Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical Pharmacology 66 (2003) 1263–1270
Biochemical Pharmacology
www.elsevier.com/locate/biochempharm

Involvement of protein kinase Cδ and extracellular signal-regulated kinase-2 in the suppression of microglial inducible nitric oxide synthase expression by *N*-[3,4-dimethoxycinnamoyl]-anthranilic acid (tranilast)

Michael Platten[a,1], Katrin Eitel[b], Jörg Wischhusen[a], Johannes Dichgans[a], Michael Weller[a,*]

[a]*Department of Neurology, University of Tübingen, 72076 Tübingen, Germany*
[b]*Department of Endocrinology, University of Tübingen, 72076 Tübingen, Germany*

Received 31 March 2003; accepted 2 June 2003

Abstract

Excess nitric oxide (NO) in the brain released by microglial cells contributes to neuronal damage in various pathologies of the central nervous system (CNS) including neurodegenerative diseases and multiple sclerosis. *N*-[3,4-Dimethoxycinnamoyl]-anthranilic acid (tranilast, TNL) is an anti-allergic compound which suppresses the activation of monocytes. We show that inducible nitric oxide synthase (iNOS) mRNA and protein expression and the release of NO from N9 microglial cells stimulated with the bacterial endotoxin lipopolysaccharide (LPS) are inhibited when the cells are exposed to TNL. TNL fails to modulate LPS-stimulated nuclear factor-κB (NF-κB) reporter gene activity and phosphorylation of inhibitory κB (IκB), indicating that NF-κB is not involved in the TNL-mediated suppression of LPS-induced iNOS expression. Moreover, TNL inhibits LPS-induced phosphorylation of extracellular signal-regulated kinase 2 (ERK-2). Finally, TNL abolishes translocation of protein kinase Cδ (PKCδ) to the nucleus and suppresses the phosphorylation of the PKCδ substrate, myristoylated alanin-rich C kinase substrate (MARCKS). We conclude that the anti-allergic compound TNL suppresses microglial iNOS induction by LPS via inhibition of a signalling pathway involving PKCδ and ERK-2.

Keywords: Tranilast; Microglia; Nitric oxide; Extracellular signal-regulated kinase-1/2; Protein kinase Cδ; Multiple sclerosis

1. Introduction

Microglial cells constitute a unique cell population within the central nervous system (CNS) ensuring brain homeostasis. With respect to morphology and function microglia closely resemble the phenotype of macrophages. Reactive microglial cells play a role in many CNS pathologies including ischemia, neurodegeneration, infection, tumorigenesis and autoimmunity [1]. Microglial cells may serve various functions including phagocytosis, antigen presentation and the release of pro- and anti-inflammatory cytokines in response to stress stimuli [2]. One of the central mediators of the microglial stress response is nitric oxide (NO). NO has been implied in the regulation of vascular integrity, neurotransmission and inflammation [3]. NO is synthesized from L-arginine by nitric oxide synthase (NOS). Of the three NOS isoforms, inducible NOS (iNOS) is expressed in glial cells as a response to bacterial endotoxins or pro-inflammatory cytokines [4]. Excess NO released by microglial cells as a consequence of iNOS induction may contribute to immunomodulation and neuronal damage in neurodegenerative disorders such as Parkinson's and Alzheimer's disease [5] and inflammatory

* Corresponding author. Tel.: +49-7071-298-7637; fax: +49-7071-295260.
E-mail address: michael.weller@uni-tuebingen.de (M. Weller).
[1] Present address: Department of Neurology, Stanford University Medical Center, Stanford, CA 94305-5316, USA.

Abbreviations: BAY, BAY 11-7082; CNS, central nervous system; DEX, dexamethasone; EDTA, ethylenediaminetetraacetic acid; ERK-1/2, extracellular signal-regulated kinase-1/2; GFX, GF 109203X; iNOS, inducible nitric oxide synthase; IFN-γ, interferon-γ; IκB, inhibitory κB; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; MARCKS, myristoylated alanin-riche kinase C substrate; NO, nitric oxide; NF-κB, nuclear factor-κB; PD, PD098059; PMSF, phenylmethylsulphonyl fluoride; PMA, phorbol 12-myristate 13-acetate; PKC, protein kinase C; ROT, rottlerin; TGF-β, transforming growth factor-β; TNL, tranilast.

0006-2952/$ – see front matter © 2003 Elsevier Inc. All rights reserved.
doi:10.1016/S0006-2952(03)00449-0

diseases such as multiple sclerosis [6,7]. Consequently, recent studies have focused on the development of pharmacological inhibitors of iNOS to attenuate neuronal damage [8] and to attenuate the course of autoimmune encephalomyelitis [9,10].

N-[3,4-dimethoxycinnamoyl]-anthranilic acid (tranilast, TNL) is an anti-allergic agent originally identified as an inhibitor of mast cell degranulation [11]. TNL has subsequently been identified as a potent inhibitor of migration, proliferation and release of transforming growth factor-β (TGF-β) from various cell types including malignant glioma cells [12]. Previous studies have attributed the suppressive effects of TNL on pulmonary fibrosis to an inhibition of macrophage activation [13]. Based on these studies, we recently showed that TNL prevents the induction of iNOS by interferon-γ (IFN-γ) via a nuclear factor-κB (NF-κB)-dependent pathway [14]. The bacterial wall component lipopolysaccharide (LPS) is a potent stimulator of iNOS in microglial cells. This study investigated the effects of TNL on LPS-mediated induction of iNOS in microglial cells and identified the signalling events involved in TNL-mediated inhibition of iNOS induced by LPS.

2. Methods

2.1. Reagents and cell culture

TNL was a generous gift of Kissei Pharmaceuticals. LPS (*Escherichia coli*, 0127:B8), phorbol 12-myristate 13-acetate (PMA) and dexamethasone (DEX) were obtained from Sigma. BAY 11-7082 (BAY), NF-κB SN50 inhibitory peptide (SN50), GF 109203X (GFX) and rottlerin (ROT) were purchased from Biomol. PD098059 (PD) was obtained from Calbiochem. Purified murine interferon-γ (IFN-γ) was purchased from Roche Diagnostics. N9 murine microglial cells were a kind gift from P. Ricciardi-Castagnoli [15]. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), 2 mM glutamine, penicillin (100 unit/mL) and streptomycin (100 μg/mL) and maintained at 37° in an atmosphere of 5% CO_2. Viability was assessed by crystal violet staining.

2.2. iNOS activity

iNOS activity was assessed by the Griess assay [14]. Briefly, conditioned supernatant was incubated with an equal volume of Griess reagent containing 1% sulphanilamide, 0.1% naphthylethylenediamine dihydrochloride and 2.5% H_3PO_4 [16] for 5 min at room temperature. The absorbance was measured at 546 nm. $NaNO_2$ diluted in DMEM served as a standard. To control for cell number, the cells were stained with crystal violet. iNOS activity is expressed as nitrite accumulated in 48 hr/10^5 cells.

2.3. Immunoblot analysis

For the preparation of whole cell lysates, the cells were lysed in 0.1 M Tris–HCl (pH 7.2) containing 0.1% NP40, 0.1 mM EDTA and 5 μg/mL phenylmethylsulphonyl fluoride. Twenty micrograms protein was separated by 10–12% SDS–PAGE and electroblotted on nitrocellulose membrane. Equal loading was controlled by Ponceau S staining. Immunodetection was performed using the following antibodies: anti-iNOS rabbit polyclonal antibody (1:1000, Cayman), antiphospo-IκBα mouse monoclonal antibody (1:1000), anti-PKCδ mouse monoclonal antibody (1:2000), antiphospho-MARCKS rabbit polyclonal antibody (1:1000), antiphospho-ERK-1/2, antiphospho-AKT, antiphospho-p38 mouse monoclonal antibodies (1:2000), anti-ERK-1/2, anti-AKT mouse monoclonal antibodies (1:500, all from Signal Transduction Laboratories). Bands were visualized using horseraddish peroxidase-conjugated anti-rabbit IgG (1:4000 Santa Cruz) or anti-mouse IgG (1:4000, Amersham).

2.4. Northern blot analysis

RNA was isolated using the RNeasy™ kit (Qiagen). Total RNA (10 μg) was separated on 1.2% agarose gels and blotted onto nylon membranes (Amersham). The filters were hybridized according to standard procedures with a ^{32}P-labeled murine cDNA probe for iNOS. For the generation of the iNOS probe, N9 cells were stimulated with IFN-γ (200 unit/mL) for 24 hr. Total RNA was subjected to reverse transcription using SuperScript II (Gibco-BRL) and oligo-dT priming (Amersham Pharmacia Biotech). iNOS fragments were PCR-amplified using primers 5′-AAGCTG-CATGTGACATCGAC-3′ and 5′-ATGTGTCTGCAGATG-TGCTG-3′ corresponding to nucleotides 386–405 and 839–858 of murine iNOS cDNA. Equal loading was controlled by ethidium bromide staining.

2.5. NF-κB reporter assay

NF-κB reporter gene activity was assayed as previously described [14]. Briefly, 8×10^3 cells/well were seeded in a 96-well plate and transfected with an NF-κB cis reporter gene plasmid (PathDetect™ No. 219077, Stratagene) encoding firefly luciferase using FuGene™ transfection reagent (Roche). pFC-MEKK (Stratagene) and pcDNA3 plasmids cotransfected with the reporter plasmid served as positive and negative controls. After freezing and thawing, the cells were lysed in 40 mM Tricine (pH 7.8) containing 50 mM NaCl, 2 mM EDTA, 1 mM $MgSO_4$, 5 mM DTT and 1% Triton X-100. Lysates were assayed using Luciferase assay reagent (40 mM Tricine, pH 7.8, containing 10 mM $MgSO_4$, 0.5 mM EDTA, 10 mM DTT, 0.5 mM coenzyme A, 0.5 M ATP and 0.5 mM beetle luciferine). Luminescence was measured in LumiNunc™ plates (Nunc) in a LumimatPlus™ (EG&G Berthold). Values are expressed as relative light units (RLU).

2.6. Immunocytochemistry

Cells grown on glass coverslips were serum-starved for 16 hr, kept in Krebs Ringer HEPES buffer (125 mM NaCl, 5 mM KCl, 1.2 mM $MgSO_4$, 2 mM $CaCl_2$, 1.2 mM KH_2PO_4, 6 mM glucose, 25 mM HEPES, pH 7·4) for 1 hr and left untreated or treated with PMA (100 nM) without or with pre-incubation (1 hr) with TNL (300 μM). After fixation in ice-cold (−20°) methanol for 20 min, the cells were washed with PBS and incubated for 15 min with 0.1% $NaBH_4$ and 0.1 M glycine in PBS to block autofluorescence. Nonspecific antibody binding was blocked for 45 min with PBG (PBS with 0.045% fish gelatin) containing 5% normal goat serum and 1% bovine serum albumin. Incubation with anti-PKCδ antibody was carried out for 24 hr at 4° after dilution in PBG containing 5% normal goat serum. After four washes with PBG, primary antibody binding was detected with an isotype-specific secondary antibody conjugated with Alexa^{TM}546 fluorescent dye. The coverslips were mounted in PermaFluor (Immunotech). Localization of the proteins was examined using confocal laser microscopy (Leica).

2.7. Statistical analyses

Experiments were usually performed in triplicate and repeated three times. The significance was evaluated by *t*-test or ANOVA at $P < 0.05$, 0.01 or 0.001.

3. Results

3.1. Inhibition of LPS-induced iNOS expression and nitrite formation in N9 microglial cells by TNL

N9 microglial cells were stimulated with LPS in the absence or presence of TNL and Northern Blot was performed to analyse iNOS mRNA expression. Fig. 1A shows that the 4 kb iNOS mRNA species was equally induced at 4 and 8 hr after LPS stimulation. This induction was unaffected by the pretreatment (1 hr) with TNL. In contrast, 16 hr after activation with LPS, there was no further increase of iNOS mRNA in TNL-treated cells as opposed to vehicle-treated cells (Fig. 1B). Immunoblot analysis showed a time-dependent accumulation of the 130 kDa iNOS protein in N9 microglial cells after stimulation with LPS which was suppressed by TNL. DEX, a known inhibitor of iNOS expression [17], was used as a positive control (Fig. 1C). Next, nitrite formation from NO was assayed in the supernatant of LPS-stimulated N9 cells. While unstimulated N9 cells did not release NO (not shown), stimulation with LPS led to the production of nitrite. Treatment with TNL and DEX resulted in a concentration-dependent decrease in nitrite formation (Fig. 1D).

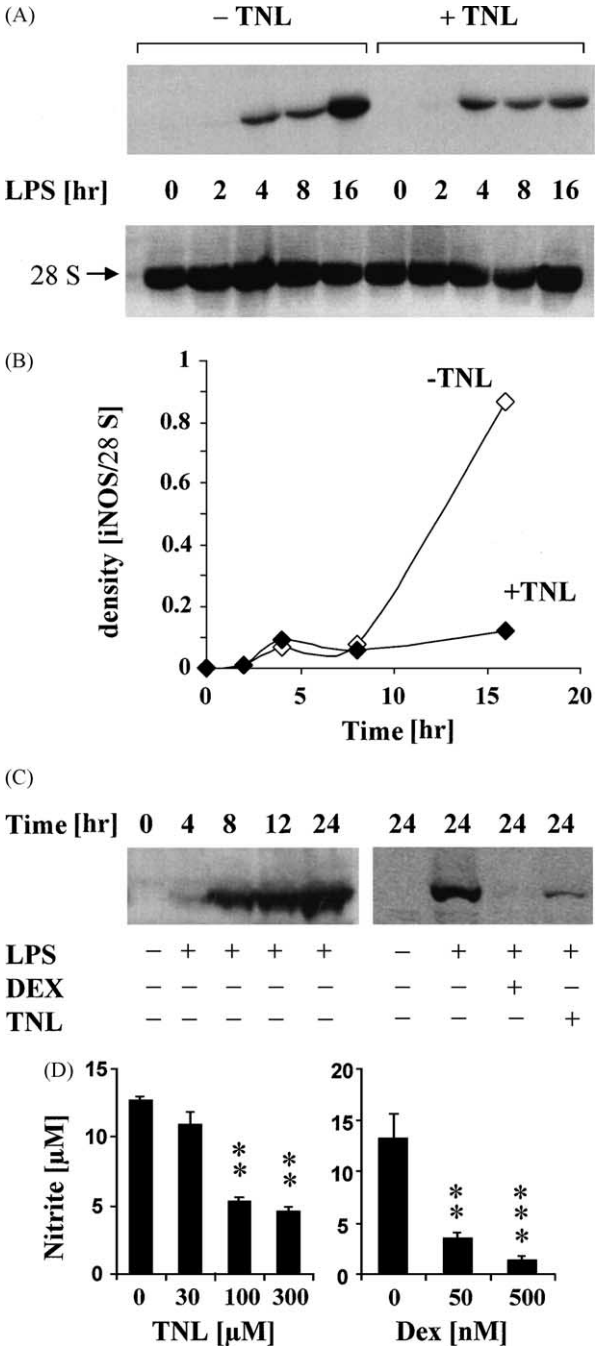

Fig. 1. TNL inhibits microglial iNOS expression and NO release. (A) N9 cells were pretreated with vehicle (−TNL) or TNL (+TNL, 300 μM) for 1 hr and stimulated with LPS (10 μg/mL) for the time indicated. iNOS mRNA expression was examined by Northern blot of total RNA (5 μg per lane). Equal loading was ascertained by ethidium bromide staining of the 28S ribosomal fractions. (B) Densitometry of iNOS mRNA and 28S ribosomal RNA signals. Values are given as ratios of the densities of iNOS and 28S signals. Data are representative of two independent experiments. (C) N9 cells were exposed to vehicle or DEX (500 nM) or TNL (300 μM) and stimulated with LPS (10 μg/mL) for the time indicated. iNOS protein expression was examined by immunoblot of whole cell protein (20 μg per lane). Data are representative of three independent experiments. (D) N9 cells were stimulated with LPS alone (10 μg/mL) or in combination with TNL or DEX. Supernatant was collected after 48 hr and nitrite was measured using the Griess assay. Values are expressed as NO_2 accumulated per 10^4 cells (mean and SEM, N = 3, $^{**}P < 0.01$, $^{***}P < 0.001$, ANOVA).

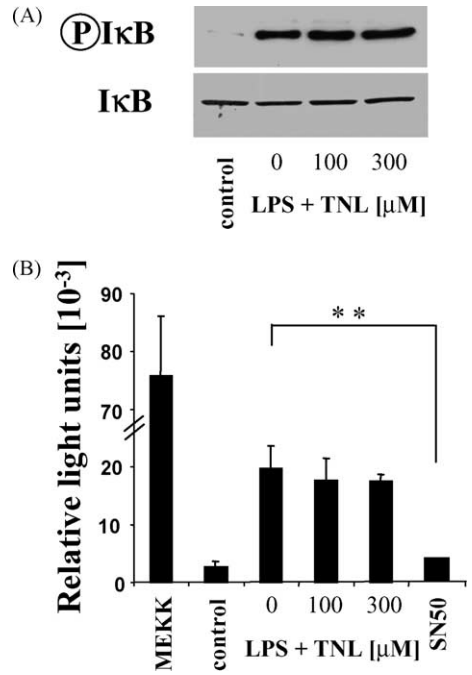

Fig. 2. TNL does not interfere with LPS-induced activation of NF-κB. (A) N9 cells were left untreated or incubated with LPS (10 mg/mL) with or without pre-incubation (1 hr) with TNL at the concentrations indicated. Whole cell protein was prepared 1 hr after stimulation and subjected to immunoblot using an antiphospho IκBα antibody revealing a band at the predicted size (40 kDa). Equal IκBα-expression was ascertained by re-hybridizing the membrane using a nonphospho-specific anti-IκBα antibody. Data are representative of two independent experiments. (B) In parallel, N9 cells were transiently transfected with an NF-κB reporter plasmid. At 24 hr after transfection cells were stimulated with LPS (10 μg/mL) for 4 hr with or without pre-incubation (1 hr) with NF-κB SN50 inhibitory peptide (SN50, 10 μg/mL) or TNL at the concentrations indicated. Transfection with a MEKK plasmid served as positive control for NF-κB activation. Luciferase activity was determined by luminometry. Values are expressed as relative light units (RLU, mean and SEM, N = 3, $^{**}P < 0.01$, *t*-test).

3.2. No interference with LPS-induced activation of NF-κB in N9 microglial cells by TNL

NF-κB is a major transcriptional activator of iNOS in microglial cells [18]. In contrast to IFN-γ-stimulated N9 cells [14], TNL did not suppress the LPS-mediated phosphorylation of inhibitory-κB (I-κB) (Fig. 2A) and the subsequent generation of NF-κB reporter gene activity in N9 cells (Fig. 2B). When N9 cells were exposed to the NF-κB inhibitor BAY, a decrease of LPS-stimulated NO release was observed at 20 μM (11.1 ± 0.7 μM/10^4 cells/48 hr vs. 14.6 ± 1.0 μM/10^4 cells/48 hr in vehicle-treated cells).

3.3. TNL inhibits the phosphorylation of ERK-1/2, but not p38 MAPK in N9 microglial cells

The mitogen-activated protein kinase (MAPK) signalling cascade—in particular p38MAPK and p44/42MAPK/extracellular signal-regulated kinase-1/2 (ERK-1/2)—has been implicated in the regulation of iNOS in LPS-activated microglial cells [19]. Therefore, we sought to investigate whether TNL interferes with the MAPK signalling pathway. Immunoblot analysis using phospho-specific antibodies demonstrated that the activation of N9 cells with LPS for 10 min resulted in the phosphorylation of p38 MAPK and p44/p42 (ERK-1/2). Treatment with TNL led to a concentration-dependent inhibition of ERK-2 phosphorylation while phospho-ERK-1 and unphosphorylated ERK-1/2 protein levels remained unchanged (Fig. 3A). Densitometry revealed that phospho-ERK-2 was reduced by 80% after exposure to 300 μM TNL (Fig. 3B).TNL did

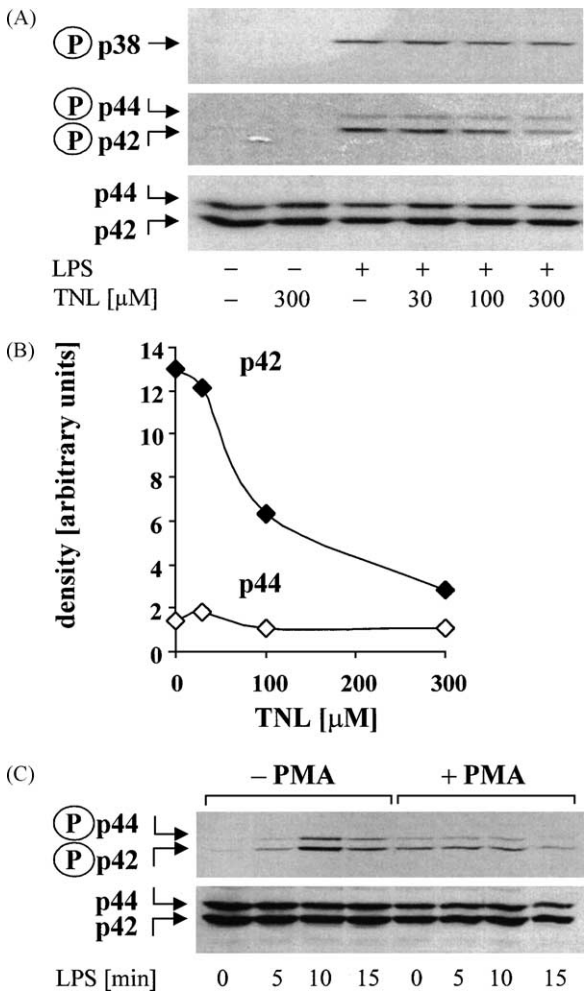

Fig. 3. TNL suppresses LPS-stimulated phosphorylation of ERK-2. (A) N9 cells were stimulated with LPS (10 μg/mL) for 10 min with or without pre-incubation (1 hr) with TNL. Whole cell protein was subjected to immunoblot using antiphospho-p38 or antiphospho-ERK-1/2 antibodies. Equal ERK-1/2-expression was ascertained by re-hybridizing the membrane using a nonphospho-specific anti-ERK-1/2 antibody. Data are representative of three independent experiments. (B) Densitometry of the phospho-ERK-1/2 protein signals. Values are given as arbitrary units. (C) For depletion of PKC, N9 cells were long-term exposed to PMA (100 nM) for 24 hr and stimulated with LPS (10 μg/mL) for the time indicated. Whole cell protein was subjected to immunoblot using antiphospho-ERK-1/2 antibody. Equal ERK-1/2-expression was ascertained by re-hybridizing the membrane with a nonphospho-specific anti-ERK-1/2-antibody. Data are representative of three independent experiments.

not alter cellular phospho-p38 levels after LPS stimulation indicating that TNL specifically suppresses iNOS activation via inhibition of ERK-2 phosphorylation (Fig. 3A). Exposure of N9 cells to the MAPK/ERK kinase-1/2 (MEK-1/2) inhibitor PD which acts upstream of ERK-1/2 resulted in a significant inhibition of LPS-mediated nitrite formation (10.7 ± 0.4 μM/10^4 cells/48 hr at 40 μM PD vs. 16.6 ± 0.3 μM/10^4 cells/48 hr compared to vehicle-treated cells). Since PKC phosphorylates Raf, which acts directly upstream of MEK [20] and since LPS-induced ERK-1/2 activation may be mediated by PKC [21], we reasoned that PKC may be involved in LPS-mediated phosphorylation of ERK-1/2 in N9 cells. Long-term treatment (24 hr) with PMA depletes PKC in various cell types including microglia [22]. Immunoblot analysis demonstrated that PKCδ was effectively depleted in N9 cells after 24 hr treatment with PMA (100 nM, data not shown). While stimulation with LPS resulted in a rapid (10 min) phosphorylation of ERK-1/2, LPS failed to induce ERK-1/2 phosphorylation in N9 cells after prolonged pretreatment with PMA, indicating that PKC is required for LPS-mediated phosphorylation of ERK-1/2 in N9 microglial cells (Fig. 3C).

3.4. Promotion of LPS-induced iNOS expression and nitrite formation in N9 microglial cells by PKC

We next analysed the role of PKC in LPS-mediated activation of N9 cells. Pretreatment (15 min) of N9 cells with PMA led to a superinduction of LPS-mediated iNOS protein expression and nitrite formation. Activation of PKC by PMA in the absence of LPS, however, did not induce iNOS protein expression (Fig. 4A). The depletion of PKC after 24-hr exposure to PMA (100 nM) resulted in a reduction of iNOS protein expression in response to LPS for 24 hr (Fig. 4B). Densitometry revealed that iNOS protein was reduced by 80% after 24-hr exposure to PMA (Fig. 4C).

3.5. Inhibition of PKCδ-activation in N9 microglial cells by TNL

We then tested the hypothesis that TNL inhibits LPS-induced iNOS activation by inhibition of PKC. Immunoblot analysis using a phospho-specific antibody demonstrated that the phosphorylation of the PKCδ substrate, MARCKS, is induced by short-term treatment with LPS. MARCKS phosphorylation was inhibited when PMA-activated N9 cells were co-exposed to TNL, supporting the notion that TNL inhibits PKC in N9 cells (Fig. 5A). Moreover, while short-term treatment with PMA superinduced iNOS protein levels in LPS-stimulated N9 cells, this superinduction was abolished by co-exposure to either the PKC inhibitor GFX or TNL (Fig. 5B). Since PKCδ appears to be a specific kinase of ERK-1/2 [23], we tested the hypothesis that PKCδ is involved in LPS-mediated iNOS activation in N9 cells. PKCδ is confined to the cytosol in resting cells but rapidly translocates to particulate fractions after activation by short-term treatment with PMA [24]. Immunocytochemistry showed that PKCδ translocates from the cytosol primarily to the nucleus following exposure to PMA (100 nM) for 15 min. Co-exposure to TNL abolished this translocation of PKCδ (Fig. 5C). In addition, ROT which at low micromolar concentrations primarily inhibits the nonclassical δ-isoform of PKC [25], strongly inhibited iNOS protein accumulation in LPS-stimulated N9 cells at concentrations as low as 5 μM (Fig. 5D).

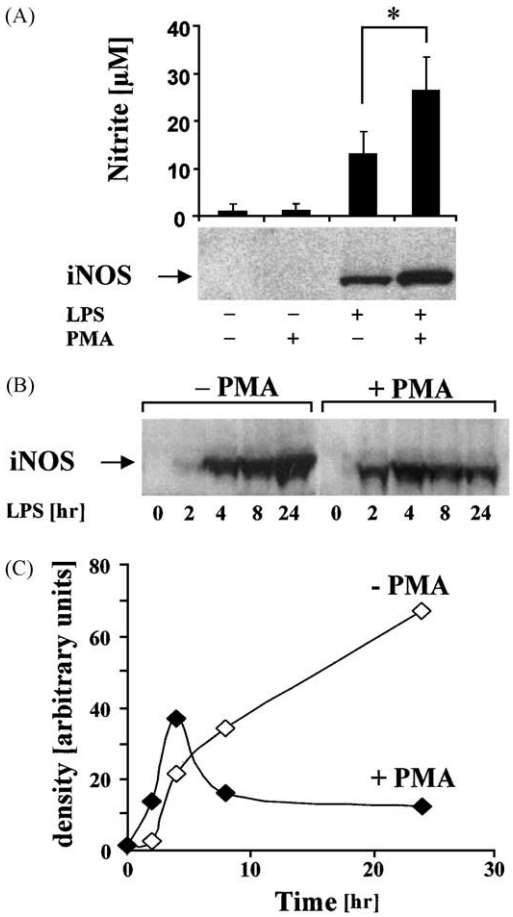

Fig. 4. PKC promotes LPS-induced iNOS expression and nitrite formation. (A) N9 cells were untreated or stimulated with LPS (10 μg/mL) without or with short-term pre-incubation (15 min) with PMA (100 nM). Supernatant was collected after 48 hr and nitrite was measured using the Griess assay (upper panel). Values are expressed as NO_2 accumulated per 10^4 cells (mean and SEM, N = 3, $^*P < 0.05$, *t*-test). In parallel, iNOS protein levels were examined 24 hr after stimulation (lower panel). Data are representative of three independent experiments. (B) N9 cells were stimulated with LPS (10 μg/mL) without or with pre-exposure to PMA (100 nM) for 24 hr. iNOS protein levels were examined at the indicated time points by immunoblot. (C) Densitometry of the iNOS protein signals. Values are given as arbitrary units. Data are representative of three independent experiments.

4. Discussion

The present study shows that TNL inhibits NO release from N9 microglial cells activated with LPS. Inhibition of

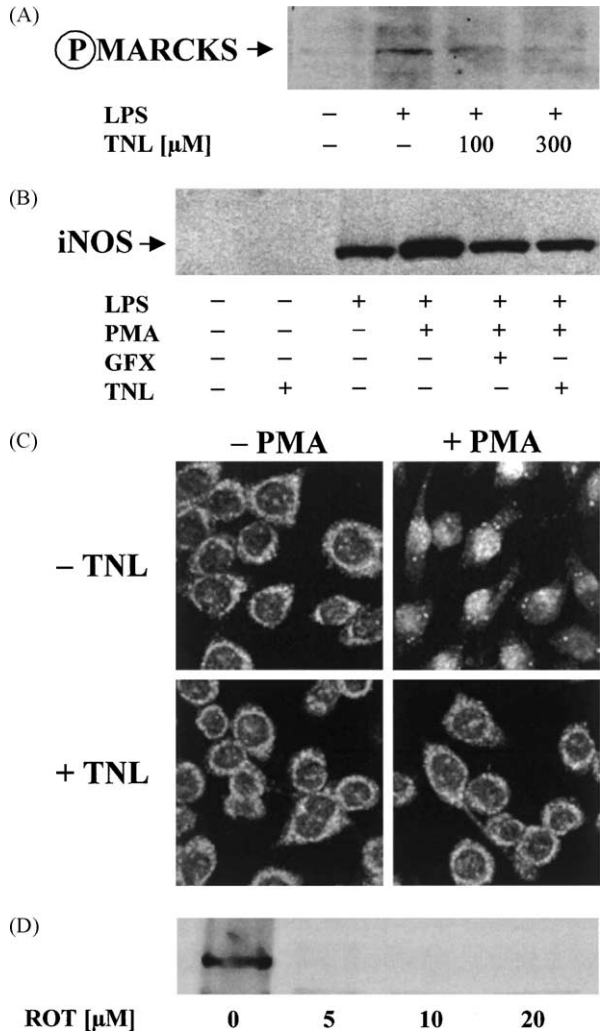

Fig. 5. TNL inhibits the activation of PKCδ. (A) N9 cells were stimulated with LPS (10 μg/mL) for 1 hr without or with pre-incubation (1 hr) with TNL at the concentrations indicated. MARCKS phosphorylation was examined by immunoblot using a phospho-specific anti-MARCKS antibody. Data are representative of three independent experiments. (B) N9 cells were untreated or stimulated with LPS (10 μg/mL) for 24 hr without or with prior activation of PKC by short-term exposure to PMA (100 nM) for 15 min in the absence or presence of TNL (300 μM) or GFX (2 μM). iNOS protein levels were assessed by immunoblot 24 hr after treatment. Data are representative of three independent experiments. (C) N9 cells were grown on coverslips and stimulated with PMA (100 nM) for 25 min without or with pre-incubation with TNL (300 μM). Cellular distribution of PKCδ was examined by immunocytochemistry. Data are representative of three independent experiments. (D) N9 cells were stimulated with LPS (10 μg/mL) for 24 hr without or with co-incubation with ROT at the concentrations indicated. iNOS protein levels were examined by immunoblot. Data are representative of three independent experiments.

NO release from LPS-stimulated N9 cells is associated with a suppressed induction of iNOS mRNA expression and iNOS protein accumulation (Fig. 1A–D). Rapid transcriptional activation of iNOS is tightly regulated by the transcription factor NF-κB [26]. After translocation to the nucleus and degradation of IκB, NF-κB targets the promoter of specific genes including iNOS to activate their transcription. Surprisingly, we found that TNL does not inhibit the activation of NF-κB or degradation of IκB in N9 cell following stimulation of N9 cells (Fig. 2A and B). LPS and cytokines may thus utilize different pathways to activate NF-κB since TNL-mediated inhibition of cytokine signalling may involve NF-κB [27]. Several lines of evidence suggest that LPS-mediated cellular effects cannot be attributed to activation of NF-κB alone. For instance, C3H/HeJ LPS-hyporesponsive mice fail to produce TNF-α, but activate normal levels of NF-κB in response to LPS [28]. Hence, TNL may interfere with the activation of other transcription factors activated by LPS, including activator protein-1 (AP-1) complex, activating transcription factor-2 (ATF-2) and tenary complex factor (TCF) [29]. Moreover, there is considerable post-transcriptional regulation of iNOS including mRNA stabilization [30]. Finally, NO release was only weakly inhibited by the NF-κB inhibitor BAY (11.1 ± 0.7 μM/10^4 cells/48 hr at 20 μM vs. 14.6 ± 1.0 μM/10^4 cells/48 hr in vehicle-treated cells), further indicating that transcriptional activation of iNOS does not solely depend on NF-κB.

We also investigated the upstream-targets that are involved in the inhibition of LPS-mediated iNOS induction by TNL. Both p38MAPK and ERK-1/2 have been identified as intracellular targets of LPS in microglial cells [19]. While studies on mesangial and smooth muscle cells demonstrated that TNL inhibits the activation of MAPK [31,32], we observed that TNL specifically inhibited the phosphorylation of ERK-2. In contrast, the phosphorylation of ERK-1 and p38 was unchanged in LPS-stimulated N9 cells (Fig. 3A and B), indicating that TNL differentially targets MAPK in these cells. Consistent with these findings, the MAPK/ERK kinase-1/2 (MEK-1/2) inhibitor PD which acts upstream of ERK-1/2 resulted in a significant inhibition of LPS-mediated nitrite formation (10.7 ± 0.4 μM/10^4 cells/48 hr at 40 μM PD vs. 16.6 ± 0.3 μM/10^4 cells/48 hr compared to vehicle-treated cells). Interestingly, p38MAPK, but not ERK-1/2, is involved in LPS-mediated activation of NF-κB [21], consistent with our observation that TNL does not inhibit LPS-induced NF-κB activation (Fig. 2B).

Several lines of evidence indicate that ERK-1/2 is phosphorylated by PKC [23,33]. Moreover, LPS-induced ERK-1/2 phosphorylation may be specifically mediated by PKC [21]. Here, the depletion of PKC by PMA precluded the activation of ERK-1/2 (Fig. 3C) and suppressed the induction of iNOS mediated by LPS (Fig. 4B and C), indicating that conventional and novel PKC isoforms are involved in LPS-mediated activation of ERK-1/2 and induction of iNOS. Moreover, activation of conventional and novel PKC isoforms by short-term treatment with PMA superinduces iNOS protein induction and nitrite formation by LPS (Fig. 4A), further supporting the hypothesis that PMA-sensitive PKC isoforms are involved in LPS-mediated iNOS activation in N9 cells. The observation that, after initial induction, iNOS protein levels decreased after stimulation with LPS in TNL-treated N9 cells (Fig. 4B and C) may again imply that TNL reduces iNOS mRNA stability.

Since there is indirect evidence that TNL inhibits PKC in endothelial cells [34], we asked whether TNL interferes with PKC activation in LPS-stimulated N9 cells. We find that TNL inhibits phosphorylation of MARCKS (Fig. 5A) and suppresses PMA-mediated superinduction of iNOS after stimulation with LPS, as does the pan-specific PKC inhibitor GFX (Fig. 5B). Of the PMA-sensitive PKC isoforms PKCδ, but not PKCα or PKCε, appears to specifically target ERK-1/2 [23]. Immunocytochemistry and confocal laser microscopy indeed showed that TNL targets PKCδ in N9 cells to inhibit its translocation from the cytosol to the nucleus following activation by PMA (Fig. 5C). While activation of PKC is classically associated with translocation from cytosol to plasma membrane, translocation, particularly of the δ-isoform of PKC, from cytosol to nucleus has been observed as a result of activation depending on the concentration of the stimulus [24,35,36]. Of note, PKCδ has been identified as a critical molecule mediating stabilization of iNOS mRNA [37]. Finally, treatment with ROT at concentrations specifically inhibiting PKCδ (5 μM) blocked iNOS expression induced by LPS (Fig. 5D), indicating that PKCδ is indeed involved in LPS-mediated induction of iNOS in microglial cells.

In summary, we show that TNL inhibits LPS-induced activation of iNOS by inhibiting the activation of PKCδ and ERK-2, thus possibly affecting post-translational modification of iNOS mRNA. Our result may have further implications for the use of TNL as a novel therapeutic agent in neurological disorders associated with activation of microglia and increased NO release, including multiple sclerosis.

Acknowledgments

We are grateful to Dr. M. Isaji and Kissei Pharmaceuticals for generously supplying TNL and Y. Nordmann for technical assistance. M.P. was a scholar of the Jung Foundation. This work was supported by the DFG grant We 1502/8-2 to M.W.

References

[1] Gonzalez-Scarano F, Baltuch G. Microglia as mediators of inflammatory and degenerative diseases. Annu Rev Neurosci 1999;22:219–40.
[2] Aloisi F. Immune function of microglia. Glia 2001;36(2):165–79.
[3] Bredt DS. Endogenous nitric oxide synthesis: biological functions and pathophysiology. Free Radic Res 1999;31(6):577–96.
[4] Colton CA, Snell J, Chernyshev O, Gilbert DL. Induction of superoxide anion and nitric oxide production in cultured microglia. Ann NY Acad Sci 1994;738:54–63.
[5] Gebicke-Haerter PJ. Microglia in neurodegeneration: molecular aspects. Microsc Res Tech 2001;54(1):47–58.
[6] Kolb H, Kolb-Bachofen V. Nitric oxide in autoimmune disease: cytotoxic or regulatory mediator? Immunol Today 1998;19(12):556–61.
[7] Willenborg DO, Staykova MA, Cowden WB. Our shifting understanding of the role of nitric oxide in autoimmune encephalomyelitis: a review. J Neuroimmunol 1999;100(1/2):21–35.
[8] Salerno L, Sorrenti V, Di Giacomo C, Romeo G, Siracusa MA. Progress in the development of selective nitric oxide synthase (NOS) inhibitors. Curr Pharm Des 2002;8(3):177–200.
[9] Okuda Y, Sakoda S, Fujimura H, Yanagihara T. Aminoguanidine, a selective inhibitor of the inducible nitric oxide synthase, has different effects on experimental allergic encephalomyelitis in the induction and progression phase. J Neuroimmunol 1998;81(12):201–10.
[10] Pozza M, Bettelli C, Aloe L, Giardino L, Calza L. Further evidence for a role of nitric oxide in experimental allergic encephalomyelitis: aminoguanidine treatment modifies its clinical evolution. Brain Res 2000;855(1):39–46.
[11] Zampini P, Riviera AP, Tridente G. *In vitro* inhibition of histamine release from mouse mast cells and human basophils by an anthranilic acid derivative. Int J Immunopharmacol 1983;5(5):431–5.
[12] Platten M, Wild-Bode C, Wick W, Leitlein J, Dichgans J, Weller M. *N*-[3,4-Dimethoxycinnamoyl]-anthranilic acid (tranilast) inhibits transforming growth factor-beta relesase and reduces migration and invasiveness of human malignant glioma cells. Int J Cancer 2001;93(1):53–61.
[13] Mori H, Tanaka H, Kawada K, Nagai H, Koda A. Suppressive effects of tranilast on pulmonary fibrosis and activation of alveolar macrophages in mice treated with bleomycin: role of alveolar macrophages in the fibrosis. Jpn J Pharmacol 1995;67(4):279–89.
[14] Platten M, Wick W, Wischhusen J, Weller M. *N*-[3,4-Dimethoxycinnamoyl]-anthranilic acid (tranilast) suppresses microglial inducible nitric oxide synthase (iNOS) expression and activity induced by interferon-gamma (IFN-gamma). Br J Pharmacol 2001;134(6):1279–84.
[15] Righi M, Mori L, De Libero G, Sironi M, Biondi A, Mantovani A, Donini SD, Ricciardi-Castagnoli P. Monokine production by microglial cell clones. Eur J Immunol 1989;19(8):1443–8.
[16] Green LC, Wagner DA, Glogowski J, Skipper PL, Wishnok JS, Tannenbaum SR. Analysis of nitrate, nitrite, and [15N]nitrate in biological fluids. Anal Biochem 1982;126(1):131–8.
[17] Minghetti L, Nicolini A, Polazzi E, Greco A, Perretti M, Parente L, Levi G. Down-regulation of microglial cyclo-oxygenase-2 and inducible nitric oxide synthase expression by lipocortin 1. Br J Pharmacol 1999;126(6):1307–14.
[18] Xie Q. A novel lipopolysaccharide-response element contributes to induction of nitric oxide synthase. J Biol Chem 1997;272(23):14867–72.
[19] Bhat NR, Zhang P, Lee JC, Hogan EL. Extracellular signal-regulated kinase and p38 subgroups of mitogen-activated protein kinases regulate inducible nitric oxide synthase and tumor necrosis factor-alpha gene expression in endotoxin-stimulated primary glial cultures. J Neurosci 1998;18(5):1633–41.
[20] Sozeri O, Vollmer K, Liyanage M, Frith D, Kour G, Mark Jr GE, Stabel S. Activation of the c-Raf protein kinase by protein kinase C phosphorylation. Oncogene 1992;7(11):2259–62.
[21] Chen CC, Wang JK. p38 but not p44/42 mitogen-activated protein kinase is required for nitric oxide synthase induction mediated by lipopolysaccharide in RAW 264.7 macrophages. Mol Pharmacol 1999;55(3):481–8.
[22] Kang J, Yang M, Jou I, Joe E. Identification of protein kinase C isoforms involved in interferon-gamma-induced expression of inducible nitric oxide synthase in murine BV2 microglia. Neurosci Lett 2001;299(3):205–8.
[23] Ueda Y, Hirai S, Osada S, Suzuki A, Mizuno K, Ohno S. Protein kinase C activates the MEK-ERK pathway in a manner independent of Ras and dependent on Raf. J Biol Chem 1996;271(38):23512–9.
[24] Wang QJ, Bhattacharyya D, Garfield S, Nacro K, Marquez VE, Blumberg PM. Differential localization of protein kinase C delta by phorbol esters and related compounds using a fusion protein with green fluorescent protein. J Biol Chem 1999;274(52):37233–9.

[25] Gschwendt M, Muller HJ, Kielbassa K, Zang R, Kittstein W, Rincke G, Marks F. Rottlerin, a novel protein kinase inhibitor. Biochem Biophys Res Commun 1994;199(1):93–8.
[26] Mattson MP, Camandola S. NF-kappaB in neuronal plasticity and neurodegenerative disorders. J Clin Invest 2001;107(3):247–54.
[27] Spiecker M, Lorenz I, Marx N, Darius H. Tranilast inhibits cytokine-induced nuclear factor kappaB activation in vascular endothelial cells. Mol Pharmacol 2002;62(4):856–63.
[28] Ding A, Hwang S, Lander HM, Xie QW. Macrophages derived from C3H/HeJ (Lpsd) mice respond to bacterial lipopolysaccharide by activating NF-kappa B. J Leukoc Biol 1995;57(1):174–9.
[29] Downey JS, Han J. Cellular activation mechanisms in septic shock. Front Biosci 1998;3:d468–76.
[30] Rodriguez-Pascual F, Hausding M, Ihrig-Biedert I, Furneaux H, Levy AP, Forstermann U, Kleinert H. Complex contribution of the 3′-untranslated region to the expressional regulation of the human inducible nitric-oxide synthase gene Involvement of the RNA-binding protein HuR. J Biol Chem 2000;275(34):26040–9.
[31] Chikaraishi A, Hirahashi J, Takase O, Marumo T, Hishikawa K, Hayashi M, Saruta T. Tranilast inhibits interleukin-1beta-induced monocyte chemoattractant protein-1 expression in rat mesangial cells. Eur J Pharmacol 2001;427(2):151–8.
[32] Watanabe S, Matsuda A, Suzuki Y, Kondo K, Ikeda Y, Hashimoto H, Umemura K. Inhibitory mechanism of tranilast in human coronary artery smooth muscle cells proliferation, due to blockade of PDGF-BB-receptors. Br J Pharmacol 2000;130(2):307–14.
[33] Chao TS, Foster DA, Rapp UR, Rosner MR. Differential Raf requirement for activation of mitogen-activated protein kinase by growth factors, phorbol esters, and calcium. J Biol Chem 1994;269(10):7337–41.
[34] Koyama S, Takagi H, Otani A, Suzuma K, Nishimura K, Honda Y. Tranilast inhibits protein kinase C-dependent signalling pathway linked to angiogenic activities and gene expression of retinal microcapillary endothelial cells. Br J Pharmacol 1999;127(2):537–45.
[35] O'Flaherty JT, Chadwell BA, Kearns MW, Sergeant S, Daniel LW. Protein kinases C translocation responses to low concentrations of arachidonic acid. J Biol Chem 2001;276(27):24743–50.
[36] Ohmori S, Shirai Y, Sakai N, Fujii M, Konishi H, Kikkawa U, Saito N. Three distinct mechanisms for translocation and activation of the delta subspecies of protein kinase C. Mol Cell Biol 1998;18(9):5263–71.
[37] Carpenter L, Cordery D, Biden TJ. Protein kinase Cdelta activation by interleukin-1beta stabilizes inducible nitric-oxide synthase mRNA in pancreatic beta-cells. J Biol Chem 2001;276(7):5368–74.