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#### **Publisher's version / Version de l'éditeur:**

<https://doi.org/10.1016/j.ejphar.2004.06.029>

*European Journal of Pharmacology*, 497, August 1, pp. 87-95, 2004

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# Activation of adenosine receptors inhibits tumor necrosis factor- $\alpha$ release by decreasing TNF- $\alpha$ mRNA stability and p38 activity

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Received 4 March 2004; received in revised form 24 May 2004; accepted 17 June 2004

Available online 20 July 2004

## Abstract

Adenosine receptor agonists have anti-inflammatory properties and modulate immune responses partly by inhibiting pro-inflammatory cytokine production by monocytes. We investigated signal transduction mechanisms by which adenosine receptor activation inhibits tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) production. Phorbol-12-myristate-13-acetate (PMA) and phytohemagglutinin treatment of human pro-monocytic U937 cells increased TNF- $\alpha$  protein release. Activation of adenosine receptors up to 1 hr following stimulation with PMA/phytohemagglutinin significantly inhibited TNF- $\alpha$  protein release indicating that inhibition of TNF- $\alpha$  occurred post-transcriptionally. The adenosine receptor agonist 2-p-(carboxyethyl)phenethylamino-5'-N-ethylcarboxamidoadenosine hydrochloride (CGS 21680) decreased stability and half-life of PMA/phytohemagglutinin-induced TNF- $\alpha$  mRNA from 80 to 37 min. p38 signaling pathways control TNF- $\alpha$  mRNA stability in macrophages and we confirmed in our cells that p38 was involved in controlling TNF- $\alpha$  release post-transcriptionally. Activation of adenosine receptors with CGS 21680 decreased phospho-p38 protein levels. These data suggest that adenosine receptor activation regulates TNF- $\alpha$  release post-transcriptionally by decreasing mRNA stability through a mechanism involving inhibition of p38 activity.

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**Keywords:** Monocyte; TNF- $\alpha$ ; Adenosine; mRNA stability; p38

## 1. Introduction

Inflammation and hyper activation of immune responses contribute to the pathology of several diseases including multiple sclerosis, Alzheimer's disease, HIV dementia, ischemic and hemorrhagic stroke, Crohn's disease and rheumatoid arthritis. Pro-inflammatory cytokines are key mediators of inflammation and immune responses that normally recruit immune cells to sites of tissue damage or infection, activate phagocytes, contribute to lymphocyte differentiation and signal cell death. Understanding how cytokine release is regulated endogenously can give important insight into immune system control and disease

pathology. The pro-inflammatory cytokine tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) in particular is protective at physiological levels and plays an important role in normal functions such as oligodendrocyte differentiation and myelination (Arnett et al., 2001); however, overproduction of this cytokine can occur during disease processes and is particularly important in Crohn's disease and rheumatoid arthritis (Kollias et al., 1999). Thus, therapeutic strategies for these diseases and other inflammatory disorders continue to be aimed at decreasing TNF- $\alpha$  levels and controlling TNF- $\alpha$  production.

Mitogen-activated protein kinase (MAPK) signaling pathways like extracellular signal-regulated kinase (ERK), p38 and cJun-N-terminal kinase (JNK) are involved in controlling cellular responses to many extracellular stimuli and play a particularly important role in responding to stress by triggering production of transiently expressed proteins like cytokines (Means et al., 2000; Rao, 2001; Zhu et al., 2000). Because cytokines like TNF- $\alpha$  are expressed transiently, their release requires transcription, protein syn-

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thesis and protein processing (Dumitru et al., 2000; Means et al., 2000; Raabe et al., 1998; Tsai et al., 2000; Zhu et al., 2000). The cell signaling pathways initiated by pro-inflammatory events converge on activation of the transcription factors nuclear factor- $\kappa$ B (NF- $\kappa$ B), Elk1, Egr-1 and activating transcription factor (ATF)-2/Jun (Dumitru et al., 2000; Guha et al., 2001; Rao, 2001; Tsai et al., 2000; Xu et al., 2001), which drive TNF- $\alpha$  transcription and production. In addition to driving TNF- $\alpha$  transcription, several recent studies have shown that MAPK signaling molecules control TNF- $\alpha$  post-transcriptionally by targeting mRNA stability and translation (Brook et al., 2000; Mahtani et al., 2001; Rutault et al., 2001). In particular, p38 regulates TNF- $\alpha$  mRNA stability in macrophages (Brook et al., 2000; Liu et al., 2002; Mahtani et al., 2001; Rutault et al., 2001) and activates protein translation (Knauf et al., 2001; Liu et al., 2002; Wang et al., 1998). Thus, control of released TNF- $\alpha$  can occur at the stages of mRNA transcription and/or mRNA stability, and in addition at later points including protein translation and/or protein processing.

Adenosine, an endogenous nucleoside, is produced at sites of inflammation and can modulate immune cell function. Adenosine may be an important regulator of pro-inflammatory cytokine production because adenosine and adenosine receptor agonists control production of cytokines involved in T<sub>H</sub>1 type immune responses and TNF- $\alpha$  production in vivo and in vitro induced by a variety of agents and experimental conditions including lipopolysaccharide, phorbol esters, congestive heart failure and intracerebral hemorrhage (Abbracchio and Cattabeni, 1999; Collis and Hourani, 1993; Hasko et al., 1998, 1996; Link et al., 2000; McWhinney et al., 1996; Wagner et al., 1998, 1999; Mayne et al., 2001). Although a wide variety of immune modulatory actions of adenosine and adenosine receptor agonists have been studied, the signaling pathways responsible for adenosine receptor-mediated regulation of TNF- $\alpha$  production and other immune modulatory actions are unclear. Accordingly, our studies were designed to identify some of these underlying signaling mechanisms and demonstrated that activation of adenosine receptors decreases TNF- $\alpha$  release by a post-transcriptional mechanism involving inhibition of p38 activity and decreased mRNA stability.

## 2. Materials and methods

### 2.1. Cultured U937 cells

Human pro-monocytic U937 cells (ATCC CRL 1593.2; batch F12641) were obtained from American Type Culture Collection (Rockville, MD) and cultured in Roswell Park Memorial Institute (RPMI)-1640 media supplemented with 10% heat-inactivated fetal bovine serum and 1% antibiotic/antimycotic as described previously (Chen et al., 1997). Cells were maintained at 37 °C in a humidified growth chamber supplemented with 5% CO<sub>2</sub>.

### 2.2. TNF- $\alpha$ assays

U937 cells were cultured to a density of 800,000 cells/ml in RPMI 1640 media and plated in 6-well or 96-well plates 1 h prior to experimentation. To induce release of TNF- $\alpha$ , cells were treated with 10 ng/ml phorbol-12-myristate-13-acetate (PMA) and 5  $\mu$ g/ml phytohemagglutinin for 4 h at 37 °C. PMA is a phorbol ester that activates protein kinase C, a well-characterized signaling pathway that transmits signals in activated immune cells. Phytohemagglutinin is a mitogen that stimulates cell proliferation and agglutination, both consequences of monocyte and lymphocyte activation. Therefore, stimulation of U937 cells with PMA/phytohemagglutinin mimics signaling events that occur following monocyte activation in vivo. Following PMA/phytohemagglutinin stimulation, cell culture supernatants were collected and TNF- $\alpha$  levels were determined by enzyme-linked immunosorbent assay (ELISA) as previously described (Chen et al., 1997). Stimulation of U937 cells for 4 h with PMA/phytohemagglutinin produced  $2.3 \pm 0.5$  pg/ $\mu$ l of TNF- $\alpha$  protein in culture supernatants as measured by ELISA.

### 2.3. Reverse transcriptase polymerase chain reaction (RT-PCR)

Total RNA was extracted from cells using a GenElute Mammalian Total RNA Purification kit (Sigma-Aldrich, Oakville, ON). RNA was reverse transcribed into cDNA using a First Strand cDNA synthesis kit (MBI Fermentas, Burlington, ON), followed by PCR amplification of 3  $\mu$ l of cDNA product using TNF- $\alpha$  (5' GACCTCTCTCTAATCAGCCC, 3' CAAAGTAGACCTGCCCAGAC or cyclophilin (5' GCTGCGTTCATTCTTTTG, 3' CTCCTGGGTCTCTGCTTTG) specific primers. TNF- $\alpha$  PCR reactions were run for 25 cycles with denaturation for 30 s at 95 °C, annealing for 30 s at 53 °C and extension for 30 s at 72 °C. Cyclophilin PCR reactions were run for 40 cycles with denaturation for 15 s at 95 °C, annealing for 15 s at 60 °C and extension for 15 s at 72 °C. RT-PCR measurements were made semi-quantitatively. To ensure that 25 cycles produced a quantity of PCR product within the linear range of amplification that would allow us to measure changes in amounts of PCR product between experimental samples, cDNA prepared from PMA/phytohemagglutinin-stimulated cells was amplified using 5, 10, 15, 18, 20, 22, 24, 26, 28, 30, 32, 35 and 40 cycles. PCR products were separated by agarose gel electrophoresis (1.5%) and imaged using ethidium bromide and a BioRad FluorS Max gel documentation system.

### 2.4. Western blots

Fifty micrograms of protein from whole cell lysates was loaded on 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gels and run at 150 mV for approximately 1 h. Gels were transferred onto nitrocellulose

membranes and placed in blocking buffer (1% skim milk powder in TBS-Tween) overnight at 4 °C. Primary antibodies were prepared in 0.5% skim milk powder in TBS-Tween (1:200 dilution) and incubated with nitrocellulose membranes at room temperature for 4 h or overnight at 4 °C. Membranes were washed and incubated for 1 h with horseradish peroxidase-conjugated secondary antibody (1:10,000 dilution). Bands were visualized and quantified using ChemiGlow chemiluminescent substrate (Alpha Innotech, San Leandro, CA) and a BioRad FluorS Max gel documentation system.

### 2.5. Statistics

All data were reported as means  $\pm$  standard errors and were analyzed using an analysis of variance (ANOVA) followed by Tukey–Kramer’s multiple-comparison test unless otherwise indicated. Statistical significance was considered at the 95% confidence interval.

### 2.6. Materials

The adenosine receptor agonist 2-*p*-(carboxyethyl)phenethylamino-5'-*N*-ethylcarboxamido adenosine hydrochloride (CGS 21680) was purchased from Research Biochemicals (Oakville, Ontario). From Tocris (Ballwin, MO), we purchased the receptor antagonist 4-(2-[7-amino-2-(furyl)[1,2,4]triazolo[2,3-*a*][1,3,5]triazin-5-ylamino]ethyl)phenol (ZM 241385). From Calbiochem, we purchased the MAPK inhibitors PD 98059, SP 600125 and SB 202190 (La Jolla, CA). All primary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Secondary horseradish peroxidase conjugated antibodies were purchased from Jackson ImmunoResearch Laboratories (Mississauga, ON). All other chemicals were purchased from standard laboratory sources.

## 3. Results

### 3.1. Adenosine receptor activation inhibits PMA/phytohemagglutinin-induced increases in TNF- $\alpha$ protein levels post-transcriptionally

Previously, we reported that the adenosine receptor agonist CGS 21680 dose-dependently inhibited PMA/phytohemagglutinin-induced increases in levels of TNF- $\alpha$  protein in U937 cells (Mayne et al., 2001). To determine whether CGS 21680 inhibited PMA/phytohemagglutinin-induced increases in levels of TNF- $\alpha$  pre- or post-transcriptionally, U937 cells were treated with CGS 21680 30 min before, simultaneously with, or 30 min, 1, 2 or 3 h following PMA/phytohemagglutinin stimulation. PMA/phytohemagglutinin-induced increases in TNF- $\alpha$  protein release were significantly inhibited ( $P < 0.001$ ) by CGS 21680 when added 30 min before (this inhibition by CGS21680 was

blocked by pretreatment with ZM241385, data not shown), simultaneously with, and 30 min or 1 h after PMA/phytohemagglutinin stimulation (Fig. 1). The anti-TNF- $\alpha$  effect of CGS 21680 was not observed when added 2 or 3 h after PMA/phytohemagglutinin stimulation. TNF- $\alpha$  mRNA was detectable after 30 min of PMA/phytohemagglutinin stimulation, whereas TNF- $\alpha$  protein was not detected until 2 h post-PMA/phytohemagglutinin stimulation (data not shown). Consequently, a conserved anti-TNF- $\alpha$  effect of CGS 21680 when added up to 1 h post-PMA/phytohemagglutinin indicates that control of TNF- $\alpha$  by adenosine receptor activation occurs post-transcriptionally.

### 3.2. Adenosine receptor activation decreased TNF- $\alpha$ mRNA stability and half-life

Post-transcriptional control of proteins occurs at the level of mRNA degradation, protein translation and processing. Typically, transiently expressed proteins like cytokines have unstable mRNAs with short half-lives. TNF- $\alpha$  mRNA half-life is 20 min in primary human monocytes (Clark, 2000). To determine changes in mRNA stability or half-life induced by adenosine receptor activation, we stimulated U937 cells with PMA/phytohemagglutinin for 2 h in the absence or presence of CGS 21680. After 2 h, cells at time 0 were collected for RT-PCR and all other cell cultures were treated with 5  $\mu$ g/ml actinomycin D to stop further transcription. Cells were then collected at time intervals ranging from 10 to 60 min following actinomycin D treatment and TNF- $\alpha$  mRNA levels were measured by semi-quantitative RT-PCR. We found that PMA/phytohemagglutinin-stimulated U937 cells showed progressively decreasing mRNA levels following addition of actinomycin D and statistically significant ( $P < 0.05$ ) decreases in mRNA were observed at 60 min (Fig. 2A and B). In cells treated with CGS 21680 in combination with PMA/phytohemagglutinin, TNF- $\alpha$  mRNA levels decreased faster following addition of actinomycin D with statistically significant decreases in mRNA observed at 30 min

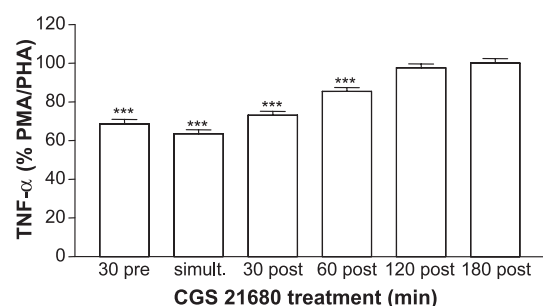


Fig. 1. CGS 21680 inhibits PMA/phytohemagglutinin-induced TNF- $\alpha$  release. Cells were untreated (untx) or were treated with CGS 21680 (1  $\mu$ M) 30 min before, simultaneously with or 30 min, 1, 2 or 3 h after PMA/phytohemagglutinin stimulation. TNF- $\alpha$  protein levels were measured by ELISA following PMA/phytohemagglutinin stimulation for 4 h ( $n=9$ ). TNF- $\alpha$  levels in PMA/phytohemagglutinin stimulated U937 cells were  $460 \pm 100$  pg/ $8 \times 10^5$  cells. \*\*\* $P < 0.001$  vs. PMA/phytohemagglutinin.



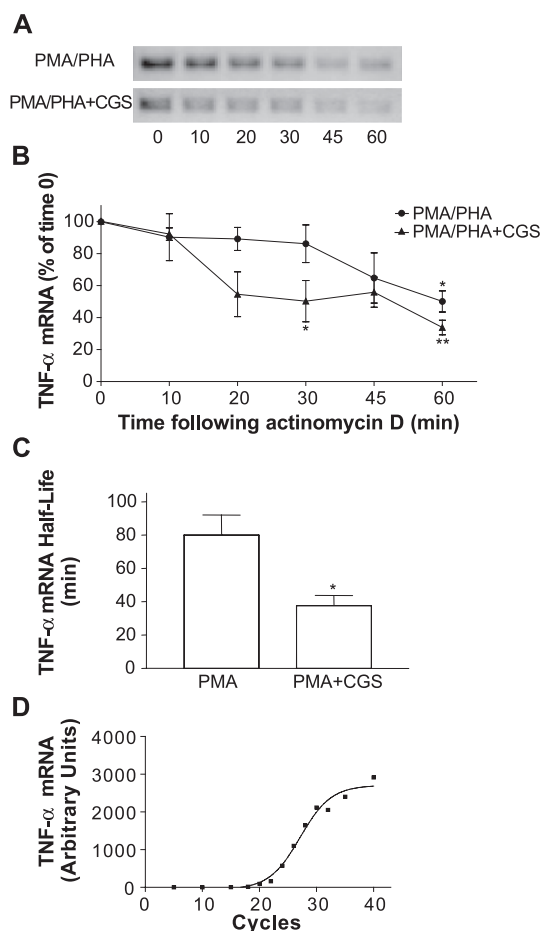


Fig. 2. Adenosine receptor activation decreases TNF- $\alpha$  mRNA stability and half-life. (A) U937 cells were treated with PMA/phytohemagglutinin alone or in combination with CGS 21680 (1  $\mu$ M) for 2 h. Actinomycin D (5  $\mu$ M) was added at time 0 and cells were collected at time intervals increasing from 10 to 60 min. TNF- $\alpha$  mRNA levels were determined by semi-quantitative RT-PCR. (B) Results from five experiments were expressed graphically and are expressed relative to cyclophilin. \* $P$ <0.05 vs. time 0 and \*\* $P$ <0.01 vs. time 0. Data from the PMA/phytohemagglutinin and PMA/phytohemagglutinin+CGS 21680 experiments were expressed as percentages of their own zero times. (C) Half-life of TNF- $\alpha$  mRNA was calculated from mRNA stability experiments (TNF- $\alpha$  expression relative to cyclophilin) and statistics were determined using a two-tailed  $t$ -test ( $n$ =5). (D) TNF- $\alpha$  mRNA levels following PCR amplification of cDNA from PMA/phytohemagglutinin stimulated U937 cells for increasing PCR cycle numbers. \* $P$ <0.05 CGS 21680 vs. PMA/phytohemagglutinin.

( $P$ <0.05) and 60 min ( $P$ <0.01), indicating that activation of adenosine A<sub>2A</sub> receptors decreased stability of PMA/phytohemagglutinin-induced TNF- $\alpha$  mRNA (Fig. 2A and B). We calculated the mRNA half-life for each experiment and compared the mean TNF- $\alpha$  mRNA half-life from PMA/phytohemagglutinin-stimulated cells with that from cells treated with PMA/phytohemagglutinin in combination with CGS 21680. TNF- $\alpha$  mRNA half-life in PMA/phytohemagglutinin-stimulated U937 cells was  $80 \pm 12$  min whereas CGS 21680 pre-treatment decreased significantly ( $P$ <0.05) mRNA half-life to  $37 \pm 8$  min (Fig. 2C). These data indicate that CGS 21680 decreased both stability and half-life of TNF- $\alpha$  mRNA.

To ensure that the semi-quantitative RT-PCR experiments for TNF- $\alpha$  performed for 25 cycles produced a quantity of PCR product within the linear range of our PCR reaction that would allow us to detect changes in amounts of PCR product between experimental samples, we ran RT-PCR with TNF- $\alpha$  primers at increasing cycle numbers with cDNA from PMA/phytohemagglutinin treated U937 cells. Twenty-five cycles produced RT-PCR product within the linear range of our PCR amplification curve and all RT-PCR reactions to detect TNF- $\alpha$  mRNA levels were conducted using 25 PCR cycles (Fig. 2D).

### 3.3. p38, ERK and JNK were involved in PMA/phytohemagglutinin-induced TNF- $\alpha$ protein release

As a first step in determining whether the effects of CGS 21680 on TNF- $\alpha$  protein release were mediated through MAPK signaling molecules, we identified which MAPK signaling molecules regulated PMA/phytohemagglutinin-induced increases in TNF- $\alpha$  release in U937 cells. Although MAPK signaling molecules regulate TNF- $\alpha$  production in other cell types including lipopolysaccharide-stimulated macrophages, it was important to determine which MAPK molecules were important for PMA/phytohemagglutinin-induced TNF- $\alpha$  release. U937 cells were stimulated with PMA/phytohemagglutinin in the presence of PD 98059 to inhibit ERK, SB 202190 to inhibit p38 or JNK inhibitor II (SP 600125) to inhibit JNK. Pre-treatment of cells for 15 min with PD 98059 inhibited PMA/phytohemagglutinin-induced TNF- $\alpha$  release by  $83 \pm 3\%$  ( $P$ <0.001), pre-treatment with SB 202190 inhibited TNF- $\alpha$  release by  $79 \pm 7\%$  ( $P$ <0.001) and pre-treatment with SP 600125 inhibited TNF- $\alpha$  release by  $71 \pm 2\%$  ( $P$ <0.001) (Fig. 3A). Because p38, ERK, and JNK are active only when phosphorylated, we next determined whether PMA/phytohemagglutinin increased the phosphorylation of these signaling molecules at treatment times ranging from 5 to 60 min. PMA/phytohemagglutinin significantly ( $P$ <0.05) increased phosphorylation of both p42 and p44 ERK isoforms within 5 min (Fig. 3B and C). However, no statistically significant changes to levels of phosphorylation of p38 or the 46- or 54-kDa isoforms of JNK were noted following PMA/phytohemagglutinin treatment (Fig. 3B and C) TNF- $\alpha$ .

We next determined if adenosine receptor activation affected PMA/phytohemagglutinin-induced increases in phospho-ERK protein levels or basal phospho-p38 and phospho-JNK levels. In the absence of PMA/phytohemagglutinin stimulation, levels of the 42- and 44-kDa isoforms of phospho-ERK were very low and were not significantly affected by either CGS 21680 or 4-(2-[7-amino-2-(furyl)[1,2,4]triazolo[2,3-a][1,3,5]triazin-5-ylamino]ethyl)phenol (ZM 241385, data not shown). Following PMA/phytohemagglutinin stimulation for 5 min, neither CGS 21680 alone nor CGS 21680 in combination with the adenosine receptor antagonist ZM 241385 affected

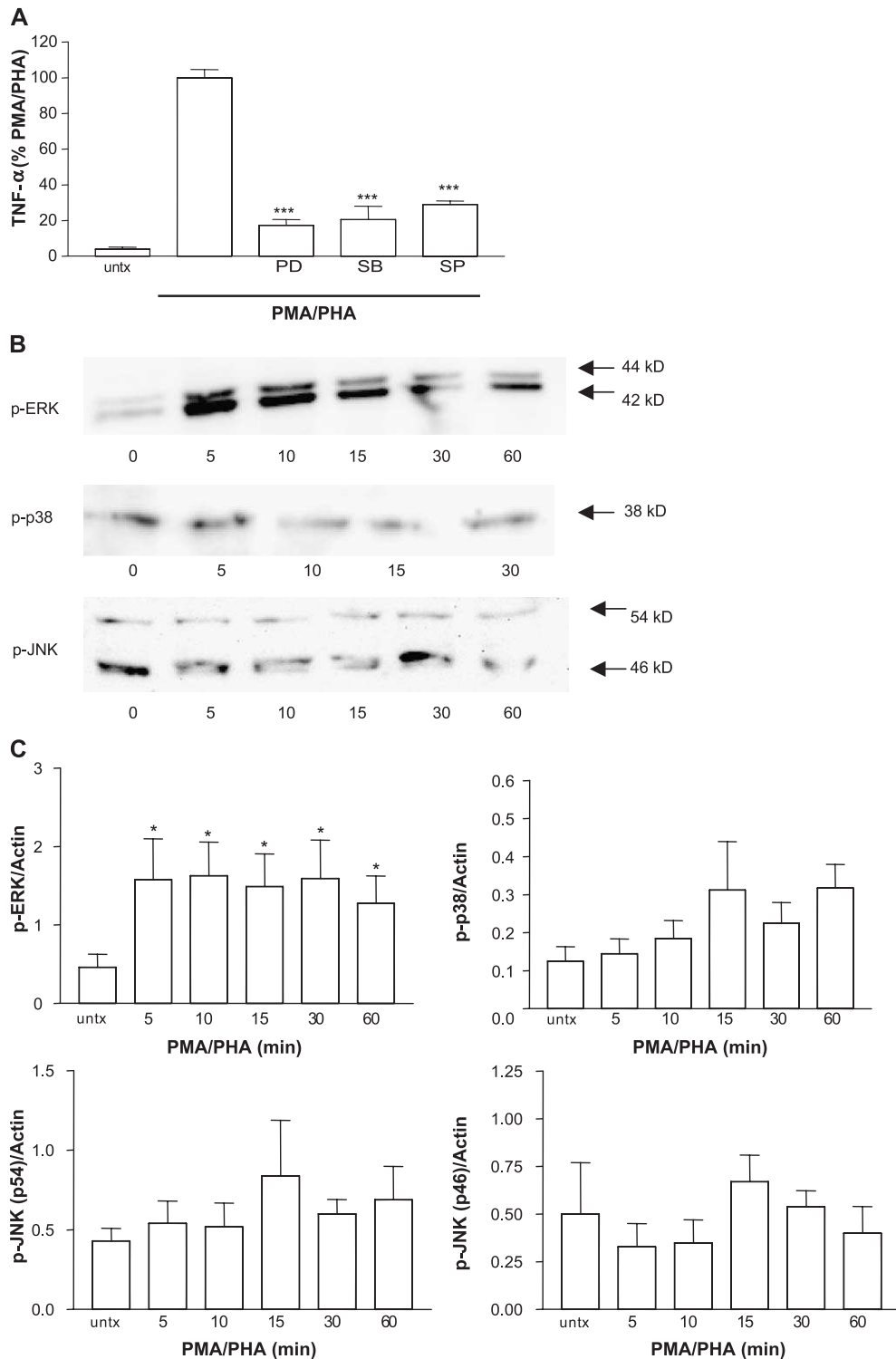


Fig. 3. MAPK signaling molecules involved in PMA/phytohemagglutinin-induced TNF- $\alpha$  release. (A) Cells were untreated (untx) or were treated with PD 98059 (10  $\mu$ M), SB 202190 (1  $\mu$ M) or SP 600125 (10  $\mu$ M) for 15 min prior to PMA/phytohemagglutinin stimulation for 4 h. TNF- $\alpha$  levels were determined by ELISA. (B) Representative Western blots showing phospho-MAPK levels in untreated (untx) cells or cells treated with PMA/phytohemagglutinin for various time points (in min). (C) Results of three Western blots expressed graphically relative to actin. \* $P$ <0.05 vs. PMA/phytohemagglutinin and \*\*\* $P$ <0.001 vs. PMA/phytohemagglutinin.

levels of phospho-ERK (Fig. 4A and B). This later result was interpreted as evidence against the involvement of phospho-ERK in the anti-TNF- $\alpha$  actions of CGS

21680. U937 cells stimulated with PMA/phytohemagglutinin in combination with the MEK inhibitor PD 98059 significantly decreased phospho-ERK levels confirming

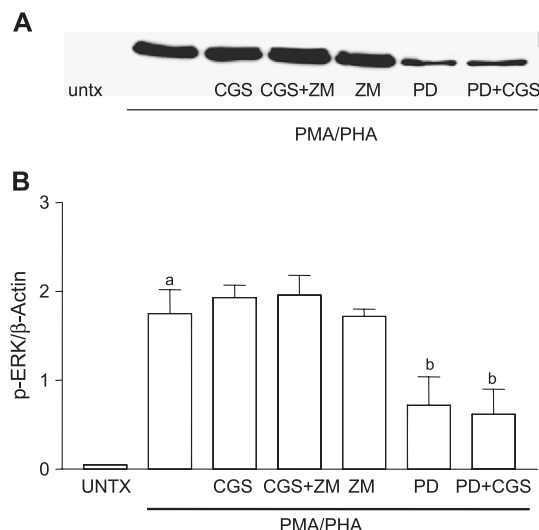


Fig. 4. CGS 21680 does not decrease PMA/phytohemagglutinin-induced phospho-ERK levels. (A) Cells were untreated (untx) or were pre-treated with CGS 21680 (1  $\mu$ M) alone or in combination with ZM 241385 (100 nM) or with PD 98059 (10  $\mu$ M) prior to stimulation with PMA/phytohemagglutinin for 5 min. Phospho-ERK levels were determined by Western blot. (B) Results of three Western blots expressed graphically relative to actin. <sup>a</sup> $P$ <0.05 vs. untreated cells and <sup>b</sup> $P$ <0.05 vs. PMA/phytohemagglutinin.

inhibition of the ERK pathway by PD 98059 (Fig. 4A and B).

### 3.4. Adenosine receptor activation decreased p38 phosphorylation

Based on findings by others that p38 promotes TNF- $\alpha$  mRNA stability and activates protein translation (Brook et al., 2000; Liu et al., 2002; Mahtani et al., 2001; Rutault et al., 2001), we tested the hypothesis that activation of adenosine receptors would decrease levels of phospho-p38 to post-transcriptionally decrease TNF- $\alpha$  protein levels. Although p38 activity was important for PMA/phytohemagglutinin-induced TNF- $\alpha$  protein release, basal phospho-p38 levels were not increased by PMA/phytohemagglutinin, indicating a basal level of p38 activity in U937 cells that was sufficient for release of TNF- $\alpha$ . Accordingly, we tested the effect of adenosine receptor activation on phospho-p38 levels without PMA/phytohemagglutinin stimulation. By western blot analysis, we found that adenosine receptor activation significantly decreased phospho-p38 levels by  $70 \pm 11\%$  ( $P$ <0.01) at 5 min and by  $71 \pm 13\%$  ( $P$ <0.001) at 10 min, phospho-p38 levels rebounded towards normal after treatment for 15 or 30 min (Fig. 5A and B). Treatment of cells with CGS 21680 did not affect total p38 protein levels (Fig. 5A). Although PMA/phytohemagglutinin stimulation did not change basal phospho-JNK levels, studies with a JNK inhibitor suggest the involvement of this pathway in controlling TNF- $\alpha$  release. CGS 21680 had no effect on basal levels of phospho-JNK, indicating that this pathway is

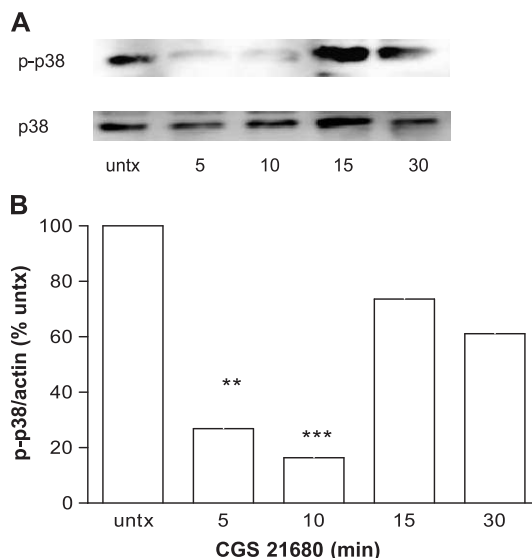


Fig. 5. CGS 21680 decreases phospho-p38 levels. (A) Cells were untreated (untx) or were treated with CGS 21680 (1  $\mu$ M) for various time points (in min) and phospho-p38 and p38 levels were detected by Western blot. (B) Results of three Western blots expressed graphically relative to actin. <sup>\*\*</sup> $P$ <0.01 vs. PMA/phytohemagglutinin and <sup>\*\*\*</sup> $P$ <0.001 vs. PMA/phytohemagglutinin.

not targeted by adenosine receptor-activated signaling events (data not shown).

### 3.5. p38-regulated TNF- $\alpha$ release post-transcriptionally

Because CGS 21680 decreased TNF- $\alpha$  mRNA stability and p38 phosphorylation, it was important to verify in our model that p38 regulates TNF- $\alpha$  release post-transcriptionally. We treated U937 cells with SB 202190 15 min prior to, simultaneously with, or 30 min, 1, 2 or 3 h after PMA/phytohemagglutinin stimulation. The anti-TNF- $\alpha$  effects of the p38 inhibitor were statistically significant ( $P$ <0.001) when added up to 2 h after PMA/phytohemagglutinin stimulation (Fig. 6). Because TNF- $\alpha$  mRNA levels are

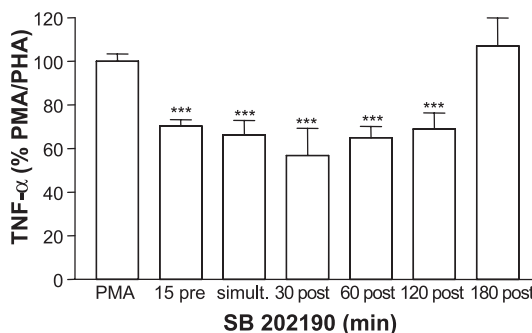


Fig. 6. p38 regulates TNF- $\alpha$  release post-transcriptionally. Cells were untreated (untx) or were treated with SB 202190 (1  $\mu$ M) for 15 min prior to simultaneously with or 30 min, 1, 2 or 3 h after PMA/phytohemagglutinin stimulation. TNF- $\alpha$  protein levels were measured by ELISA following 4 h with PMA/phytohemagglutinin ( $n$ =9). <sup>\*\*\*</sup> $P$ <0.001 vs. PMA/phytohemagglutinin.

detectable after 30 min treatment with PMA/phytohemagglutinin and TNF- $\alpha$  protein can be measured after 1 h (data not shown), these data indicate that p38 controls TNF- $\alpha$  release post-transcriptionally.

#### 4. Discussion

Studies designed to understand endogenous regulators of immune responses give us insight into both normal immune system control and disease pathologies. Adenosine, adenosine receptor agonists and adenosine regulatory agents have anti-inflammatory and immune modulatory properties and may represent an important endogenous system to regulate immune responses. However, to date, only limited information has appeared detailing the mechanisms by which adenosine systems control these events. Accordingly, our studies were aimed to test specific hypotheses about the involvement of pre- and post-transcriptional events and members of the MAP kinase family in the regulation of TNF- $\alpha$  following activation of adenosine receptors.

We showed that the adenosine receptor agonist CGS 21680 decreased PMA/phytohemagglutinin-induced increases in TNF- $\alpha$  protein levels post-transcriptionally. Consistent with our data, adenosine blocked TNF- $\alpha$  protein production when added before, simultaneously with, or after LPS stimulation in human monocytes (Le Moine et al., 1996), in THP-1 monocytic cells (Bshesh et al., 2002) and in neonatal rat myocytes (Wagner et al., 1998). The notion that adenosine regulates TNF- $\alpha$  post-transcriptionally is also supported by studies showing no effect of adenosine receptor agonists on the binding activity and nuclear translocation of NF- $\kappa$ B, a transcription factor important for TNF- $\alpha$  production (Bshesh et al., 2002; Hasko et al., 1996; Sajjadi et al., 1996). Some studies have shown decreased steady-state TNF- $\alpha$  mRNA levels by 2-chloroadenosine in RAW 264.1 macrophages (Firestein et al., 1994) and in PMA-differentiated U937 cells (Sajjadi et al., 1996). Adenosine and the A<sub>2</sub> receptor agonist *N*(6)-[2-(3, 5-dimethoxyphenyl)-2-(2-methylphenyl)]ethyl adenosine (DPMA) decreased steady-state TNF- $\alpha$  mRNA levels in cardiomyocytes and failing human heart (Wagner et al., 1999). Decreased steady-state mRNA levels indicate either adenosine-induced decreases in TNF- $\alpha$  transcription or TNF- $\alpha$  mRNA stability. Although CGS 21680 under the conditions used here was not selective for any particular adenosine receptor subtype, the A<sub>2A</sub> receptor selective antagonist ZM 241385 did block the effects of CGS 21680 on TNF- $\alpha$  levels and thus suggests that the effects of CGS 21680 were due to A<sub>2A</sub> receptor activation.

Our finding that CGS 21680 decreases TNF- $\alpha$  mRNA stability follows a recent study by Nemeth et al. which found that adenosine decreased TNF- $\alpha$  protein production in a post-transcriptional manner in RAW 264.7 macrophages (Nemeth et al., 2003). TNF- $\alpha$  protein production

is controlled by mRNA half-life and stability. mRNA encoding transiently expressed proteins like TNF- $\alpha$  contain adenosine/uridine-rich elements (AREs) in the 3' untranslated region (UTR) that destabilize mRNA leading to an average half-life of approximately 20 min for TNF- $\alpha$  in human monocytes (Clark, 2000; MacKenzie et al., 2002). Stimulation of immune cells with LPS increased slightly TNF- $\alpha$  mRNA stability and half-life thereby favoring TNF- $\alpha$  protein production (Clark, 2000; MacKenzie et al., 2002). Our data showed that TNF- $\alpha$  mRNA had a half-life of 80 min in PMA/phytohemagglutinin-treated cells. Thus, PMA/phytohemagglutinin like LPS can increase TNF- $\alpha$  mRNA stability and promote TNF- $\alpha$  release, and by decreasing mRNA stability and mRNA half-life, CGS 21680 can efficiently block post-transcriptionally TNF- $\alpha$  protein release induced by PMA/phytohemagglutinin.

Our studies were designed to identify signaling mechanisms that mediate the anti-TNF- $\alpha$  actions of CGS 21680 and those involved in regulating TNF- $\alpha$  post-transcriptionally by controlling mRNA stability. Production of TNF- $\alpha$  induced by inflammatory stimuli involves de novo protein synthesis and activation of MAPK signaling pathways (Dumitru et al., 2000; Guha et al., 2001; Means et al., 2000; Raabe et al., 1998; Tsai et al., 2000; Zhu et al., 2000). Although MAPK proteins activate transcription factors including NF- $\kappa$ B, Elk1, Egr1 and ATF-2/Jun (Guha et al., 2001; Paludan et al., 2001; Rao, 2001) that drive transcription of inflammatory genes including TNF- $\alpha$  (Guha et al., 2001; Paludan et al., 2001), p38 has been implicated in regulating TNF- $\alpha$  RNA stability (Brook et al., 2000; Liu et al., 2002; Mahtani et al., 2001; Rutault et al., 2001) and protein translation (Knauf et al., 2001; Liu et al., 2002; Wang et al., 1998). In human monocytes, p38 promoted stabilization of TNF- $\alpha$  mRNA through the 3' UTR ARE

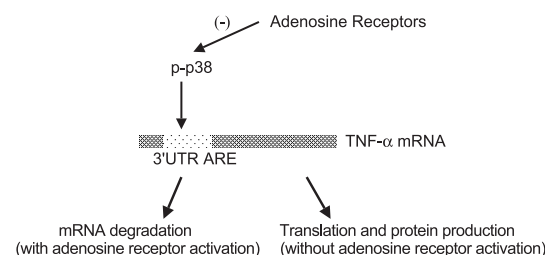


Fig. 7. Schematic of proposed signaling pathways mediating adenosine-receptor control of TNF- $\alpha$  release. mRNAs encoding transiently expressed proteins like TNF- $\alpha$  contain adenosine-uridine rich elements (ARE) in their 3' UTR, which decrease mRNA stability and half-life. This allows tight control of protein expression by increasing or decreasing mRNA degradation. MAPK signaling pathways, particularly p38, are involved in controlling mRNA stability through 3' UTR AREs on TNF- $\alpha$  mRNA. By decreasing p38 signaling, adenosine receptors can efficiently control endogenous signaling pathways that regulate mRNA degradation. Decreasing mRNA stability and half-life of TNF- $\alpha$  mRNA results in decreased protein release. By targeting TNF- $\alpha$  mRNA in this fashion, adenosine receptors may control production of transiently expressed proteins whose mRNA stability is under similar ARE regulation.



(Kontoyiannis et al., 1999), and the p38 inhibitor SB 202190 blocked TNF- $\alpha$  production by destabilizing LPS-induced mRNA (Wang et al., 1999). Emphasizing further the involvement of different levels of p38 signaling in cytokine regulation are results that the upstream p38 activator MKK6 and the downstream p38 target MAPKAPK-2 regulate TNF- $\alpha$  production and TNF- $\alpha$  mRNA stability (Clark, 2000). In our model, the p38 inhibitor SB 202190 inhibited PMA/phytohemagglutinin-induced TNF- $\alpha$  protein release post-transcriptionally. In combination with studies by others, these data indicate that p38 likely regulates TNF- $\alpha$  mRNA stability without the necessity of activating transcription factors and initiating TNF- $\alpha$  gene expression.

Finally, we showed that CGS 21680 decreased basal phospho-p38 levels, suggesting decreased p38 activity as a potential mechanism for post-transcriptional regulation of TNF- $\alpha$  by adenosine receptor activation. Although the effect of CGS 21680 on phospho-p38 levels was transient and began to recover to basal levels after 30 min, the downstream effects of this decrease in p38 activity may be longer lasting. Our proposed signaling model is outlined in Fig. 7. Adenosine receptor activation decreases phospho-p38 levels, TNF- $\alpha$  mRNA stability, and TNF- $\alpha$  protein release. Because phospho-p38 signaling is important in controlling stability of transiently expressed protein mRNAs containing ARE sequences in their 3' untranslated regions, decreased phospho-p38 signaling following adenosine receptor activation may efficiently control production of transiently expressed proteins by promoting mRNA degradation.

By modulating immune responses and shaping patterns of cytokine production, adenosine may be an important metabolite that regulates damage associated with various pathological events including stroke, septic shock, and congestive heart failure (Bouma et al., 1994; Mayne et al., 2001; Wagner et al., 1999). Because adenosine and its receptors are immune modulatory, further elucidation of the signaling pathways mediating these effects should increase our understanding of immune-related disease mechanisms and should aid in designing effective and novel therapeutic strategies. Finally, by targeting a signaling pathway such as p38 normally involved in stabilizing mRNAs encoding transiently expressed proteins, adenosine may efficiently control stress responses by regulating production of proteins like TNF- $\alpha$ . The identification of this signaling pathway suggests that adenosine is an efficient endogenous regulator of proteins specifically produced in a transient fashion as occurs during inflammation.

## Acknowledgements

Operating grants from the Canadian Institutes for Health Research (JDG, MBM) and the National Institutes on Aging (AG17628, JDG) supported this work. The Alzheimer's

Society of Canada and the Manitoba Health Research Council provided pre-doctoral salary support for JF.

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