Vitamin E (α -tocopherol) attenuates cyclo-oxygenase 2 transcription and synthesis in immortalized murine BV-2 microglia

Tamara EGGER*, Rufina SCHULIGOI†, Andrea WINTERSPERGER*, Rainer AMANN†, Ernst MALLE* and Wolfgang SATTLER*¹

*Institute of Medical Biochemistry and Molecular Biology, University Graz, Harrachgasse 21, 8010 Graz, Austria, and †Institute of Experimental and Clinical Pharmacology, University Graz, 8010 Graz, Austria

One of the immediate early microglial genes that are up-regulated in response to proinflammatory stimuli is cyclo-oxygenase 2 (COX-2). In the present study, we have investigated the effects of α -tocopherol (α TocH), an essential constituent of the nervous system, on the activation of COX-2 in lipopolysaccharide (LPS)-stimulated mouse BV-2 microglia. In unstimulated BV-2 cells, COX-2 mRNA and protein were almost undetectable but were strongly up-regulated in response to LPS. Activation of COX-2 protein synthesis in LPS-stimulated BV-2 cells involved activation of the extracellular-signal-regulated kinase 1/2 (ERK1/2) and p38 mitogen-activated protein kinase (MAPK) pathway and was sensitive to the protein kinase C (PKC) inhibitors staurosporine and chelerythrine, and the MAP kinase/ERK kinase 1/2 inhibitors PD98059 and U0126. Supplementation of BV-2 cells with α TocH before LPS stimulation resulted in pronounced up-regulation of protein phosphatase 2A (PP2A)

activity, down-regulation of PKC activity, ERK1/2 phosphorylation and nuclear factor κ B (NF κ B) activation. As a result, COX-2 protein levels and prostaglandin E₂ production were significantly lower in α TocH-supplemented cells. The effects of α TocH on PKC activity could be reverted by calyculin A and okadaic acid, two PP inhibitors. In summary, our results suggest that α TocH activates microglial PP2A activity and thereby silences an LPS-activated PKC/ERK/NF κ B signalling cascade resulting in significantly attenuated COX-2 protein synthesis. These *in vitro* results imply that α TocH could induce quiescence to pathways that are associated with acute or chronic inflammatory conditions in the central nervous system.

Key words: extracellular signal-regulated kinase, mitogen-activated protein kinase, nuclear factor κ B, prostaglandin E₂.

INTRODUCTION

The activation of microglia that occurs after acute central nervous system (CNS) injury is accompanied by a characteristic pattern of cellular and metabolic changes that are generally considered as beneficial and reflect an attempt to cope with a drastically altered microenvironment of the CNS [1]. In contrast, chronic neuropathological conditions as observed in multiple sclerosis, HIV infection or Alzheimer's disease are characterized by sustained up-regulation of microglial genes related to inflammation and prolonged production of proinflammatory compounds [1]. Once activated chronically, microglia are capable of producing a variety of proinflammatory mediators and potentially neurotoxic compounds. These include complement, cytokines, reactive oxygen and nitrogen species, proteases, excitatory amino acids and prostaglandins (PGs) [2].

PGH₂, the proximal precursor of PG biosynthesis, is produced by either of the cyclo-oxygenases (COXs), isoforms 1 and 2. The *COX-1* gene has properties commonly associated with house-keeping genes and is constitutively expressed. In contrast, the *COX-2* promoter contains a TATA box and several transcription factor-binding sites that are implicated in the regulation of COX-2 expression [3]. This is of importance under neuropathological conditions where COX-2 not only catalyses the synthesis of proinflammatory PGs but is itself rapidly induced by an array of other inflammatory mediators [4]. This is consistent with the identification of *COX-2* as an immediate early response gene and studies demonstrating that transcriptional regulation is a key mechanism for control of COX-2 levels [3,5]. *In vitro* experiments

revealed that COX-2 induction in response to proinflammatory stimuli is observed in neurons, astrocytes and microglia, resulting in elevated PG synthesis [4]. *In vitro*, PGE₂ induces the production of proinflammatory stimuli such as interleukin 6, chemokines or amyloid precursor protein [6,7]. Accumulating evidence suggests that COX-2 levels are increased in Alzheimer's disease brain and correlate with the expression levels of β -amyloid [8,9]. Thus COX-2 is located in a pivotal amplifying position under inflammatory conditions.

The above-mentioned findings make excess PG production under neuropathological conditions a promising target for COX-inhibiting non-steroidal anti-inflammatory drugs (NSAIDs). Epidemiological studies have confirmed a decrease in prevalence of Alzheimer's disease among patients undergoing NSAIDs treatment [10]. However, the neuroprotective effect of NSAIDs is also mediated by peroxisome proliferator-activated receptors [11,12] and modulation of γ -secretase activity [13]. A class of natural compounds that received considerable attention as neuroprotective agents are members of the vitamin E family. 'Vitamin E' is comprised of eight naturally occurring isomers, the tocopherols and the tocotrienols. α -Tocopherol (α TocH), the biologically most active compound of the vitamin E family [14], is an indispensable micronutrient for normal functioning of the nervous system [15]. In addition to its role as physiological antioxidant, α TocH is able to induce a ramified morphology of rat microglia and to suppress adhesion molecule expression, both effects are indications of immunological deactivation [16]. Recently [17], we could demonstrate that α TocH attenuates microglial superoxide anion radical production by interference

Abbreviations used: CNS, central nervous system; COX, cyclo-oxygenase; DTT, dithiothreitol; EMSA, electrophoretic mobility-shift assay; ERK1/2, extracellular signal-regulated kinase 1/2; FCS, foetal calf serum; JNK, c-Jun N-terminal kinase; LPS, lipopolysaccharide; MAP, mitogen-activated protein; MAPK, MAP kinase; MEK, MAP kinase/ERK kinase; NF κ B, nuclear factor κ B; NSAIDs, non-steroidal anti-inflammatory drugs; PG, prostaglandin; PKC, protein kinase C; PP, protein phosphatase; SAPK, stress-activated protein kinase; α TocH, α -tocopherol.

¹ To whom correspondence should be addressed (e-mail wolfgang.sattler@kfunigraz.ac.at).

with the assembly of the NADPH-oxidase complex. Li et al. [18] have shown that α TocH protects neurons from the assault of activated microglia via interference with the p38 mitogen-activated protein kinase (MAPK)-dependent signalling cascade. Taken together, these findings indicate that α TocH can exert specific neuroprotective properties, including immunological deactivation of microglia.

The present study aimed at characterizing the effects of α TocH supplementation on COX-2 expression in murine immortalized BV-2 microglia. We have used specific inhibitors of intracellular-signalling pathways to pinpoint the site(s) of action where α TocH interferes with COX-2 transcription, synthesis and with PGE₂ production in response to lipopolysaccharide (LPS), a potent activator of the inflammatory response.

EXPERIMENTAL

Materials

Chemicals were obtained from Sigma (Vienna, Austria) or Merck (Vienna, Austria). RPMI 1640 medium and foetal calf serum (FCS) were obtained from PAA Laboratories (Linz, Austria). L-glutamine, gentamicin and trypsin were obtained from Biochrom (Berlin, Germany). Synthetic PGE₂, EGTA, EDTA, NaF, deoxycholate, Triton X-100, dithiothreitol (DTT), staurosporine, leupeptin, PMSF, all-*rac*- α TocH, glycerol and LPS from *Escherichia coli* were obtained from Sigma. Plastic ware for cell culture was from Greiner (Linz, Austria). [5,6,8,11,12,14,15(*N*)-³H]PGE₂ and the enhanced chemiluminescence (ECL[®]) detection kit were obtained from Amersham Pharmacia (Vienna, Austria). Calyculin A, okadaic acid, PD98059 and Na₃VO₄ were purchased from Calbiochem–Novabiochem (La Jolla, CA, U.S.A.). [³²P]γ-ATP was obtained from NEN (Vienna, Austria). Chelerythrine was obtained from Alomone Laboratories (Jerusalem, Israel). Opti-Fluor scintillation cocktail was obtained from Packard Canberra (Vienna, Austria). COX-1 and COX-2 antibodies, and the COX-2 cDNA probe were obtained from Cayman Chemicals (Ann Arbor, MI, U.S.A.). Peroxidase-conjugated anti-rabbit IgG was purchased from Merck. The antibodies raised against extracellular signal-regulated kinase 1/2 (ERK1/2) and active, phosphorylated ERK1/2 (pERK1/2) and the gel shift assay kits were from Promega (Mannheim, Germany). Phospho-MAPK family antibody sampler kit was purchased from Cell Signalling Technology (Hertfordshire, U.K.). The Malachite Green protein phosphatase 2A (PP2A) kit was obtained from Upstate Biotechnology (Lake Placid, NY, U.S.A.). A polyclonal goat antiserum raised against the catalytic subunit of PP2A (sc-6110) and horseradish peroxidase-conjugated rabbit anti-goat IgG (sc-2922) were obtained from Santa Cruz Biotechnologies (Santa Cruz, CA, U.S.A.). PVDF membrane was obtained from Pall (Vienna, Austria).

Cell culture

The BV-2 cell line was produced by immortalization of murine microglia by infection with a v-raf/v-myc carrying J2 retrovirus and retains properties of microglia [19].

Murine BV-2 microglia were maintained in RPMI 1640 medium supplemented with 10% (w/v) FCS, gentamicin (50 µg/ml) and glutamine (2 mM) as described previously [17]. Before some experiments, cells were incubated in RPMI 1640 medium containing α TocH (added as ethanolic solution, final concentration 25–100 µM, up to 24 h), staurosporine (1 µM), chelerythrine (7 µM [20]), U0126 (10 µM) or PD98059 (10 µM [21]). Where indicated, the cells were activated with LPS

(10 µg/ml) in the presence or absence of calyculin A (10 nM [22]) or okadaic acid (500 nM [23]). α TocH levels in RPMI 1640 containing 10% FCS were below the absolute limit of detection (9 pmol) of the method used.

PGE₂ measurements

Where indicated, cells were cultured (24 h) in α TocH-containing (100 µM) medium before the experiments. Thereafter the medium was changed and cells were preincubated in the presence of the chosen compounds. After this preincubation, the cells were stimulated with LPS (10 µg/ml, 24 h) in the presence of the chosen inhibitors. PGE₂ levels were analysed by RIA using [5,6,8,11,12,14,15(*N*)-³H]PGE₂ as tracer and synthetic PGE₂ as standard. The detection limit (defined as 10% inhibition of binding) was 4 pg, corresponding to 147 pg/ml of sample; the effective dose 50 (ED₅₀) was 29 pg with an interassay variation of 3%.

PP2A assay

BV-2 cells were grown to confluency in 6-well trays. Before the experiments, the cells were supplemented with α TocH (100 µM, 24 h) wherever indicated. Thereafter the cells were stimulated with LPS (10 µg/ml; 30 min). Before PP2A measurements, the cells were washed with Hanks balanced salt solution and scraped in RIPA buffer (500 µl; containing leupeptin, aprotinin, pepstatin and PMSF). PP2A activity was measured with a Ser/Thr phosphatase assay kit in accordance with the manufacturer's instructions. Briefly, samples containing 5 µg of cell protein were added to a solution containing the K-R-pT-I-R-R peptide (final concentration 100 µM) in a 96-well microtitre plate, incubated for 20 min, and Malachite Green solution was added (100 µl). After 15 min, measurements (560 nm) were performed on a Victor 1420 (Wallac Oy, Sweden) plate reader. Phosphate release was determined by comparing absorbance with the standard curve.

Lysis procedures

COX-2

BV-2 cells were cultured and pretreated exactly as described above for PGE₂ measurements. Cells (20 × 10⁶ cells) were then washed twice with PBS (10 mM, pH 7.4) and scraped off in 1 ml extraction buffer (50 mM Tris/10 mM EDTA/1% Triton X-100/1 mM PMSF/50 µM pepstatin A/0.2 mM leupeptin) as described previously [24]. The cells were lysed for 10 min, sonicated twice for 30 s and centrifuged (5000 g, 10 min at 4 °C). The cell extract (supernatant) was diluted (1:1, v/v) with sample buffer [0.1 M Tris/HCl (pH 6.8)/4% (w/v) SDS/15% (w/v) glycerol/1% mercaptoethanol], and equal amounts (100 µg protein/lane) were loaded on to 8% SDS gels.

MAPK

Cells were cultured (24 h) in α TocH-containing (100 µM) medium before the experiments. Thereafter the medium was changed and cells were preincubated in the presence of the indicated compounds. After this preincubation, the cells were stimulated with LPS (10 µg/ml, 10–20 min) in the presence of the compounds chosen for the purpose. After washing with PBS, 20 × 10⁶ cells were lysed in 500 µl ice-cold buffer [1% Triton X-100/20 mM Tris (pH 7.5)/100 mM NaCl/1 mM Na₃VO₄/40 mM NaF/5 mM EGTA/0.2% SDS/0.5% deoxycholate/0.2 mM PMSF] for 15 min [25]. Debris was sonicated (twice at 4 °C for 30 s), centrifuged (13000 g, 10 min at 4 °C) and diluted (1:1, v/v) with

sample buffer. Equal amounts (120 μ g of protein/lane) were loaded on to 10% SDS gels. The cells were not kept serum-free before pERK analysis.

SDS/PAGE and immunoblotting procedures

SDS/PAGE was performed on 8 or 10% gels at 150 V for 90 min in a Bio-Rad Miniprotein Chamber. For Western-blot analysis, protein fractions were electrophoretically transferred on to PVDF membranes (150 mA, 60 min at 4 °C). Immunochemical detection of COX isoforms was performed with sequence-specific polyclonal rabbit anti-COX-1 (1:500) and rabbit anti-COX-2 (dilution 1:1000) antibodies. Immunochemical detection of ERK1/2, pERK1/2, pp38 MAPK and pSAPK (phosphorylated stress-activated protein kinase)/JNK (c-Jun N-terminal kinase) were performed with sequence-specific rabbit anti-mouse IgGs as primary antibodies (1:5000). Detection of the catalytic subunit of PP2A (PP2Ac) in nuclear BV-2 extracts (prepared as described below) was performed with a polyclonal goat antiserum (dilution 1:2500). Immunoreactive bands were visualized with peroxidase-conjugated anti-rabbit IgGs (dilution 1:5000) or rabbit anti-goat IgG (1:2500) and the ECL[®] detection system.

Nuclear factor κ B (NF κ B)

Cells were cultured (24 h) in α TocH-containing (100 μ M) medium before the experiments. Thereafter the medium was changed and cells were preincubated in the presence of the indicated compounds. After this preincubation, the cells were stimulated with LPS (10 μ g/ml, 0.25 or 6 h) in the presence of the indicated compounds. Immunochemical detection of the p50 subunit of NF κ B in nuclear extracts [26] of BV-2 cells was performed with sequence-specific rabbit anti-mouse IgGs as primary antibody (1:2000).

For electrophoretic mobility-shift assays (EMSAs), cells were lysed in buffer A [10 mM Hepes (pH 7.9)/1.5 mM MgCl₂/10 mM KCl/0.5 mM DTT/0.2 mM PMSF containing 0.5% Nonidet P40] on ice for 15 min, followed by vortex-mixing for 10 s as described previously [26]. Nuclei were pelleted, and nuclear extracts were obtained by high salt extraction after incubating nuclei in 20 mM Hepes (pH 7.9), containing 25% (v/v) glycerol, 420 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM DTT and 0.2 mM PMSF for 20 min. Nuclei were pelleted again and the supernatant containing the nuclear extract was stored at -70 °C. Double-stranded DNA oligonucleotides, containing the consensus-binding site for NF κ B (5'-AGTTGAGGGGAC-TTCCAGGC-3') were used for gel shift assays. The oligonucleotide was end-labelled with [γ -³²P]ATP (3000 Ci/mmol at 10 mCi/ml) and T4 polynucleotide kinase and purified on a Sephadex G-50 column. Protein (3 μ g) from each sample was incubated with ³²P-labelled double-stranded oligonucleotide. The nuclear extract-DNA complexes were resolved in 4% (w/v) polyacrylamide gels. The gels were then dried and autoradiographed. Competition experiments were performed to confirm the identity of NF κ B-binding proteins.

Northern-blot analysis

Total cellular RNA was isolated from confluent 9 cm Petri dishes by RNA-easy kit (Qiagen) followed by purification of mRNA using the polyadenylated [poly(A)⁺] Purist[™] kit (Ambion). mRNA (1 μ g) was applied in each lane, separated in formaldehyde-containing agarose gels (1%) and transferred on to HybondX membranes by pressure blotting. Membrane-bound mRNA was UV-fixed, and then hybridized with a ³²P-labelled

murine COX-2 cDNA probe. After hybridization, the membranes were washed and analysed on a PhosphorImager system (Molecular Dynamics, Sunnyvale, CA, U.S.A.).

Statistical analysis

Results are expressed as means $\pm$ S.D. or means $\pm$ S.E.M. Student's *t* test was applied to calculate statistical significance among different groups using the GraphPad Prism package.

RESULTS

α TocH inhibits PGE₂ production in LPS-activated BV-2 microglia

Exposure of BV-2 cells to LPS increased PGE₂ approx. 10-fold over baseline levels (390 $\pm$ 98 versus 4139 $\pm$ 525 pg/mg of cell protein; basal versus LPS-stimulated respectively; Figure 1A).

Figure 1 Effects of α TocH on PGE₂ levels and COX-2 gene expression in LPS-stimulated BV-2 microglia

Cells were grown to confluency in 6-well plates in RPMI 1640 medium. After α TocH supplementation (25–100 μ M, 24 h) the cells were stimulated with LPS for 24 h in the absence (–) or presence (+) of the compounds as described in the Methods section. (A) The medium was collected and PGE₂ concentrations were measured by RIA. Data represent means $\pm$ S.D. from one representative experiment (performed in triplicates) out of four. Asterisks denote values statistically different from LPS-stimulated cells (****P* < 0.0005). (B) Cells were cultivated in 9 cm Petri dishes in RPMI 1640 in the absence (–) or presence (+) of α TocH (24 h). Thereafter, the cells were stimulated with LPS for 24 h in the presence of the above compounds. poly(A)⁺ RNA was isolated as described in the Materials section and the Methods section and subjected to Northern-blot analysis using a specific murine COX-2 probe. The relative amount of COX-2 mRNA was measured by image analysis after normalization with that of 28 S rRNA. The mRNA content of the LPS-stimulated sample was set as 100%. The densitograph represents means $\pm$ S.E.M. for three independent experiments.

To determine the effects of α TocH, cells were precultured in medium containing α TocH (25–100 μ M). Under these conditions, cells acquire α TocH in a nearly linear manner from 1.5 ng of α TocH/mg of cell protein (unsupplemented cells) to 11 μ g of α TocH/mg of cell protein (medium containing 100 μ M α TocH) after 24 h supplementation [17]. Increasing the concentration of α TocH addition led to a significant reduction of detectable PGE₂ in the cellular supernatant of LPS-stimulated cells almost back to baseline levels (432 ± 129 pg/mg of cell protein at 100 μ M α TocH).

To determine whether α TocH interferes with *COX-2* transcription, Northern-blot analyses were performed. In unstimulated BV-2 cells, no *COX-2* mRNA was detectable, whereas LPS stimulation led to a strong induction of *COX-2* transcripts (Figure 1B). In line with effects of α TocH on PGE₂ synthesis, the levels of *COX-2* mRNA in LPS-stimulated cells were significantly decreased in α TocH-supplemented cells (Figure 1B), indicating that α TocH at concentrations ≥ 50 μ M interferes with *COX-2* transcription in BV-2 microglia.

Effect of α TocH on cellular PP2A activities

α TocH inhibits protein kinase C (PKC) activity in BV-2 cells, an effect that could be counteracted by okadaic acid and by calyculin A, two PP inhibitors [17]. To explore this phenomenon in more detail, we have directly analysed PP2A activity in unstimulated and stimulated cells that were cultured in the presence or absence of α TocH. There was a general tendency towards higher PP2A activities in the regimens that were treated with α TocH (this was reflected by decreased PKC activities; results not shown and [17]; Figure 2A). In unstimulated cells, PP2A activities were not significantly different [1212 ± 16 versus 1375 ± 44 pmol $\cdot$ (mg of cell protein)⁻¹ $\cdot$ min⁻¹, absence versus presence of α TocH]. In LPS-stimulated cells, the addition of α TocH resulted in significantly up-regulated PP2A activities [1159 ± 21 versus 1637 ± 82 pmol $\cdot$ (mg of cell protein)⁻¹ $\cdot$ min⁻¹, absence versus presence of α TocH]. The vehicle [0.03 % (v/v) ethanol] alone had no effect on PP2A activity [1104 ± 94 pmol $\cdot$ (mg of cell protein)⁻¹ $\cdot$ min⁻¹]. The addition of okadaic acid significantly decreased PP2A activities in LPS-stimulated cells; however, this effect was not counteracted by α TocH (results not shown).

Besides stimulating PP2A activity, α TocH treatment resulted in significantly increased levels of immunoreactive PP2Ac (36 kDa) in nuclear extracts of BV-2 cells (Figure 2B). In response to α TocH supplementation, PP2Ac levels were increased approx. 2-fold in unstimulated and LPS-stimulated cells respectively. The addition of the vehicle (0.03 % ethanol) had no effect on PP2Ac signals. Immunoblot analysis of cellular plasma membrane fractions revealed a tendency towards decreased levels of PP2Ac after α TocH treatment; this effect was, however, statistically not significant (results not shown).

Effects of α TocH on LPS-induced MAPK activation

In the next set of experiments, the effects of LPS and α TocH on the activation of the MAPK pathways (ERK1/2, p38 MAPK, SAPK/JNK), a signalling cascade contributing to *COX-2* transcription, were studied (Figure 3). Vehicle controls revealed that ethanol (0.03 %) neither activated nor interfered with LPS-induced activation of the MAPK pathways. The exposure of BV-2 microglia to LPS resulted in rapid phosphorylation of ERK1/2 (Figure 3A; 'pERK1/2'). ERK1/2 phosphorylation in response to LPS was strongly inhibited by α TocH and U0126 (reduction by 60 and 95 % respectively). A consistent observation throughout the present study was that ERK2 (42 kDa) phosphorylation was always more pronounced than phosphorylation of ERK1

Figure 2 Effects of α TocH on PP2A in BV-2 microglia

(A) Cells were grown to confluency in 6-well trays in RPMI 1640 medium in the absence (—) or presence (+) of α TocH (24 h), thereafter the cells were stimulated with LPS (30 min). Where indicated, the cells were incubated in the presence of ethanol (EtOH) as vehicle control. After stimulation, the cells were washed twice with Hanks balanced salt solution and lysed. PP2A activities were measured as described in the Materials section and the Methods section. Data shown represent mean values from three independent experiments and are represented as Box Whiskers plots (***) $P = 0.0001$ in comparison with cells that were stimulated with LPS in the absence of α TocH. (B) Cells were cultivated in 9 cm Petri dishes in RPMI 1640 in the absence (—) or presence (+; 24 h) of α TocH. EtOH was used as vehicle control. Thereafter fresh medium with or without α TocH and LPS was added. After 10 min, the cells were washed and nuclear extracts were prepared as described in the Materials section and the Methods section. Nuclear proteins (30 μ g/lane) were separated on SDS/PAGE (10% gels), transferred on to PVDF membranes and detected with a polyclonal goat anti-PP2Ac antiserum. Immunoreactive bands were visualized with peroxidase-conjugated rabbit anti-goat IgGs (1:2500) using ECL[®] development. The immunoblots are from one representative experiment and the histograms represent means $\pm$ S.D. for three independent experiments.

(44 kDa). Time-dependent experiments revealed that ERK1/2 phosphorylation in response to LPS was a nearly linear process (Figure 3B). In the presence of α TocH, ERK1/2 phosphorylation showed a lag phase of 10 min and was then inhibited by approx. 50 %. LPS stimulation resulted in rapid phosphorylation of p38 MAPK (Figure 3C; 'pp38 MAPK') and was inhibited by approx. 40 % in the presence of α TocH. The addition of ethanol and U0126 had no effect on p38 phosphorylation. Also time-dependent p38 MAPK phosphorylation was inhibited between 35 and 50 % in α TocH-supplemented BV-2 cells (Figure 3D). Under

Figure 3 Effect of α TocH on MAPK pathways in BV-2 microglia

Cells were cultivated in 9 cm Petri dishes in RPMI 1640 in the absence (—) or presence (+) of α TocH for 24 h. The medium was changed, fresh medium with or without α TocH or U0126 was added. After a 30 min preincubation period LPS was added. The cells were washed and lysed, transferred to sample buffer, separated on 10% gels (120 µg of total protein/lane) and blotted to PVDF membranes. Detection of pERK1/2 (**A, B**), and pp38 MAPK (**C, D**) were performed with sequence-specific rabbit anti-mouse IgGs (dilution 1:5000; from the MAPK-antibody sampler kit). Immunoreactive bands were visualized with peroxidase-conjugated goat anti-rabbit IgGs (1:5000) using ECL[®] development. The immunoblots are from one representative experiment and the histograms represent means $\pm$ S.D. from four independent experiments (*** P < 0.0005 in comparison with LPS-stimulated cells). For more clarity, the effects of α TocH (●) on time-dependent phosphorylation of ERK1/2 (**B**) and p38 MAPK (**D**) are shown as line graphs and represent means $\pm$ S.D. for four independent experiments.

these experimental conditions, no pSAPK/pJNK (phosphorylated JNK) was detectable (results not shown).

To establish a possible linkage between PKC activity and activation of the ERK1/2 pathway, the cells were cultivated in the absence or presence of α TocH, staurosporine (a PKC inhibitor) and PD98059 and U0126 before LPS stimulation. In response to these compounds, the levels of immunoreactive COX-2 protein were significantly down-regulated by 60–95%

(α TocH), 95% (staurosporine), 96% (PD98059) and 98% (U0126; results not shown).

Effects of α TocH on NF κ B activation

In non-stimulated cells, NF κ B dimers are present as cytoplasmic latent complexes due to the binding of inhibitory κ B that mask the nuclear localization signal. On stimulation, inhibitory κ Bs

Figure 4 Effect of α TocH, PKC, MEK and protein phosphatase inhibitors on NF κ B activation in BV-2 microglia

(A) Cells were grown to confluency in 75 cm² flasks in RPMI 1640 medium. Expression of p50 was assessed under basal conditions (control), or after LPS stimulation under the conditions described in the Methods section. Nuclear lysates were transferred on to SDS sample buffer, separated on 10% gels (80 µg of total protein/lane) and electrophoretically transferred on to PVDF membranes. Detection of immunoreactive p50 was performed with sequence-specific rabbit IgG (dilution 1:1000), peroxidase-conjugated goat anti-rabbit IgG (1:5000) and ECL[®] development. One representative experiment out of three is shown and densitographs represent means $\pm$ S.E.M. for three independent experiments ($^{***}P < 0.0005$ in comparison with LPS-stimulated cells). (B) Cells were cultivated in RPMI 1640 medium on 9 cm Petri dishes until confluency in the absence (—) or presence (+) of the indicated compounds. NF κ B activation was measured after LPS stimulation (0.25 or 6 h) in the presence of the chosen compounds. EMSAs were performed as described in the Methods section. To ensure specific binding, excess amount of an unlabelled oligonucleotide ('competitor', 5 ng) was added to the reaction mixture, and disappearance of the shifted band was examined. The arrow indicates the position of the NF κ B-specific oligonucleotide–protein complex. One representative experiment out of three is shown. Densitographs represent means $\pm$ S.E.M. for three independent experiments ($^{**}P < 0.005$, $^{***}P < 0.0005$).

are phosphorylated and subjected to rapid proteasomal degradation. This unmask the nuclear localization signal in NF κ B, permitting nuclear translocation and activation of the target

Figure 5 Effect of α TocH on PGE₂ levels (A) and COX-2 (B) protein synthesis in BV-2 microglia

Cells were grown to confluency in 6-well plates in RPMI 1640 medium. Where indicated, cells were preincubated (24 h) in α TocH-containing (100 µM) medium before the experiments. Thereafter the medium was changed and cells were preincubated in the presence of compounds. After this preincubation, the cells were stimulated with LPS in the presence of inhibitors. (A) After LPS stimulation, the medium was collected and PGE₂ levels were measured by RIA. Data represent means $\pm$ S.D. from one representative experiment (out of three) performed in triplicates ($^{**}P < 0.005$, $^{***}P < 0.0005$). (B) For COX-2 immunoblots, the cells were lysed as described in the Materials section and the Methods section. Detection of COX-2 (15 µg of total protein/lane) was performed with a sequence-specific rabbit anti-COX-2 IgG (1:1000), peroxidase-conjugated goat anti-rabbit IgG (dilution 1:5000) and ECL[®] development. The arrow indicates the position of COX-2 (molecular mass $\approx$ 73 kDa). Immunoblots of one representative experiment (out of three) are shown. Densitographs represent means $\pm$ S.E.M. for three independent experiments ($^{***}P < 0.0005$ in comparison with the LPS-stimulated cells).

genes [27]. In line, the p50 subunit of NF κ B was barely detectable in nuclear extracts of unstimulated cells (Figure 4A). Stimulation with LPS showed a strong induction of immunoreactive p50 ($\approx$ 8-fold higher than baseline levels). When the culture medium was supplemented with α TocH, the nuclear p50 levels were decreased by 60%. Similar observations were made in the presence of staurosporine (decrease by 70%).

To confirm the observations of decreased immunoreactive p50 subunit, we have performed EMSAs (Figure 4B). In the presence

Figure 6 Effect of chelerythrine on pERK1/2, COX-1 and COX-2 protein levels in BV-2 microglia

Cells were grown to confluency on 9 cm Petri dishes in RPMI 1640 medium. After stimulation with LPS (20 min for ERK1/2 phosphorylation and 24 h for COX-2 stimulation) in the absence (—) or presence (+) of chelerythrine (7 µM) the cells were washed and lysed as described in the Materials section and the Methods section. The lysate was transferred on to SDS sample buffer, separated on 10% gels (120 µg of total protein/lane; ERK1/2 and pERK1/2) or 8% gels (15 µg of total protein/lane; COX-1 and -2) and transferred on to PVDF membranes. Western-blot analysis was performed as described in Figure 5(B). Results from one representative experiment out of two are shown.

of a specific mouse NFκB peptide as competitor, no NFκB signal was observed. Analysis of unstimulated cells resulted in a weak, but detectable band. Stimulation with LPS for the indicated time points resulted in significant activation of NFκB. EMSA performed on αTocH-supplemented cells confirmed decrease in NFκB levels (on average by 45%). The addition of staurosporine and the MAP kinase/ERK kinase (MEK) inhibitor U0126 significantly decreased signal intensities (on average a decrease by 85 and 70% was observed in the presence of staurosporine and U0126). The addition of okadaic acid restored normal NFκB activation in response to LPS.

Effects of αTocH, staurosporine, chelerythrine, PP and MEK inhibitors on LPS-induced PGE₂ levels, COX-2 and COX-1 protein synthesis

Finally, we have verified findings from above (Figure 4) on the product level (Figure 5A). Supplementation of the culture medium with αTocH (100 µM) before LPS stimulation significantly attenuated PGE₂ levels after 24 h (82% inhibition; 3897 ± 555 versus 694 ± 141 pg PGE₂/mg of cell protein). Incubation of BV-2 in the presence of staurosporine suppressed PGE₂ levels to background levels. The addition of okadaic acid and calyculin A to αTocH-supplemented cells partially, but not completely, restored PGE₂ levels (2698 ± 350 and 3253 ± 1555 pg PGE₂/mg of cell protein respectively). The MEK inhibitors PD98059 and U0126 impaired PGE₂ levels by 86 and 70% in comparison with LPS-stimulated BV-2 cells. The addition of the vehicle (0.03% ethanol) had no effect on PGE₂ levels in both unstimulated and stimulated cells.

On protein levels, no expression of COX-2 was observed in the absence of LPS (Figure 5B). αTocH supplementation decreased immunoreactive COX-2 levels in LPS-stimulated cells by 57% (quantitatively similar observations were made after 6 h LPS stimulation; results not shown). In line with an involvement of PKC during COX-2 activation, staurosporine inhibited COX-2 expression, whereas okadaic acid and calyculin A restored COX-2 protein expression in αTocH-supplemented cells. Both MEK inhibitors strongly suppressed COX-2 expression. The fact that PD98059 more efficiently inhibited PGE₂ levels than U0126 (Figure 5A) was also reflected on COX-2 protein level (Figure 5B). In line with the proposed housekeeping function, microglial COX-1 levels were unaffected by LPS stimulation, αTocH, staurosporine, calyculin A, or PD98059 and U0126 (results not shown).

Finally, we have tested the effects of the specific (but not isoenzyme selective) PKC inhibitor chelerythrine [belonging to a new class of inhibitors that interfere with the phosphate-acceptor site and non-competitively inhibit the ATP-binding site [20]] on pERK1/2 COX-1 and COX-2 expression (Figure 6). The addition of chelerythrine had no effect on the total levels of ERK1/2 and COX-1 but significantly inhibited ERK1/2 phosphorylation and COX-2 synthesis respectively.

DISCUSSION

The results obtained during the present study imply that in BV-2 microglia, PKC-dependent activation of the ERK1/2 pathway converges on NFκB activation and subsequent COX-2 transcription, COX-2 protein synthesis and PGE₂ levels respectively. The supplementation of BV-2 cells with αTocH stimulates cellular PP2A activity, and, as a result, inhibits COX-2 transcription and protein synthesis by silencing a PKC/ERK/NFκB-signalling cascade. However, despite pronounced down-regulation of COX-2 mRNA in response to αTocH, COX-2 protein was still detectable, findings that may reflect the half-life time of COX-2 protein. These findings are in line with previous observations where inhibition of PKCα by αTocH is linked with the activation of a PP, which in turn dephosphorylates PKCα thereby inhibiting its activity [28].

There is ample evidence that αTocH exerts specific neuroprotective properties and rescues neurons from the assault of a variety of agents inducing oxidative stress that are suggested in neurological disorders [16–18,29]. In the case of COX-2 it was shown that αTocH attenuates COX-2 transcription and COX-2 activity: in murine macrophages, αTocH significantly decreased LPS-induced COX-2 mRNA expression, COX-2 protein synthesis and PGE₂ release [30]. As observed during the present study, the effects of αTocH supplementation of J774 macrophages were more pronounced on COX-2 mRNA than protein level [30]. In a rat model of cyclosporine-induced nephrotoxicity, it was demonstrated that αTocH reduces COX-2 transcription [31]. αTocH supplementation results in reduced COX-2 activity and PGE₂ levels in macrophages [32] and normalizes COX-2 activity in senescent mice by direct inhibition of its activity [33].

The present study revealed that αTocH significantly stimulates PP2A activity in LPS-elicited BV-2 microglia. The PP2A holoenzyme is a heterotrimer consisting of a core heterodimer, which is composed of a scaffold and catalytic subunit that associates with different regulatory subunits (see [34,35] for reviews). PP2A is able to inactivate PKC by dephosphorylation [36,37] and is a negative regulator of the MAPK cascade [38]. Ricciarelli et al. [28] have shown that PKCα phosphorylation in smooth-muscle cells is strongly diminished in response to αTocH treatment. Our

studies have also indicated that PKC is subject to down-regulation in response to α TocH supplementation, resulting in defective assembly of the microglial NADPH-oxidase complex [17].

However, the mechanism(s) of PP2A activation by α TocH in microglia or other cellular systems are far from being clear. Although the heterotrimeric PP2A complex was first described as soluble activity, a membrane-associated form has been identified in T-cells [39] and in COS cells [40]. In the latter system, PP2A is physically associated with PKC α [40], probably in a manner similar to that for mast cells [23].

Dephosphorylation of PKC α was found in the membrane compartment [23], indicating that PP2A, after translocation to the membrane, modulates PKC activity by dephosphorylation. In model lipid membranes, α TocH appears to cluster into α TocH-enriched domains [41]. Thus it is intriguing to speculate that, similar to other signal transducing elements [42], membrane located PP2A is targeted to specialized membrane microdomains, whose affinity for the PP2A holoenzyme (or particular subunits of the enzyme) is modulated by the α TocH content. Interestingly, the results of the present study show that α TocH treatment enhances the levels of immunodetectable PP2Ac in nuclear extracts, although the underlying mechanisms that contribute to these observations are currently not clear. Our observations favour the existence of α TocH-enriched microdomains in the cellular plasma membrane, since a substantial amount of cellular α TocH in brain capillary endothelial cells is recovered in a caveolae-enriched fraction (W. Sattler, unpublished work). Findings that part of COX-2 in fibroblasts co-localizes with caveolin-1 in human fibroblasts [43] might support such a microdomain hypothesis.

The present results favour an important quantitative contribution of PP2A and PKC to transcriptional activation of COX-2 expression in BV-2 microglia, findings compatible with other reports: LPS-induced COX-2 transcription in primary rat microglia involves PKC and NF κ B activation [5] and PKC activation is an obligatory pathway for COX-2 transcription in TNF α -elicited human lung epithelial cells [44]. Furthermore, it was established that the activation of PKC δ contributes to MEK and ERK phosphorylation in a Ras-independent manner [45]. PKC-dependent ERK activation was also demonstrated in endotoxin-tolerant mice where direct activation of PKC re-established ERK phosphorylation [46]. Studies using the human COX-2 promoter [47] demonstrated that LPS up-regulates COX-2 transcription via either MAPK or PKC pathways. At the promoter level, the presence of at least two *cis*-acting elements (NF κ B, NF-IL6 and cAMP-response element) is necessary to achieve maximal induction of transcription [47].

During the present study, we have also observed down-regulation of the p38MAPK pathway in response to α TocH, findings in line with a recent study [18], where α TocH significantly attenuated the expression of pp38, but not total p38 in N9 microglia. Interestingly and of relevance for the present study, the levels of pp38 did not correlate with NF κ B activation after treatment of N9 cells with α TocH [18]. This might be a reflection of cell-specific differences of direct NF κ B activation, comparable with observations that the p38 MAPK pathway directly regulates the NF κ B pathway in neutrophils [48] but not in monocytes [49]. However, it is reasonable to assume that p38 activation could be responsible for the part of COX-2 activation that is not sensitive to α TocH supplementation. In contrast with ERK1/2 and p38 MAPK pathways, the activation of JNK and activator protein 1 was not responsive to LPS stimulation in BV-2 microglia (results not shown). Supplementation of BV-2 microglia with α TocH at concentrations of $\geq 50 \mu$ M (approx. 2-fold higher than plasma

levels [50]) resulted in similar increases in the cellular α TocH concentrations as reported previously [51] and attenuated PKC activity, ERK1/2 phosphorylation and NF κ B activation during the present study. These α TocH concentrations can be achieved in brain, as the α TocH content of various regions of rat brain ranges from 12 to 20 μ g/g of wet tissue [52] corresponding to approx. 40–65 μ M α TocH (calculated on the basis of a water content of 70%).

In conclusion, the present study provides insights into mechanisms by which α TocH (at concentrations that are achievable *in vivo*) diminishes microglial COX-2 expression, an enzyme involved in various neurodegenerative diseases. Our *in vitro* findings indicate that α TocH could have an important silencing function on COX-2 transcription in response to inflammatory conditions in the CNS.

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