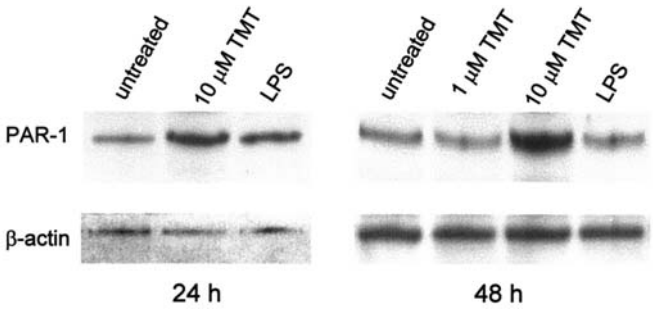

Figure 3. Western blot analysis of PAR-1 in rat primary astrocytes. After exposure to 10 μM TMT, PAR-1 expression increases at 24 h (left) and 48 h (right) with respect to the basal level. Doses below 10 μM TMT (1 μM in this experiment) were ineffective. LPS induces an upregulation of PAR-1 at 24 h (left) whereas, at 48 h (right), PAR-1 expression returns to the level of the untreated control cells. ß-actin was used as an internal control. The experiments were repeated five times with similar results.

Immunohistochemical localization. In primary cultures of astrocytes, weak diffuse immunostaining for PAR-1 occurred in untreated astroglial cells (Fig. 2). This immunoreactivity was highly specific as demonstrated by control experiments (not shown) performed with equivalent amounts of non-specific Igs or blocking peptide (for details see Materials and methods). TMT treatment (at 24 and 48 h) induced changes in both cellular morphology as well as in PAR-1-related immunoreactivity. In particular, at 10 μM TMT, strong specific immunostaining for PAR-1 was observed in several activated astrocytes, which showed a rounding up of the cell body and a retraction of cell processes (Fig. 2). The percentage of rounded and intensely positive cells increased at higher doses of TMT, reaching a maximum (100%) at 100 μM (Fig. 2).

Western blot analysis. Immunoblotting was performed on lysates of astroglial cultures after exposure to 1 and 10 μM TMT for 24 and 48 h. According to the above results, 10 μM TMT corresponded to the minimal dose exerting cytotoxic activity on glial cells and determining increased specific immunostaining for PAR-1. In astrocyte lysates, the anti-PAR-1 antibody recognised a band of 48 kDa corresponding to the unglycosylated form of the receptor (33). After treatment with 10 μM TMT, the intensity of this specific band was increased at 24 h as well as 48 h compared to controls (Fig. 3).

To check if the observed PAR-1 upregulation after TMT treatment in astroglial cells was an effect specifically induced

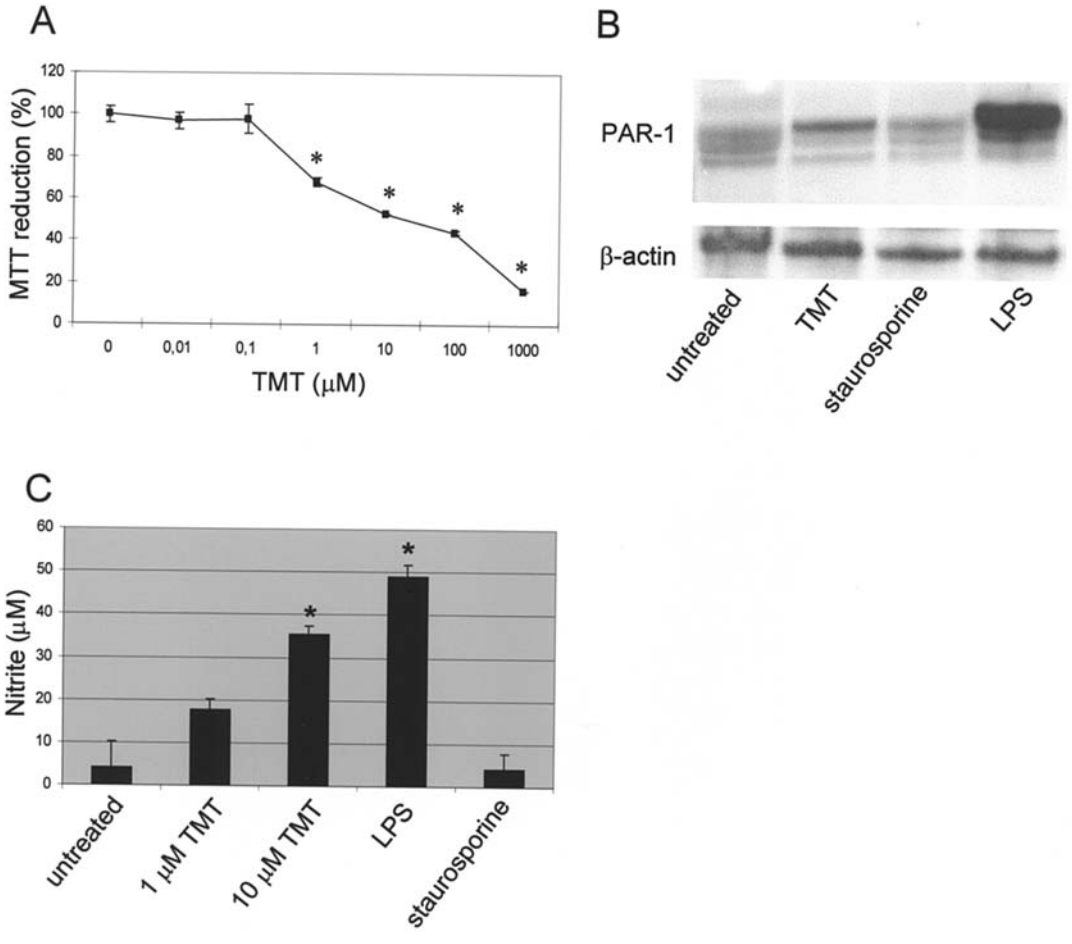

Figure 4. Effects of TMT and LPS treatment on the rat C6 astrocytoma cell line. (A) MTT assay demonstrates that TMT exerts cytotoxic activity in a concentration-dependent manner. n=3 experiments; *p<0.001 versus untreated cultures; ANOVA with Bonferroni-corrected t-test. (B) Western blot analysis shows that TMT and LPS determine an evident upregulation of PAR-1 at 24 h whereas no variations of PAR-1 were observed following staurosporine treatment. ß-actin was used as an internal control. Western blot experiments were repeated five times with similar results. (C) Nitrite assay reveals that TMT and LPS (but not staurosporine) induce an increased production of nitrite in the culture medium at 48 h in comparison to control (untreated cells). n=3 experiments; *p<0.001 versus untreated cultures; ANOVA with Bonferroni-corrected t-test.

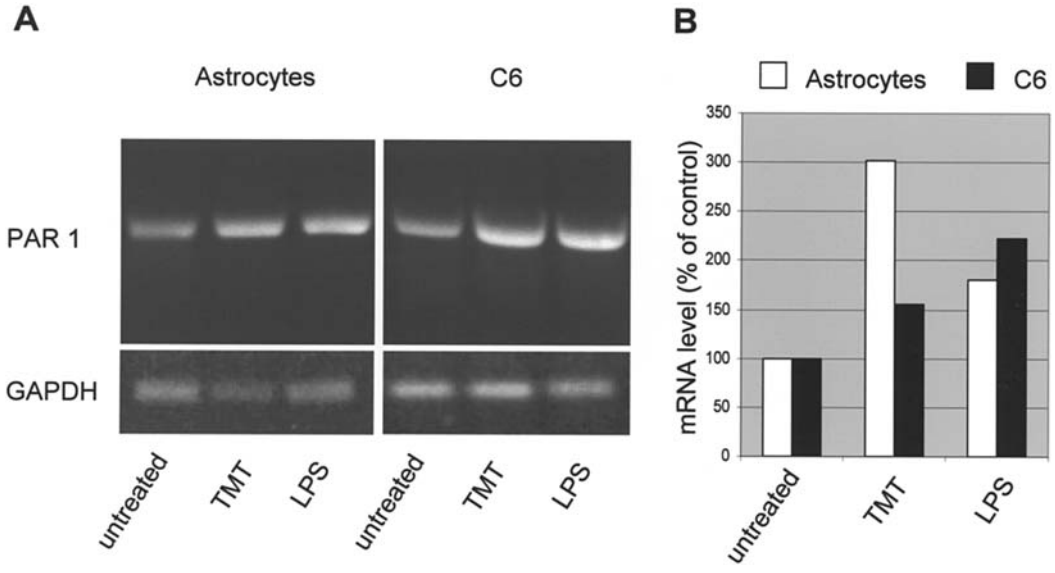

Figure 5. Semiquantitative RT-PCR analysis on rat primary astrocytes (left) and C6 astrocytoma cells. (A) The gels show mRNA levels of PAR-1 in untreated cells and in cells exposed for 12 h to TMT or LPS. It is evident that both TMT and LPS upregulate PAR-1 mRNA. GAPDH mRNA was used as an internal standard. RT-PCR experiments were repeated four times with similar results. (B) Densitometric analysis of gels shown in panel A. The mRNA signal was normalized to the GAPDH signal from the same RT product.

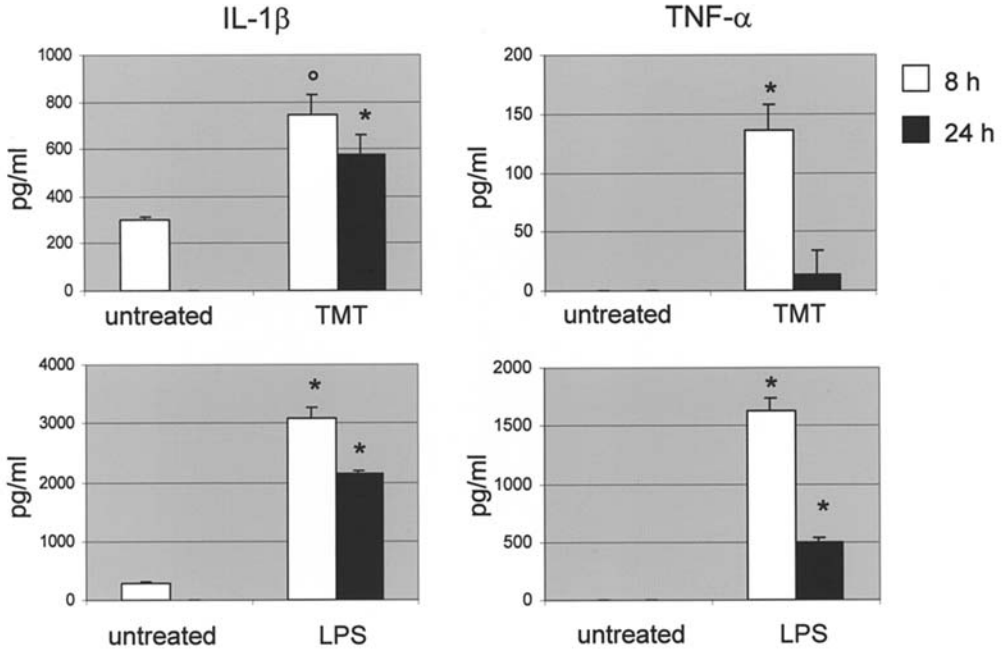

Figure 6. IL-1ß and TNF-α synthesis from rat primary astrocytes assessed by ELISA. Both LPS and TMT determine an increased production of IL-1ß measured in cell lysates and TNF-α detected in culture medium of astrocytes at 8 and 24 h. n=3 experiments; °p<0.05, *p<0.001 versus untreated cultures; ANOVA with Bonferroni-corrected t-test.

by this neurotoxicant or represented a common reaction to other stress agents causing cell death or glial cell activation, we decided to analyze PAR-1 expression in astrocytes treated with staurosporine, hydrogen peroxide, sodium azide, and LPS. We found that, while staurosporine, hydrogen peroxide and sodium azide were ineffective in modifying PAR-1 expression at both 24 and 48 h (not shown), an evident upregulation of this receptor appeared following astrocyte exposure to LPS. This increased expression of PAR-1 due to LPS was transient in time, returning to the control level at 48 h (Fig. 3).

In order to exclude that our results were due to eventually contaminating microglia, which also contains PAR-1 (4), the previous experiments were repeated on a rat astrocytoma cell line (C6). As shown in Fig. 4, a clear upregulation of PAR-1 occurred also in this cell line following TMT and LPS treatment (Fig. 4b). According to literature data (34), this rat astrocytoma cell line was sensitive to TMT-treatment as shown by MTT assay (Fig 4a). Furthermore, in both TMT- and LPS-treated C6 astrocytoma cells, an increased concentration of nitrite was measured in the culture medium at 48 h (Fig. 4c).

RT-PCR. Semiquantitative RT-PCR experiments were performed at different times (4, 8, 12 and 24 h) after exposure of astrocytes and C6 astrocytoma cells to TMT and LPS. Both these treatments significantly upregulated PAR-1 at 12 h (Fig. 5). Besides, the amount of PAR-1 mRNA dropped to the basal level after 24 h of TMT and LPS treatment. The expression of the house-keeping gene, GAPDH, was used as an internal standard.

Cytokine release. TNF-α and IL-1ß levels were measured by ELISA in both LPS- and TMT-stimulated astrocyte cultures at 8 and 24 h of treatment. The results showed that TMT determines an increased production of IL-1ß at 8 h, which remains constant up to 24 h (Fig. 6). Furthermore, the release of TNF-α in TMT-treated astrocytes peaks at 8 h and then declines at 24 h (Fig. 6). The levels of IL-1α and TNF-α in LPS-treated astrocytes at 8 and 24 h are shown for comparison (Fig. 6).

Discussion

Proteinases and their cognate proteinase-activated receptors (PARs) are known to play important roles in the ontogeny and pathophysiology of the nervous system (7,8). Recently, we studied the behaviour of thrombin system components in the rat hippocampus following administration of trimethyltin (TMT) (30), a potent neurotoxicant that causes a loss of pyramidal neurons associated to astrogliosis and microglia response (25-29). In particular, by RT-PCR and immunohistochemistry we were able to demonstrate that PARs, namely PAR-1, were upregulated in reactive astrocytes of CA3 and hylus regions (30), i.e. hippocampal areas that represent the main target of TMT injury (26,29). In order to gain further insights into this phenomenon, we extended our study to *in vitro* TMT-treated rat astrocytes.

In particular, Western blot and RT-PCR experiments showed that, in primary cultures of neonatal rat astrocytes also, PAR-1 was upregulated after exposure to TMT. This effect was unlikely due to eventually contaminating microglial cells, as similar PAR-1 upregulation also occurred in a C6 rat astrocytoma cell line, which is free of other contaminating cells. Immunohistochemical analysis indicated that TMT-activated astroglial cells, which presented a rounded body and a reduction of processes, contained more PAR-1 with respect to control resting cells (showing a normal morphology). These immunocytochemical results are in line with our Western blot and RT-PCR data (Figs. 3 and 5) and strongly support the view that the PAR-1 upregulation that we observed in previous *in vivo* experiments following TMT administration (30) is caused by direct TMT action on astrocytes.

In the present experiments, we also detected PAR-1 upregulation (mRNA and protein) following exposure of astrocytes to LPS whereas no changes were found following treatment with staurosporine, hydrogen peroxide and sodium azide. Interestingly, while staurosporine, hydrogen peroxide and sodium azide are all neurotoxic factors inducing apoptosis or necrosis of cells (35-37), LPS is a compound that causes mainly glial cell activation with production of proinflammatory cytokines (such as IL-1ß and TNF-α) and nitric oxide release (present results and refs. 38-40). It is known from the literature (41,27), and we have confirmed in the present experiments, that TMT also induces the synthesis of IL-1ß and TNF-α by astrocytes. Therefore, the PAR-1 upregulation we detected in TMT-treated astroglial cells is probably a reflection of glial cell activation rather than of cell death.

Concerning this connection, it should be noted that: a) PAR-1-mediated activation of astrocytes is known to induce the synthesis of inflammatory mediators, such as IL-1ß and iNOS (16); b) IL-1ß and other proinflammatory cytokines have been shown to induce PAR-1 overexpression in some organs, such as skeletal muscle (42) and liver (43). Although data concerning point b) are lacking at present for glial cells, the following events may be hypothesised: TMT and LPS may activate glial cells according to their own mechanisms (44-46). This glial activation involves a series of complex morphological and biochemical processes including the binding of thrombin and related proteases to their physiological receptor, PAR-1. This PAR-1 activation would induce cytokine release from astroglial cells (see point a), which would in turn cause PAR-1 upregulation (see point b). If so, this upregulation would represent a mechanism amplifying astrocytic activation and glial inflammatory response. Further studies are necessary to substantiate this hypothesis.

Acknowledgements

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