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## Dual TNF $\alpha$ -Induced Effects on NRF2 Mediated Antioxidant Defence in Astrocyte-Rich Cultures: Role of Protein Kinase Activation

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### Abstract

Tumor necrosis factor- $\alpha$  (TNF $\alpha$ ) is a pleiotropic molecule that can have both protective and detrimental effects in neurodegeneration. Here we have investigated the temporal effects of TNF $\alpha$  on the inducible Nrf2 system in astrocyte-rich cultures by determination of glutathione (GSH) levels,  $\gamma$ glutamylcysteine ligase ( $\gamma$ GCL) activity, the protein levels of Nrf2, Keap1, the catalytic and modulatory subunit of  $\gamma$ GCL ( $\gamma$ GCL-C and  $\gamma$ GCL-M respectively). Astrocyte-rich cultures were exposed for 24 or 72 h to different concentrations of TNF $\alpha$ . Acute exposure (24 h) of astrocyte-rich cultures to 10 ng/mL of TNF $\alpha$  increased GSH,  $\gamma$ GCL activity, the protein levels of  $\gamma$ GCL-M,  $\gamma$ GCL-C and Nrf2 in parallel with decreased levels of Keap1. Antioxidant responsive element (ARE)-mediated transcription was blocked by inhibitors of ERK1/2, JNK and Akt whereas inactivation of p38 and GSK3 $\beta$  further enhanced transcription. In contrast treatment with TNF $\alpha$  for 72 h decreased components of the Nrf2 system in parallel with an increase of Keap1. Stimulation of the Nrf2 system by  $\alpha$ BHQ was intact after 24 h but blocked after 72 h treatment with TNF $\alpha$ . This down-regulation after 72 h correlated with activation of p38 MAPK and GSK3 $\beta$ , since inhibition of these signalling pathways reversed this effect. The upregulation of the Nrf2 system by TNF $\alpha$  (24 h treatment) protected the cells from oxidative stress through elevated  $\gamma$ GCL activity whereas the down-regulation (72 h treatment) caused pronounced oxidative toxicity. One of the important implications of the results is that in a situation where Nrf2 is decreased, such as in Alzheimer's disease, the effect of TNF $\alpha$  is detrimental.

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## Keywords

Neuroinflammation; Nrf2; Antioxidant system; TNF $\alpha$ ; Glutathione

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## Introduction

Cytokines such as tumor necrosis factor  $\alpha$  (TNF $\alpha$ ) are elevated in the brain in a number of acute and chronic neurodegenerative diseases [31]. The functional significance of these changes is not fully clear and TNF $\alpha$  can exert both protective and detrimental effects on brain cells. The protective effects include increased levels of the anti-oxidant glutathione (GSH) [59], increased levels of the anti-apoptotic protein Bcl-2 [55], maintained Ca<sup>2+</sup>-homeostasis [5] and elevated protective enzymes such as MnSOD [2]. However, detrimental effects such as decreased anti-oxidant defense and oxidative stress that initiate apoptosis or necrosis have also been described [34]. Lowered levels of growth factors by TNF $\alpha$ , for example nerve growth factors, have also been reported [14]. The circumstances that determine whether TNF $\alpha$  have toxic or protective effects are at large unknown, but it is likely that the concentration of TNF $\alpha$ , the chemical environment (neurotrophic factors and other cytokines), the receptor distribution in different brain areas and the duration of elevated TNF $\alpha$  levels are important [13, 38]. For example it has been shown that chronic treatment with low levels of TNF $\alpha$  in the substantia nigra by microinjection of an adenoviral vector expressing TNF $\alpha$  causes neuronal cell death after 14 days but not after 7 days [9], favoring that a temporal aspect is important in TNF $\alpha$  effects.

Astrocytes constitute the main support cell for neurons [1]. This support includes shuttling of glutathione (GSH) to the extracellular space, breakdown of GSH and neuronal uptake of cysteine, which leads to elevated neuronal GSH levels [11]. This antioxidant system can be induced to increase astroglial and neuronal GSH concentrations by the transcription factor Nrf2 [50]. Interestingly it is sufficient to overexpress Nrf2 levels in astrocytes to protect neurons in animal models of neurodegeneration [56]. Reduced levels of Nrf2 makes the astroglial cells more vulnerable to oxidative stress [6], and reduced astroglial support sensitizes neurons to normally non-toxic insults [50]. Deletion of Nrf2 makes the animals over-sensitive to oxidative stress and they also develop white matter damage and retinopathy spontaneously [18, 19, 64]. Interestingly, brains from Alzheimer patients have low levels of Nrf2 in hippocampal astrocytes [41] indicating poor astroglial support in this disease, at least in the hippocampus. The reason for this decrease is at present not known but one possibility is inflammation-induced down-regulation of Nrf2 function, i.e. soluble effectors such as cytokines secreted by activated microglia [6].

TNF $\alpha$  treatment has earlier shown to elevate MnSOD in astrocytes and protect astrocytes from 3-nitropropionic acid induced superoxide accumulation and loss of mitochondrial transmembrane potential [2]. Likewise TNF $\alpha$  increased neuroprotective BDNF synthesis in astrocytes most likely via activation of ERK1/2 [44]. Long term incubation (72 h) of astrocytes with TNF $\alpha$  (30 ng/ml) induced  $\gamma$ -glutamyl transpeptidase [43] whereas a depletion of GSH was observed rapidly after adding TNF $\alpha$  (50 ng/ml) to the culture medium [51]. TNF $\alpha$  can also change the cytokine profile of the astrocyte. It has, for example, been demonstrated that TNF $\alpha$  can lead to induction of other cytokines such as IL-6 [46]. TNF $\alpha$  can also elevate HO-1 expression in astroglia [32]. As expression of HO-1 is partly regulated by Nrf2 this effect implies activation of Nrf2 by TNF $\alpha$ .

We have shown that astrocyte-rich cultures treated with medium from LPS-activated microglia can either up-regulate or down-regulate the astrocytic anti-oxidant defense via the transcription factor Nrf2 [6]. The major determinants on the astrocyte anti-oxidant defense

were the original concentration of LPS used to activate microglia and the time to which the astrocyte-rich culture was subjected to the medium from activated microglia. TNF $\alpha$  is a major cytokine released by activated microglia, is increased in CSF of patients suffering from Alzheimer's disease [21] and systemic TNF $\alpha$ , derived from peripheral inflammation, could possibly enter the brain and induce direct effects on brain cells [38]. Here we subjected astrocyte-rich cultures to TNF $\alpha$ , and determined the effects on the Nrf2 system and the possible signaling pathways involved.

## Materials and Methods

### Reagents

Lithium chloride (LiCl), buthionine sulfoxide (BSO),  $\alpha$ BHQ, GSH, cysteine, glutamic acid, 5-sulfosalicylic acid (5-SSA), naphthalene-2,3-dicarboxaldehyde (NDA) and hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) were from Sigma (Stockholm, Sweden). U0126 was from Cell Signaling Technology (Beverly, USA). SP600125, SB203085 and Ly294002 were from Calbiochem (Solna, Sweden). Dulbecco's modified Eagle medium, poly-D-lysine, foetal bovine serum (FBS) and penicillin/streptomycin solution were from Gibco/Invitrogen (Merelbeke, Belgium). Other common reagents were purchased from standard suppliers.

### Ethics Statement

All experiments were carried out in accordance with institutional (ethical approval number 395-2008 issued by the Animal Ethical Committee of Gothenburg) and national guidelines for the care and use of experimental animals and the European Communities Council Directive of 24 November 1986 (86/609/EEC).

### Astrocyte-Rich Primary Cultures and Treatments

Cortical astrocyte-rich primary cultures were prepared from cortex of newborn (P1–P2) Sprague–Dawley rats as previously described [17, 37]. In brief, the rats were decapitated and cortices were carefully dissected. The tissue was mechanically passed through a nylon mesh (80  $\mu$ m mesh size) into culture medium. The medium consisted of MEM supplemented to the following composition: 20 % (v/v) FBS, 1 % penicillin-streptomycin, 1.6 times the concentrations of amino acids and 3.2 times the concentration of vitamins (in comparison to MEM), 1.6 mM L-glutamine, 7.15 mM glucose and 48.5 mM NaHCO<sub>3</sub>. The cells were cultured in a humidified atmosphere of 95 % air and 5 % CO<sub>2</sub>. The medium was changed after 3 days in culture and thereafter three times a week. This procedure results in an astrocyte-rich culture with ca 10 % microglia. Cells were used after 7–10 days in culture when a near-confluent monolayer had been formed.

For short-term experiments (24 h), 1 h before treatments, culture medium was replaced by fresh DMEM and then exposed to the different treatments for 24 h. After that time, cultures were washed with ice-cold PBS and used for Western Blot determinations. For the 72 h experiments, cultures were exposed to DMEM plus 1 % FBS with or without different concentrations of TNF $\alpha$  for 48 h after which media was replaced with serum-free DMEM with or without different doses of TNF $\alpha$  and incubation was continued for 24 h to complete the 72 h in vitro. The 48 h incubation in presence of 1 % FBS was to avoid cell death by prolonged trophic factor deprivation.

### Western Blot Analysis

After treatments, cultures were washed with ice-cold PBS and lysed in Tris-buffered saline pH 7.6 (TBS), 1 % Triton X-100, EDTA 1 mM, EGTA 1 mM plus complete protease inhibitors cocktail (Roche; Stockholm, Sweden). Cell lysates were mixed with 5 $\times$  Laemmli sample buffer and boiled for 5 min. Then equal amount of protein (30  $\mu$ g) were resolved on

10 % SDS-PAGE in a MOPS or MES buffer (Invitrogen; Carlsbad, USA) and electroblotted at 40 V for 70 min at 4 °C to nitrocellulose (Bio-Rad; Hercules, USA). The membranes were blocked for 1 h at room temperature (RT) in 5 % (w/v) dry skimmed milk (Semper Mjöl; Sundryberg, Sweden) in TBS with 0.1 % Tween 20 (TBST). Then, the membranes were incubated overnight at 4 °C with the corresponding primary antibodies (anti-phospho-p38 and anti-phospho-Ser9-GSK3 $\beta$  were from New England Biolabs (Beverly, USA). TNF $\alpha$  and anti-Nrf2 were from R&D Diagnostics (Minneapolis, USA). Anti-Keap1, anti- $\alpha$ -tubulin, anti- $\gamma$ GCL-C and anti- $\gamma$ GCL-M antibodies were from Santa Cruz Biotechnology (Heidelberg, Germany)) in 5 % bovine serum albumin (BSA)-TBST, extensively washed with TBST solution and incubated with the correspondent secondary antibodies (peroxidase-conjugated anti-rabbit and anti-mouse secondary antibodies were from Vector Laboratories (Burlingame, USA)) for 1 h at RT. Finally, the blots were rinsed and the peroxidase reaction was developed by enhanced chemiluminescence SuperSignal® West Dura Extended Duration Substrate (Thermo Scientific; Rockford, USA). Blots were stripped in Restore™ Plus Western Blot Stripping Buffer (Thermo Scientific; Rockford, USA) and were reprobated sequentially.

Images were captured with a Fujifilm Image Reader LAS-1000 Pro v2.6 (Stockholm, Sweden) and the different band intensities (density arbitrary units) corresponding to immunoblot detection of protein samples were quantified using the Fujifilm Multi Gauge v3.0 software (Stockholm, Sweden).

### Cytotoxicity and Viability Assays

Cell death was quantified by measurement of lactate dehydrogenase (LDH) release into the medium. LDH levels were determined using a commercial kit (Roche; Stockholm, Sweden). The LDH level corresponding to complete cell death was determined in sister cultures exposed to Triton X-100 (1 % final concentration) for 24 h. In the case of 72 h treatments, after 48 h of incubation with DMEM with 1 % FBS with or without different concentrations of TNF $\alpha$ , the media were changed to fresh serum-free DMEM with or without different concentrations of TNF $\alpha$  and incubation was carried out to complete the 72 h in vitro. An aliquot of media was obtained for measuring LDH levels to establish if different treatments for 24 or 72 h had any toxic effects on these cultures. Immediately after the 24 or 72 h treatment with TNF $\alpha$ , media was replaced with fresh serum-free DMEM. Cell cultures were then exposed to H<sub>2</sub>O<sub>2</sub> 250  $\mu$ M for 3 h, after which an aliquot of media was taken to measure LDH levels. Background LDH levels were determined in untreated sister cultures and subtracted from experimental values to yield the signal specific for experimentally-induced injury. Percentage of cell death in experimental conditions was calculated using the formula: [% of cell death = ((experimental value – BK)/(FK – BK))\*100], where BK stands for “blank” (sham wash) and FK stands for “full kill” (complete cell death).

### Transfections and Reporter Gene Analysis

The ARE reporter gene vector along with a *Renilla* luciferase expression vector from the Signal™ Antioxidant Response Reporter Kit (SABiosciences; Frederick, USA) were transiently transfected into 10<sup>5</sup> astroglial cells using Lipofectamine™ Reagent (Invitrogen; Merelbeke, Belgium) according to the manufacture’s recommendation. After 18 h medium was removed and changed with fresh serum-free DMEM and 2 h later, cells were stimulated as described in each case. Stimulation was allowed to proceed for another 18 h before cells were harvested, washed with phosphate saline buffer pH 7.4 (PBS) and lysed in cell lysis buffer (Promega; Nacka, Sweden). Luciferase activity (both firefly and *Renilla* luciferase activity) were evaluated using the Dual-Luciferase® Reporter Assay System (Promega). Values were normalized to the *Renilla* luciferase activity (Promega). The Dual-Luciferase® Reporter Assay System refers to the simultaneous expression and measurement of two

individual reporter enzymes within a single system. Thus, the “experimental” reporter (firefly luciferase) is correlated with the effect of specific experimental conditions whereas the activity of the cotransfected “control” (*Renilla* luciferase) reporter provides an internal control for the efficiency of the transfection. Firefly and *Renilla* luciferase activity were measured as light emission over a period of 10 s each time in a VICTOR<sup>2</sup> Multilabel Counter (Wallac; Turku, Finland).

### siRNA Mediated Knock-Down of Nrf2

Nrf2 expression was down-regulated by using siRNA technique as previously described [26]. Briefly, astrocyte-rich cultures were transiently transfected using ON-TARGET-plus SMARTpool siRNA against rat Nrf2 (Thermo Scientific Dharmacon, Rockford, USA). ON-TARGET plus scrambled sequence pool (Thermo Scientific Dharmacon, Rockford, USA) was used as negative control. The transfection into 10<sup>5</sup> astroglial cells was initiated by incubating the cultures with OptiMEM (Invitrogen, Merelbeke, Belgium) for 30 min. Nrf2 ON-TARGETplus SMART-pool siRNA or ON-TARGET plus scrambled sequence (100 nM, final concentration) was mixed with Lipofectamine 2000 (Invitrogen, Merelbeke, Belgium) in Opti-MEM and incubated for 20 min prior to addition to the astrocyte-rich cultures. After 5 h, OptiMEM containing 20 % FBS was added to the transfection mixture and the astrocyte-rich cultures were incubated for 19 h. The cultures were thereafter further incubated in serum-free DMEM with or without TNF $\alpha$ . The efficiency of the knockdown was evaluated by western blot (Fig. 2c) using anti-Nrf2 antibody. The optical densities of Nrf2 blot was correlated to the densities of tubulin and showed a decrease in the level of Nrf2 by approximately 80 % compared to untreated samples (Fig. 2d).

### Statistical Analysis

Results are presented as mean  $\pm$  standard error mean (SEM) of at least three separate experiments with different cell preparations. One way ANOVA followed by the Bonferroni's post hoc test for multiple comparison were used to determine statistical significance (95 %;  $p < 0.05$ ).

## Results

### Effect of 24 h TNF $\alpha$ on the Astroglial Nrf2 System

First we evaluated the effects of 24 h exposure of astrocyte-rich cultures to TNF $\alpha$  on the components of the inducible Nrf2 system,  $\gamma$ GCL activity and GSH content (Fig. 1). As shown in Fig. 1a, b, 10 ng/mL of TNF $\alpha$  induced an increased expression of Nrf2,  $\gamma$ GCL-C and  $\gamma$ GCL-M but reduced the level of Keap1. Next, the effects of different doses of TNF $\alpha$  on the  $\gamma$ GCL activity (Fig. 1c) and the GSH content (Fig. 1d) were evaluated. Together these experiments showed that the highest dose of TNF $\alpha$  tested (10 ng/mL) increased the enzymatic activity of  $\gamma$ GCL and the content of GSH likely via activation of Nrf2.

The phenolic compound  $\alpha$ BHQ has been shown to increase both GSH levels and  $\gamma$ GCL activity in astrocytes [12, 27] and its protective effects have been associated with the activation of the Nrf2 system [23, 52]. We therefore investigated whether  $\alpha$ BHQ could further increase the intracellular levels of GSH and  $\gamma$ GCL activity in astrocyte-rich cultures treated for 24 h with 10 ng/mL of TNF $\alpha$ . Indeed, and as shown in Fig. 1e, f, co-treatment with TNF $\alpha$  (10 ng/mL) and  $\alpha$ BHQ (20  $\mu$ M) increased both the  $\gamma$ GCL activity and GSH content in astrocyte-rich cultures in comparison to treatment with either  $\alpha$ BHQ or TNF $\alpha$  alone.

## Oxidative Stress Response of Astrocyte-Rich Cultures Treated for 24 h with TNF $\alpha$

Next we wanted to evaluate if 24 h exposure to TNF $\alpha$  increased the astroglial resistance to oxidative stress. As shown in Fig. 2a, treatment with 10 ng/mL TNF $\alpha$  for 24 h resulted in an increased protection against the oxidative stress induced by 250  $\mu$ M H<sub>2</sub>O<sub>2</sub>. This correlates well with the increased levels of GSH and  $\gamma$ GCL activity in cells treated with TNF $\alpha$  (10 ng/mL) for 24 h (see Fig. 1c, d). Next we investigated if the protective effects of TNF $\alpha$  were due to increased production of GSH. This was performed by the use of a  $\gamma$ GCL-inhibitor, BSO, that blocks de novo synthesis of GSH. Treatment of the cultures with 1 mM of BSO for 24 h completely reversed the protective effects of TNF $\alpha$  against the oxidative stress (Fig. 2b). To confirm that the activation of the Nrf2 system was involved in the protective effects of TNF $\alpha$ , we used the siRNA technology to knock-down Nrf2 expression (Fig. 2c) by about 80 % (Fig. 2d). As shown in Fig. 2e TNF $\alpha$  has no protective effect on astrocyte-rich cultures against oxidative stress when Nrf2 is down-regulated by siRNA. Interestingly, co-treatment with  $\alpha$ BHQ (20  $\mu$ M) and 10 ng/mL TNF $\alpha$  of astrocyte-rich cultures subjected to H<sub>2</sub>O<sub>2</sub>-induced oxidative stress caused enhanced protection compared to treatment with only  $\alpha$ BHQ or TNF $\alpha$  (Fig. 2f). This indicates that the Nrf2 activation is not saturated by either 20  $\mu$ M  $\alpha$ BHQ or 10 ng/mL TNF $\alpha$ . Supplementary Fig. 1 shows the LDH levels of astroglial-enriched cultures subjected to different treatments for 24 h or 72 h before the exposure to H<sub>2</sub>O<sub>2</sub> 250 $\mu$ M.

## Signalling Pathways Involved in TNF $\alpha$ Activation of the Nrf2-System

In order to elucidate the signalling pathways involved in the effects of 10 ng/mL TNF $\alpha$  on Nrf2 transcriptional activity, we transiently transfected astrocyte-rich cultures with a commercial ARE-LUC reporter gene vector along with a *Renilla* luciferase expression vector. Transfected cells were treated for 24 h with TNF $\alpha$  in the presence or absence of various signalling pathway inhibitors (Fig. 3). Treatment with 10 ng/mL of TNF $\alpha$  for 24 h activated ARE-mediated transcription, as reflected by the higher luciferase activity compared to control. Inhibition of the ERK1/2 MAPK (Fig. 3a), JNK MAPK (Fig. 3b) and Akt (Fig. 3d) signalling pathways blocked the increment in the transcriptional activity induced by TNF $\alpha$ . In contrast, when the transiently transfected astrocyte-rich cultures were treated with TNF $\alpha$  for 24 h and the p38 MAPK (Fig. 3c) or the GSK3 $\beta$  inhibitor (Fig. 3e) higher luciferase activities were detected. Interestingly, when the two inhibitors (SB203580 and lithium chloride) were added together, the activating effects on ARE-mediated transcription were additive (Fig. 3f). In summary, the results in Fig. 3 show that ERK1/2, JNK and Akt pathways can enhance ARE-mediated transcription whereas activation of p38 and GSK3 $\beta$  has opposite negative effects.

## Effect of Prolonged TNF $\alpha$ Treatment on the Astroglial Nrf2 System

In our earlier studies we showed that medium from LPS activated microglia can have positive effects after 24 h but negative effects after 72 h on the Nrf2-system in astrocyte-rich cultures [6]. Similar dual effects of LPS with protection after 24 h and sensitisation after 72 h to an hypoxic-ischemic insult was demonstrated in neonatal rats [57]. Since the 24 h exposure of astrocyte-rich cultures to TNF $\alpha$  induced a similar increase in the antioxidant defence in the astrocyte-rich cultures, we decided to investigate the effects of prolonged (72 h) exposure of astrocyte-rich cultures to TNF $\alpha$  on components of the inducible Nrf2 system. We first analysed the effects of various doses of TNF $\alpha$  on astroglial Nrf2, Keap1,  $\gamma$ GCL-C and  $\gamma$ GCL-M protein expression (Fig. 4a). In contrast to the effects after 24 h, TNF $\alpha$  treatment for 72 h decreased the expression of Nrf2,  $\gamma$ GCL-C and  $\gamma$ GCL-M but increased the level of Keap1 (Fig. 4a). These effects were observed at both 1 and 10 ng/mL TNF $\alpha$  concentrations, whereas treatment with 0.5 ng/mL resulted in decreased levels of  $\gamma$ GCL-C and  $\gamma$ GCL-M. Next, the effects of different doses of TNF $\alpha$  on the  $\gamma$ GCL activity (Fig. 4c) and the GSH content (Fig. 4d) were evaluated. In agreement with the decreased protein

levels of  $\gamma$ GCL-C and  $\gamma$ GCL-M, TNF $\alpha$  at all concentrations decreased both the enzymatic activity of  $\gamma$ GCL and the content of GSH.

Interestingly, and in contrast to the results from cultures treated for 24 h with TNF $\alpha$ , addition of  $\alpha$ BHQ (20  $\mu$ M) to cultures treated for 48 h with 10 ng/mL TNF $\alpha$  did not elevate the activity of  $\gamma$ GCL activity and the GSH content (Fig. 4e). As suspected from the detrimental effects on the Nrf2 system by TNF $\alpha$  treatment for 72 h the vulnerability to oxidative stress (250  $\mu$ M H<sub>2</sub>O<sub>2</sub>) was enhanced (Fig. 5a). As shown in Fig. 5b treatment with  $\alpha$ BHQ (20  $\mu$ M) protected non-treated cells from oxidative stress but this positive effect was lost in cultures treated with TNF $\alpha$  for 72 h (Fig. 5b). To sum up, prolonged treatment (72 h) of astrocyte-rich cultures with TNF $\alpha$  results in decreased anti-oxidant defense, increased vulnerability to oxidative stress and inability to activate the protective Nrf2-system.

The results from the treatment of the astrocytes for 24 h with TNF $\alpha$  and inhibitors of ERK1/2, JNK, p38 MAPK, Akt, and GSK3 $\beta$  phosphorylation (Fig. 3) showed that activation of ERK1/2, JNK and Akt have positive effects whereas activation of p38 and GSK3 $\beta$  have negative effects on the Nrf2 system. This is in agreement with our previous study showing that ERK1/2 and JNK MAPKs pathways are involved in maintaining and/or increasing the levels of Nrf2 on astrocytes exposed to microglial-conditioned medium [7]. However, the activation of ERK1/2 and JNK were lost after 72 h and the negative p38 MAPK and GSK3 $\beta$  effects on Nrf2 levels become more prevalent. This agrees well with earlier studies on the participation of p38 MAPK and GSK3 $\beta$  in the modulation of Nrf2-mediated expression of antioxidant enzymes (Cui et al. 2007; [35, 42, 52]). Thus, we inhibited the p38 and GSK3 $\beta$  signalling pathways and evaluated the levels of Nrf2 and  $\gamma$ GCL-M protein expression after 72 h treatment with TNF $\alpha$  (Fig. 6). These experiments showed that inhibition of p38 MAPK with SB203580 (20  $\mu$ M) and GSK3 $\beta$  with LiCl (5 mM) restored the down-regulated levels of both Nrf2 and  $\gamma$ GCL-M (Fig. 6c, d).

## Discussion

Treatment of astrocyte-rich cultures with TNF $\alpha$  (10 ng/ml) caused upregulation of Nrf2,  $\gamma$ GCL-M and  $\gamma$ GCL-C after 24 h and down-regulation following 72 h treatment (1, 10 ng/ml). These effects agree well with the finding that TNF $\alpha$  increased GSH content in rat hepatocytes by regulating the expression of  $\gamma$ GCL-C [33] and that TNF $\alpha$  at 10 ng/ml after 24 h increased HO-1 mRNA in human macrophages [16]. It is important to note that none of the treatments with TNF $\alpha$  exerted toxicity per se. However, when the cells were challenged with oxidative stress (H<sub>2</sub>O<sub>2</sub>), increased toxicity was observed in cells treated for 72 h with TNF $\alpha$  whereas 24 h treatment protected against oxidative stress. The protection after 24 h was related to the increased protein levels of  $\gamma$ GCL as an inhibitor of this enzyme, BSO, blocked the protective effect. The protective effect of TNF $\alpha$  may partly involve the NF- $\kappa$ B and AP-1 transcription factors. The promoter of  $\gamma$ GCL-C contains binding sites for NF- $\kappa$ B subunits and AP-1 [33, 59]. The promoter for rat  $\gamma$ GCL-M contains an ARE sequence but no AP-1 or NF- $\kappa$ B binding sites [59]. Activation of  $\gamma$ GCL-M may still be related to AP-1 mediated transcription since c-Jun, which is dependent on AP-1, is a binding partner that enhances transcription by Nrf2 [59]. However, the requirement and importance of Nrf2 as a mediator of the protective effects of TNF $\alpha$  was confirmed by Nrf2 down-regulating by siRNA technology, which blocked the TNF $\alpha$ -mediated protection against H<sub>2</sub>O<sub>2</sub> after 24 h treatment. In an earlier study on the effects of TNF $\alpha$  (20 ng/mL) on astrocyte metabolism, no changes in content and release of GSH was observed after 48 h [15]. In that study normal serum containing medium was used which will likely decrease the availability and free concentration of TNF $\alpha$  in comparison to our study where low serum was used. Moreover, in the mentioned study [15], 21 day cultures whereas we used 7–10 day cultures which may be

important for the response of the cells. For example it has been shown that the Nrf2-system is less responsive in cultures older than 10 days in culture [49].

The receptors activated by TNF $\alpha$  and responsible for the up-and down-regulation of the Nrf2-system were not elucidated in this study. However, TNFR1 could be involved in both these effects of TNF $\alpha$  as time-related sensitization and protection against oxygen-glucose deprivation in organotypic slices by TNF $\alpha$  was elicited via the TNFR1 subtype and not the TNFR2 subtype [30]. Interestingly, TNF $\alpha$  can induce TNFR2 receptors which imply that these receptors could be involved in the dual effects reported here [29]. Another factor that could be of importance is that TNF $\alpha$  can induce synthesis and release of other cytokines such as IL-6 [46]. However, it should be noted that the dual effects by TNF $\alpha$  treatment for 24 or 72 h in organotypic slices was lost by genetic deletion of TNFR1, indicating that TNF $\alpha$  indeed is the major player when added exogenously [30]. Studies are in progress to determine if TNF $\alpha$  induce synthesis of other proinflammatory cytokines and/or changes in the receptor population of TNFR1 and TNFR2 that could be involved in the reported modulation of the neuroprotective Nrf2-inducible antioxidant system in astrocytes.

The ability of  $\alpha$ BHQ to induce the Nrf2 system, elevate GSH and  $\gamma$ GCL activity and protect astrocyte-rich cultures against H<sub>2</sub>O<sub>2</sub> was preserved after 24 h also in the presence of TNF $\alpha$ . Moreover, the effects of TNF $\alpha$  and  $\alpha$ BHQ were in fact additive. The reasons for this additive effect were not investigated further but could be due to higher levels of GSH with both treatments via Nrf2, i.e. the activation of Nrf2 is not saturated with TNF $\alpha$  or  $\alpha$ BHQ alone. Alternatively, complementary protective functions are elevated by  $\alpha$ BHQ and TNF $\alpha$ , possibly via AP-1 and/or NF- $\kappa$ B as discussed above.

The effects after 24 h treatment with TNF $\alpha$  on ARE-mediated transcription after 24 h were evaluated by the use of a commercial plasmid containing multiple ARE-sequences (but no AP-1 or NF- $\kappa$ B binding sites) that was coupled to a sequence coding for luciferase. From the experiments using the ARE-LUC plasmid it was obvious that TNF $\alpha$  mediates a robust increase in ARE-mediated transcription. Using different inhibitors of kinases we found that blockers of JNK, ERK1/2 and Akt completely inhibited the TNF $\alpha$ -induced increase in ARE-mediated transcription after 24 h. In contrast inhibiting GSK3 $\beta$  and p38 MAPK had positive and additive effects. The finding that ERK1/2 and JNK have positive effects on ARE-dependent transcription is in agreement with earlier findings [58]. These results are also in agreement with our earlier reports [6, 7]. It is not fully clear if Nrf2 itself is phosphorylated, as mutations of phosphorylation sites on Nrf2 make little difference concerning stability of Nrf2 and transactivation potency [48, 53]. It is possible that some other co-factors are activated or co-repressors are inactivated by these kinases [48, 53]. For example, CREB binding protein (CBP) is a cofactor that, when phosphorylated, binds to Nrf2 and increase transactivation [47]. Although the full biochemical background for the positive effects of TNF $\alpha$  on the Nrf2 system was not elucidated here, it is clear that TNF $\alpha$  activates ARE-mediated transcription via Nrf2, although this does not rule out indirect participation of other transcription factors such as AP-1 and NF- $\kappa$ B [59].

Inhibitors of p38 MAPK and GSK3 $\beta$  together increased ARE-stimulated transcription additively after 24 h treatment with TNF $\alpha$  indicating that different sites and/or proteins were phosphorylated. The mechanisms are likely due to that both activated GSK3 $\beta$  and p38 MAPK can cause export of Nrf2 from the nucleus leading to enhanced breakdown via the proteasome pathway [45, 63].

The decrease in Keap1 protein levels after 24 h treatment with TNF $\alpha$  could be one reason for the increased levels of Nrf2, as this will result in less Nrf2 directed for ubiquitination and proteasomal degradation [8]. The decreased level of Keap1 is similar to that found in a



recent *in vivo* study showing that Keap1 is decreased after MCAO occlusion in the peri-infarct region [54]. We reported earlier that inhibition of the proteasome in astrocyte-rich cultures increased the levels of Keap1 in astrocyte-rich cultures treated for 24 h with medium from microglia activated with 10 ng/mL of LPS [6]. Thus, Keap1 itself can be targeted for proteasomal degradation and one important factor that determines degradation of Keap1 is dephosphorylation, which increases instability and elevates proteasomal breakdown [20].

Treatment of the astrocyte-rich cultures with TNF $\alpha$  (1 and 10 ng/ml) for 72 h dramatically reduced Nrf2/ $\gamma$ GCL-M/ $\gamma$ GCL-C levels whereas Keap1 levels were increased. This agrees well with an earlier long-term study on inflamed kidney [24]. In our recent report on the effects on astrocyte-rich cultures treated for 72 h with medium from LPS-activated microglia we found decreased levels of Nrf2/ $\gamma$ GCL-M but here Keap1 was also down-regulated [6]. The factors behind the dynamic changes in Keap1, which may relate to phosphorylation [20], are highly interesting as Keap1 has been shown to regulate the activity of IKKB/NF- $\kappa$ B activity [26] and the degree of Bcl-2 degradation by the proteasome [36].

The decreased levels of Nrf2/ $\gamma$ GCL in astrocyte-rich cultures after 72 h treatment with TNF $\alpha$  were counteracted by blockers of p38 MAPK and GSK3 $\beta$ . The negative effect of p38 MAPK on the Nrf2 system is in corroboration with earlier studies in cell lines [63], in murine embryonic fibroblast [35] and we earlier showed that treatment of astrocyte-rich cultures for 72 h with medium from LPS-activated microglia down-regulated the levels of proteins in the Nrf2 system in a p38 MAPK-sensitive fashion [6]. Inhibition of p38 MAPK activation was protective and restored the inducibility of the Nrf2 system [6]. The reason for the negative effects of activated p38 MAPK may be related to a decreased nuclear localization and increased degradation of Nrf2 [35]. An alternative and intriguing explanation is that p38 MAPK could indirectly decrease the acetylation levels of Nrf2, leading to nuclear export and degradation. Recent studies have shown that CBP/p300, which has acetylase transferase activity, elevate Nrf2-acetylation and Nrf2-mediated transcription [22]. Interestingly, activation of p38 MAPK can initiate degradation of p300 [39] and thus decrease the acetylation levels and binding efficacy of Nrf2 to ARE-sequences. In accordance, we showed that the non-selective HDAC inhibitors valproate and trichostatin-A restored Nrf2 and levels of  $\gamma$ GCL-M strongly indicating that acetylation levels, that partly appear to depend on p38 MAPK activity, is an important factor in Nrf2 stability [7]. A similar theoretical explanation for the down-regulatory effects of GSK3 $\beta$  is that increased activation of Akt, which decreases activation of GSK3 $\beta$  via phosphorylation, decreases HDAC activity whereas activation of GSK3 $\beta$  has the opposite effect [4]. A putative mechanism in our case is thus that activated GSK3 $\beta$  decreases acetylation of Nrf2, which leads to elevated degradation of Nrf2.

Concerning the long-term negative effects of TNF $\alpha$  on Nrf2/ $\gamma$ GCL-M/ $\gamma$ GCL-C, NF  $\kappa$ B may also be involved. Thus, it has been shown that the p65 subunit of NF  $\kappa$ B can decrease Nrf2 mediated-transcription via elevated levels of nuclear Keap1 with dissociates Nrf2 from ARE-sequences [62]. This may be partly due to the deprivation of CBP (CREB-binding protein), which facilitates recruitment of HDAC3 to small Maf-proteins [28]. The effect being deacetylation of local histones and Nrf2, followed by decreased ARE-mediated transcription.

The strong effects of kinase activity on ARE-activated transcription after treatment with TNF $\alpha$  thus indicate that the “background” kinase activation in combination with effects of TNF $\alpha$  on these kinases can be deterministic for transactivation by Nrf2. The kinase activity may thus be one key to understanding the various effects of TNF $\alpha$ , neuroinflammation and its opposite effects in normal and diseased brain as it has been discussed earlier [38].

When hypoxia-ischemia in 8-day rats was induced 24 h after LPS injection a preconditioning protective effect on brain damage was demonstrated, whereas sensitization occurred 72 h following the LPS-injection [57]. This correlates well with our in vitro studies showing time-dependent up- and down-regulation of the Nrf2-system in astrocyte-rich cultures by inflammatory mediators from microglia [6]. It is interesting to note that earlier studies have shown decreased Akt and GSK3 $\beta$  phosphorylation after hypoxia-ischemia induced brain damage in rats [3, 60]. The activation of GSK3 $\beta$  by both inflammation and hypoxia-ischemia may, in addition to cause export of Nrf2 from the nucleus [42], have long-lasting effects via activation of HDACs and DNA methyltransferases that may be important factors for the long-term outcome after an insult [7, 40].

In conclusion, treatment of astrocyte-rich cultures with TNF $\alpha$  for 24 h increased Nrf2 mediated transcription and protected against oxidative stress in an Nrf2-dependent way. In contrast treatment for 72 h decreased the Nrf2-system and made the cells more vulnerable to oxidative stress. The elevated Nrf2-mediated transcription was dependent on activation of ERK1/2, JNK and Akt, whereas down-regulation could be restored by inhibitors of GSK3 $\beta$  and p38 MAPK signaling pathways. The implications include that TNF $\alpha$  can protect astrocytes only if the Nrf2-system is functioning. In disease states where the Nrf2 system is dysfunctional, i.e. for example in Alzheimer's disease the protective effect of TNF $\alpha$  is lost [41].

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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## Abbreviations

<b>ARE</b>	Antioxidant responsive element
<b>BSO</b>	Buthionine sulfoxide
<b>DMEM</b>	Dulbecco's Modified Eagle's Medium
<b>FBS</b>	Foetal bovine serum
<b><math>\gamma</math>GCL</b>	Gamma-glutamylcysteine ligase
<b><math>\gamma</math>GCL-C</b>	Gamma-glutamylcysteine ligase catalytic subunit
<b><math>\gamma</math>GCL-M</b>	Gamma-glutamylcysteine ligase modulatory subunit
<b>GSH</b>	Glutathione
<b>GSK3<math>\beta</math></b>	Glycogen synthase kinase-3 beta
<b>Keap1</b>	Kelch-like ECH-associated protein 1
<b>ERK1/2</b>	Extracellular regulated kinase
<b>FBS</b>	Foetal bovine serum
<b>JNK</b>	c-Jun N-terminal kinase

<b>LPS</b>	Lipopolysaccharide
<b>MAPKs</b>	Mitogen-activated protein kinases
<b>MCM</b>	Microglia-conditioned medium
<b>MEK</b>	Mitogen-activated protein kinase kinase
<b>MEM</b>	Modified Eagle's Medium
<b>MnSOD</b>	Manganese superoxide dismutase
<b>NDA</b>	Naphthalene-2,3-dicarboxaldehyde
<b>NF-<math>\kappa</math>B</b>	Nuclear factor kappa-light-chain-enhancer of activated B cells
<b>Nrf2</b>	Nuclear factor-erythroid 2-related factor 2
<b>PBS</b>	Phosphate buffered saline
<b>5-SSA</b>	5-Sulfosalicylic acid
<b>tBHQ</b>	Tert-butylhydroquinone
<b>TNF<math>\alpha</math></b>	Tumor necrosis factor-alpha

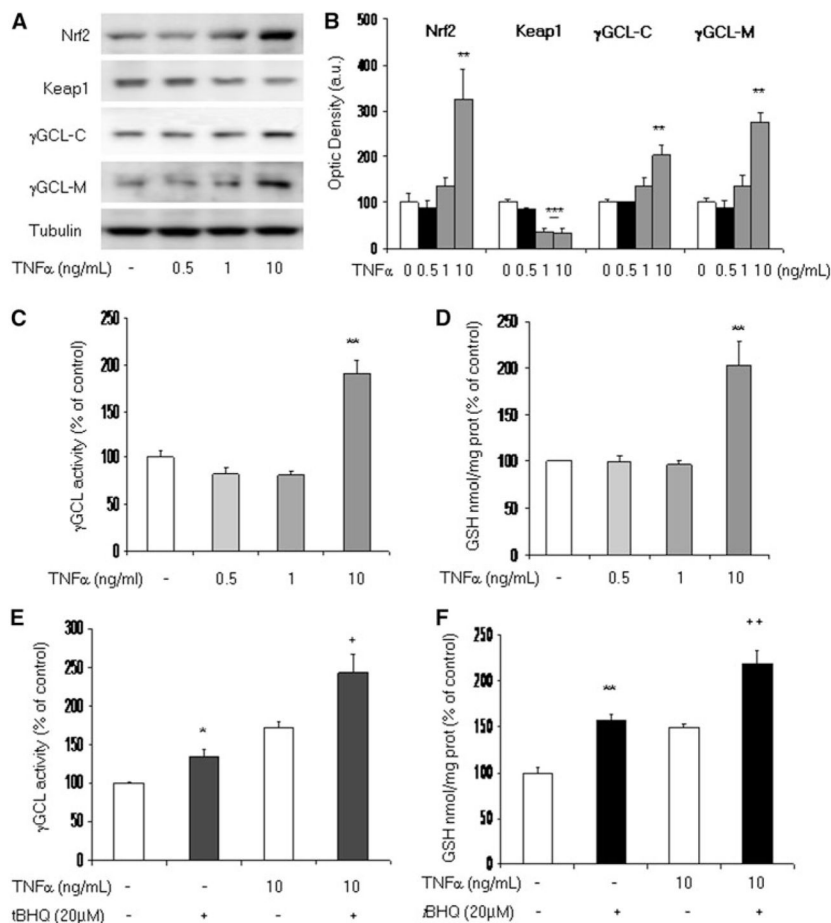
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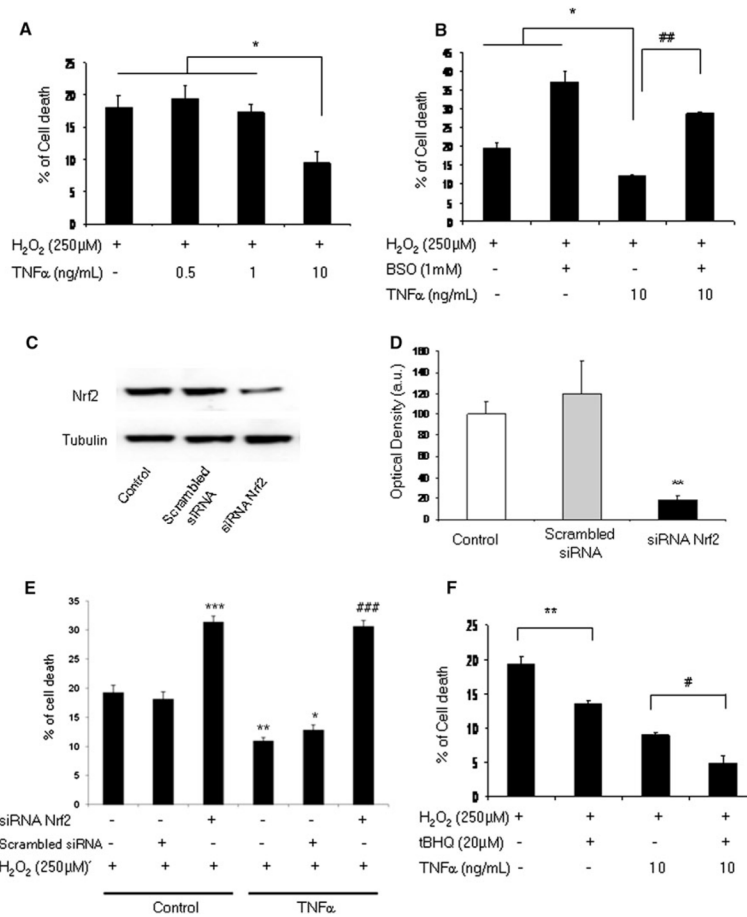
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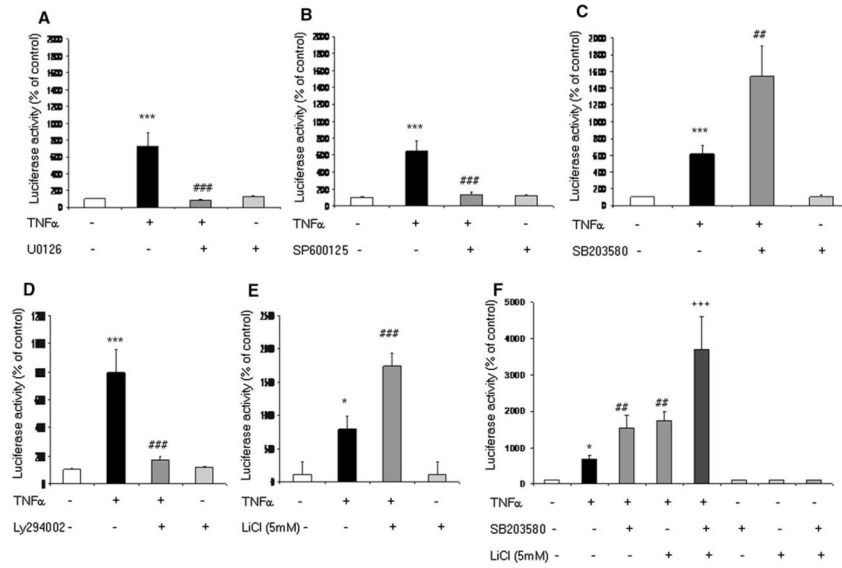


**Fig. 1.** TNF $\alpha$  (24 h treatment) increased astroglial antioxidant defense system. Astrocyte-rich cultures were exposed to different concentrations of TNF $\alpha$  (0.5, 1 and 10 ng/mL) for 24 h followed by the protein expression analysis of Nrf2, Keap1,  $\gamma$ GCL-C and  $\gamma$ GCL-M subunits (a). For the western blot, a representative experiment of four independent experiments is shown. In (b), the densitometric analysis is shown. Statistics: \*\* $p$  < 0.01 versus control; \*\*\* $p$  < 0.005 versus control. TNF $\alpha$  (10 ng/mL) induced an increase in  $\gamma$ GCL activity (c) and GSH (d). Results are shown as mean  $\pm$  SEM and expressed as percentage of control. Statistics: \*\* $p$  < 0.01 versus control. Astrocyte-rich cultures were treated with TNF $\alpha$  in the presence or absence of 20  $\mu$ M tBHQ and the activity of  $\gamma$ GCL (e) and GSH levels (f) were determined. In both cases, results shown are the mean  $\pm$  SEM and expressed as percentage of control. Statistics: \* $p$  < 0.05 versus control; \*\* $p$  < 0.01 versus control; + $p$  < 0.05 versus TNF $\alpha$  10 ng/mL; ++ $p$  < 0.01 versus TNF $\alpha$  10 ng/mL. (n = 4–6)

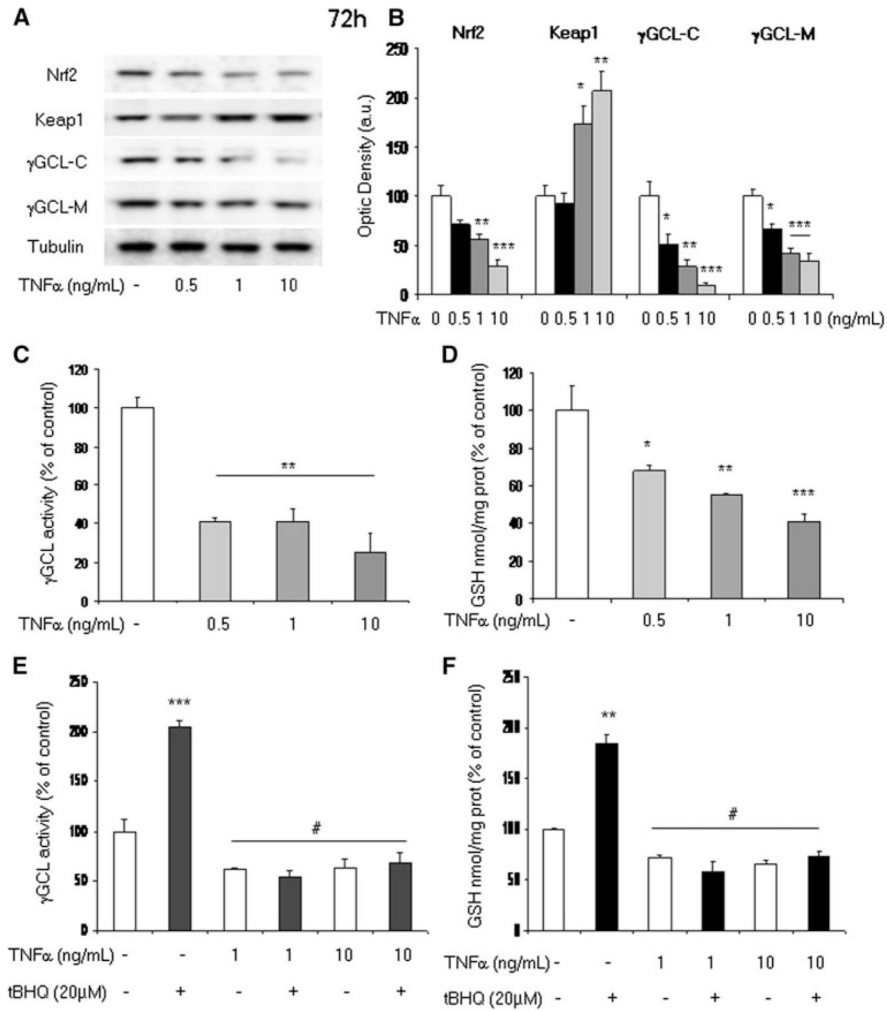


**Fig. 2.** TNF $\alpha$  (24 h treatment) protected from cell death induced by 3 h exposure to 250  $\mu$ M hydrogen peroxide. Astrocyte-rich cultures pre-treated for 24 h with 10 ng/mL TNF $\alpha$  showed higher resistance to oxidative stress (a). Inhibition of  $\gamma$ GCL activity with 1 mM BSO reversed the protective effects of 10 ng/mL of TNF $\alpha$  (b). Treatment with siRNA directed against Nrf2 lowered the expression of the Nrf-2 protein by approximately 80 % (c). Densitometric analysis of Nrf2 protein expression in astrocyte-rich cultures treated with siRNA directed against Nrf-2. Data are plotted as ratio of the Nrf2/tubulin obtained in each condition (d). Treatment with siRNA against Nrf2 reversed the protective effects of 10 ng/mL of TNF $\alpha$  (24 h) against 250  $\mu$ M hydrogen peroxide (e). Co-treatment with the Nrf2-inducer tBHQ 20  $\mu$ M potentiated the protective effect of 10 ng/mL TNF $\alpha$  (f). In all cases, results are shown as the mean  $\pm$  SEM. Statistics: \* $p$  < 0.05 versus control; \*\* $p$  < 0.01 versus control; \*\*\* $p$  < 0.005 versus control; # $p$  < 0.05 versus TNF $\alpha$  10 ng/mL; ## $p$  < 0.01 versus TNF $\alpha$  10 ng/mL; ### $p$  < 0.005 versus TNF $\alpha$  10 ng/mL (n = 4–8)

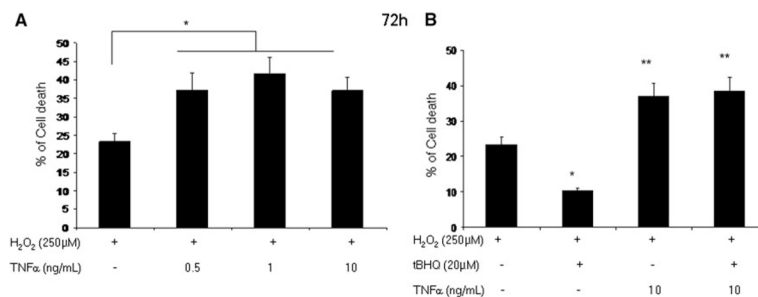


**Fig. 3.**

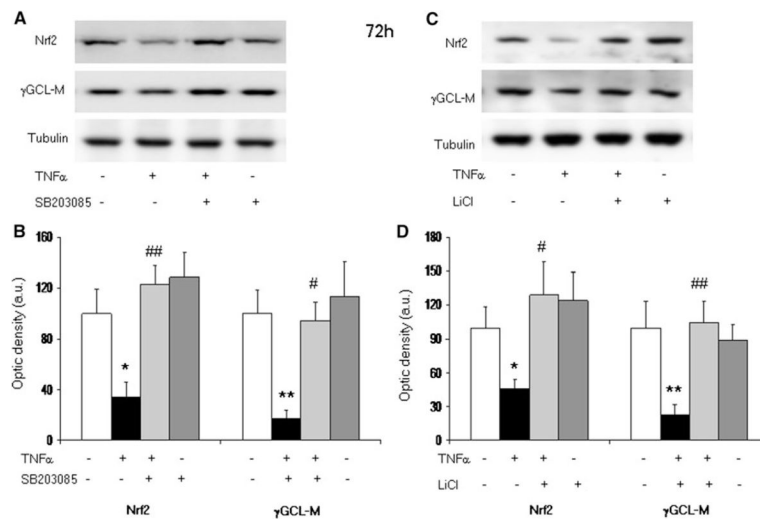
Effect of the inhibition of ERK1/2, JNK, p38 MAPK, Akt and GSK3 $\beta$  signalling pathways on the ARE-driven luciferase activity induced by 10 ng/mL TNF $\alpha$ . Astrocyte-rich cultures were transfected with ARE-Luc reporter gene construct and treated for 24 h with 10 ng/mL TNF $\alpha$  in the presence or absence of 10  $\mu$ M U0126 (a), 10  $\mu$ M SP600125 (b), 20  $\mu$ M SB203580 (c), 10  $\mu$ M Ly294002 (d), 5 mM LiCl (e) and a combination of 5 mM LiCl and 20  $\mu$ M SB203580 (f). Mean  $\pm$  SEM of the luciferase activity of astroglial cells transiently transfected with the reporter plasmid ARE-Luc. Data are plotted as percentage of the experimental relative light units/basal relative light units ratio obtained in untreated conditions. Statistics: \* $p$  < 0.05 versus control; \* $p$  < 0.05 versus control; \*\*\* $p$  < 0.005 versus control; ### $p$  < 0.01 versus TNF $\alpha$ ; ### $p$  < 0.005 versus TNF $\alpha$ ; +++ $p$  < 0.005 versus TNF $\alpha$  + SB203580 or TNF $\alpha$  + LiCl (n = 9)

**Fig. 4.**

TNF $\alpha$  (72 h treatment) decreased the astroglial antioxidant defense system. Astrocyte-rich cultures were exposed to different concentrations of TNF $\alpha$  (0.5, 1 and 10 ng/mL) for 72 h followed by protein expression analysis of Nrf2, Keap1,  $\gamma$ GCL-C and  $\gamma$ GCL-M subunits (a). For the western blot, a representative experiment of four independent experiments is shown. In (b), the densitometric analysis is shown. Statistics: \* $p$  < 0.05 versus control; \*\* $p$  < 0.01 versus control; \*\*\* $p$  < 0.005 versus control. TNF $\alpha$  (10 ng/mL) induced a reduction in  $\gamma$ GCL activity (c) as well as on the levels of GSH (d). Results are shown as mean  $\pm$  SEM and expressed as percentage of control. Statistics: \* $p$  < 0.05 versus control; \*\* $p$  < 0.01 versus control; \*\*\* $p$  < 0.005 versus control. Astrocyte-rich cultures were treated with TNF $\alpha$  (10 ng/mL) for 72 h in the presence or absence of 20  $\mu$ M tBHQ and the activity of  $\gamma$ GCL (e) and GSH levels (f) were determined. In this case, tBHQ was unable to restore the TNF $\alpha$ -induced down-regulation of  $\gamma$ GCL activity and GSH levels. In both cases, results shown are the mean  $\pm$  SEM and expressed as percentage of control. Statistics: \*\* $p$  < 0.01 versus control; \*\*\* $p$  < 0.005 versus control; # $p$  < 0.05 versus control (n = 4–6)



**Fig. 5.** TNF $\alpha$  (72 h treatment) increased cell death induced by 3 h exposure to 250  $\mu$ M hydrogen peroxide. Astrocyte-rich cultures pre-treated for 72 h with 10 ng/mL TNF $\alpha$  showed higher cell death levels when challenged to oxidative stress (a). Treatment with *α*BHQ 20  $\mu$ M was unable to protect from the effects of 72 h treatment with 10 ng/mL of TNF $\alpha$  (b). Results are shown as the mean  $\pm$  SEM. Statistics: \* $p$  < 0.05 versus control + H<sub>2</sub>O<sub>2</sub>-treatment; \*\* $p$  < 0.01 versus control + H<sub>2</sub>O<sub>2</sub>-treatment (n = 4–6)

**Fig. 6.**

Effect of the inhibition of p38MAPK and GSK3 $\beta$  on the down-regulated expression of Nrf2 and  $\gamma$ GCL-M induced by 10 ng/mL of TNF $\alpha$  (72 h). Inhibition of p38 MAPK activation with the specific inhibitor SB203580 (20  $\mu$ M) resulted in a restoration in the down-regulated expression of Nrf2 and  $\gamma$ GCL-M induced by 72 h treatment with TNF $\alpha$  (a). In (b), the densitometric analysis is shown. Statistics: \* $p$  < 0.05 versus control; \*\* $p$  < 0.01 versus control; # $p$  < 0.05 versus TNF $\alpha$ ; ## $p$  < 0.01 versus TNF $\alpha$ . Inhibition of GSK3 $\beta$  activation with LiCl (5 mM) resulted in a reversal of the down-regulated expression of Nrf2 and  $\gamma$ GCL-M induced by 72 h treatment with TNF $\alpha$  (c). In (d), the densitometric analysis is shown. Statistics: \* $p$  < 0.05 versus control; \*\* $p$  < 0.01 versus control; # $p$  < 0.05 versus TNF $\alpha$ ; ## $p$  < 0.01 versus TNF $\alpha$  (n = 6)