BJP BRITISH JOURNAL OF PHARMACOLOGY

BPS BRITISH PHARMACOLOGICAL SOCIETY

British Journal of Pharmacology (2010), 160, 1754–1764

0007-1188/10
www.brjpharmacol.org

RESEARCH PAPER

Spinal glial TLR4-mediated nociception and production of prostaglandin E_2 and TNF

O Saito[1]*, CI Svensson[1†], MW Buczynski[2], K Wegner[1‡], X-Y Hua[1], S Codeluppi[3], RH Schaloske[2], RA Deems[2], EA Dennis[2] and TL Yaksh[1]

[1]*Department of Anesthesiology, University of California San Diego, La Jolla, USA,* [2]*Department of Chemistry and Biochemistry and Department of Pharmacology, University of California San Diego, La Jolla, USA, and* [3]*Department of Neuroscience, Karolinska Institute, Stockholm, Sweden*

Background and purpose: Toll-like receptor 4 (TLR4) expressed on spinal microglia and astrocytes has been suggested to play an important role in the regulation of pain signalling. The purpose of the present work was to examine the links between TLR4, glial activation and spinal release of prostaglandin E_2 (PGE_2) and tumour necrosis factor (TNF), and the role these factors play in TLR4-induced tactile allodynia.
Experimental approach: Toll-like receptor 4 was activated by intrathecal (i.t.) injection of lipopolysaccharide (LPS) and KDO_2-Lipid A (KDO_2) to rats. Tactile allodynia was assessed using von Frey filaments and cerebrospinal fluid collected through spinal dialysis and lumbar puncture. PGE_2 and TNF levels were measured by mass spectometry and ELISA. Minocycline and pentoxifylline (glia inhibitors), etanercept (TNF-blocker) and ketorolac (COX-inhibitor) were given i.t. prior to injection of the TLR4-agonists, in order to determine if these agents alter TLR4-mediated nociception and the spinal release of PGE_2 and TNF.
Key results: Spinal administration of LPS and KDO_2 produced a dose-dependent tactile allodynia, which was attenuated by pentoxifylline, minocycline and etanercept but not ketorolac. Both TLR4 agonists induced the spinal release of PGE_2 and TNF. Intrathecal pentoxifylline blunted PGE_2 and TNF release, while i.t. minocycline only prevented the spinal release of TNF. The release of PGE_2 induced by LPS and KDO_2 was attenuated by i.t. administration of ketorolac.
Conclusions and implications: Activation of TLR4 induces tactile allodynia, which is probably mediated by TNF released by activated spinal glia.
British Journal of Pharmacology (2010) **160,** 1754–1764; doi:10.1111/j.1476-5381.2010.00811.x

Keywords: spinal cord; intrathecal; astrocytes; microglia; tumour necrosis factor; prostaglandin; lipopolysacaride; Toll-like receptor 4; pain

Abbreviations: CSF, cerebrospinal fluid; i.p., intraperitoneal; i.t., intrathecal; KDO_2, KDO_2-Lipid A; LPS, lipopolysaccharide; PGE_2, prostaglandin E_2; TLR4, Toll-like receptor 4; TNF, tumour necrosis factor

Introduction

Classically, astrocytes and microglia have been viewed as serving mostly supportive and nutritive roles for neurones. Although widely accepted, other concepts have challenged this perspective, pointing to the fact that glia can respond to changes in their local microenvironment and influence neuronal excitability (Galambos, 1961). There is an interest in the role played by these non-neuronal cells in pain processing, in particular in persistent pain states which occur following peripheral tissue and nerve injury. Studies demonstrating that spinal glia are activated in response to peripheral stimuli and that behaviourally defined hypersensitivity is attenuated when glial activity is blocked using glia inhibitors support this notion (for review see Milligan and Watkins, 2009).

There is an ongoing effort to elucidate the mechanisms through which glia are activated in response to tissue and nerve injury. Chemokines (e.g. fractalkine, CCL2), cytokines (e.g. IL-6, IL-18) and ATP acting on their respective receptors expressed on glia have been suggested to play important roles.

Correspondence: Camilla I Svensson, Department of Physiology and Pharmacology, Karolinska Institute, Von Eulers vag 8, 171 77 Stockholm, Sweden. E-mail: camilla.svensson@ki.se
O.Saito and CI Svensson contributed equally to this manuscript.
*Current address: Department of Anesthesiology and Pain Clinic, Konodai Hospital, International Medical Center of Japan, Japan.
†Current mail address: Department of Physiology and Pharmacology, Karolinska Institutet, 171 77 Stockholm, Sweden.
‡Current mail address: Section of Anesthesia and Critical Care, Department of Clinical Studies, University of Pennsylvania, New Bolton Center, 382 West Street Road, Kennett Square, PA 19348 (610) 925-6469.
Received 10 August 2009; revised 18 March 2010; accepted 24 March 2010

More recently, the Toll-like family of receptors (TLRs) has also been implicated in this process. TLRs are noted for recognizing chemical structures like cell membrane products, DNA and mRNA expressed by various classes of microbes. In addition to bacterial and viral antigens, several endogenous ligands such as heat shock proteins (Gay and Gangloff, 2007; Miyake, 2007) and saturated fatty acids (Lee *et al*., 2001) have also been proposed to activate TLRs. Most TLR-initiated signalling leads to nuclear translocation of NF-κB and production of a number of pro-inflammatory factors, such as prostaglandins, cytokines and chemokines (Muller-Ladner *et al*., 2002; Takeda and Akira, 2004). Nine TLRs have been identified in humans. In the central nervous system, TLRs1–9 are expressed on microglia and TLRs 1, 3, 4, 5 and 9 have been found on astrocytes (Jack *et al*., 2005; Miyake, 2007).

Toll-like receptor 4 is activated by lipopolysaccharide (LPS) from Gram-negative bacteria (Miyake, 2004; Raetz *et al*., 2006). Intrathecal (i.t.) injection of LPS induces nociception (pain), which is perceived as tactile allodynia (non-painful stimulus perceived as painful) (Meller *et al*., 1994; Reeve *et al*., 2000; Kehl *et al*., 2004). Further, allodynia observed following nerve and tissue injury is attenuated in mice lacking functional TLR4 (Tanga *et al*., 2005), in rats following TLR4 knockdown by intrathecally delivered TLR4 antisense (Tanga *et al*., 2005) and in rats receiving TLR4 antagonists (Bettoni *et al*., 2008; Hutchinson *et al*., 2009a). Importantly, preventing TLR4-mediated signalling suppresses spinal microglial activation and decreases nerve injury-induced spinal release of pro-inflammatory cytokines (Tanga *et al*., 2005). Hence, TLR4 on spinal glia appear to be activated by endogenous ligands and to play an important role in spinal nociceptive processing.

Lipopolysaccharide is commonly used in experimental studies of TLR4 functions. However, the micro-heterogeneity of wild-type Gram-negative bacteria LPS often constitutes a problem and requires careful batch comparison and determination of equipotent doses. This variability is thought to depend on differences in the length and composition of the terminal glycan chains of different LPS species (Raetz *et al*., 2006). Hence, in order to establish a TLR4-induced model of hypersensitivity avoiding these problems, we assessed the allodynic effect of KDO_2-Lipid A (KDO_2), a chemically defined LPS molecule (Raetz *et al*., 2006). This substance is purified from *Escherichia coli* and consists of lipid A anchor and a 3-deoxy-D-*manno*-octulosonic acid disaccharide, and is fully active as an endotoxin and is highly selective for TLR4. Additional aims of this study were to examine if TLR4 activation induces spinal release of prostaglandin E_2 (PGE_2) and tumour necrosis factor (TNF) and to investigate the role of spinal glia in the release of these mediators as well as TLR-4-mediated allodynia. Our studies showed that i.t. injection of KDO_2 induced allodynia comparable to that observed after i.t. injection of LPS. In addition, our findings suggest that TLR4-associated tactile allodynia is mediated by TNF, but not PGE_2 release, and that activation of both microglia and astrocytes play a role in TLR4-induced nociception.

Methods

All experiments were carried out according to protocols approved by the Institutional Animal Care Committee of the University of California, San Diego. Nomenclature conforms to BJP's Guide to Receptors and Channels (Alexander *et al*., 2008).

Animals and surgery

Male Holzman Sprague-Dawley rats (300–400 g) were housed individually in micro isolator filter cages and maintained on a 12 h light/dark cycle with free access to food and water. To permit bolus i.t. drug delivery, chronic lumbar i.t. injection catheters (single lumen PE-5, 8.5 cm in length, Spectranetics, Colorado Springs, CO, USA) were implanted through a cisternal exposure under isoflurane anaesthesia (2–4%) and externalized as described elsewhere (Yaksh and Rudy, 1976). To permit dialysis of the lumbar i.t. space, rats were prepared with chronic triple lumen loop dialysis catheters advanced 8.5 cm through a cisternal incision to the lumbar enlargement under isoflurane anaesthesia and externalized (Marsala *et al*., 1995; Koetzner *et al*., 2004). The i.t. portion of the dialysis probe consists of a tubular 3 cm cellulose dialysis fibre (Filtral AN69HF, Cobe Laboratories) bent double and connected at its ends to 7 cm of two lumen of the triple lumen catheter, and the third lumen permits the delivery of i.t. drug without interrupting dialysis. Rats were monitored daily and removed from the study if any neurological dysfunction was noted, if there was greater than 10% weight loss over 5 days or if the catheter was occluded. Fewer than 5% of the animals prepared were so excluded. Studies involving rats with chronic dialysis catheters or single lumen injection catheter were undertaken 4–5 days after surgery.

Drug administration

The following drugs were delivered i.t. in 10 μL saline followed by 10 μL saline flush. LPS [1–30 μg, LPS *E. coli* O111:B4, Calbiochem, USA, Cat# 437627 (Lot # B67875, was used for studies presented in this manuscript)], KDO_2 (0.1–10 μg, Advanti Polar Lipids Inc. Alabaster, AL, USA), pentoxifylline (60–120 μg, Sigma), minocycline (10–120 μg, Sigma), etanercept (Enbrel, 30–100 μg, Amgen, Thousand Oaks, CA, USA) and ketorolac (50 μg, Allergan, Irvine, CA, USA). In addition, ibuprofen (30 $mg \cdot kg^{-1}$, Sigma) was delivered intraperitoneal (i.p.) in saline.

Behavioural analysis

For assessment of tactile allodynia, rats were placed in individual Plexiglas compartments with wire mesh bottoms. Following a 30-min acclimatization period, mechanical allodynia was assessed using von Frey filaments and the Dixon up-down method as described by Chaplan *et al*. (1994). Briefly, calibrated filaments (Stoelting, Wood Dale, IL, USA) with buckling forces between 0.41 and 15.2 g were applied perpendicularly to the mid-paw plantar surface until the filament was slightly bent and held there for 4–6 s. Stimuli were separated by several seconds or until the animal was calm with both hind paws placed on the grid. A positive response was noted if the paw was sharply withdrawn. Testing always began with the 2.0 g filament and tests were performed on animals prior to and 15, 30, 60, 90 and 120 min after injec-

tion of LPS/KDO_2. The 50% probability withdrawal threshold was determined and plotted versus time; the data were also expressed as the area under the curve (AUC) of allodynic index for the time period 0–120 min. This resulting value has the units percentage change × time. The formula for calculating the percentage change is 100 × (baseline tactile threshold – post-drug tactile threshold)/baseline tactile threshold, where tactile threshold is expressed in g. Increasing values indicates increasing tactile allodynia.

Dialysis

A syringe pump (Harvard, Natick, MA, USA) was connected and dialysis tubing was perfused with artificial cerebrospinal fluid (ACSF) at a rate of 10 μL·min^{-1}. The ACSF contained (mM) 151.1 Na^+, 2.6 K^+, 0.9 Mg^{2+}, 1.3 Ca^{2+}, 122.7 Cl^-, 21.0 HCO_3 and 2.5 HPO_4, and it was bubbled with 95% O_2/5% CO_2 before each experiment to adjust the final pH to 7.2. The efflux (30 min per fraction) was collected in an automatic fraction collector (Eicom, Kyoto, Japan) at 4°C. Two baseline samples were collected after the initial 30 min washout, and additional five fractions were collected after i.t. injection of LPS (1 μg in 10 μL saline followed by 10 μL of saline to flush injection line). Minocycline, pentoxifylline, ibuprofen were injected i.t. or i.p. 15 min prior to LPS. The concentration of PGE_2 in spinal dialysate was measured by ELISA using a commercially available kit (Assay Designs 90001, Assay Designs, Ann Arbor, MI, USA). The antibody is selective for PGE_2 with less than 2.0% cross-reactivity to $PGF_{1\alpha}$, $PGF_{2\alpha}$, 6-keto$PGF_{1\alpha}$, PGA_2 or PGB_2, but cross-reacts with PGE_1 and PGE_3

Lumbar CSF collection

Cerebrospinal fluid collection micropipettes (0.8–1.1 × 100 mm, KIMAX-51, Kimble Products) were prepared in advance using a micropipette puller (Industrial Science Associates, Ridgewood, NY, USA) to obtain a fine injection tip on one end of the micropipette. Isoflurane-anaesthetized rats were placed in sternal recumbancy and the tips of the wings of the ilium were identified as a tactile landmark. A midline skin incision was started 1 cm caudal from the landmark and made posterior to a length of 3 cm. The muscles were bluntly dissected and retracted laterally to expose the interspinous space at L4/L5. The L4/L5 interspinous ligament and L5 spinous process were carefully removed. The L4 spinous process was elevated using forceps to widen the interspinous space for access to the dura. Careful attention was paid to remove tissue from the dura and eliminate any pooling of blood at the site of collection. The tip of the pulled microcapillary tube was obliquely introduced into the i.t. space. The jugular veins were compressed to increase the i.t. pressure and 40–50 μL of clear CSF was collected by capillary action. The CSF was transferred to Eppendorf tubes and either immediately frozen on dry ice and stored at −70°C until assayed for TNF by ELISA according to the manufacturer's instructions (R & D Systems, Minneapolis, MN, USA) or extracted for LC-MS/MS analysis. Inhibitors were injected i.t. or i.p. 15 min prior to LPS or KDO2 and CSF collected 2 h post TLR4-agonist injection. Animals were killed following CSF collection.

Eicosanoid analysis by LC-MS/MS

The analysis of eicosanoids from CSF was performed by LC-MS/MS using a tandem quadrupole mass spectrometer (ABI 4000 Q Trap®, Applied Biosystems). LC-grade solvents were purchased from EMD Biosciences. Strata-X solid phase extraction columns were purchased from Phenomenex (Torrance, CA, USA). All eicosanoids were purchased from Cayman Chemicals (Ann Arbor, MI, USA). Briefly, 25 μL of CSF was supplemented with 100 μL of internal standards (100 pg·μL^{-1}, EtOH) and diluted to 10% EtOH (by volume). Eicosanoids were then purified by solid phase extraction and prepared for analysis by LC-MS/MS as previously described (Buczynski *et al*., 2007; Deems *et al*., 2007). Samples were reconstituted in 50 μL of LC buffer and 80% (40 μL) was injected on column. Quantitative PGE_2 determination was performed by stable isotope dilution using the following multiple reaction monitoring transitions: (d_4) PGE_2 355→193 and PGE_2 351→189. Results are reported as pg of eicosanoid·μL^{-1} CSF (mean ± SEM).

Primary astrocyte cultures from spinal cord

Purified cultures of adult rat spinal astrocytes were prepared using a method described previously (Schwartz and Wilson, 1992) with some modifications. The spinal cords were ejected from the vertebral column using a saline-filled syringe and immediately placed in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Grand Island, NY, USA). The meningies were removed and the cord cut into small pieces using a sterile razorblade. The tissue was chemically dissociated by 0.5% Trypsin-EDTA for 10 min followed by mechanical trituration in DMEM. After being centrifuged at 450× *g* for 5 min, the cells were suspended in DMEM containing 10% foetal bovine serum (Gibco) and plated in a flask coated with poly-L-lysine (Sigma). The cultures were maintained in a humidified atmosphere of 95% air/5% CO_2 at 37°C for 10 days, with the media being changed media on days 4 and 7. On days 10 and 11, cells growing on top of the confluent astrocyte layer were removed by shaking at 200 rpm for 2 h at 37°C and replacing the media. On day 12 the cells were trypsinized and 40 000 cells replated in 6-well plates. These cultures were used for studies when confluent, typically within 4–6 days. Prior to stimulation experiments, cells were deprived of serum for 24 h in DMEM containing 0.1% foetal bovine serum. Drugs were added to 2 mL of media in a total volume of 20 μL PBS. Pentoxifylline (100 μM) was added 30 min prior to KDO_2 (1 μg·mL^{-1}) and the media was transferred to Eppendorf tubes 24 h later and either immediately frozen on dry-ice and stored at −70°C until assayed for TNF by ELISA according to the manufacturer's instructions (R & D Systems, Minneapolis, MN, USA) or extracted for LC-MS/MS analysis. The amount of protein in each well was determined after the cells had been lysed and the TNF and PGE_2 concentration were normalized to amount of protein in respective well.

In order to determine the purity of the cell cultures, cells cultured on chamber glass slides (Lab-TekII Chamber slides, Nalge Nunc Int. Naperville, IL, USA) were fixed in 4% formaldehyde in 0.01 M phosphate buffered saline (PBS), pH 7.4, for 15 min, made permeable in PBS buffer with 0.1% Triton X-100 for 5 min, blocked for 1 h in PBS buffer with 5% goat

serum, incubated for 1 h with antibodies against GFAP (Invitrogen, 1:1000), S100β (Abcam, 1:1000, nestin (Millipore, 1:200), 3G10 (Seikagaku Corporation, 1:100), Iba-1 (Wako Chemicals, 1:1000) and NeuN (Millipore, 1:1000) in PBS buffer with 5% goat serum, and incubated with Alexa Fluor-conjugated secondary antibodies (1:300) in PBS buffer with 5% goat serum. After being washed with PBS, the chambers were removed and the glass slides covered with coverlids using ProLong Gold antifade mounting media containing the nuclear stain DAPI (Invitrogen). The number of nuclei and number of GFAP, S100β, nestin, 3G10, Iba-1 and NeuN-positive cells were counted and the purity of the culture expressed as the percentage of cells immunopositive for respective marker compared with total number of cells.

Quantitative real-time PCR

After the astrocyte cell cultures had been subjected to 24 h of serum deprivation, mRNA was extracted from them using Trizol (Invitrogen) according to the manufacturer's protocol. Complementary DNA was prepared and quantitative real-time PCR performed with TaqMan Gene Expression Assays (both according to the manufacturer's instructions, Applied Biosystems, Foster City, CA, USA) to determine relative mRNA levels, using the GeneAmp 7500 Fast Sequence Detection system (Applied Biosystems). Pre-developed specific primers were used to detect GFAP (Assay ID Rn00566603), glutamate transporter-1 (GLT-1) (Assay ID Rn00568080), S100β (Assay ID Rn00566139), glutamine synthetase (Assay ID Rn00567121) and HPRT1 (Assay ID Rn01527838) (Applied Biosystems). Sample threshold cycle (Ct) values in standard curve samples containing GFAP, GLT-1, S1000β, glutamine synthetase and HPRT1 mRNA were used to calculate the cDNA concentration equivalents in the test samples. The data were then normalized to HPRT1 gene expression to obtain relative concentration and presented as relative gene expression.

Statistics

All the data are presented as mean ± SEM. Differences were assessed by one-way ANOVA followed by Bonferroni *post-hoc* test for multiple groups, or Student's *t*-test for two groups, with a criterion of $P < 0.05$ for significance (Prism statistical software, CA, USA).

Results

Intrathecal injection of LPS and KDO_2 induced tactile allodynia

In order to confirm previous work demonstrating that spinal delivery of LPS induces allodynia (Meller *et al*., 1994; Reeve *et al*., 2000; Kehl *et al*., 2004) 1–30 μg LPS was injected i.t and tactile allodynia assessed by von Frey filaments. Administration of 10 μg of LPS gave rise to a pronounced allodynia 30 and 60 min after injection (Figure 1A). Calculation of AUC of the allodynic index for the time period 0–120 min showed that only the 10 μg dose of LPS caused a significant increase in hypersensitivity, as compared with the vehicle group (LPS 10 μg: 372 ± 88 vs. vehicle: 98 ± 49, $P < 0.05$, $n = 5$)

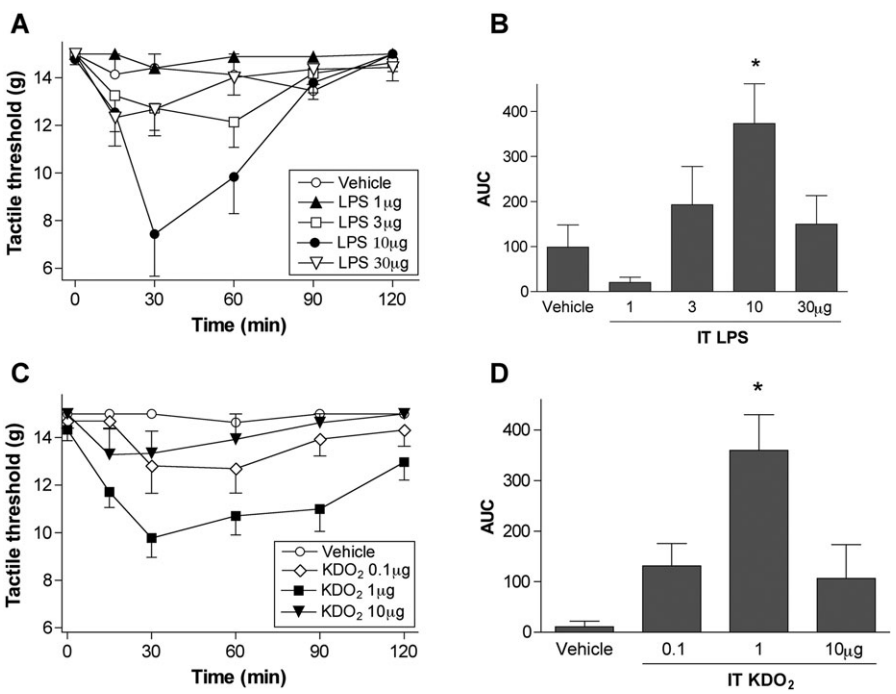

Figure 1 Intrathecal injection of LPS and KDO_2 induced tactile allodynia. Graphs display dose-response curves for induction of allodynia by i.t. injection of (A) LPS (1–30 μg) and (C) KDO_2 (0.1–10 μg). The histograms represent the allodynic index calculated as area under curve (AUC) for 0–120 min for the different i.t. doses of (B) LPS and (D) KDO_2 indicating that 10 μg LPS and 1 μg KDO_2 induce significant hypersensitivity. The data are presented as mean ± SEM of 5–6 rats per group. *$P < 0.05$ for LPS and KDO_2 versus vehicle (PBS). KDO_2, KDO_2-Lipid A; LPS, lipopolysaccharide.

(Figure 1B). Intrathecal injection of a higher dose of LPS, 30 μg, was not associated with tactile allodynia (LPS 30 μg: 150 ± 64 vs. vehicle: 98 ± 49, $P > 0.05$, $n = 5$) (Figure 1B). Of importance, the rats receiving 30 μg of LPS appeared to be sick (piloerection, vocalization upon touch and reduced activity). Based on the bell-shaped dose-response relationship for i.t. LPS, all follow-up studies were conducted using 10 μg LPS.

For comparison, KDO_2 was injected i.t. at doses ranging from 0.1 to 10 μg. Intrathecal administration of 1 μg KDO_2 gave rise to tactile allodynia 30 and 60 min after injection (Figure 1C), with a pattern similar to that observed with i.t. LPS. Although not as pronounced as in the LPS group, the animals appeared to be sick after i.t. injection of KDO_2. Intrathecal injection of 1 μg KDO_2 induced allodynia, as compared with vehicle control group (KDO_2 1μg 359 ± 71 vs. vehicle 11 ± 11, $P < 0.01$, $n = 5$–6) (Figure 1D), while a higher dose KDO_2 (10 μg) did not ($P > 0.05$) (Figure 1D). These data are in accordance with the observations for i.t. LPS, i.e. the dose-response curves for allodynia induced by i.t. LPS and i.t. KDO_2 are both bell-shaped. Based on these results, 1 μg KDO_2 was given i.t. in the follow-up experiments.

Activation of spinal TLR4 receptors induces spinal release of PGE_2, which was attenuated by i.t. injection of pentoxifylline, ketorolac and i.p. injection of ibuprofen, but not by i.t. injection of minocycline

Based on previous work showing that microglia and astrocyte release PGE_2 in response to LPS stimulation (Fontana *et al*., 1982; Slepko *et al*., 1997), we injected LPS i.t. to determine whether it induces the release of PGE_2 *in vivo*. Release of PGE_2 was assessed in CSF collected by spinal dialysis and by lumbar puncture. The PGE_2 concentration was measured by ELISA in the dialysate and by LC/MS in the CSF, withdrawn by insertion of a microcapillary tube into the i.t. space.

In the dialysis study, a 30 min washout preceded collection of two 30-min baseline samples. The basal PGE_2 level in the i.t. fluid was 176 ± 20 fmol·100 μL^{-1} perfusate ($n = 17$) (Figure 2A). Intrathecal injection of LPS (1 μg, $n = 5$), produced a profound increase in PGE_2 concentration (approximately 14-fold higher over baseline at 120 min) in the dialysate (Figure 2A). In order to assess the role of glia in this process, two 'glia inhibitors' pentoxifylline and minocycline were injected i.t. prior to i.t. LPS. Minocycline is thought to act specifically on microglia, while pentoxifylline exerts its action both on microglia and astrocytes; however, the exact mechanism of these two drugs has not yet been defined. LPS-evoked PGE_2 release was partially blocked by i.t. injection of pentoxifylline (120 μg), while i.t. injection of minocycline (120 μg) did not alter the effect of i.t. LPS (Figure 2A). Comparison between groups showed that LPS-evoked release of PGE_2 was significantly lower in the group that received i.t. pentoxifylline prior to LPS (61 ± 13 vs. 118 ± 19 fmol·100 μL^{-1}·min^{-1}, $P < 0.05$, $n = 6$) (Figure 2B). In addition, LPS-evoked PGE_2 release was significantly attenuated by i.p. pretreatment (30 mg·kg^{-1}) with the non-selective COX inhibitor ibuprofen (vehicle + LPS, 89 ± 27; ibuprofen + LPS, 32 ± 19 fmol·100 μL^{-1}·min^{-1}, $P < 0.05$, $n = 5$) (Figure 2C and D).

Next, we extracted CSF by lumbar puncture in order to assay PGE_2 and TNF levels in CSF by LC/MS and ELISA respectively. CSF was collected 2 h after i.t. injection of 1 μg KDO_2 and 1–10 μg of LPS. Intrathecal injection of both KDO_2 and LPS induced a significant increase in PGE_2 concentration as compared with i.t. injection of PBS (KDO_2 1 μg: 44.8 ± 13 vs. 0.8 ± 0.3 pg·μL^{-1}, $P < 0.05$, $n = 6$; LPS 1 μg: 50.4 ± 12.2 vs. 0.8 ± 0.2 pg·μL^{-1}, $P < 0.05$, $n = 11$) (Figure 3A). There was no difference between the effects of the two doses of LPS (10 μg: 53.0 ± 10.2; 1 μg: 50.4 ± 12.2 pg·μL^{-1}, $P > 0.05$, $n = 4$–11) (Figure 3B). Intrathecal injection of pentoxifylline (120 μg)

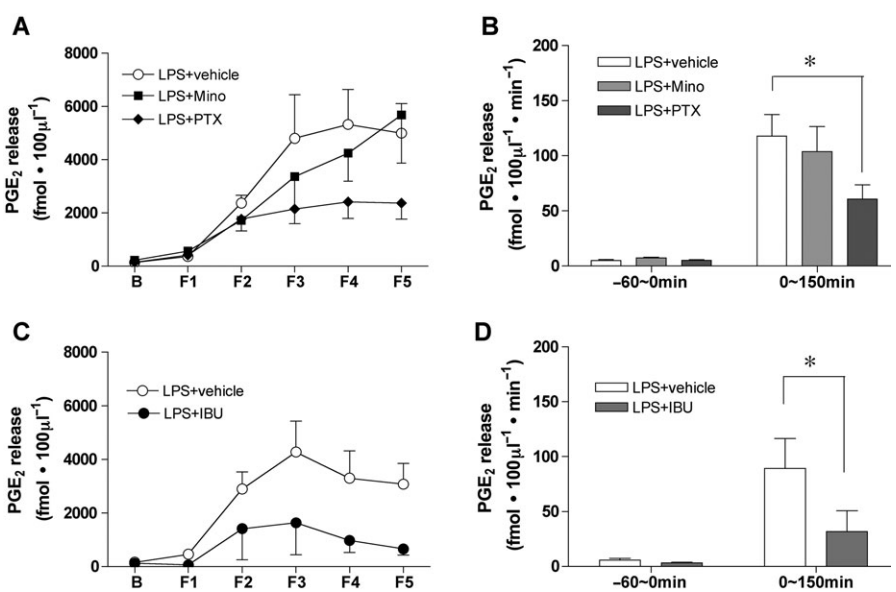

Figure 2 LPS-evoked spinal PGE_2 release was blocked by i.t. injection of pentoxifylline and ibuprofen but not minocycline. (A) PGE_2 measured by ELISA in cerebrospinal fluid collected by spinal dialysis in 30 min fractions after i.t. injection of LPS with our without pretreatment (15 min) with minocycline (Mino), pentoxifylline (PTX) or (C) ibuprofen (IBU). (B, D) PGE_2 release presented as area under curve (AUC) calculated for the baseline (60 min prior to LPS injection) and 0–150 min after i.t. injection of LPS. The results represent the mean ± SEM ($n = 5$–6 rats per group). *$P < 0.05$. LPS, lipopolysaccharide; PGE_2, prostaglandin E_2.

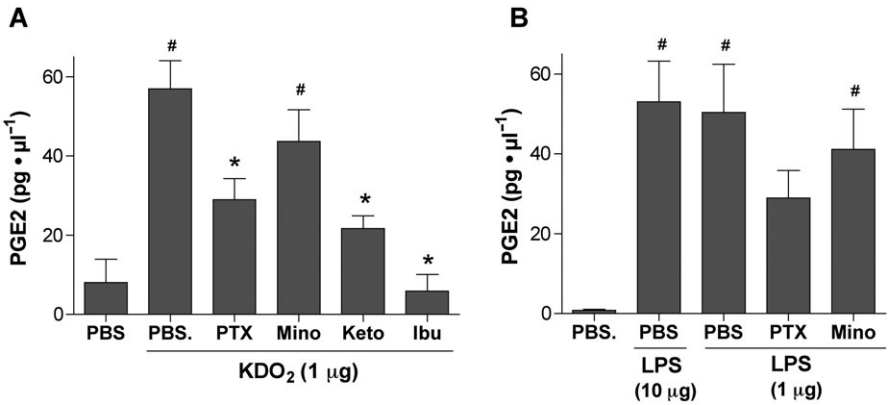

Figure 3 Spinal PGE_2 release evoked by KDO_2 and LPS was blocked by i.t. injection of pentoxifylline, ketorolac and i.p. injection of ibuprofen but not i.t. injection of minocycline. PGE_2 measured by LC/MS in cerebrospinal fluid collected by lumbar puncture 2 h after i.t. injection of (A) KDO_2 and (B) LPS with our without pretreatment (15 min) with minocycline (Mino, i.t. 60 µg), pentoxifylline (PTX, i.t. 120 µg), ketorolac (Ket, i.t. 50 µg) or ibuprofen (Ibu, i.p. 30 $\mu g \cdot kg^{-1}$). Each column represents the mean ± SEM (n = 4–11 rats per group). $^{\#}P < 0.05$ as compared with i.t. injection of vehicle (PBS) and $^{*}P < 0.05$ as compared with i.t. injection of KDO_2 or LPS alone. KDO_2, KDO_2-Lipid A; LPS, lipopolysaccharide; PGE_2, prostaglandin E_2.

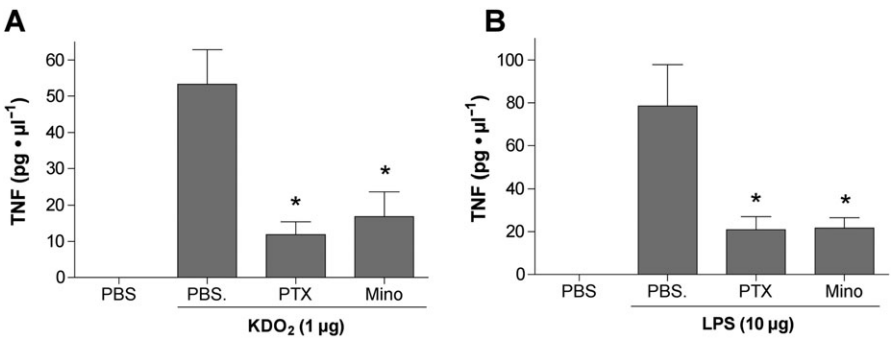

Figure 4 Spinal TNF release evoked by KDO_2 and LPS was blocked by i.t. injection of pentoxifylline and minocycline. TNF was measured by ELISA in cerebrospinal fluid collected by lumbar puncture 2 h after i.t. injection of (A) KDO_2 and (B) LPS with our without pretreatment (15 min, i.t.) with minocycline (Mino, 60 µg), pentoxifylline (PTX, 120 µg) Each column represents the mean ± SEM (n = 6–9 rats per group). $^{*}P < 0.05$ as compared with i.t. injection of KDO_2 or LPS. KDO_2, KDO_2-Lipid A; LPS, lipopolysaccharide; TNF, tumour necrosis factor.

($P < 0.05$, $n = 8$) and ketorolac (50 µg) ($P < 0.05$, $n = 6$) partially blocked and i.p. injection of ibuprofen (30 $mg \cdot kg^{-1}$, $P < 0.05$, $n = 5$) completely blocked KDO_2-induced PGE_2 release, while i.t. minocycline (60 µg) had no effect ($P > 0.05$, $n = 6$) (Figure 3A). In agreement with the results from the dialysis-ELISA study, extracting CSF by lumbar puncture and assessing PGE_2 by LC/MS showed that i.t. injection of minocycline had no effect on i.t. LPS-induced PGE_2 release ($P > 0.05$, $n = 9$) (Figure 3B). Intrathecal injection of pentoxifylline tended to reduce LPS-induced PGE_2 release; however, statistical significance was not reached (28.9 ± 6.9 vs. 53.0 ± 10.2 $pg \cdot \mu L^{-1}$, $P > 0.05$, n = 8–10) (Figure 3B).

Intrathecal injection of LPS and KDO_2-induced spinal release of TNF, which was attenuated by i.t. injection of pentoxifylline and minocycline

Tumour necrosis factor may be an important means of communication between glia and neurones. Recent work has shown that TNF receptors are expressed on spinal glia as well as neurones. Moreover, there is evidence that i.t. injection of LPS and peripheral inflammation stimulate the spinal production of TNF (Bianchi *et al.*, 2007; Shen *et al.*, 2009). Here, we assessed the concentration of TNF in CSF, by ELISA, 2 h after i.t. injection of the TLR4 agonists, as well as the effect of i.t. pretreatment with pentoxifylline and minocycline. CSF was collected by lumbar puncture, as TNF is too large to diffuse across the dialysis-membrane. Intrathecal injection of the vehicle (PBS) did not cause a detectable release of TNF, but in contrast, a pronounced TNF release was detected 2 h after i.t. injection of 1 µg KDO_2 (53.3 ± 9.6 $pg \cdot \mu L^{-1}$, n = 9, Figure 4A) and 10 µg of LPS (78.4 ± 19.4 $pg \cdot \mu L^{-1}$, n = 6, Figure 4B). Both pentoxifylline and minocycline significantly attenuated the spinal TNF release induced by i.t. KDO_2 (pentoxifylline 120 µg: 11.8 ± 3.6 $pg \cdot \mu L^{-1}$, $n = 7$, $P < 0.05$; minocycline 60 µg: 16.8 ± 6.9 $pg \cdot \mu L^{-1}$, $P < 0.05$, $n = 5$) and i.t. LPS (pentoxifylline: 20.8 ± 6.0 $pg \cdot \mu L^{-1}$, $P < 0.05$, $n = 6$; minocycline: 21.6 ± 4.9 $pg \cdot \mu L^{-1}$, $P < 0.05$, $n = 6$), indicating that TNF was released from microglia and possibly also from astrocytes.

Intrathecal treatment with pentoxifylline, minocycline and etanercept attenuated the tactile allodynia induced by i.t. LPS and i.t. KDO_2

As LPS and KDO_2 act on TLR4 receptors expressed on microglia and astrocytes, the anti-allodynic effect of minocycline

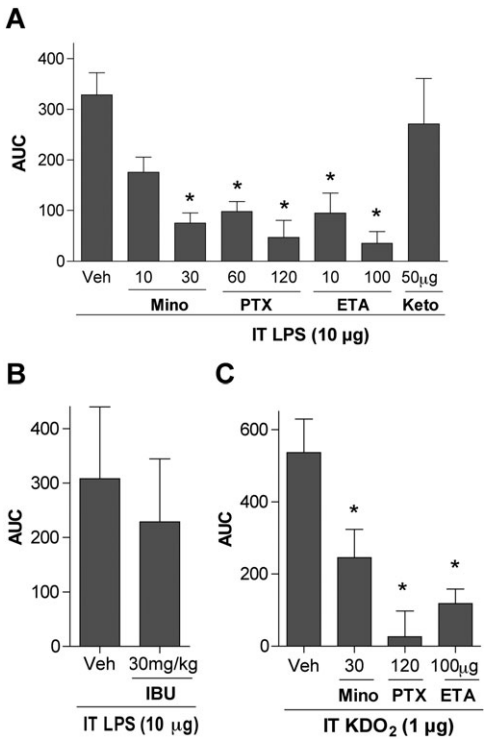

Figure 5 The tactile allodynia induced by intrathecal injection of LPS and KDO_2 was attenuated by i.t. injection of pentoxifylline, minocycline and etanercept, but not by i.t. ketorolac or i.p. ibuprofen. The histograms represent the allodynic index calculated as area under curve (AUC) for 0–120 min for (A) minocycline (Mino), pentoxifylline (PTX), etanercept (ETA) and ketorolac (Keto) injected i.t and (B) ibuprofen (IBU) injected i.p. 15 min prior to injection of LPS. (C) Area under curve (AUC) calculated for the time frame 0–120 min after i.t. injection of KDO_2 with or without pretreatment (15 min) with minocycline, pentoxifylline and etanercept. The data are presented as mean ± SEM of 5–12 rats per group. *$P < 0.05$, as compared with i.t. injection of LPS and KDO_2 alone. KDO_2, KDO_2-Lipid A; LPS, lipopolysaccharide.

and pentoxifylline was also assessed. Calculation of AUC over the 0–120 min time period showed that i.t. injection of 30 μg, but not 10 μg, minocycline (15 min pretreatment) attenuated tactile allodynia induced by 10 μg i.t. LPS, as compared with vehicle injected rats (75 ± 20 vs. 329 ± 43, $P < 0.01$, $n = 5–6$) (Figure 5A). Pentoxifylline, 60–120 μg i.t. (15 min pretreatment), also attenuated i.t. LPS-induced tactile allodynia as compared with the vehicle control group (60 μg: 98 ± 20, $P < 0.05$; 120 μg: 47 ± 33 $P < 0.01$ vs. vehicle: 329 ± 43, $n = 5–6$) (Figure 5A).

It has been demonstrated in glia cell cultures that both microglia and astrocytes release TNF in response to LPS stimulation (Sawada *et al*., 1989). As we observed that i.t. LPS evoked spinal release of TNF, we examined if blocking the action of TNF attenuates i.t. LPS-induced hypersensitivity. For this purpose, we used the soluble TNF decoy receptor etanercept, which binds TNF and prevents it from acting on TNF-receptors. Etanercept (10–100 μg) was administered i.t. 1 h prior to injection of LPS and both doses had an anti-allodynic effect as compared with injection of vehicle (10 μg: 95 ± 39, $P > 0.05$; 100 μg: 36 ± 23 $P < 0.01$ vs. vehicle: 329 ± 43, $n = 5$) (Figure 5A). The effect of the higher doses of minocycline, pentoxifylline and etanercept on tactile allodynia induced by i.t. KDO_2 generated similar results. All three drugs blocked i.t. KDO_2-induced hypersensitivity, as compared with vehicle injected animals (minocycline: 245 ± 78, $P < 0.05$; pentoxifylline: 26 ± 71, $P < 0.01$, etanercept: 118 ± 40, $P < 0.01$ vs. vehicle: 536 ± 93, $n = 5–12$) (Figure 5C).

COX-inhibition through i.t. administration of ketorolac and i.p. injection of ibuprofen did not block i.t. LPS-induced allodynia

In order to assess the role of prostaglandins in LPS-induced allodynia, the COX-1/2 inhibitors ketorolac and ibuprofen were utilized. Ketorolac was administered i.t. (50 μg) and ibuprofen i.p. (30 $mg \cdot kg^{-1}$) 15 min prior to injection of LPS. Although the two agents at the given doses blocked the spinal PGE_2 release evoked by LPS and KDO_2, neither of them significantly affected LPS-induced hypersensitivity ($P > 0.05$, $n = 5$, Figure 5A and B).

KDO_2 stimulation induced PGE_2 and TNF release from primary astrocyte cultures, which was partly blocked in the presence of pentoxifylline

In order to examine whether spinal astrocytes have the capacity to release TNF and PGE2, and if pentoxifylline attenuates such release, we used primary cell cultures of astrocytes from adult rat spinal cords. After 24 h of serum deprivation, the astrocytes were subjected to KDO_2 (1 $\mu g \cdot mL^{-1}$) for 24 h and the media collected for TNF analysis with ELISA and PGE_2 analysis by LC/MS. KDO_2 stimulated the release of both TNF (Figure 6A) and PGE_2 (Figure 6B) from the primary cell cultures as compared with PBS controls (TNF, 6.2 ± 0.6 vs. 0.7 ± 0.2 $ng \cdot mL^{-1}$, $P < 0.05$, $n = 6$; PGE_2, 1.4 ± 0.3 vs. 0.1 ± 0.03 $ng \cdot mL^{-1}$, $P < 0.05$, $n = 6$). When pentoxifylline (100 μM) was added to the cultures 30 min prior to KDO_2 stimulation, a partial, but statistically significant, reduction of TNF and PGE_2 release was observed (TNF, 4.0 ± 0.4 vs. 6.2 ± 0.6 $ng \cdot mL^{-1}$, $P < 0.05$; $n = 6$; PGE_2, 0.6 ± 0.06 vs. 1.4 ±0.3 $ng \cdot mL^{-1}$, $P < 0.05$, $n = 6$) (Figure 6A and B). We have previously shown that our protocol generates cell cultures that stain positive for the astrocye-associated proteins GFAP, S100β, nestin, vimentin and heparan sulphate proteoglycan (Codeluppi *et al*., 2009). In order to determine the purity of the cultures used in the current work the cells were stained with markers for astrocytes (GFAP, S100β, heparan sulphate proteoglycans (3G10), nestin), microglia (Iba-1) and neurones (NeuN). The cultures consisted of 97% GFAP, 97% S100β, 96% 3G10, 87% nestin, 3% Iba-1 and 0% NeuN-positive cells (Figure 6C–E). We also assessed gene expression and found that GFAP, GLT-1, S100β and glutamine synthetase, common astrocyte-associated genes, were all readily detectable in the control cell cultures and increased in cells stimulated with KDO2 (1 $\mu g \cdot mL^{-1}$) for 24 h (Figure 6G).

Discussion

Our results show that activation of spinal TLR4 by i.t. injection of LPS, and the LPS analogue KDO_2, induces tactile allodynia. This allodynia was attenuated by i.t. injection of the

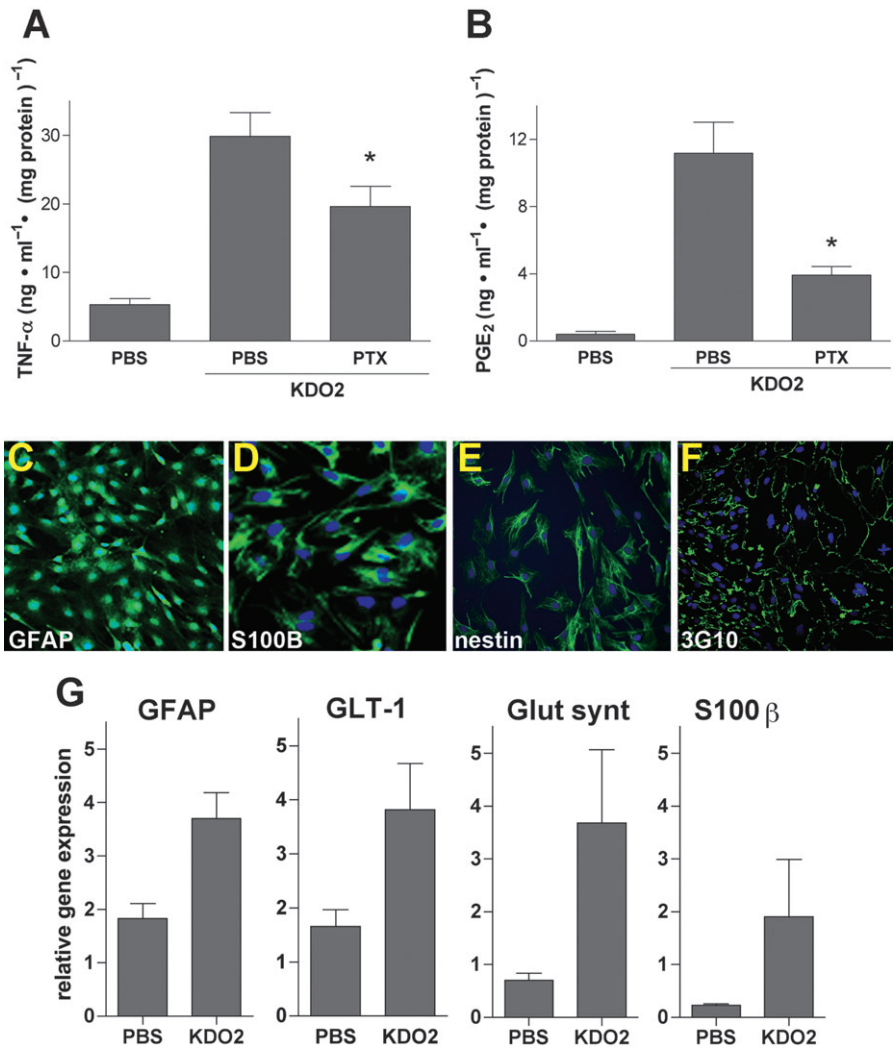

Figure 6 KDO_2-induced TNF and PGE_2 release from primary astrocyte cultures was partly prevented by pentoxifylline. (A) TNF and (B) PGE_2 were measured by ELISA in media collected from spinal primary astrocyte cultures after 24 h stimulation with KDO_2 with our without pretreatment (30 min) with pentoxifylline (PTX, 100 mM). PGE_2 and TNF concentrations were normalized to amount of protein (mg) in corresponding well. Each column represents the mean ± SEM ($n = 6$ different astrocyte cultures per group). $^{\#}P < 0.05$ as compared with vehicle (PBS) control and $^{*}P < 0.05$ as compared with KDO_2-stimulated cultures. Representative images depicting GFAP (C), S100β (D), 3G10 (E), nestin (G) immunolabelling of the astrocytes. (G) Quantitative real-time PCR showing gene expression for GFAP, GLT-1, S100β and glutamine synthetase (Glut synt) in control cultures (PBS) and cultures stimulated with KDO2 (1 $\mu g \cdot mL^{-1}$) for 24 h. Each column represents the mean ± SEM ($n = 3$ different astrocyte cultures per group). KDO_2, KDO_2-Lipid A; PGE_2, prostaglandin E_2; TNF, tumour necrosis factor.

glia inhibitors pentoxifylline and minocycline, and the TNF-blocker etanercept, while inhibition of COX-1/2 using ketorolac and ibuprofen had no effect. Intrathecal injection of LPS and KDO_2 evoked spinal release of TNF and PGE_2 and pretreatment with pentoxifylline reduced TLR4-induced release of both TNF and PGE_2, while minocycline only blocked the TNF release. Hence, these findings support the hypothesis that spinal TLR4 is involved in pain signal processing. Activation of glia, and subsequent release of cytokines such as TNF appear to be one of the underlying mechanisms for TLR4-mediated nociception. The role of spinal PGE_2 in this scenario is complex, and the results raise the possibility of a separation between anti-inflammatory effects and anti-nociceptive activity of COX inhibitors under our experimental conditions (see discussion below).

Over the past decade, a variety of mediators of glial activation and potential links between activated glia and enhanced and/or prolonged neuronal excitability have been reported. One such exciting group of factors are the Toll-like receptors and in particular TLR4 (Tanga *et al*., 2005). TLR4 is a transmembrane receptor protein that has extracellular leucine-rich repeated domains and an intracellular cytoplasmic signalling domain. TLR4 is involved predominantly in innate immune responses, but is also associated with triggering of the adaptive immune system. While LPS is well recognized for its ability to bind TLR4 through interaction with CD14, it is also likely that there are endogenous ligands for TLR4 receptors. In this context heat-shock proteins, fibronectin, hyaluronan, high mobility group box protein 1 (Gay and Gangloff, 2007; Miyake, 2007) and saturated fatty acids (Lee *et al*., 2001) have been proposed to act as TLR4 agonists. A role for endogenous TLR4 ligands in pain transmission is supported by studies showing that TLR4 knockout, spinal TLR4 knock-down and administration of a TLR4 antagonist attenuate nerve injury-

induced pain, in the absence of bacterial infection. A role of TLR4 in other types of pain has not yet been reported. However, like peripheral nerve injury (Raghavendra *et al*., 2004), peripheral inflammation leads to an increase in spinal gene expression of TLR4 and the TLR4 accessory protein CD14 (Tanga *et al*., 2004), pointing to the possibility that TLR4 may also participate in inflammation-induced hypersensitivity.

In this study, two TLR4 agonists, LPS and KDO_2, were used. Both agonists showed a robust induction of tactile allodynia after i.t. injection, and interestingly, both agonists displayed a characteristic bell-shaped dose-response curve. At doses over 10 μg LPS and 1 μg KDO_2, the allodynic effect was lost, and we believe this is due to the general 'sickness response' commonly generated by endotoxins (Maier *et al*., 1993). Rats receiving the highest doses of TLR4 agonists showed reduced spontaneous activity (e.g. lethargy). These rats frequently vocalized upon testing, suggesting discomfort, but did not respond to stimulation by paw withdrawal. Similar biphasic patterns, with hypersensitivity induced at lower doses and no effect at higher doses, have been reported by others after application of cytokines, for example following intracerebroventricular injection of IL-1β or PGE_2 (Hori *et al*., 1998) and after intranureal injection of TNF to the sciatic nerve (Zelenka *et al*., 2005). Of note, in a recent report no allodynic effect was observed after i.t. injection of 2 and 100 μg LPS (Hutchinson *et al*., 2009b). This is in one way consistent with our data, as we failed to detect tactile allodynia subsequent to i.t. injection of 3 and 30 μg LPS, and only the 10 μg LPS dose lead to an increased sensitivity to application of von Frey filaments. In previous work, we have experienced variations in the nociceptive response between different LPS batches, and hence strictly used the same batch for any given related series of experiments, and were forced to assess equinociceptive dosing between batches. This is most likely due to the heterogeneity associated with LPS. Accordingly, using the more homogeneous KDO_2 for TLR4-associated studies may have a significant advantage; indeed, macrophages from TLR4-knockout mice show no inflammatory response with KDO_2, arguing for the specificity of KDO_2 for the TLR4 receptor (Raetz *et al*., 2006).

Intrathecal injection of LPS and KDO_2 induced the release of both PGE_2 and TNF. We assessed PGE_2 release in CSF collected by both spinal dialysis and through lumbar puncture, and found that the two methods gave similar results. The dialysis approach has the advantage of giving a temporal resolution, but the disadvantage of a relatively low recovery (approximately 10%) and size cut-off of the dialysis membrane. Hence this method is not suitable for the assessment of TNF. We also analysed the levels of PGE_2 with two different methods, ELISA and LC/MS, and established that both methods are appropriate for PGE_2 measurement in small volumes of CSF.

The role of glia in TLR4-evoked PGE_2 release was examined by injecting two glia activation inhibitors, minocycline and pentoxifylline, i.t. prior to injection of LPS or KDO_2. Minocycline has been shown to exert biological effects distinct from its antimicrobial action (Klein and Cunha, 1995). It has emerged as a potent inhibitor of microglial activation and proliferation, without any known direct action on astrocytes or neurones (Amin *et al*., 1996; Tikka *et al*., 2001a; Tikka and Koistinaho, 2001b). Pentoxifylline, like its analogue propentofylline, is a methylxanthine derivative that inhibits phosphodiesterase activity and adenosine uptake and decreases the synthesis of pro-inflammatory cytokines. Pentoxifylline and propentofylline prevent the activation and release of cytokines from spinal glia, both astrocytes and microglia (Chao *et al*., 1992; Schubert *et al*., 1998; Tawfik *et al*., 2008), and attenuate mechanical allodynia induced by nerve injury (Sweitzer *et al*., 2001; Liu *et al*., 2007; Tawfik *et al*., 2008). Interestingly, while both minocycline and pentoxifylline blocked TNF release, only pentoxifylline had an effect on the spinal release of PGE_2 evoked by LPS and KDO_2. This could be due to minocycline and pentoxifylline interfering with different TLR4-mediated intracellular signalling pathways or the result of pentoxifylline acting on both microglia and astrocytes, given that PGE_2 was synthesized in response to activation of astrocytic TLR4.

Inhibition of COX, either by i.t. injection of ketorolac or by systemic (i.p.) injection of ibuprofen blocked TLR4-mediated spinal PGE_2 release. While this is expected, it is noteworthy that i.p. injection of ibuprofen blocked spinal release of PGE_2 evoked by a spinal stimulus, i.e. i.t. injection of KDO_2. This indicates that constitutively expressed spinal COX is an important source for central PGE_2 synthesis and that systemic delivery of COX inhibitors influence spinal COX activity.

Intrathecal injection of pentoxifylline, minocycline and etanercept attenuated the allodynia induced by LPS and KDO_2, suggesting that glial activation and TNF release are part of the TLR4-mediated events that lead to spinal sensitization. In contrast, the COX inhibitors, which both systemically and spinally efficiently suppressed TLR4-mediated spinal PGE_2 release, had no effect on TLR4-induced tactile allodynia. Moreover, minocycline readily attenuated allodynia, while it did not affect spinal release of PGE_2. These observations suggest that TLR4 activation-associated COX products do not play a role in TLR4-mediated nociception. Rather, cytokines such as TNF (present study) and interleukin 1β (Clark *et al*., 2006), both released rapidly from glial cells following TLR4 activation, are the potential mediators for TLR4-triggered pain signals. PGE_2 and related eicosanoids may participate in other responses to TLR4 activation, e.g. illness symptoms, regulation of blood circulation and cell migration (for review see (Buczynski *et al*., 2009). Nevertheless, the present finding, that the anti-inflammatory action of COX inhibitors could be dissociated from their anti-nociceptive activity during TLR4 activation, indicates that blocking spinal COX activity may not be effective in relieving the pain that is associated primarily with pro-nociceptive cytokines.

In the central nervous system, TLR4 is expressed predominantly on microglia (Lehnardt *et al*., 2002); however, a low-level constitutive expression of TLR4 in astrocytes has also been reported in culture. The functional expression of these receptor proteins in astrocytes is further supported by the ability of TLR4 ligands to induce both mRNA expression and protein secretion of pro-inflammatory cytokines from astrocytes (Bowman *et al*., 2003; Jack *et al*., 2005). Accordingly, we found that primary cultures of adult rat spinal astrocytes responded to stimulation with the TLR4 agonist KDO_2 by releasing TNF and PGE_2. Pentoxifylline prevented KDO2-

induced release of TNF and PGE_2 from these cells. However, TNF release, in particular, was only modestly reduced in the presence of pentoxifylline and this indicates that pentoxifylline only interferes with activation of some of the several intracellular signalling pathways leading to TNF release (NF-κB, p38 mitogen activated protein kinase, etc.). It is also possible that a higher concentration of pentoxifylline is needed to completely attenuate TNF release. While it is clear that microglia play an important role in the regulation of TNF release evoked by i.t. injection of both LPS and KDO_2, it is not possible at this time to determine if astrocytes contributed to the TNF release observed in our *in vivo* studies.

In summary, these *in vivo* and *ex vivo* data affirm that TLR4 plays an important role in pain processing and that glia activity is critical in TLR4-induced hypersensitivity and TNF release. With more TLR4 antagonists becoming available, the role of TLR4 in pain processing can be explored in further detail and such studies will most certainly reveal new avenues for interfering with glia activity in order to provide pain relief.

Acknowledgements

This work was supported by the Arthritis Foundation (CIS) and NIH grants DA21654 (CIS), Swedish Research Council (CIS), NS16541 (TLY) and GM64611 and U54 GM069338 (EAD) and Wenner-Gren Foundations (SC).

Conflict of interest

None to declare.

References

Alexander SP, Mathie A, Peters JA (2008). Guide to Receptors and Channels (GRAC). 3rd edn. *Br J Pharmacol* **153** (Suppl. 2): S1–S209.

Amin AR, Attur MG, Thakker GD, Patel PD, Vyas PR, Patel RN *et al*. (1996). A novel mechanism of action of tetracyclines: effects on nitric oxide synthases. *Proc Natl Acad Sci USA* **93**: 14014–14019.

Bettoni I, Comelli F, Rossini C, Granucci F, Giagnoni G, Peri F *et al*. (2008). Glial TLR4 receptor as new target to treat neuropathic pain: efficacy of a new receptor antagonist in a model of peripheral nerve injury in mice. *Glia* **56**: 1312–1319.

Bianchi M, Martucci C, Ferrario P, Franchi S, Sacerdote P (2007). Increased tumor necrosis factor-alpha and prostaglandin E2 concentrations in the cerebrospinal fluid of rats with inflammatory hyperalgesia: the effects of analgesic drugs. *Anesth Analg* **104**: 949–954.

Bowman CC, Rasley A, Tranguch SL, Marriott I (2003). Cultured astrocytes express toll-like receptors for bacterial products. *Glia* **43**: 281–291.

Buczynski MW, Stephens DL, Bowers-Gentry RC, Grkovich A, Deems RA, Dennis EA (2007). TLR-4 and sustained calcium agonists synergistically produce eicosanoids independent of protein synthesis in RAW264.7 cells. *J Biol Chem* **282**: 22834–22847.

Buczynski MW, Dumlao DS, Dennis EA (2009). Thematic review series: proteomics. An integrated omics analysis of eicosanoid biology. *J Lipid Res* **50**: 1015–1038.

Chao CC, Hu S, Close K, Choi CS, Molitor TW, Novick WJ *et al*. (1992). Cytokine release from microglia: differential inhibition by pentoxifylline and dexamethasone. *J Infect Dis* **166**: 847–853.

Chaplan SR, Bach FW, Pogrel JW, Chung JM, Yaksh TL (1994). Quantitative assessment of tactile allodynia in the rat paw. *J Neurosci Methods* **53**: 55–63.

Clark AK, D'Aquisto F, Gentry C, Marchand F, McMahon SB, Malcangio M (2006). Rapid co-release of interleukin 1beta and caspase 1 in spinal cord inflammation. *J Neurochem* **99**: 868–880.

Codeluppi S, Svensson CI, Hefferan MP, Valencia F, Silldorff MD, Oshiro M *et al*. (2009). The Rheb-mTOR pathway is upregulated in reactive astrocytes of the injured spinal cord. *J Neurosci* **29**: 1093–1104.

Deems R, Buczynski MW, Bowers-Gentry R, Harkewicz R, Dennis EA (2007). Detection and quantitation of eicosanoids via high performance liquid chromatography-electrospray ionization-mass spectrometry. *Methods Enzymol* **432**: 59–82.

Fontana A, Kristensen F, Dubs R, Gemsa D, Weber E (1982). Production of prostaglandin E and an interleukin-1 like factor by cultured astrocytes and C6 glioma cells. *J Immunol* **129**: 2413–2419.

Galambos R (1961). A glia-neural theory of brain function. *Proc Natl Acad Sci USA* **47**: 129–136.

Gay NJ, Gangloff M (2007). Structure and function of Toll receptors and their ligands. *Annu Rev Biochem* **76**: 141–165.

Hori T, Oka T, Hosoi M, Aou S (1998). Pain modulatory actions of cytokines and prostaglandin E2 in the brain. *Ann N Y Acad Sci* **840**: 269–281.

Hutchinson MR, Lewis SS, Coats BD, Skyba DA, Crysdale NY, Berkelhammer DL *et al*. (2009a). Reduction of opioid withdrawal and potentiation of acute opioid analgesia by systemic AV411 (ibudilast). *Brain Behav Immun* **23**: 240–250.

Hutchinson MR, Ramos KM, Loram LC, Wieseler J, Sholar PW, Kearney JJ *et al*. (2009b). Evidence for a role of heat shock protein-90 in toll like receptor 4 mediated pain enhancement in rats. *Neuroscience* **164**: 1821–1832.

Jack CS, Arbour N, Manusow J, Montgrain V, Blain M, McCrea E *et al*. (2005). TLR signaling tailors innate immune responses in human microglia and astrocytes. *J Immunol* **175**: 4320–4330.

Kehl LJ, Kovacs KJ, Larson AA (2004). Tolerance develops to the effect of lipopolysaccharides on movement-evoked hyperalgesia when administered chronically by a systemic but not an intrathecal route. *Pain* **111**: 104–115.

Klein NC, Cunha BA (1995). Tetracyclines. *Med Clin North Am* **79**: 789–801.

Koetzner L, Hua XY, Lai J, Porreca F, Yaksh T (2004). Nonopioid actions of intrathecal dynorphin evoke spinal excitatory amino acid and prostaglandin E2 release mediated by cyclooxygenase-1 and -2. *J Neurosci* **24**: 1451–1458.

Lee JY, Sohn KH, Rhee SH, Hwang D (2001). Saturated fatty acids, but not unsaturated fatty acids, induce the expression of cyclooxygenase-2 mediated through Toll-like receptor 4. *J Biol Chem* **276**: 16683–16689.

Lehnardt S, Lachance C, Patrizi S, Lefebvre S, Follett PL, Jensen FE *et al*. (2002). The toll-like receptor TLR4 is necessary for lipopolysaccharide-induced oligodendrocyte injury in the CNS. *J Neurosci* **22**: 2478–2486.

Liu J, Feng X, Yu M, Xie W, Zhao X, Li W *et al*. (2007). Pentoxifylline attenuates the development of hyperalgesia in a rat model of neuropathic pain. *Neurosci Lett* **412**: 268–272.

Maier SF, Wiertelak EP, Martin D, Watkins LR (1993). Interleukin-1 mediates the behavioral hyperalgesia produced by lithium chloride and endotoxin. *Brain Res* **623**: 321–324.

Marsala M, Malmberg AB, Yaksh TL (1995). The spinal loop dialysis catheter: characterization of use in the unanesthetized rat. *J Neurosci Methods* **62**: 43–53.

Meller ST, Dykstra C, Grzybycki D, Murphy S, Gebhart GF (1994). The possible role of glia in nociceptive processing and hyperalgesia in the spinal cord of the rat. *Neuropharmacology* **33**: 1471–1478.

Milligan ED, Watkins LR (2009). Pathological and protective roles of glia in chronic pain. *Nat Rev Neurosci* **10**: 23–36.

Miyake K (2004). Innate recognition of lipopolysaccharide by Toll-like receptor 4-MD-2. *Trends Microbiol* **12**: 186–192.

Miyake K (2007). Innate immune sensing of pathogens and danger signals by cell surface Toll-like receptors. *Semin Immunol* **19**: 3–10.

Muller-Ladner U, Gay RE, Gay S (2002). Role of nuclear factor kappaB in synovial inflammation. *Curr Rheumatol Rep* **4**: 201–207.

Raetz CR, Garrett TA, Reynolds CM, Shaw WA, Moore JD, Smith DC *et al*. (2006). Kdo2-Lipid A of Escherichia coli, a defined endotoxin that activates macrophages via TLR-4. *J Lipid Res* **47**: 1097–1111.

Raghavendra V, Tanga FY, DeLeo JA (2004). Complete Freunds adjuvant-induced peripheral inflammation evokes glial activation and proinflammatory cytokine expression in the CNS. *Eur J Neurosci* **20**: 467–473.

Reeve AJ, Patel S, Fox A, Walker K, Urban L (2000). Intrathecally administered endotoxin or cytokines produce allodynia, hyperalgesia and changes in spinal cord neuronal responses to nociceptive stimuli in the rat. *Eur J Pain* **4**: 247–257.

Sawada M, Kondo N, Suzumura A, Marunouchi T (1989). Production of tumor necrosis factor-alpha by microglia and astrocytes in culture. *Brain Res* **491**: 394–397.

Schubert P, Ogata T, Miyazaki H, Marchini C, Ferroni S, Rudolphi K (1998). Pathological immuno-reactions of glial cells in Alzheimer's disease and possible sites of interference. *J Neural Transm Suppl* **54**: 167–174.

Schwartz JP, Wilson DJ (1992). Preparation and characterization of type 1 astrocytes cultured from adult rat cortex, cerebellum, and striatum. *Glia* **5**: 75–80.

Shen A, Zhou D, Shen Q, Liu HO, Sun L, Liu Y *et al*. (2009). The expression of tumor necrosis factor-alpha (TNF-alpha) by the intrathecal injection of lipopolysaccharide in the rat spinal cord. *Neurochem Res* **34**: 333–341.

Slepko N, Minghetti L, Polazzi E, Nicolini A, Levi G (1997). Reorientation of prostanoid production accompanies "activation" of adult microglial cells in culture. *J Neurosci Res* **49**: 292–300.

Sweitzer SM, Schubert P, DeLeo JA (2001). Propentofylline, a glial modulating agent, exhibits antiallodynic properties in a rat model of neuropathic pain. *J Pharmacol Exp Ther* **297**: 1210–1217.

Takeda K, Akira S (2004). TLR signaling pathways. *Semin Immunol* **16**: 3–9.

Tanga FY, Raghavendra V, DeLeo JA (2004). Quantitative real-time RT-PCR assessment of spinal microglial and astrocytic activation markers in a rat model of neuropathic pain. *Neurochem Int* **45**: 397–407.

Tanga FY, Nutile-McMenemy N, DeLeo JA (2005). The CNS role of Toll-like receptor 4 in innate neuroimmunity and painful neuropathy. *Proc Natl Acad Sci USA* **102**: 5856–5861.

Tawfik VL, Regan MR, Haenggeli C, Lacroix-Fralish ML, Nutile-McMenemy N, Perez N *et al*. (2008). Propentofylline-induced astrocyte modulation leads to alterations in glial glutamate promoter activation following spinal nerve transection. *Neuroscience* **152**: 1086–1092.

Tikka TM, Koistinaho JE (2001b). Minocycline provides neuroprotection against N-methyl-D-aspartate neurotoxicity by inhibiting microglia. *J Immunol* **166**: 7527–7533.

Tikka T, Fiebich BL, Goldsteins G, Keinanen R, Koistinaho J (2001a). Minocycline, a tetracycline derivative, is neuroprotective against excitotoxicity by inhibiting activation and proliferation of microglia. *J Neurosci* **21**: 2580–2588.

Yaksh TL, Rudy TS (1976). Chronic catheterization of the spinal subarachnoid space. *Physiol Behav* **17**: 1031–1036.

Zelenka M, Schafers M, Sommer C (2005). Intraneural injection of interleukin-1beta and tumor necrosis factor-alpha into rat sciatic nerve at physiological doses induces signs of neuropathic pain. *Pain* **116**: 257–263.