DISSECTION OF TUMOR-NECROSIS FACTOR-α INHIBITION OF LONG-TERM POTENTIATION (LTP) REVEALS A p38 MITOGEN-ACTIVATED PROTEIN KINASE-DEPENDENT MECHANISM WHICH MAPS TO EARLY—BUT NOT LATE—PHASE LTP

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Abstract—The pro-inflammatory cytokine tumor-necrosis factor-α (TNF-α) is elevated in several neuropathological states that are associated with learning and memory deficits. Previous work has reported that TNF-α inhibits the induction of LTP in areas CA1 [Neurosci Lett 146 (1992) 176] and dentate gyrus [Neurosci Lett 203 (1996) 17]. The mechanism(s) underlying this process of inhibition have not to date been addressed. Here, we show that perfusion of TNF-α prior to long-term potentiation (LTP) inducing stimuli inhibited LTP, and that in late LTP (3 h post-tetanus) a depression in synaptic field recordings was observed (68±5%, n=6 versus control 175±7%, n=6, P<0.001). We investigated the involvement of the mitogen-activated protein kinase (MAPK) p38 in the inhibition of LTP by TNF-α as p38 MAPK has previously been shown to be involved in interleukin-1β inhibition of LTP in the dentate gyrus [Neuroscience 93 (1999b) 57]. Perfusion of TNF-α led to an increase in the levels of phosphorylated p38 MAPK detectable in the granule cells of the dentate gyrus. The p38 MAPK inhibitor SB 203580 (1 μM) was found by itself to have no significant effect on either early or late phase LTP in the dentate gyrus. SB 203580 was found to significantly reverse the inhibition of early LTP by TNF-α (SB/TNF-α 174±5%, n=6 versus TNF-α 120±7%, n=6, P<0.001, 1 h post-tetanus) to values comparable to control LTP (control 175±7%, n=6). Interestingly however, the depressive effects of TNF-α on late LTP (2–3 h) were clearly not attenuated by p38 MAPK inhibition (SB/TNF-α 132±5%, n=6 versus control LTP 175±7%, n=6, P<0.001, 3 h post-tetanus). This work suggests that TNF-α inhibition of LTP represents a biphasic response, a p38 MAPK-dependent phase that coincides with the early phase of LTP and a p38 MAPK independent phase that temporally maps to late LTP. © 2004 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: cytokines, long-term potentiation, memory, MAPK.

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Abbreviations: aCSF, artificial cerebrospinal fluid; EPSP, field excitatory post-synaptic potential; HFS, high frequency stimulation; IL-1β, interleukin-1β; JNK, c-Jun N terminal kinase; LTD, long-term depression; LTP, long-term potentiation; MAPK, mitogen-activated protein kinase; NMDA, N-methyl-D-aspartate; TNF-α, tumor-necrosis factor-α; VDCC, voltage-dependent calcium channel.
shown to lead to an enhancement of synaptic transmission through increasing the surface expression of AMPA receptors (Beattie et al., 2002). Altogether these findings show that the neuromodulatory effects of TNF-α/H9251 are varied and complex.

This study aimed to initially examine the effects of TNF-α on both the early (approximately 1 h post-induction) and late phases of LTP (3 h post-tetanus), and then to assess the role of p38 MAPK in mediating TNF-α inhibitory effects on LTP. These studies for the first time show that TNF-α inhibits early-LTP by a p38 MAPK-dependent mechanism, similar to that found for IL-1β. However late-LTP is inhibited by a mechanism independent of p38 MAPK.

**EXPERIMENTAL PROCEDURES**

Transverse hippocampal slices (350 μm) were prepared by standard methods from adult male Wistar rats (50–80 g). Briefly, slices were equilibrated for at least 1 h in a holding chamber at room temperature, pH 7.4, in oxygenated artificial cerebrospinal fluid (aCSF; composition in mM: NaCl, 120; KCl, 2.5; MgSO4, 2; CaCl2, 2; NaHCO3, 26; NaH2PO4, 1.25; α-glucose, 10) before being transferred to a recording chamber perfused with aCSF at a flow rate of 5–7 ml/min at 29–30 °C. Extracellular recordings of field excitatory post-synaptic potentials (fEPSP) were elicited by stimulation of the medial perforant path of the dentate gyrus by a monopolar glass electrode at a frequency of 0.05 Hz. Responses were recorded by a glass electrode placed in the middle third of the molecular layer in the presence of 100 μM of the GABA A receptor antagonist picrotoxin (Sigma-Aldrich Ireland Ltd, Dublin, Ireland) and stimulus strength was adjusted to give a response 35% of maximal. Stable baseline recordings were made for at least 10 min prior to application of drugs. LTP was induced by high frequency stimulation (HFS) consisting of eight trains of eight pulses at 200 Hz at 2-s intervals at a stimulus strength corresponding to 75% of maximal. Stable baseline recordings were made for at least 10 min prior to application of drugs. LTP was induced by high frequency stimulation (HFS) consisting of eight trains of eight pulses at 200 Hz at 2-s intervals at a stimulus strength corresponding to 75% of maximal. Recordings were analyzed off-line using the Strathclyde electrophysiology software (J. Dempster, Edinburgh, UK).

Recombinant rat TNF-α (R & D Systems, UK) was prepared in sterile PBS containing 0.1% BSA (Sigma-Aldrich Ireland Ltd.). Stable baseline recordings (20 min) were obtained before application of TNF-α (4.5 ng/ml). Additional drugs used included SB 203580 (Calbiochem, UK) dissolved in dimethylsulphoxide to a final concentration of 1 μM.

For immunostaining, the hippocampus was dissected out following bath application of the drugs and immersed in 4% paraformaldehyde at 4 °C for overnight fixation. Slices were then re-
moved and passed through a series of graded sucrose solutions (5%, 15%, 30%) to facilitate cryoprotection. Sections were removed from the sucrose solution, embedded in OCT and then cut at a thickness of 10 μm by a cryostat set at a cutting temperature of −16 °C. Sections were then mounted onto silanized slides. For detection of active p38 MAP kinase, phosphospecific anti-p38 (mouse monoclonal IgG2 subclass; 5 μg/ml; Sigma), that reacts specifically with the active doubly phosphorylated form of p38 MAPK and its related isoforms was used (Sigma, St Louis, USA). It does not recognize the non-phosphorylated and mono-phosphorylated forms of the p38 MAPK molecule or the non-, mono- or diphosphorylated forms of JNK and ERK MAPK. The binding of the antibody to its respective epitope was visualized using the Vectastain Elite ABC kit (Vector Laboratories, CA, USA) according to the manufacturer’s recommendations with the exception of an additional step. For phospho-p38 MAPK the concentration of the secondary biotinylated antibody was 2.5 μg/ml. The chromagen diaminobenzidine tetrahydrochloride (Sigma-Aldrich Ireland Ltd.) was used which produces a brownish precipitate, the counterstain used was hematoxylin, which stains nuclear chromatin blue. For non-immune control, a mouse IgG antibody (Chemicon International Ltd., UK) was used as the primary antibody. In such slides no brown staining corresponding to p-p38 MAPK was seen, with only counterstaining apparent by hematoxylin. Slides were analyzed on a microscope equipped with Axiovision software.

All data are expressed as mean±S.E.M. In all experiments examining LTP, all data points represent 5 min averages. For statistical analysis of data, two-tailed paired Student’s t-tests were employed to test significance between baseline and post-treatment values, and two-tailed unpaired Student’s t-test was employed for comparisons between different test and control slices.

RESULTS

Effects of TNF-α on tetanically induced LTP

LTP was generated by applying HFS (eight trains of eight pulses at 200 Hz) to the medial perforant path of the dentate gyrus that led to a robust LTP persisting for at least 3 h post-tetanus (fEPSP 175±7%, n=6, 3 h post-tetanus versus 1 h post-tetanus 175±6%, n=6; Fig. 1A). In some slices, this potentiation lasted for up to 6 h (data not shown). When TNF-α (4.5 ng/ml) was applied to the hippocampal slice 20 min pre-tetanus early-LTP (TNF-α/LTP fEPSP 120±7%, n=6 versus control LTP 175±6%, n=6; Fig. 1A), was significantly impaired as previously published (Butler et al., 2002). The LTP was followed in these TNF-α-treated slices for 3 h post-tetanus corresponding to late LTP, and the fEPSP recordings were seen to decrease below baseline fEPSP values and plateaued at approximately 3 h post-tetanus (TNF-α/LTP fEPSP 68±5%, n=6 versus control LTP 175±7%, n=6, P<0.001).

Effects of TNF-α on levels of p-p38 MAPK in the dentate gyrus

In order to explore the potential role of p38 MAPK in mediating the inhibitory effects of TNF-α on LTP it was first necessary to show that the latter could activate p38. We
first examined the levels of diphosphorylated p38 MAPK in the dentate granule cells of untreated rat hippocampal slices. As can be seen (Fig. 2A, 2C) prominent immunostaining was observed (40×/63× magnification). Therefore phospho-p38 MAPK is present at high basal levels in the granule cells of rats aged 4–5 weeks. Next, we perfused TNF-α (4.5 ng/ml) through hippocampal slices and examined the activation status of p38 MAPK 30 min post-drug addition. TNF-α led to more intense and wide-spread immunostaining for p38 MAPK in the granule cells (Fig. 2B, 2D) when compared with untreated slices.

Effects of TNF-α on baseline synaptic transmission
The effects of TNF-α (4.5 ng/ml) on baseline synaptic transmission was studied in order to determine if TNF-α depressive effects on late-LTP could be mirrored by TNF-α application alone (Fig. 3A). TNF-α was added at time 20 min and recordings of the fEPSPs measured for 200 min post-drug addition, this time matched the time-course of TNF-α application in the LTP studies. A comparison was made between TNF-α and test control on baseline synaptic transmission, no significant difference was found at the time-points examined (TNF-α baseline 86±3%, n=6 versus control baseline 96±3%, n=6, at time 220 min, P>0.05; Fig. 3A). Control baseline was found to be significantly different from TNF-α/LTP (TNF-α/LTP 68±5%, n=6 versus control baseline 96±3% n=6, P<0.01).

Effects of SB 203580 on LTP in the dentate gyrus
The p38 MAPK inhibitor SB 203580 (1 μM) when added 50 min prior to application of tetanic stimulation led to a small, although not significant increase in baseline synaptic transmission (P>0.05; Fig. 3B). Following high-frequency stimulation, the magnitude of LTP in SB 203580-treated slices as measured 60 min post-tetanus, was found to be indistinguishable from control LTP (SB-LTP 178±9% 1 h post-tetanus, n=5 versus control 175±6%, n=6). The effects of SB 203580 on late-LTP was examined and again no significant difference was observed between control and SB-treated slices (SB-LTP 163±7% 3 h post-tetanus, n=5 versus control LTP 175±7%, n=6, P>0.2).

![Fig. 3](image-url)

(A) Application of TNF-α alone does not mimic the depression seen in HFS–TNF-α pre-treated hippocampal slices. TNF-α (4.5 ng/ml) was added at time 20 min and remained in for the duration of the experiment (220 min total). At time 220 min the fEPSP response in TNF-α baseline was significantly different from TNF-α/LTP slices (TNF-α Baseline 86±3%, (▲), n=6 versus 68±5%, n=6 in TNF-α/LTP slices, P<0.05). TNF-α baseline is not significantly different from control baseline over the time period investigated (TNF-α baseline 86±3%, n=6 versus control baseline 96±3%, (□), n=6, at time 220 min P>0.05). Control baseline, □; TNF-α Baseline, ▲. Open bars represent the time period of cytokine perfusion. (B) The p38 MAPK inhibitor SB 203580 (1 μM) does not affect early (1 h) or late (approximately 3 h) phase LTP in the dentate gyrus. SB 203580 (1 μM) was added at time 10 min and was present throughout the duration of the experiment. It led to a small though not significant increase in baseline synaptic transmission. SB 203580 was found not to affect either early-LTP (178±9% 1 h post-tetanus (■), n=5 in SB 203580 treated slices compared with control 175±6% 1 h post-tetanus, (Δ), n=6, P>0.7) or late-LTP (163±7% 3 h post-tetanus in SB 203580 treated slices, n=5 compared with 175±7% in control slices, P>0.2). SB 203580 LTP, ■; Control LTP, Δ; open bar represents the time of drug perfusion.
Effects of SB 203580 on inhibition of LTP by TNF-α

When slices were incubated with SB 203580 (1 μM) 30 min prior to TNF-α addition and for the remainder of the experiment the impairment of early-LTP by TNF-α was significantly attenuated (Fig. 4A; SB/TNF-α 174 ± 5%, n = 6, 1 h post-tetanus compared with 120 ± 7% in TNF-α-treated slices, P < 0.001). Indeed at 1 h post-tetanus the SB/TNF-α LTP was found to be indistinguishable from control LTP (SB/TNF-α 174 ± 5%, n = 6 versus control 175 ± 7%, n = 6). A decline in the magnitude of the fEPSP slope was seen in the SB/TNF-α-treated slices (Fig. 4B), relative to control LTP, which emerged at 1.5 h post-tetanus (SB/TNF-α 165 ± 6%, n = 6 compared with control 181 ± 6%, n = 6) and became significant at 2 h post-tetanus (SB/TNF-α 153 ± 5%, n = 6 compared with control LTP 181 ± 7%, n = 6, P < 0.01) and which continued to further in significance by 3 h post-tetanus (SB/TNF-α 132 ± 5%, n = 6 versus control LTP 175 ± 7%, n = 6, P < 0.001). These results suggest that TNF inhibits the early phase of LTP by a p38-dependent mechanism and negatively affects late-LTP by a mechanism independent of p38. A summary of the effects of SB203580 on inhibition of LTP by TNF-α is shown in Fig. 5.

DISCUSSION

LTP is known to consist of distinct temporal phases that involve different molecular mechanisms for their expression in the CA1 and dentate gyrus regions of the hippocampus (Sweatt, 1999). There is an initial phase generally referred to as short-term potentiation that is independent of protein kinase activity and lasts approximately 30 min followed by early phase, designated early LTP that is over by approximately 1–2 h that is independent of protein synthesis, and dependent on such kinases as CaMKII and PKC. A later more persistent phase designated late LTP requires new protein synthesis and is PKA dependent (Abel et al., 1997; Frey et al., 1993; Huang and Kandel, 1998).
The neurobiological impact of the pro-inflammatory cytokine TNF-α is increasingly being recognized because of the plethora of information now available on its neuro-pathological as well as its physiological effects. We show in this study that TNF-α at pathophysiological concentrations (4.5 ng/ml) inhibited the induction of LTP, as reported previously in the CA1 and dentate gyrus region of the hippocampus (Cunningham et al., 1996; Tancredi et al., 1992). However these earlier studies did not address later stages (>1 h after induction). In this study we decided to examine the effects of TNF-α on early and late-LTP by following the electrophysiological response for 3 h post-tetanus. In so doing, a synaptic depression was unveiled which stabilized at approximately 3 h post-tetanus. This has not been previously reported in TNF-α- or IL-1β-treated slices. However a similar electrophysiological depressive phenomenon has been reported previously with the cytokine interferon-α in the CA1 region (Mendoza-Fernandez et al., 2000). In order to address whether this depression could be effected by bath application of this cytokine without application of HFS we followed the electrophysiological response of slices perfused with TNF-α for approximately 3 h. We found that TNF-α baseline responses were statistically different from TNF-α LTP at points corresponding to 3 h post-tetanus. No significant difference was noted between control and TNF-α baseline responses for the same time-point investigated. As such we can conclude that HFS must activate signaling mechanisms that facilitate the depression observed in TNF-α-treated hippocampal slices.

TNF-α has previously been shown to activate a family of mitogen-activated protein kinases (MAPK), which include p42/44, p38 and c-Jun N-terminal kinase (JNK; Vandenabeele et al., 1995). The p42 MAPK has been shown to be important in the induction of NMDA-receptor-dependent LTP in the CA1 (English and Sweatt, 1996) and dentate gyrus (Coogan et al., 1999a). In contrast, it has been shown that p38 MAPK is an integral signaling component in metabotropic glutamate receptor dependent LTD, first demonstrated in the CA1 of neonates (Bolshakov et al., 2000) and later in the dentate gyrus region of the rat hippocampus (Murray and O’Connor, 2003). The p38 MAPK is not involved in the induction of NMDA receptor dependent early LTP, as reported previously (Coogan et al., 1999b), nor is it involved in the induction of late LTP (3 h post-tetanus), as SB/LTP fEPSP values were found to be comparable to control LTP at 1 h and 3 h post-tetanus. Another stress-activated protein kinase, JNK, has recently been shown by this laboratory to be involved in LTD in the dentate gyrus, and similar to p38 MAPK was shown not to influence LTP (Curran et al., 2003). Therefore p42 MAPK and p38/JNK appear to have contrasting physiological roles in the hippocampal synaptic plasticity processes of LTP and LTD.
We set out to investigate whether p38 MAPK could possibly be involved in mediating TNF-α inhibition of LTP. First, we examined the levels of activated p38 MAPK in the dentate gyrus region of the hippocampus of young rats (4 weeks). Using antibody that recognizes dually phosphorylated p38 MAPK, strong immunostaining of the granule cells in the dentate gyrus was observed (Fig. 2A, 2C), indicating high basal levels, as well as marked immunostaining of the pyramidal neurons in the CA1 and CA3 regions (data not shown). TNF-α was perfused through hippocampal slices and led to an increase in the level of immunostaining (Fig. 2B, 2D). This is not surprising since TNF-α is known to lead to the activation of p38 MAPK. The specific p38 MAPK inhibitor SB 203580 (IC50 = 600 nM in cells) was used in this study at a final concentration of 1 μM, as doses of 0.6 μM were found to prevent the phosphorylation of heat-shock protein-27 in response to IL-1β, cellular stresses and bacterial endotoxins in vivo (Cuenda et al., 1995). We found that p38 MAPK is involved in the inhibition of LTP by TNF-α but importantly that p38 MAPK inhibition does not reverse the impairment of late-LTP by TNF-α (>80 min post-tetanus). This only became apparent due to the long follow-up of 3 h post-tetanus undertaken in this study. The synaptic response measured by fEPSP recordings were seen to decline from approximately 1 h and at 2 h post-tetanus a statistical significant difference between control LTP and SB/TNF-α-LTP was observed (P < 0.01), which furthered in significance by the 3 h time-point (P < 0.001). Could this later-phase possibly represent a protein-synthesis dependent mechanism of inhibition of LTP by TNF-α? Indeed, the time-course coincides with late LTP, which has now been firmly established to be dependent on new protein synthesis (Frey et al., 1993; Huang and Kandel, 1994; Nguyen and Kandel, 1996; Otani and Abraham, 1989). What are the targets of p38 MAPK in mediating TNF-α inhibition of early-LTP? Possible substrates include heat shock protein 27, that has been shown to affect actin dynamics (Rousseau et al., 1997), the microtubule-associated protein tau (Reynolds et al., 1997), and cytoplasmic phospholipase A2 (Kramer et al., 1996), the enzyme that liberates arachidonic acid from membrane phospholipids. Coogan et al. (1999) have already given some insight into this question by the finding that inhibition of the cyclooxygenase pathway by indomethacin attenuates IL-1β inhibition of LTP, and more specifically cyclooxygenase-2 (Murray and O’Connor, 2001) therefore suggesting a link between arachidonic acid metabolites and the impairment of LTP by IL-1β.

In summary this paper reports that p38 MAPK detected at high basal levels in the dentate gyrus, does not have a physiological role in early or late-LTP. The role of p38 MAPK in TNF-α inhibition of LTP has been delineated. This work shows that p38 MAPK kinase is involved in mediating the inhibitory effects of TNF-α on early LTP (approximately 1 h). Interestingly however, the depressive effects of TNF-α on late LTP (approximately 1–3 h) proved to be independent of p38 MAPK. Therefore we can conclude that TNF-α inhibition of LTP represents a biphasic response, where the later phase may represent a protein-synthesis dependent phase.

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