British Journal of Pharmacology (1997) 122, 1483–1489

Lipopolysaccharide induces expression of tumour necrosis factor alpha in rat brain: inhibition by methylprednisolone and by rolipram

†M. Buttini, *A. Mir, K. Appel, K.H. Wiederhold, S. Limonta, *P.J. Gebicke-Haerter & [1]H.W.G.M. Boddeke

Novartis Ltd, Preclinical Research S-360.402, CH-4002 Basel, and *Psychiatric University Clinic, Freiburg University Medical School, Hauptstr. 5, D-79104 Freiburg-i.-Br

1 We have investigated the effects of the phosphodiesterase (PDE) type IV inhibitor rolipram and of the glucocorticoid methylprednisolone on the induction of tumour necrosis factor alpha (TNF-α) mRNA and protein in brains of rats after peripheral administration of lipopolysaccharide (LPS).

2 After intravenous administration of LPS, a similar time-dependent induction of both TNF-α mRNA and protein was observed in rat brain. Peak mRNA and protein levels were found 7 h after administration of LPS.

3 *In situ* hybridization experiments with a specific antisense TNF-α riboprobe suggested that the cells responsible for TNF-α production in the brain were microglia.

4 Intraperitoneal administration of methylprednisolone inhibited the induction of TNF-α protein in a dose-dependent manner. A maximal inhibition of TNF-α protein production by $42.9 \pm 10.2\%$ was observed at a dose regimen consisting of two injections of each 30 mg kg^{-1} methylprednisolone.

5 Intraperitoneal administration of rolipram also inhibited the induction of TNF-α protein in a dose-dependent manner. The maximal inhibition of TNF-α protein production was $96.1 \pm 12.2\%$ and was observed at a dose regimen of three separate injections of each 3 mg kg^{-1} rolipram.

6 *In situ* hybridization experiments showed that the level of TNF-α mRNA induced in rat brain by LPS challenge was reduced by intraperitoneal administration of methylprednisolone (2×15 mg kg^{-1}) and of rolipram (3×3 mg kg^{-1}).

7 We suggest that peripheral administration of LPS induces a time-dependent expression of TNF-α in rat brain, presumably in microglial cells, and that methylprednisolone and rolipram inhibit LPS-induced expression of TNF-α in these cells via a decrease of TNF-α mRNA stability and/or TNF-α gene transcription.

Keywords: TNF-α; rolipram; methylprednisolone; bacterial lipopolysaccharide; microglia; rat brain

Introduction

The cytokine tumour necrosis factor a (TNF-α) plays a critical role in a variety of host defence as well as tumour cytotoxicity mechanisms (for a review, see Beutler & Cerami, 1988). As a proinflammatory cytokine, TNF-α is involved in the regulation of inflammatory responses, such as synthesis of prostaglandins (Dayer *et al*., 1985), activation of neutrophils (Shalaby *et al*., 1985), modulation of the function of vascular endothelial cells (Stolpen *et al*., 1986) and septic shock (Beutler & Cerami, 1986). TNF-α is a peptide secreted by macrophages upon stimulation with lipopolysaccharide (LPS), an endotoxin of Gram-negative bacteria (Carswell *et al*., 1975; Beutler *et al*., 1985). The mature TNF-α, which is derived from a 26 kDa propeptide, is a homotrimer with a subunit molecular weight of 17 kDa (Pennica *et al*., 1985; Jue *et al*., 1990).

Intracerebral occurrence of TNF-α has been reported in variety of pathological states or experimental disease models, such as infectious meningitis (Frei *et al*., 1990; Hunter *et al*., 1992), cerebral malaria (Lucas *et al*., 1992), administration of endotoxin (Gatti & Bartfai, 1993; Laye *et al*., 1994), multiple sclerosis (Hofman *et al*., 1989; Sharief & Hentges, 1991), AIDS dementia complex (Merrill & Chen, 1991) and experimental trauma (Taupin *et al*., 1993). Central effects of TNF-α include fever and induction of slow-wave sleep (Dinarello *et al*., 1986), stimulation of the hypothalamo–pituitary–adrenal axis and inhibition of the hypothalamo–pituitary–gonadal axis (Rivier, 1993). Possible cellular sources of TNF-α in the brain could be microglia (Righi *et al*., 1989; Ganter *et al*., 1992; Buttini *et al*., 1996), astrocytes (Liebermann *et al*., 1989) or neurons (Breder *et al*., 1994; Liu *et al*., 1994). *In vitro*, TNF-α has been shown to mediate damage to oligodendrocytes and myelin (Selmaj & Raine, 1988) and to induce astrocytic proliferation (Selmaj *et al*., 1990), thus probably contributing to demyelination and reactive gliosis after brain injury.

In the present study, we have investigated the induction of TNF-α mRNA and protein in the rat brain after peripheral administration of bacterial lipopolysaccharide. Additionally, we have assessed the effect of rolipram, a specific inhibition of the type IV family of phosphodiesterases (Beavo & Reifsnyder, 1990) which has been shown to selectively inhibit LPS-stimulated TNF-α release *in vitro* (Semmler *et al*., 1993) and *in vivo* (Sekut *et al*., 1995), and of the synthetic corticosteroid methylprednisolone on the LPS-induced TNF-α expression in rat brain.

Methods

Chemicals

Bacterial lipopolysaccharide (*E. Coli* 055:B5) was purchased from Westphal Difco (Detroit, MI). Methylprednisolone 21-

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[1] Author for correspondence.

†Present address: Gladstone Molecular Neurobiology Program and Department of Neurology, University of California, San Francisco, CA 94141-9100, U.S.A.

hemisuccinate was purchased from Sigma (St Louis, MS) and rolipram was synthesized in house. Methylprednisolone was dissolved in H_2O and rolipram was dissolved in a 1:1 mixture of polyethyleneglycol 400 (PEG)/H_2O).

Animal treatments

Male Sprague-Dawley rats (180–200 g) were injected intravenously (i.v.) with a sublethal dose of 5 mg kg^{-1} LPS dissolved in sterile saline. Control animals received injections of 0.9% saline alone. To determine the time-dependent induction of TNF-α mRNA and protein in the brain, animals were killed at different time points ($n=3-7$ per time point) after the injection of LPS. Animals treated with methylprednisolone received a first intraperitoneal (i.p.) injection of methylprednisolone 30 min before the i.v. injection of LPS, and a second i.p. injection of methylprednisolone 3 h after the LPS injection. Three different doses of methylprednisolone were administered: 3 mg kg^{-1} ($n=3$), 15 mg kg^{-1} ($n=5$) and 30 mg kg^{-1} ($n=5$). Animals treated with rolipram received a first i.p. injection of rolipram 30 min before the LPS injection, a second i.p. injection of rolipram 2 h, and a third i.p. injection of rolipram 5.5 h after the LPS injection. Three different doses of rolipram were administered: 0.03 mg kg^{-1} ($n=3$), 0.3 mg kg^{-1} ($n=5$) and 3 mg kg^{-1} ($n=4$). Control animals received injections of LPS and of vehicle (0.9% saline for methylprednisolone, $n=11$; and a 1:1 mixture of PEG 400/H_2O for rolipram, $n=7$). All the animals assessed for the effect of rolipram or methylprednisolone on LPS-induced TNF-α expression were killed 7 h after LPS injection. The control experiments and experiments with compounds were performed in a pooled modus.

Cloning of TNF-α cDNA from isolated microglial cells

Microglia were isolated from mixed astroglia cultures and treated with lipopolysaccharide as described previously (Gebicke-Haerter *et al.*, 1989). Based on morphological, immunological and pharmacological criteria, the purity of microglia isolated by this procedure has been shown to be >99%. Microglial RNA was extracted according to Chomczynski & Sacchi (1987). Then 1.0 μg of total RNA was reverse transcribed for 60 min and cDNA was specifically amplified by addition of oligonucleotide primers and DNA polymerase from Thermophilus aquaticus (Gene Amp RNA PCR Kit, Perkin Elmer Cetus, U.S.A.). The following primer pairs were used: 5′-ATGAGCACAGAAAGCATGATC and 5′-CAGAGCAATGACTCCAAAGTA (Estler *et al.*, 1992). The polymerase chain reaction (PCR) was run in 30 cycles with denaturation at 94°C for 1 min, annealing at 60°C for 1 min and extension at 72°C for 1.30 min on a programmable thermal cycler (Mini-Cycler, MJ Research, Inc., U.S.A). The 704 bp PCR-product was cloned into pCRTMII plasmid according to the supplier's instructions (TA Cloning Kit, Invitrogen, U.S.A.). Partial sequencing of the obtained cDNA on an automatic sequencer (ALF, Pharmacia, Sweden) confirmed the identity with rat cDNA for TNF-α (Estler *et al.*, 1992).

RNA probes and in vitro *transcription*

Antisense and sense riboprobes to TNF-α mRNA were generated from the pCRTMII plasmid containing rat TNF-α cDNA (see above). Linearization of this plasmid with NotI, followed by transcription with Sp6 RNA polymerase (Boehringer Mannheim) generates an TNF-α antisense riboprobe; linearization with *Bam*HI and transcription with T7 RNA polymerase generates the corresponding sense probe.

For radioactive *in situ* hybridization, the riboprobes were synthesized *in vitro* using [33P]UTP (DuPont-NEN) according to Promega's recommended protocol. labelled probes were purified on a Bio-Spin 30 column (Biorad) according to manufacturer's instructions. For non-radioactive *in situ* hybridization, digoxigenin-labelled probes were synthesised in an *in vitro* transcription reaction containing 2 μg linearized DNA template, 500 mM digoxigenin-labelled UTP (Boehringer Mannheim), 500 mM CTP, 500 mM GTP, 500 mM ATP, 50 mM UTP, 100 mM dithiothreitol (DTT), 40 U RNase inhibitor, 40 mM Tris-HCl (pH 7.5), 6 mM $MgCl_2$, 2 mM spermidine, 5 mM NaCl and 1000–2000 U DNA-dependent RNA polymerase. The transcription reaction was run for 45 min at 37°C, then additional 1000 U of the appropriate polymerase were added and the reaction mixture was incubated for an another 45 min. The DNA template was degraded by addition of 2 U DNAse I and incubation at 37°C for 10 min. The DNAse reaction was stopped by addition of 80 μl 25 mM EDTA. The digoxigenin-labelled probes were then purified on a Bio-Spin 30 column (Biorad) according to manufacturer's instructions. Before being used for hybridization, radioactively-labelled and digoxigenin-labelled transcripts were ethanol precipitated, degraded to an average length of 150 bp by partial alkaline hydrolysis, ethanol precipitated again and resuspended in 80 ml TE buffer (1 mM EDTA, 10 mM Tris-HCl, pH 7.4) containing 0.1 M DTT.

In situ *hybridization*

At different time points after injection of LPS (1 h, 3 h, 6 h, 7 h, 8 h, 1 day, 3 days; 2 animals per time point), rats were anaesthetized and killed by decapitation. Rats treated with LPS and 15 mg kg^{-1} methylprednisolone (2 injections, see above) and rats treated with LPS and 3 mg kg^{-1} rolipram (3 injections, see above) were anesthetized and killed by decapitation 7 h after i.v. injection of LPS (3 animals per treatment group). The brains of all the rats were quickly removed and frozen on dry ice. Twenty micron cryostat sections from brains of control and LPS-treated rats were thaw-mounted onto Vectabond-R-coated slides and stored at −70°C until used. Prior to *in situ* hybridization, sections were thawed, air-dried and fixed by immersion for 20 min at room temperature in 4% paraformaldehyde in 1 × PBS (2.6 mM KCl, 1.4 mM KH_2PO_4, 136 mM NaCl, 8 mM Na_2HPO_4, pH 7.4), washed once in 3 × PBS, twice in 1 × PBS, 5 min each, and then incubated for 10 min in 0.1 M triethanolamine, pH 8, containing 0.25% acetic anhydride. After two washes in 1 × PBS, 5 min each, sections were processed for radioactive or non-radioactive *in situ* hybridization. In case of radioactive *in situ* hybridization, sections were additionally dehydrated through a graded series of ethanol (60, 80, 95 and 100%, 2 min each), immersed for 10 min in chloroform, partially rehydrated again by immersion in 100% ethanol and 95% ethanol, 2 min each, and air-dried.

Radioactively labelled transcripts were used at a concentration of 2 to 2.5×10^7 cpm ml^{-1} hybridization buffer, and the digoxigenin-labelled transcripts at a concentration of 400–500 ng ml^{-1} hybridization buffer (4 × SSC (1 × SSC: 150 mM NaCl, 15 mM Na-citrate), 50% formamide, 10% dextran sulfate, 1 × Denhardt's (0.02% polyvinylpyrrolidone, 0.02% Ficoll, 0.02% bovine serum albumin), 250 μg ml^{-1} yeast tRNA, 400 μg ml^{-1} salmon sperm DNA, 500 μg ml^{-1} heparin sodium salt). In case of non-radioactive *in situ* hybridization, prehybridization was performed for 3 h at room temperature. No prehybridization was necessary in the case of radioactive *in situ* hybridization. For hybridization, each slide was overlaid with 70 μl hybridization solution containing the appropriate amount of labelled transcript and incubated overnight at 55°C in a chamber humidified with 4 × SSC. After hybridization, sections were washed for 10 min in two changes of 2 × SSC at room temperature and 30 min in 0.1 × SCC at 70°C. They were then treated with RNaseA (20 μg ml^{-1} in RNase buffer: 0.5 M NaCl, 1 mM EDTA, 10 mM Tris-HCl, pH 7.4) for 30 min at 37°C. After a wash in RNase buffer for 30 min at room temperature, sections were subjected to two additional high stringency washing steps in 0.1 × SSC at 70°C, 40 min each. Sections hybridized with radioactively labelled transcripts were dehydrated and exposed to β-max film (Amersham) for 1 month. Sections hybridized with digoxigenin-labelled transcripts were rinsed in 2 × SSC and subjected to immunological detection as described below.

Immunological detection of digoxigenin-labelled hybrids

Slides were washed in 1 × PBS for 10 min at room temperature. Non-specific binding sites were blocked by incubation in PBS containing 3% sheep serum and 0.3% Triton X-100 for 45 min at room temperature. Slides were then incubated in the same buffer with 1:500 diluted alkaline-phosphatase conjugated anti-digoxigenin antibody for 2 h at 37°C or overnight at 4°C. After three washes in PBS, 10 min each and incubation for 5 min in TBS, pH 9.2, containing 50 mM $MgCl_2$, colour development was carried out for 14–16 h at room temperature with 0.3 mg ml^{-1} nitroblue tetrazolium (NBT) and 0.1 mg ml^{-1} 5-bromo-4-chloro-3-indolyl phosphate (BCIP) in the same buffer containing 0.24 mg ml^{-1} levamisole at room temperature. The colour reaction was stopped by transferring the slides into distilled water. Sections were then covered with Crystal-Mount medium (Biomedia), dried for 20 min at 50°C in the dark and coverslipped with Depex (Merck).

Preparation of brain extracts

At different time points after injection of LPS (1 h, 4 h, 7 h, 13 h, 18 h and 24 h, 3–7 animals per time point), rats were deeply anaesthetized with 50 mg kg^{-1} pentobarbital and subsequently flush-perfused with PBS (100 ml per rat) to remove the blood. Rats treated with LPS and either methylprednisolone (2 injections, see above) or rolipram (3 injections, see above) were processed the same way at 7 h after i.v. injection of LPS. Brains were removed, roughly minced with scissors and then homogenized with a Potter-Elvehjem homogenizer in PBS at 4°C. Homogenates were centrifuged for 10 min at 1000 *g* and 4°C to remove debris. The pellets were discarded, and the protein content of the supernatant was determined using the Bradford Protein Assay from Biorad according to manufacturer's instructions. Brain homogenates were snap-frozen in liquid nitrogen and kept at −80°C until further use.

TNF-α-specific ELISA assay

Brain homogenates were quickly thawed, centrifuged (500 *g* for 1 min), and TNF-α protein content of the supernatants was determined without any dilution using an ELISA. Nunc maxi sorp 96-well plates were coated with 3 μg ml^{-1} PBS monoclonal hamster antimurine TNF-α (Genzyme: 1221-00). After coating the plates were washed four times with washing solution (PBS, Tween 0.02% sodium azide). Subsequently the plates were incubated for 2 h at 37°C with PBS containing 2% BSA and 0.02% sodium azide. Samples or a dilution series of 62.5–8000 pg ml^{-1} recombinant TNF-α (Innogenetics: CY-044) were added and the plates were incubated overnight at room temperature. The plates were washed four times with washing solution and incubated 2 h at 37°C with PBS containing 3 μg ml^{-1} polyclonal rabbit-anti-rat TNF-α (Innogenetics CY-051) and 2% BSA. The plates were washed four times with washing solution and incubated 2 h at 37°C with a 1:500 dilution of monoclonal anti-rabbit IgG labelled with alkaline phosphatase (Sigma A-2556) in PBS containing 2% BSA. The plates were washed four times with PBS and incubated 30 min with a 10% diethanolamine buffer (pH = 9.8)) containing 1 mg ml^{-1} *p*-Nitrophenyl phosphate (104 Sigma) and 0.5 mM $MgCl_2$. The absorbance at 405 nm was determined.

Statistical analysis

Data were expressed as means ± s.e.mean and were analysed by one-way analysis of variance. Post-hoc analyses were carried out by Dunnet's multiple comparison test. *P* values less than 0.05 were considered significant.

Results

LPS-induced expression of TNF-α in rat brain

Whereas no TNF-α mRNA hybridization signals were detected in brains of saline injected rats (Figure 1A), intravenously administered LPS induced a time-dependent expression of TNF-α mRNA in the rat brain. The first TNF-α mRNA signals appeared 3 h after injection of LPS in the rostral parts of the brain (brainstem, cerebellum, mesencephalic structures, thalamus) (Figure 1B). From 6 to 8 h after LPS administration, TNF-α mRNA hybridization signals were visible throughout the rat brain (Figure 1C). The strongest hybridi-

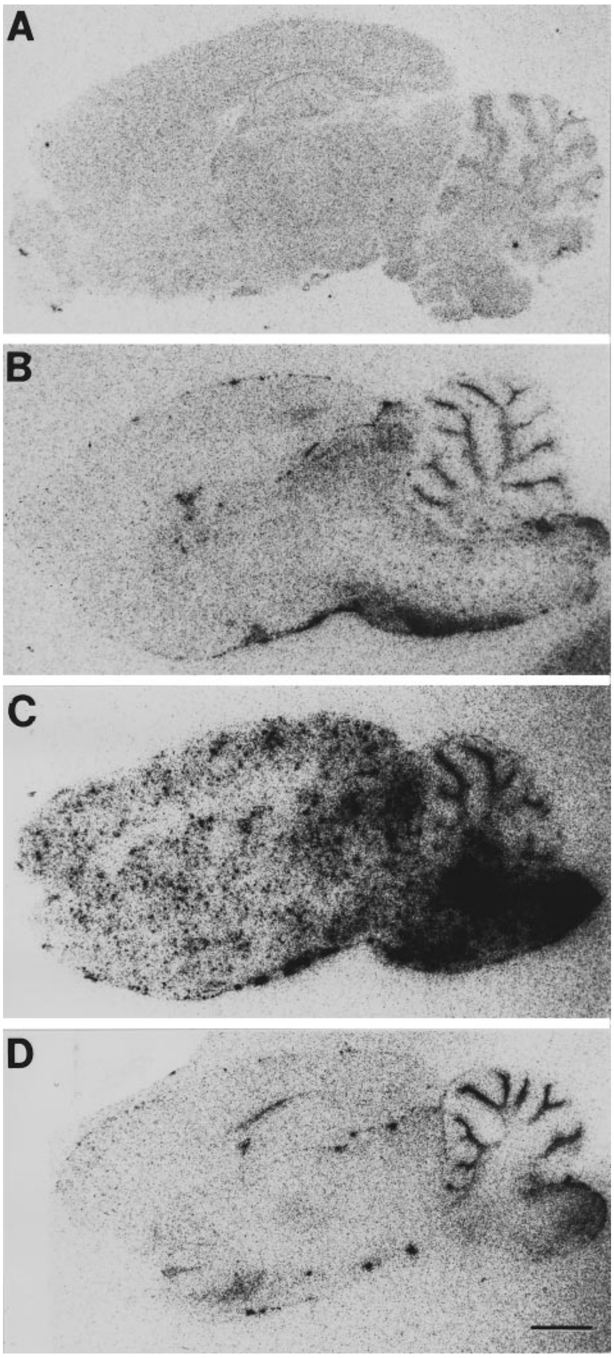

Figure 1 Induction of TNF-α mRNA in rat brain following i.v. injection of 5 mg kg^{-1} LPS as visualized by radioactive *in situ* hybridization with a ^{33}P-UTP labelled cRNA. TNF-α hybridization pattern in the brain of a saline-injected rat (A) and in the brains of LPS-treated rats 3 h (B), 8 h (C) and 1 day (D) after LPS administration. Scale bar = 0.2 cm.

zation signals were detected in the brainstem and the cerebellum. One day after administration of LPS, TNF-α mRNA hybridization signals were still visible in the brainstem and the cerebellum, and scattered signals were still present in the rest of the brain (Figure 1D). Two days after administration of LPS, TNF-α mRNA could no longer be detected (data not shown). Experiments using TNF-α mRNA sense riboprobes for hybridization on brain sections from LPS-treated rats showed no signals at all, thus confirming the specificity of the *in situ* hybridization procedure (data not shown). Non-radioactive *in situ* hybridization with a digoxigenin-UTP labelled cRNA showed that the TNF-α mRNA positive cells in the brains of LPS-treated rats displayed a ramified and branched morphology, which is typical for microglia (Figure 2).

To perform quantitative analysis of TNF-α protein expression in brain extracts of LPS-treated rats, we have used a TNF-α-specific ELISA assay. A similar time curve as observed for TNF-α mRNA expression was found for TNF-α protein expression (Figure 3). Whereas under control conditions (in saline-injected animals) no TNF-α protein could be detected, a maximal increase of TNF-α protein was observed 7 h after administration of LPS (Figure 3).

Inhibition of LPS-induced TNF-α production in rat brain by methylprednisolone and rolipram

The effect of the antiinflammatory compound methylprednisolone and of the phosphodiesterase (PDE) IV inhibitor rolipram on TNF-α expression in the brain was evaluated 7 h after i.v. administration of LPS.

Intraperitoneal administration of methylprednisolone induced a dose-dependent inhibition of TNF-α protein production, which however was only significant at a dose of 2×30 mg kg^{-1}; $P<0.05$ (Figure 4). The maximal inhibition induced by methylprednisolone (2×30 mg i.p.) was $42.9\pm10.3\%$ ($n=5$). Administration of the methylprednisolone vehicle (0.9% saline) was without effect on LPS-induced TNF-α expression in the brain (Figure 4). However, administration of the rolipram vehicle (H_2O/PEG) consistently enhanced the LPS-induced TNF-α protein expression by $160\pm34.7\%$ when compared to injection of 0.9% saline/LPS (methylprednisolone control) or to injection of LPS alone (Figure 5). The PDE IV inhibitor rolipram, at a dose regimen of 3×3 mg kg^{-1} administered i.p., induced a significant reduction of $96.1\pm12.2\%$ of TNF-α expression when compared to vehicle-injected (H_2O/PEG) controls ($P<0.05$; $n=4$) (Figure 5).

To determine whether the inhibition of TNF-α production by methylprednisolone and rolipram was due to decreased levels of TNF-α mRNA, we performed *in situ* hybridization on brain sections of rats treated with LPS and the maximally effective doses of methylprednisolone or rolipram. Both, methylprednisolone and rolipram markedly reduced the TNF-α mRNA levels in comparison to vehicle-injected controls (Figure 6).

Discussion

The present study demonstrates that peripheral administration of LPS induces a transient and time-dependent expression of TNF-α presumably in microglial cells throughout the rat brain, which is dose-dependently inhibitable by administration of methylprednisolone or rolipram.

The time curve of induction of TNF-α mRNA in the rat brain following peripheral stimulation with LPS is comparable to that described for IL-1β mRNA (Buttini & Boddeke, 1995). However, as apparent from the comparatively long exposure

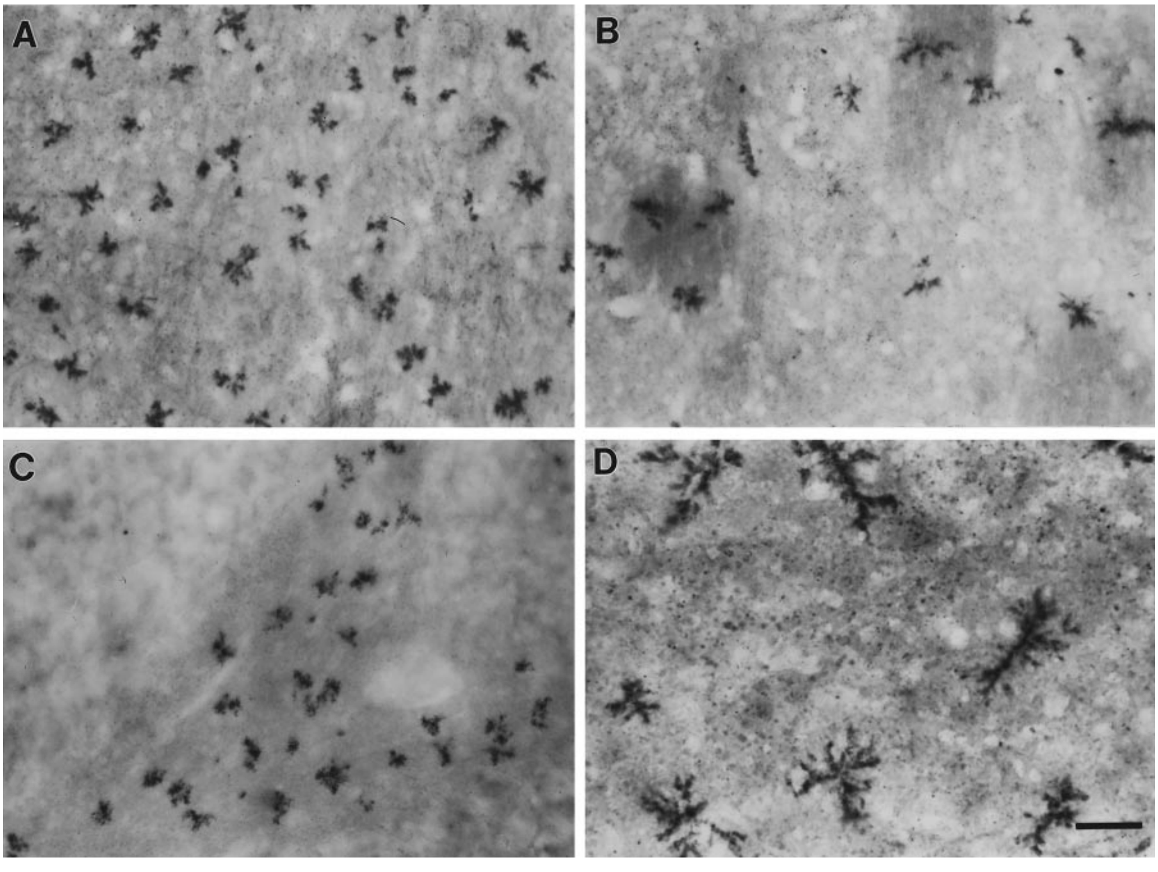

Figure 2 Visualization of TNF-α mRNA positive cells in different brain regions 8 h after i.v. injection of 5 mg kg^{-1} LPS using non-radioactive *in situ* hybridization with a digoxigenin-labelled cRNA. (A) thalamus, (B) caudate putamen, (C) cerebellum, and (D) high magnification of TNF-α mRNA positive cells in the caudate putamen. Note the ramified morphology typical for microglial cells. Scale bars = 50 μm (a–c), 15 μm (d).

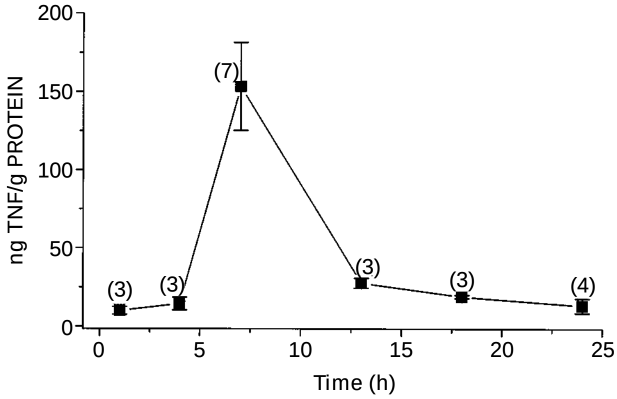

Figure 3 Time-dependent expression of TNF-α protein in the rat brain following i.v. injection of 5 mg kg^{-1} LPS. At different time-points following LPS injection, rats were killed and brain extracts were prepared as described in Methods. TNF-α protein content was determined using a specific ELISA. The values represent means ±s.e.mean. The number of animals tested at each time point is indicated in the graph.

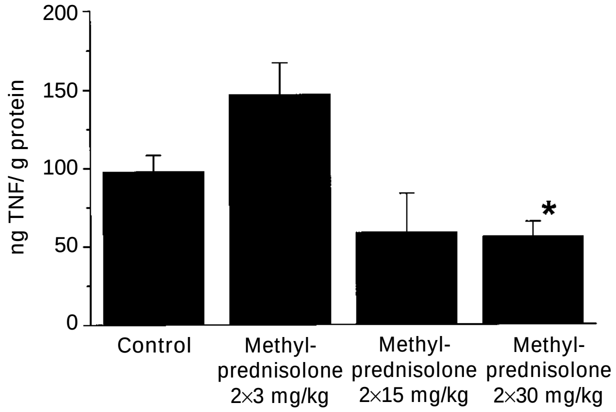

Figure 4 Inhibition of LPS-induced TNF-α protein production in rat brain by methylprednisolone. Animals received 2 i.p. injections of methylprednisolone, one before and one after LPS injection (see Methods). Data represent TNF-α protein concentrations measured in control animals injected with vehicle ($n=11$), in animals injected with 2×3 mg kg^{-1} methylprednisolone ($n=5$), in animals injected with 2×15 mg kg^{-1} ($n=5$) and in animals injected with 2×30 mg kg^{-1} methylprednisolone ($n=4$) and are expressed as means±s.e.mean. The asterisk (*) indicates significant inhibition of TNF-α production by methylprednisolone. ($P<0.05$).

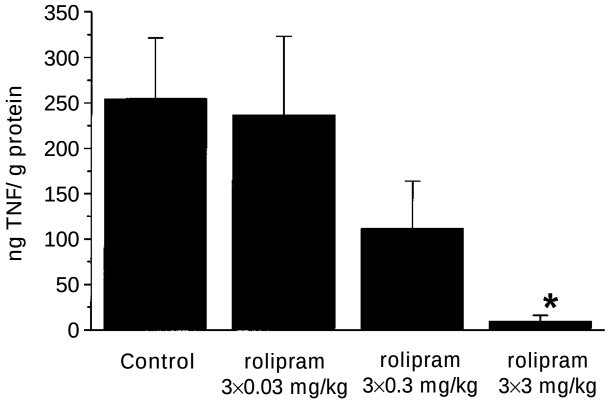

Figure 5 Inhibition of LPS-induced TNF-α production in rat brain by rolipram. Animals received 3 i.p. injections of rolipram, one before and two after LPS injection (see Methods). Data represent TNF-α protein concentrations measured in control animals injected with vehicle ($n=7$), in animals injected with 3×0.03 mg kg^{-1} rolipram ($n=3$), in animals injected with 3×0.3 mg kg^{-1} rolipram ($n=5$) and in animals injected with 3×3 mg kg^{-1} rolipram ($n=4$) and are expressed as means±s.e.mean. The asterisk (*) indicates significant inhibition of TNF-α production by rolipram ($P<0.05$).

times which were necessary for visualization of TNF-α mRNA hybridization signals (4 weeks for the detection of TNF-α mRNA versus 12 days for the detection of IL-1β mRNA), we conclude that TNF-α mRNA was expressed at much lower levels than IL-1β mRNA. The time curve for TNF-α protein expression, measured by an TNF-α-specific ELISA assay, was similar to that observed for the expression of TNF-α mRNA. Maximal increase of TNF-α protein was observed 7 h after administration of LPS, which fitted well with the time point of maximal expression of TNF-α mRNA.

The spot-like appearance of the TNF-α mRNA hybridization signals in autoradiograms of brain sections hybridized with a radioactively labelled TNF-α mRNA antisense riboprobe were comparable to the IL-1β mRNA hybridization signals observed in the rat brain after focal ischaemia (Buttini *et al*., 1994) or after peripheral LPS administration (Buttini & Boddeke, 1995), suggesting that these two cytokines are synthesized by the same cells. In the case of IL-1β mRNA, we have shown that these cells are microglial cells (Buttini & Boddeke, 1995). Non-radioactive *in situ* hybridization with a digoxigenin-UTP labelled cRNA, showed that the TNF-α

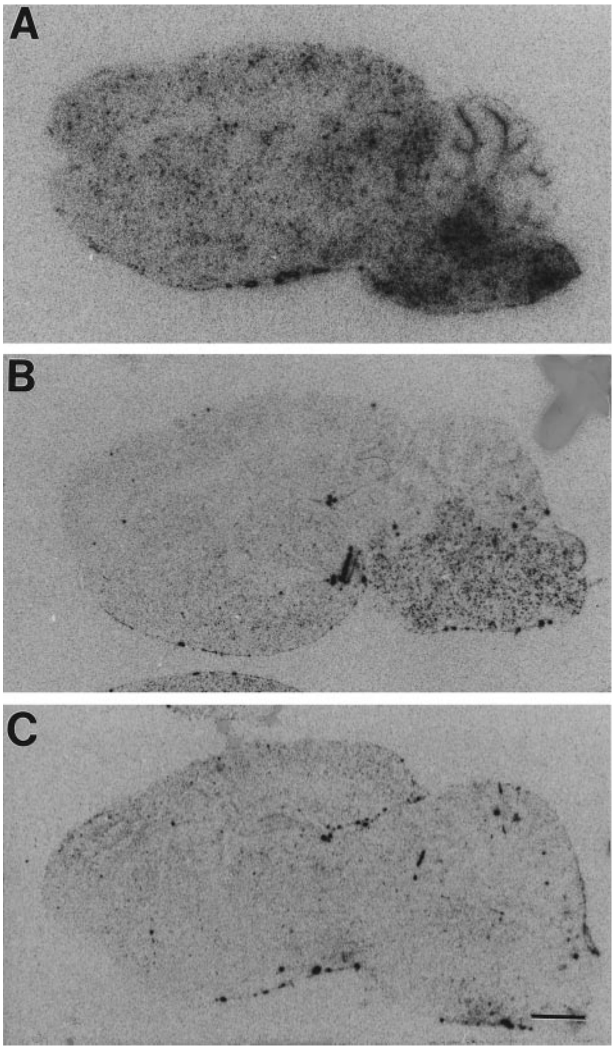

Figure 6 Inhibition of LPS-induced TNF-α mRNA expression in rat brain by methylprednisolone and by rolipram. Radioactive *in situ* hybridisation experiment for TNF-α mRNA. (A) Brain section of a control, LPS-treated animal which received 2 i.p. injections of 0.9% saline, (B) brain section of a LPS-treated animal which received 2 i.p. injections of 30 mg kg^{-1} methylprednisolone, (C) brain section of a LPS-treated animal which received 3 i.p. injections of 3 mg kg^{-1} rolipram. The detailed animal treatment procedure is described in Methods. All the animals were killed by decapitation 7 h after i.v. administration of 5 mg kg^{-1} LPS and brains were processed for *in situ* hybridization as described in Methods.

mRNA positive cells in the brains of LPS-treated rats displayed the morphology of microglia. No TNF-α mRNA could be detected in cells with the morphology of neurons or astrocytes. In addition, cells with similar morphology have been labelled by specific markers for microglia (OX-42 antibody and isolectin B4) on tissue sections adjacent to the sections used for *in situ* hybridization (data not shown).

Treatment with two different anti-inflammatory compounds, the synthetic glucocorticoid, methylprednisolone and the PDE IV inhibitor, rolipram, inhibited LPS-induced brain expression of TNF-α in a dose-dependent manner. Curiously, a significant (Student's *t* test $P<0.05$) potentiation of the response to LPS by the rolipram vehicle H_2O/PEG was observed. We have no clear explanation for this finding. It is possible that PEG acts directly on TNF-α production and/or facilitates the entry of LPS into the brain. Using *in situ* hybridization, we showed that both compounds reduced TNF-α mRNA levels in the brain of LPS-treated rats. Both compounds have already been shown to inhibit TNF-α production by cultured microglial cells (Chao *et al*., 1992). Glucocorticoids are known to decrease the stability of cytokine mRNAs in macrophages *in vitro* (Amano *et al*, 1993). A similar mechanism may underlie the effect of methylprednisolone on TNF-α production by microglial cells.

PDE inhibitors inhibit TNF-α production by increasing intracellular levels of cAMP, which represses TNF-α gene transcription (Katakami *et al*., 1988; Ulrich-Schade & Schudt, 1993), inhibitors of PDE IV isoenzymes being the most potent mediators of his effect. PDE IV inhibitors have been reported to inhibit TNF-α gene transcription and reduce disease severity in different models of peripheral inflammation (Klemm *et al*., 1995; Sekut *et al*., 1995) as well as in brain inflammatory processes (Genain *et al*., 1995; Sommer *et al*., 1995). Treatment with the type IV phosphodiesterase inhibitor rolipram inhibited production of TNF-α and prevented development of EAE in marmosets (Genain *et al*., 1995). Additionally, it was reported that TNF-α production in autoreactive T cells from EAE affected animals and from MS patients was strongly inhibited by rolipram (Sommer *et al*., 1995). In another study demyelination and neuronal loss, resulting from experimental lentiviral encephalitis, were strongly reduced after treatment with the unspecific PDE inhibitor pentoxifylline. In this study it was clearly demonstrated that pentoxifylline reduced the production of TNF-α mRNA, leading to decreased transcription of IL-1β and NO-synthase (Philippon *et al*., 1994). The role of TNF-α in inflammation-induced CNS pathology remains controversial. Increase in TNF-α expression has been reported in a wide variety of CNS diseases and CNS disease models, including ischemia (Buttini *et al*., 1996), multiple sclerosis (Hofman *et al*., 1989), AIDS dementia (Price *et al*., 1988), HIV-encephalitis (Persidsky *et al*., 1997) bacterial meningitis (Tunkel *et al*., 1990) and cerebral malaria (Lucas *et al*., 1997). *In vitro*, TNF-α causes demyelination and death of oligodendrocytes (Selmaj & Raine, 1988). The PDE-inhibitor pentoxifylline and TNF-α binding protein, a physiological inhibitor of TNF-α activity, have recently been reported to decrease oedema and facilitate motor function recovery in a rat model of head injury (Shohami *et al*., 1996). On the other hand, however, TNF-α was shown to protect isolated neurons from metabolic and excitoxic injury (Cheng *et al*., 1994). In addition, in mice where the p55 and p75 TNF receptors have been deactivated by targeted gene disruption, pathological CNS alterations induced by focal cerebral ischemia and excitotoxic injury were exacerbated, suggesting that tumour necrosis factors may have a protective role in these models of brain injury (Bruce *et al*., 1996).

In our model of LPS-induced central upregulation of TNF-α mRNA and protein, no parenchymal tissue injury due to the local inflammatory reaction could be detected. Further studies should show how interference with synthesis, secretion or action of TNF-α will affect the pathological outcome in different models of brain injury and inflammation.

In summary, our data demonstrate that peripheral administration of LPS induces a rapid and transient expression of TNF-α, presumably in microglial cells, in rat brain. Brain expression of TNF-α can be inhibited by the two anti-inflammatory compounds methylpredisolone and rolipram. These data are consistent with the notion that the mechanisms underlying the regulation of cytokine production in peripheral macrophages and in microglial cells are similar, and suggest a potential therapeutic use of TNF-α inhibitors for prevention and treatment of immune-mediated CNS injury.

M.B. was a recipient of a predoctoral fellowship from the Ministry of Culture, Luxembourg.

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(Received May 27, 1997
Revised August 18, 1997
Accepted August 28, 1997)