EXCLI Journal 2012;11:373-385 – ISSN 1611-2156
Received: April 08, 2012, accepted: July 05, 2012, published: July 09, 2012

BIDIRECTIONAL EFFECTS OF SERUM TNF ALPHA LEVEL AND SPINAL P38MAPK PHOSPHORYLATION ON HYPERALGESIA VARIATION DURING CFA-INDUCED ARTHRITIS

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ABSTRACT

Regarding the role of TNFα in the induction of hyperalgesia, the dual suggested roles of the Pp38 MAPK intracellular pathway in the emergence of symptomatic inflammation, we aimed to investigate the bidirectional effects of serum TNFα level and p38 MAPK phosphorylation on hyperalgesia variation during different stages of adjuvant-induced arthritis. Hyperalgesia and edema were assessed at 0, 3, 7, 14, and 21 days of study after arthritis induction by CFA. Anti-TNFα and Pp38 inhibitor were administered during the 21 days of study. Receptor and intra-cellular enzyme expression were detected by western blotting. Anti-TNFα administration in the AA group decreased paw volume and hyperalgesia until the 14th day of study; on the 21st day, those symptoms increased. Daily administration of anti-TNFα antibody caused significant decrease in spinal mOR protein and Pp38/p38 MAPK enzyme level expression on the 14th and 21st days compared to the AA control group. Our data suggested that phosphorylation of spinal p38 MAPK enzyme played an important role in bidirectional effects of serum TNFα on inflammatory symptoms via spinal mOR expression variation.

Keywords: TNFα, inflammation, hyperalgesia, mu opioid receptor, p38 MAPK

INTRODUCTION

Inflammation is the primary and, essentially, a salutary response that normally resolves with the restoration of normal tissue structure and function; however, when inflammation persists (chronic inflammation), it can cause tissue damage and loss of function (Rodriguez-Vita and Lawrence, 2010). It results in rapid elevation of the secretion of inflammatory mediators, chemokines, and cytokines, such as interleukins 1 and 6 (IL-1 and IL-6), and tumour necrosis factors α (TNFα) (Zaringhalam et al., 2010). Pain and disability are the principal clinical features of inflammation. This situation is associated with sensitisation of specialised sensory neurons that comprise the nociceptive (pain) pathway (Inglis et al., 2005). Rheumatoid arthritis (RA) is the most common form of inflammatory arthritis, characterised by chronic synovitis, progressive joint damage, and significant pain disability. Although the etiology of RA remains elusive, there is evidence that cytokines play a critical role in its pathogenesis (Garfield et al., 2005). Complete Freund's adjuvant (CFA) has been utilised to induce an arthritic immunopathological disease that displays many of the pathological features of human RA. This arthritis model has

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been used extensively, not only to analyse the cellular and molecular aspects of RA, but also to evaluate the anti-inflammatory/ anti-nociceptive effects of newly developed drugs on chronic arthritis (Lee et al., 2009). The use of biological therapies targeting these key pro-inflammatory molecules and their receptors has emerged as a powerful tool for the control of many systemic inflammatory disorders in the last few years (Garfield et al., 2005). TNFα is a potent cytokine that exerts pleiotropic functions in immunity, inflammation, control of cell proliferation, differentiation, and apoptosis (Caminero et al., 2011). TNFα is a key cytokine involved in the pathogenesis of RA, resulting in a chronic inflammatory state in which the synovial membrane is the primary site of attack. Therapies directed against tumour necrosis factor (TNF) are effective for treatment of rheumatoid arthritis and reduced pain scores associated with this condition (Inglis et al., 2005). However, the precise molecular mechanisms by which TNFα exerts its destructive effect in RA are not known, and the mechanisms by which TNFα antagonists exert their therapeutic effect are not completely understood (Garfield et al., 2005). TNFα signalling involves various pathways and signalling molecules, and this makes it an interesting and complex process to investigate. The binding of TNFα to its receptors initiates a cascade of events involving the activation of a series of mitogen-activated protein kinase kinase (MAP2K) that further phosphorylates and activates a dual-specificity protein kinase (MEK). This, in turn, activates a mitogen-activated protein (MAP) kinase (e.g. p38 MAPK); then, activated p38 MAPK phosphorylates downstream kinases. Measuring levels of phosphorylated p38 (i.e. Pp38) often provides a measure of cellular responses to inflammation (Zhang et al., 2008). Pp38 MAPK is primarily activated within cells involved in the inflammatory process, which in turn induces the synthesis of key inflammatory mediators, such as TNFα, IL-1, IL-6, and IL-8; this production can play a key role in the pathogenesis of many chronic inflammatory disorders, such as RA (Cuenda and Rousseau, 2007). Moreover, it is clear that sustained inflammation causes physiological and pharmacological changes in the pain inhibitory system, and that increases in the potency of opioid agonists alleviate hyperalgesia during inflammation. Opioid receptors are involved in the pain mandatory system during inflammation, and the μ-opioid receptor (mOR) is the most commonly associated with analgesic therapy in chronic pain (Martin et al., 2001). Our previous studies also indicated that decreased hyperalgesia during chronic inflammation was related to an increase in spinal mOR expression (Zaringhalam et al., 2008; Tekieh et al., 2011). Scientists have established that p38MAPK also might play a key role in the variation of the expression of cellular plasma membrane receptors during different stages of inflammation. Some studies have indicated that p38 MAPK enzyme activation modulates opioid receptor endocytosis (Mace et al., 2005). Therefore, regarding the role of TNFα in hyperalgesia induction via intra-cellular signalling pathways, the dual suggested roles of the p38 MAPK enzyme in the emergence of symptomatic inflammation, and the importance of developing effective and safer painkilling drugs with fewer side effects, we aimed to investigate the relationships among serum TNFα level, p38 MAPK enzyme activity, and hyperalgesia variation during different stages of CFA-induced arthritis in male Wistar rats.

MATERIALS AND METHODS

Laboratory animals

Adult male Wistar rats, weighing 200–220 g at the beginning of the experiments, were used in the present study. The animals were housed at a room temperature of 22.0 ± 1 °C with a 12 h light–dark cycle (lights on, 08:00 am to 8:00 pm). Food and water were available. The study protocol was approved by the local ethics committee for the use of animals in research, and we followed the guidelines of ethical standards for in-

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vestigation of experimental pain in animals (Zimmermann, 1983).

Local paw inflammation induction

A model of persistent inflammatory pain was produced by unilateral intraplantar injection of complete Freund's adjuvant (CFA) (heat-killed and dried *mycobacterium Tuberculosis*) in a volume of 100 µl into the plantar surface of the right hind paw (ipsilateral side) of rats under light anaesthesia with methoxy isoflurane. Control rats were given saline in a volume of 100 µl into the plantar surface of the right hind paw (Rezazadeh et al., 2009).

Measurement of paw edema

The intensity of inflammatory edema was measured on days 0, 3, 7, 14, and 21 after the injection of CFA and other interventions. Paw swelling (edema) was assessed by measuring the volume of the injected hind paw by plethysmometer (model 7141; Ugo Basile, Comerio, VA, Italy), before and after intervention. The results are expressed as the algebraic difference between the volume (ml) of the injected paw before CFA injection (day 0) and during different stages of study. All experiments were repeated three times, and the average per paw was determined (Bianchi et al., 2008).

Measurement of thermal hyperalgesia

To assess sensitivity to thermal stimulation, each of the rats' hind paws was tested individually using a thermal stimulus apparatus (Ugo Basilar; Verse, Italy). The intensity of the thermal stimulus was adjusted to achieve an average baseline paw withdrawal latency of approximately 20–22 s in naive rats. Only quick hind paw movements (with or without licking of the hind paws) away from the stimulus were considered a withdrawal response. Paw movements associated with locomotion or weight-shifting were not counted as a response. The paws were measured alternating between left and right, with an interval of more than 5 min between measurements (Narita et al., 2008). The mean latency of the withdrawal responses for each foot was calculated. Then, the value for the affected paw (CFA-injected paw) was subtracted from that of the other paw, and the result was considered the hyperalgesia sign in the injured paw (Tekieh et al., 2011).

Blood sampling and serum TNFα measurements

The serum TNFα levels of the rats were assayed by a rat standard ELISA kit (Koma Biotech, Seoul, Korea) on day 0 (before CFA injection) and at different phases of study according to the manufacturer's protocol. The rats were lightly anaesthetised and retro-orbitally bled into heparinised tubes. The samples were centrifuged and stored at -80 °C. The collected serum showed 100 % cross-reactivity with the ELISA kit (Zaringhalam et al., 2010).

Chemicals and reagents

To evaluate further the roles of TNFα and Pp38 MAPK in inflammatory symptoms, the rats were treated with TNFα antibody and p38 MAPK phosphorylation inhibitor to deplete serum TNFα and Pp38 levels. The anti-rat TNFα antibody was obtained from Abcam/CA (# ab9755), and the Pp38 MAPK inhibitor, SB203580, was purchased from Promega/USA. According to the manufacturer, the neutralisation dose 50 (ND50) for this anti-rat TNFα antibody was approximately 0.145–0.165 µg/ml in the presence of 2.0 ng/ml rat TNFα. SB203580 (p38 MAPK inhibitor) re-suspended 1 mg in 265 µl of vehicle to produce a stock solution of 10 mM; for inhibition of p38 phosphorylation, 70 nM/100 µM of that stock was administered via I.P. on a daily basis (Zaringhalam et al., 2010). The anti-TNFα neutralising antibody and SB203580 were dissolved in sterile phosphate-buffered saline (PBS), and the control animals received only PBS as a vehicle. The primary antibodies—p38 MAPK antibody (#ab31828), phospho-p38 MAP Kinase (Thr180Tyr182) antibody (#ab32557), and mOR antibody (#ab10275) were obtained from Abcam/

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UK. Other reagents were all of analytical grade. All solutions were prepared fresh a maximum of 30 min prior to injection and were equilibrated at room temperature.

Western blot analysis

After behavioural tests, western blot was used to examine the variations of spinal mOR, p38 MAPK, and Pp38 MAPK enzyme expression detection during different stages of study. Briefly, the lumbar spinal cord was quickly removed after the rat was decapitated, and homogenised in RIPA buffer containing 50 mM Tris-HCL (pH 7.4), 1mM EDTA, NP40 1 %, NaCl 150 mM, aprotinin 10 mg/ml, leupeptin 1 µl, and PMSF 2 mM. Equal amounts of proteins (50 µg) were loaded on 10 % sodium dodecyl sulphate – polyacrylamide gel electrophoresis (SDS-PAGE) gels and run at 100mv for approximately 1 h. Proteins were transferred to Immobilon-P PVDF membranes (Millipore, Bedford, MA) using mini-PROTEAN II (Bio-Rad) at 100 V for 80 min. Nonspecific binding sites on the membrane were blocked by incubation (90 min at 24 °C or overnight at 4 °C) in blocking buffer (0.2 % Aurora Blocking Reagent; 1X Phosphate Buffered Saline: 0.058 M Na_2HPO_4, 0.017 M NaH_2PO_4, 0.068 M NaCl; 0.05 % Tween-20 from ICN Biomedicals, Costa Mesa, CA) followed by incubation (1 h, 24 °C) with primary antibodies in blocking buffer (rabbit polyclonal mOR, rabbit monoclonal SB203580, and mouse monoclonal SB203580 (1:1000; Abcam plc UK). The membranes were washed twice with blocking buffer and then incubated (1 h, 24°C) with secondary antibodies in blocking buffer (anti-rabbit and anti-mouse IgG [1:10000; Abcam]). They were then washed three times with blocking buffer, followed by two quick rinses with assay buffer (20 mM Tris-HCl, pH 9.8, 1 mM $MgCl_2$). The immunoreactivity of the proteins on the membranes was visualised using a chemiluminescence detection system (ECL, Amersham). The membranes were then incubated in stripping buffer (100 µM 2-mercaptoethanol, 2 % SDS, 62.5 mM Tris [pH 6.7]) at 50 °C for 30 min and reprobed with beta-actin primary antibody (1:5000; Cell Signalling) as a loading control. Band intensity was measured densitometrically using NIH Image (1.60) and expressed as the ratio of the intensity of the mOR band to that of β-actin; Pp38 MAPK band to p38 MAPK to account for any differences in starting spinal mOR and phosphorylated p38 MAPK enzyme proteins, respectively. Each experiment was replicated three times with new groups of rats (Kurien and Scofield, 2006).

Experimental procedure

To recognise the effect and relation of serum TNFα level and Pp38 MAPK enzyme on inflammation, after single local hind paw CFA injection and ipsilateral paw inflammation induction, anti-TNFα antibody and p38 phosphorylation inhibitor, SB203580, were administered daily from day 0 to day 21 of the study. Behavioural tests, blood TNFα level assessment, and spinal protein expression variation detection were made during the different phases of inflammation (Zaringhalam et al., 2008), at 0 (before CFA injection), 3, 7, 14, and 21 days. To achieve these different time point measurements during this study, different experimental groups (as control and experimental groups) were identified - each experimental group was comprised of six adult rats. At the end of each session in each study group, the animals were deeply anaesthetised and decapitated; then, the lumbar spinal cords were quickly removed on iced saline and stored at -80 ºC until selected protein detection was conducted by western blotting.

Statistical analysis

Results are presented as the mean ± SEM. Data was analysed by one-way analysis of variance (ANOVA), followed by Tukey's test for multiple comparison. Unpaired Student's *t*-test was used to determine significant differences between the groups. An effect was determined to be significant if the p-value was less than 0.05.

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RESULTS

Variation of serum TNFα levels during different stages of study

To determine the magnitude of change following a CFA-induced inflammation, and the role of some mediators in this process, serum TNFα concentrations were examined by rat-specific ELISA kit. A remarkable increase in TNFα concentration after CFA treatment was observed. Serum TNFα levels significantly increased on the 3rd, 7th, 14th, and 21st days, compared to day 0, in the AA rats ($p<0.001$). Our findings revealed that continuation of inflammation caused significant decreases in TNFα levels on the 14th and 21st days, compared to the 3rd and 7th days of the study ($p<0.001$); however, they were higher than on day 0 ($p<0.001$). Anti-TNFα administration in the AA rats returned serum TNFα levels to those of day 0 (before CFA injection), and the AA+ anti-TNFα rats indicated a significant reduction in serum TNFα levels compared to the same days in the AA group ($p<0.001$). Moreover, blocking of p38 MAPK enzyme phosphorylation by SB203580 caused a significant reduction in serum TNFα levels on the 7th, 14th, and 21st days in the AA rats, compared with the AA control group ($p<0.05$, $p<0.01$, and $p<0.01$, respectively). In the AA rats, serum TNFα levels decreased significantly on days 14 and 21 of the study, compared to day 7 ($p<0.05$). There was no significant difference in TNFα level between days 0 and 3 during the administration of the p38 MAPK enzyme activity inhibitor (Figure 1).

Paw volume variation due to long-term anti-TNFα administration in AA rats

CFA injection into the right hind paw caused a marked increase in paw volume (edema). Right injected paw volume showed significant increase on days 3, 7, 14, and 21 after CFA injection compared to day 0 in the AA control group ($p<0.001$). Our results indicated that CFA-injected paw volume was significantly reduced by anti-TNFα administration on days 3, 7, and 14 compared to the same days in the AA control group ($p<0.001$). Paw volume in the AA+ anti-TNFα group on the 21st day was not only significantly greater than that of the AA control group ($p<0.01$), but also significantly greater than the previous days in the same group ($p<0.001$). Furthermore, our data showed that SB203580 administration during 21 days after CFA injection caused a meaningful decrease in paw volume on the 3rd, 7th, 14th, and 21st days (Figure 2). There were no significant differences in paw volume variation between the AA+ PBS group (as vehicle) and the AA control group (data not shown).

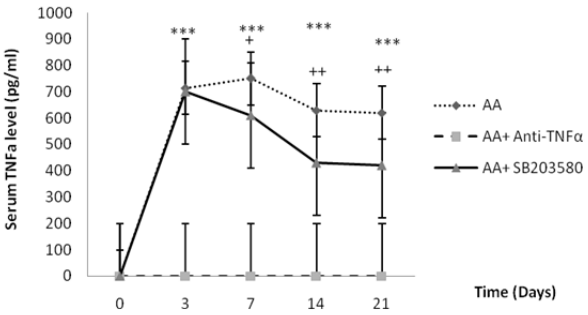

Figure 1: ELISA analysis of serum TNFα level during different stages of CFA-induced inflammation. A significant decrease of serum TNFα levels were observed in AA+ anti–TNFα and AA+ SB203580 groups. Data are presented as mean ± S.E.M (n=6/group). *** $p<0.0001$: For comparing the variation of serum TNFα level between different days of study with day 0. ++ $p<0.001$, + $p<0.01$: Comparison of serum TNFα level between AA and AA+ SB203580 rats. AA: adjuvant-induced arthritis; SB203580: p38 MAPKinases inhibitor

Thermal hyperalgesia variation due to long-term anti-TNFα administration in AA rats

The CFA-injected rats displayed varying degrees of thermal hyperalgesia in the injected paw during different stages of AA. Hyperalgesia significantly increased on days 3 and 7 after CFA injection compared to day 0 in the AA control group ($p<0.001$). Our results also indicated that inflammation continuation caused hyperalgesia to decrease on days 14 and 21, compared with days 3 and 7 ($p<0.001$). Moreover, there was a significant decrease of hyperalgesia

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on day 21 of the study compared to day 14 in the AA control group ($p< 0.001$). Anti-hyperalgesic effects of daily TNFα antibody neutralising dose administration in the AA rats had been seen in the first week of the study, but this effect reversed on the 14th and 21st days after CFA injection. Thermal hyperalgesia declined significantly with daily anti-TNFα antibody administration on the 3rd and 7th days of intervention in the AA+ anti-TNFα group compared to the same days in the AA control group ($p<0.001$ for both). Furthermore, long-term daily neutralising doses of TNFα antibody treatment caused a significant increase in hyperalgesia on days 14 and 21 of the study in the AA+ anti-TNFα group compared with the same days in the AA control group ($p<0.001$ for both). SB203580 administration during 21 days of study caused a significant decrease of hyperalgesia on the 7th day of this study; however, long-term daily administration caused a reversal of this effect on the 21st day and remarkably increased thermal hyperalgesia (Figure 3). In addition, no significant discrepancy was identified between the AA+ PBS group and the AA control group (data not shown).

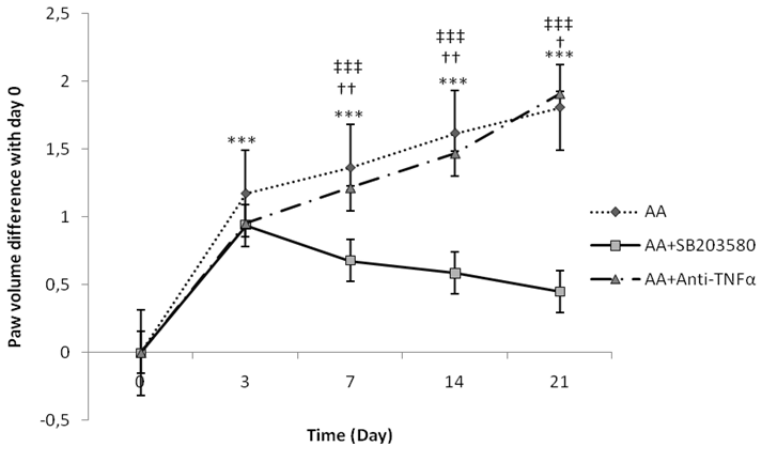

Figure 2: Paw edema variation due to anti-TNFα antibody and SB203580 administration during 21 days of CFA-induced inflammation, which was assessed by plethysmometer. Data are expressed as mean ± S.E.M (n=6/group). *** p<0.001: For comparing of paw volume variations between different days of study and day 0 in AA rats. †† p<0.001, † p<0.01: Comparison of paw volume variation between AA and AA+ anti-TNFα antibody treated rats. ‡‡‡ p<0.0001: Comparison of paw variation between AA and AA+ SB203580 treated rats. AA: adjuvant-induced arthritis; SB203580: p38 MAPKinases inhibitor

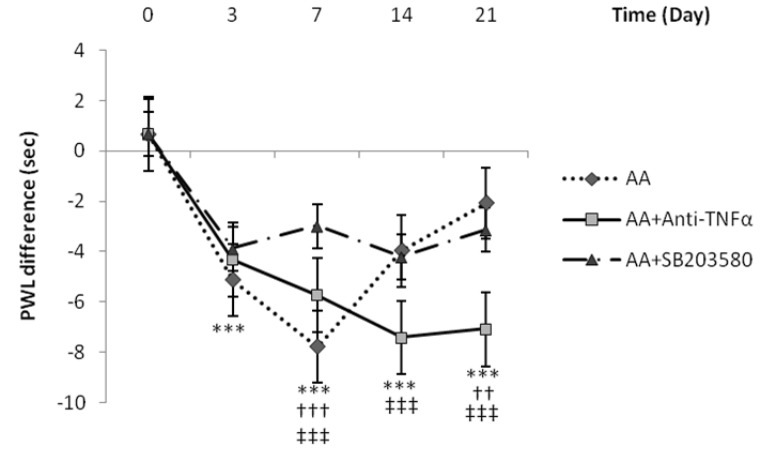

Figure 3: Anti-TNFα antibody and SB203580 administration caused significant variation on CFA-induced hyperalgesia during 21 days. Data are expressed as mean ± S.E.M (n=6/group). *** p<0.0001: For comparing the hyperalgesia variation between day 0 and other days in AA rats. ‡‡‡ p<0.0001: For comparing hyperalgesia variations between AA and AA+ anti-TNFα antibody treated groups. ††† p<0.0001, †† p<0.001: For comparing hyperalgesia variations between AA and AA+ SB203580 treated groups. AA: adjuvant-induced arthritis; SB203580: p38 MAPKinases inhibitor

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Spinal mOR expression variation due to long-term anti-TNFα antibody administration in AA rats

In order to check mOR protein expression in the lower back part of the spinal cords of the experimental groups, we used polyclonal antibody to detect mOR protein in the spinal cord. Analysis by densitometry showed that AA time-dependently caused a significant increase in spinal mOR protein expression when compared with the control group; in addition, there was no considerable increase in spinal mOR expression until the 7th day of inflammation in the AA group, compared to day 0 (Figure 4A, B, C).

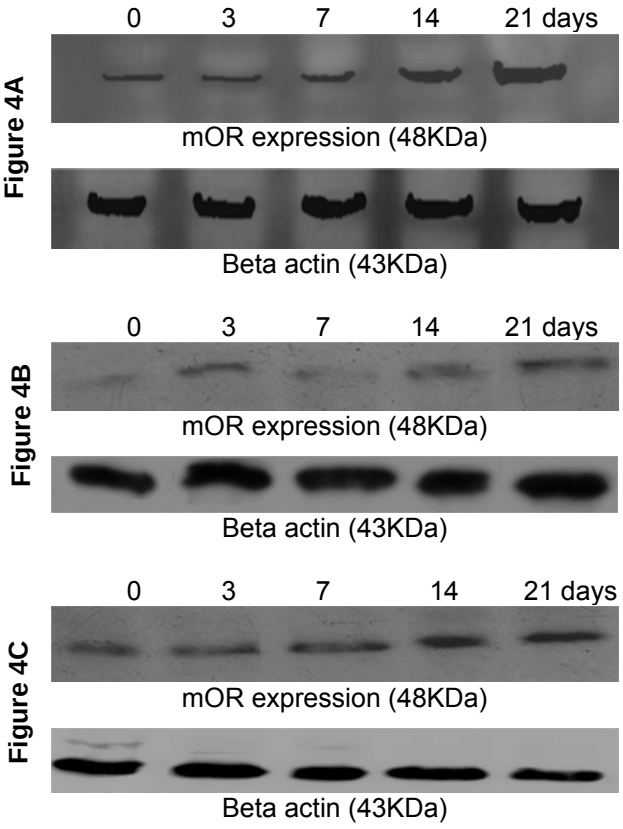

Figure 4A, B, C: Immunoblots of mOR extracted from the lower back part of spinal cords, which normalised to beta actin during different stages of study (days 0, 3, 7, 14, 21). **A:** Variation of lower back spinal mOR expression during different days in AA, **B**: Variation of lower back spinal mOR expression during different days in AA+ SB203580 group **C**: Variation of lower back spinal mOR expression during different days in AA+ anti-TNFα group. AA: adjuvant-induced arthritis; SB203580: p38 MAP-Kinases inhibitor

Our results confirm that, on the lower back part of the spinal cords obtained from the AA rats, spinal mOR protein expression observably increased on days 14 and 21 compared to day 0 of the study ($p<0.001$). On day 21 day of AA treatment, spinal mOR protein expression increased significantly higher from day 14 ($p<0.01$). Daily administration of anti-TNFα antibody in the AA rats caused a significant decrease in spinal mOR protein expression on the 14th and 21st days of the study compared with the AA control group ($p<0.001$). Comparison of spinal mOR protein expression between the AA+ anti-TNFα and AA control groups indicated that mOR expression decrease was significantly higher on day 21 compared with day 14 ($p<0.001$) (Figure 5). Our data also showed that the ratio of spinal mOR protein band intensity in the AA+ SB203580 group significantly decreased on days 14 and 21 compared with the AA control group ($p<0.001$ for both). The decrease of spinal mOR protein band intensity in the AA+ SB203580 group on day 21 was significantly higher than on day 14 ($p<0.001$) (Figure 6). PBS administration (as a vehicle) in the AA rats did not alter spinal mOR protein expression compared with the AA control group (data not shown).

Spinal mOR expression variation due to long-term anti-Pp38 MAPK enzyme antibody administration in AA rats

For the sake of investigative quantity of Pp38 MAPK enzyme activity in the lower back part of the spinal cord, we utilised a monoclonal antibody that detected the Pp38 MAPK enzyme. Afterward, the data was explained by densitometry (Figure 7A, B, C). Our data showed that in the lower back segment of the spinal cords obtained from the AA rats, p38 MAPK enzyme phosphorylation was noticeably raised during different days of study. Pp38 to p38 MAPK enzyme levels in the AA rats indicated significant increases on days 3, 7, 14, and 21, compared with day 0 (3rd day: $p<0.01$; other days: $p<0.001$). However, the levels of phosphorylated p38 MAPK enzyme in-

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creased on days 14 and 21 compared with day 0 in the AA rats; however, this increase was significantly higher on the 21st day ($p<0.01$). Our results also showed that Pp38/p38 MAPK enzyme levels in the lower back segment of spinal cord of the AA+ anti-TNFα group significantly decreased on days 7, 14, and 21 of the study compared with the AA control group ($p<0.01$, $p<0.001$, $p<0.001$, respectively). The decrease in Pp38/p38MAPK enzyme levels on day 21 was significantly greater than on day 14 when comparing between the AA+ anti-TNFα and AA groups ($p<0.001$ for both) (Figure 8). PBS administration in the AA group as a vehicle of anti-TNFα antibody did not change p38 MAPK enzyme phosphorylation compared to the AA control group (data not shown).

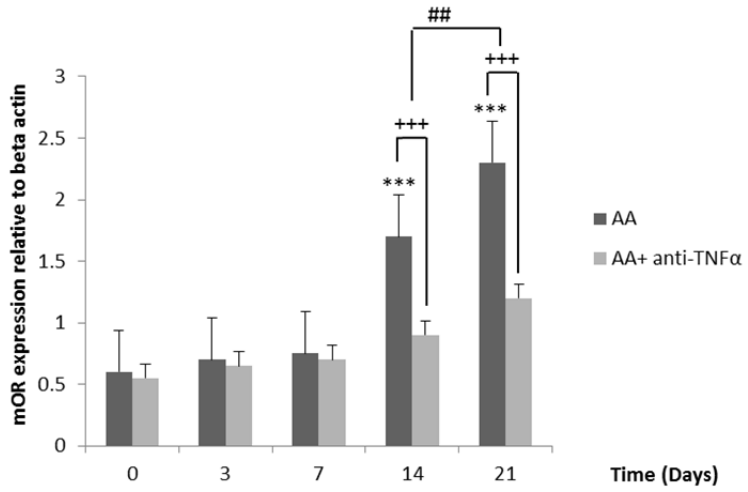

Figure 5: Anti-TNFα antibody administration significantly reduced spinal mOR expression during different stages of AA. Data are presented as mean ± SEM (n=6/group). ***p<0.001 Comparison of ratio of spinal mOR protein band intensity between different days of AA. +++p<0.001: Comparison of spinal mOR protein band intensity variation between AA and AA+ anti-TNFα antibody treated rats on 14th and 21st days. ##p<0.01 Comparison of spinal mOR protein band intensity variation in AA and AA+ anti-TNFα antibody treated rats between 14th and 21st days. AA: adjuvant-induced arthritis; TNFα: tumour necrosis factor α

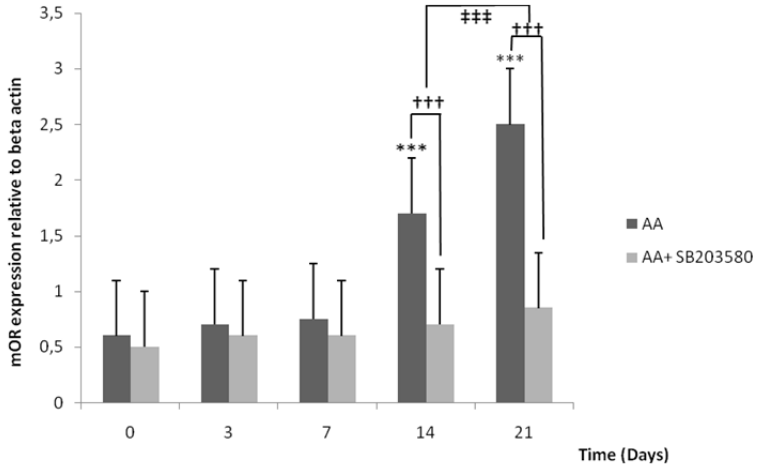

Figure 6: Effects of SB203580 administration on spinal mOR expression. SB203580 administration significantly reduced spinal mOR expression during different stages of AA. Data are presented as mean ± SEM (n=6/group). ***p<0.001 Comparison of ratio of spinal mOR protein band intensity between different days of AA. ††† p<0.001 Comparison of spinal mOR protein band intensity between different days in AA and AA+SB203580 treated rats. ‡‡‡p<0.001 Comparison of spinal mOR protein band intensity between AA and AA+ SB203580 treated rats. AA: adjuvant-induced arthritis; SB203580: p38 MAP-Kinases inhibitor

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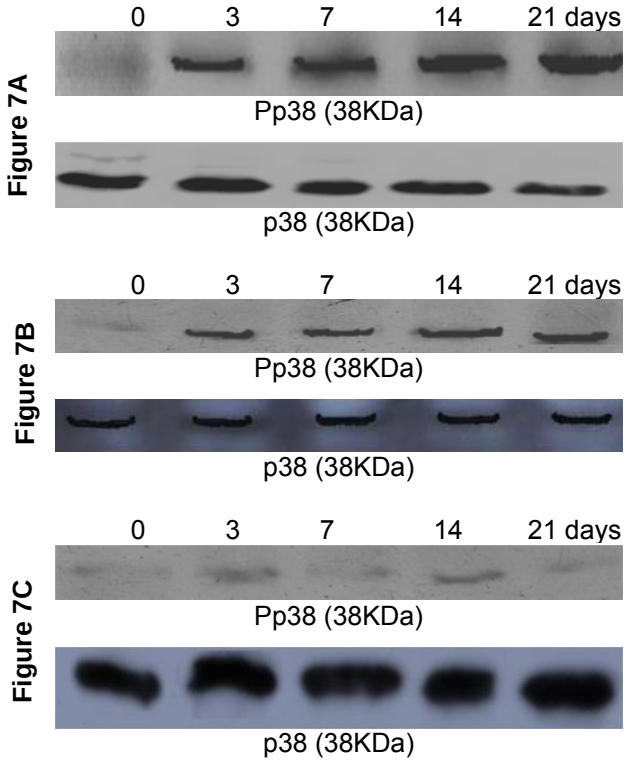

Figure 7A, B, C: Immunoblots of Pp38 MAPK enzyme extracted from the lower back part of spinal cord, which normalised to p38 MAPK enzyme during different stages of study (days 0, 3, 7, 14, 21). **A:** Variation of Pp38 MAPK enzyme expression in AA rats. **B:** Variation of Pp38 MAPK enzyme expression in AA+ anti-TNFα rats. **C:** Variation of Pp38 MAPK enzyme (38KDa) expression in AA+ SB203580 rats. AA: adjuvant-induced arthritis; SB203580: p38 MAPKinases inhibitor; TNFα: tumour necrosis factor α

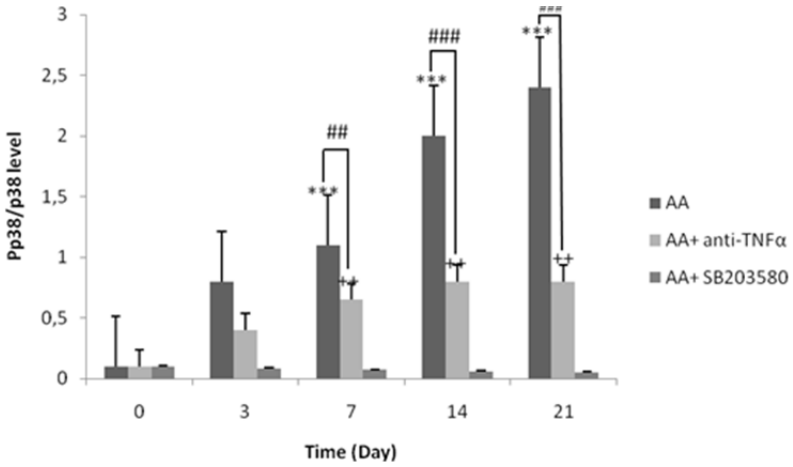

Figure 8: Anti-TNFα antibody and SB203580 administration significantly decreased p38 MAPK enzyme phosphorylation during different stages of AA. Data are presented as mean ± SEM (n=6/group). ***p<0.001: Comparison of ratio of Pp38/p38 MAPK enzyme band intensity between different days of AA with day 0. ++p<0.001: Comparison of Pp38/p38 MAPK enzyme band intensity between different days of study and day 0 in AA+ anti-TNFα antibody treated rats. ###p<0.001: Comparison of Pp38/p38 MAPK enzyme protein band intensity between AA+ SB203580 and AA+ anti-TNFα antibody treated rats. AA: adjuvant-induced arthritis; SB203580: p38 MAPKinases inhibitor; TNFα: tumour necrosis factor α.

DISCUSSION

Coordination between the immune and nervous systems is essential for managing and modulating the immune neurobiological responses to inflammation (Zöllner et al., 2003). It is clear that cytokines are involved in different stages of inflammation by inducing time-dependent pro-/anti-inflammatory effects. TNFα is a key pro-inflammatory cytokine involved in the pathogenesis of RA (Bingham, 2002). Some studies suggest that therapy with anti-TNFα agents provides a marked improvement in patients with RA (Baumgartner, 2004). However, our results demonstrated that long-term (21 days) anti-TNFα administration can elevate hyperalgesia during chronic CFA-induced arthritis.

In the first phase of this study, our results indicated that the CFA-injected paw was typified by a rapid onset of inflammation evident within 24 h that continued to increase up to day 21. The findings, in agreement with others, not only indicated

an increase of serum TNFα levels due to the CFA injection, but also indicated that the levels remain elevated until the 21st day after CFA intervention. A number of studies have documented that CFA-induced mono-arthritis has two phases; in the first phase (inflammatory phase), an increase in pro-inflammatory cytokines, such as IL-1β, IL-6, and TNFα secretion, cause hyperalgesia and edema. The pro-inflammatory cytokine TNFα is a systemic marker of inflammation (Zaringhalam et al., 2010). In acute situations, TNFα has been reported to sensitise nociceptive neurons indirectly via the induction of a pro-inflammatory cytokine cascade involving IL-1β, IL-6, and IL-8, resulting in the release of prostaglandins and other mediators from immune cells (Hackett et al., 2008). Furthermore, the application of TNFα enhances calcium currents and increases neuron sensitivity to the neurotoxin capsaicin in cultures of sensory neurons (Khan et al, 2008). Thus, the anti-hyperalgesia and anti-inflammatory effects of anti-TNFα antibody administration during the acute phase of CFA-induced inflammation were expected, as they were mentioned by some other studies.

Chronic inflammation in arthritis is driven by a variety of pro-inflammatory cytokines, chemokine, and other mediators that outnumber or outperform their anti-inflammatory counterparts (Choy and Panay, 2001). CFA-induced arthritis is the most widely used chronic model in which the physiological and pathological changes are comparable with those seen in human rheumatoid arthritis (Möller and Villiger, 2006). Although the chronic phase of this study (arthritic phase) is manifested as a progressive increase in the volume of the injected paw, hyperalgesia significantly decreased compared to the acute phase. The long-term administration of anti-TNFα neutralising doses in this study also enhanced hyperalgesia in CFA-injected rats. Previous studies have reported the significant efficacy of anti-TNFα antibody Etanercept treatment of inflammatory symptoms in short-term studies (Brandt et al., 2004). It has been shown that the transition from acute to chronic inflammation is likely to involve several systems, including changes in cytokine signalling, both centrally and peripherally; however, the mechanisms involved in these changes are poorly understood. Recent studies indicate that chronic inflammation is associated with physiological and pharmacological changes in the pain inhibitory system. The analgesic effects of peripherally applied opioids are augmented under conditions of tissue injury and inflammation. Our previous studies clarified the important role of spinal mOR expression increase on hyperalgesia decrease during the chronic phase of inflammation, which was blocked by naloxone (Zaringhalam et al., 2008). Assessment of long term anti-TNFα administration effects on spinal mOR expression by western blotting in this study showed a significant reduction of expression of these opioid receptors during chronic CFA-induced inflammation (14th and 21st days). It was presumed that an increase of TNFα levels can change cellular peptide synthesis via different pathways, directly or indirectly (Medeiros et al., 2010). TNFα signalling involves various pathways and signalling molecules, and this makes it an interesting and complex process to investigate. It has been reported that elevated level of TNFα increased extracellular regulated kinases and MAPK phosphorylation. In addition, recent studies indicate that phosphorylation of p38MAPK was decreased in response to TNFα inhibitors. Consistent with some studies, our findings showed that spinal Pp38 MAPK enzyme levels increased during CFA inflammation, that the increase was related to serum TNFα levels, and that anti-TNFα administration led to a decrease of phosphorylation of spinal p38 MAPK. On the other hand, MAPK signalling pathways, like ERK, p38, and JNK are involved in controlling cellular responses to many stimuli, and they play a particularly important role in responding to inflammation by triggering production of transiently expressed protein synthesis and protein processing. In line with this, our

results also indicate that long-term inhibition of p38 MAPK enzyme phosphorylation reduced spinal mOR expression during chronic-phase CFA-induced inflammation. Taken together, it seems that a long-term increase in serum TNFα level can have a direct effect on spinal mOR expression via p38 MAPK enzyme phosphorylation.

Furthermore, it has been shown that the biological responses induced by TNFα might be mediated by other cytokines, such as IL-6 and IL-10. Some of these cytokines have been shown to elicit both pro- and anti-inflammatory effects. IL-6 was shown to induce analgesia in an animal model of inflammation by contributing to the activation of the endogenous opioid system, which is induced in response to peripheral inflammation (Zöllner et al., 2003). Our previous study also revealed that administration of anti-IL-6 antibody causes hyperalgesia by decreasing spinal mOR expression during the chronic phase of CFA-induced inflammation. Therefore, it is predictable that long-term anti-TNFα administration, by decreasing the secretion and activation of pro-/anti-inflammatory cytokines, such as IL-6, may change spinal mOR expression, and via that effect on hyperalgesia, variation during chronic CFA-induced inflammation. In summary, this study confirmed the time-dependent and bidirectional effects of serum TNFα level on CFA-induced hyperalgesia, at least part of which may be mediated via spinal mOR expression variation. Our data also suggest that phosphorylation of p38 MAPK enzyme plays an important role in serum TNFα effects on mOR expression, which is inhibited by anti-TNFα and SB203580. Therefore, it seems that passing the initiating factors in human rheumatoid arthritis and manipulating the cytokine balance may be an effective therapeutic means by which chronic inflammation can be managed; however, this subject requires further investigation.

ACKNOWLEDGMENT

This project was supported by Iran National Science Foundation (INSF) and Neuroscience Research Centre of Shahid Beheshti University of Medical Sciences.

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