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Dual TNFα-Induced Effects on NRF2 Mediated Antioxidant Defence in Astrocyte-Rich Cultures: Role of Protein Kinase Activation

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Abstract

Tumor necrosis factor-α (TNFα) is a pleiotropic molecule that can have both protective and detrimental effects in neurodegeneration. Here we have investigated the temporal effects of TNFα on the inducible Nrf2 system in astrocyte-rich cultures by determination of glutathione (GSH) levels, γglutamylcysteine ligase (γGCL) activity, the protein levels of Nrf2, Keap1, the catalytic and modulatory subunit of γGCL (γGCL-C and γGCL-M respectively). Astrocyte-rich cultures were exposed for 24 or 72 h to different concentrations of TNFα. Acute exposure (24 h) of astrocyte-rich cultures to 10 ng/mL of TNFα increased GSH, γGCL activity, the protein levels of γGCL-M, γGCL-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 GSK3β further enhanced transcription. In contrast treatment with TNFα for 72 h decreased components of the Nrf2 system in parallel with an increase of Keap1. Stimulation of the Nrf2 system by *t*BHQ was intact after 24 h but blocked after 72 h treatment with TNFα. This down-regulation after 72 h correlated with activation of p38 MAPK and GSK3β, since inhibition of these signalling pathways reversed this effect. The upregulation of the Nrf2 system by TNFα (24 h treatment) protected the cells from oxidative stress through elevated γGCL 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 TNFα is detrimental.

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Keywords

Neuroinflammation; Nrf2; Antioxidant system; TNFα; Glutathione

Introduction

Cytokines such as tumor necrosis factor α (TNFα) 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 TNFα 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^{2+}-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 TNFα, for example nerve growth factors, have also been reported [14]. The circumstances that determine whether TNFα have toxic or protective effects are at large unknown, but it is likely that the concentration of TNFα, the chemical environment (neurotrophic factors and other cytokines), the receptor distribution in different brain areas and the duration of elevated TNFα levels are important [13, 38]. For example it has been shown that chronic treatment with low levels of TNFα in the substantia nigra by microinjection of an adenoviral vector expressing TNFα causes neuronal cell death after 14 days but not after 7 days [9], favoring that a temporal aspect is important in TNFα 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].

TNFα 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 TNFα increased neuroprotective BDNF synthesis in astrocytes most likely via activation of ERK1/2 [44]. Long term incubation (72 h) of astrocytes with TNFα (30 ng/ml) induced γ-glutamyl transpeptidase [43] whereas a depletion of GSH was observed rapidly after adding TNFα (50 ng/ml) to the culture medium [51]. TNFα can also change the cytokine profile of the astrocyte. It has, for example, been demonstrated that TNFα can lead to induction of other cytokines such as IL-6 [46]. TNFα 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 TNFα.

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. TNFα is a major cytokine released by activated microglia, is increased in CSF of patients suffering from Alzheimer's disease [21] and systemic TNFα, derived from peripheral inflammation, could possibly enter the brain and induce direct effects on brain cells [38]. Here we subjected astrocyte-rich cultures to TNFα, and determined the effects on the Nrf2 system and the possible signaling pathways involved.

Materials and Methods

Reagents

Lithium chloride (LiCl), buthionine sulfoxide (BSO), *t*BHQ, GSH, cysteine, glutamic acid, 5-sulfosalicylic acid (5-SSA), naphthalene-2,3-dicarboxaldehyde (NDA) and hydrogen peroxide (H_2O_2) 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 μm 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 mML-glutamine, 7.15 mM glucose and 48.5 mM NaHCO3. The cells were cultured in a humidified atmosphere of 95 % air and 5 % CO_2. 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 TNFα for 48 h after which media was replaced with serum-free DMEM with or without different doses of TNFα 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× Laemmeli sample buffer and boiled for 5 min. Then equal amount of protein (30 μg) 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 Mjölk; Sundyberg, 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-GSK3β were from New England Biolabs (Beverly, USA). TNFα and anti-Nrf2 were from R&D Diagnostics (Minneapolis, USA). Anti-Keap1, anti-α-tubulin, anti-γGCL-C and anti-γGCL-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 RestoreTM Plus Western Blot Stripping Buffer (Thermo Scientific; Rockford, USA) and were reprobed 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 TNFα, the media were changed to fresh serum-free DMEM with or without different concentrations of TNFα 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 TNFα, media was replaced with fresh serum-free DMEM. Cell cultures were then exposed to H_2O_2 250 μM 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 Cignal™ Antioxidant Response Reporter Kit (SABiosciences; Frederick, USA) were transiently transfected into 10^5 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^2 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 10^5 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 TNFα. 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 ± 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 TNFα on the Astroglial Nrf2 System

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

The phenolic compound *t*BHQ has been shown to increase both GSH levels and γGCL 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 *t*BHQ could further increase the intracellular levels of GSH and γGCL activity in astrocyte-rich cultures treated for 24 h with 10 ng/mL of TNFα. Indeed, and as shown in Fig. 1e, f, co-treatment with TNFα (10 ng/mL) and *t*BHQ (20 μM) increased both the γGCL activity and GSH content in astrocyte-rich cultures in comparison to treatment with either *t*BHQ or TNFα alone.

Oxidative Stress Response of Astrocyte-Rich Cultures Treated for 24 h with TNFα

Next we wanted to evaluate if 24 h exposure to TNFα increased the astroglial resistance to oxidative stress. As show in Fig. 2a, treatment with 10 ng/mL TNFα for 24 h resulted in an increased protection against the oxidative stress induced by 250 μM H_2O_2. This correlates well with the increased levels of GSH and γGCL activity in cells treated with TNFα (10 ng/mL) for 24 h (see Fig. 1c, d). Next we investigated if the protective effects of TFNα were due to increased production of GSH. This was performed by the use of a γGCL-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 TNFα against the oxidative stress (Fig. 2b). To confirm that the activation of the Nrf2 system was involved in the protective effects of TNFα, we used the siRNA technology to knock-down Nrf2 expression (Fig. 2c) by about 80 % (Fig. 2d). As shown in Fig. 2e TNFα has no protective effect on astrocyte-rich cultures against oxidative stress when Nrf2 is down-regulated by siRNA. Interestingly, co-treatment with *t*BHQ (20 μM) and 10 ng/mL TNFα of astrocyte-rich cultures subjected to H_2O_2-induced oxidative stress caused enhanced protection compared to treatment with only *t*BHQ or TNFα (Fig. 2f). This indicates that the Nrf2 activation is not saturated by either 20 μM *t*BHQ or 10 ng/ml TNFα. 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_2O_2 250μM.

Signalling Pathways Involved in TNFα Activation of the Nrf2-System

In order to elucidate the signalling pathways involved in the effects of 10 ng/mL TNFα 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 TNFα in the presence or absence of various signalling pathway inhibitors (Fig. 3). Treatment with 10 ng/mL of TNFα for 24 h activated ARE-medieated 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 TNFα. In contrast, when the transiently transfected astrocyte-rich cultures were treated with TNFα for 24 h and the p38 MAPK (Fig. 3c) or the GSK3β 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 GSK3β has opposite negative effects.

Effect of Prolonged TNFα 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 TNFα 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 TNFα on components of the inducible Nrf2 system. We first analysed the effects of various doses of TNFα on astroglial Nrf2, Keap1, γGCL-C and γGCL-M protein expression (Fig. 4a). In contrast to the effects after 24 h, TNFα treatment for 72 h decreased the expression of Nrf2, γGCL-C and γGCL-M but increased the level of Keap1 (Fig. 4a). These effects were observed at both 1 and 10 ng/mL TNFα concentrations, whereas treatment with 0.5 ng/mL resulted in decreased levels of γGCL-C and γGCL-M. Next, the effects of different doses of TNFα on the γGCL activity (Fig. 4c) and the GSH content (Fig. 4d) were evaluated. In agreement with the decreased protein

levels of γGCL-C and γGCL-M, TNFα at all concentrations decreased both the enzymatic activity of γGCL and the content of GSH.

Interestingly, and in contrast to the results from cultures treated for 24 h with TNFα, addition of *t*BHQ (20 μM) to cultures treated for 48 h with 10 ng/mL TNFα did not elevate the activity of γGCL activity and the GSH content (Fig. 4e). As suspected from the detrimental effects on the Nrf2 system by TNFα treatment for 72 h the vulnerability to oxidative stress (250 μM H_2O_2) was enhanced (Fig. 5a). As shown in Fig. 5b treatment with *t*BHQ (20 μM) protected non-treated cells from oxidative stress but this positive effect was lost in cultures treated with TNFα for 72 h (Fig. 5b). To sum up, prolonged treatment (72 h) of astrocyte-rich cultures with TNFα 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 TNFα and inhibitors of ERK1/2, JNK, p38 MAPK, Akt, and GSK3β phosphorylation (Fig. 3) showed that activation of ERK1/2, JNK and Akt have positive effects whereas activation of p38 and GSK3β 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 GSK3β effects on Nrf2 levels become more prevalent.This agrees well with earlier studies on the participation of p38 MAPK and GSK3β in the modulation of Nrf2-mediated expression of antioxidant enzymes (Cui et al. 2007; [35, 42, 52]). Thus, we inhibited the p38 and GSK3β signalling pathways and evaluated the levels of Nrf2 and γGCL-M protein expression after 72 h treatment with TNFα (Fig. 6). These experiments showed that inhibition of p38 MAPK with SB203580 (20 μM) and GSK3β with LiCl (5 mM) restored the down-regulated levels of both Nrf2 and γGCL-M (Fig. 6c, d).

Discussion

Treatment of astrocyte-rich cultures with TNFα (10 ng/ml) caused upregulation of Nrf2, γGCL-M and γGCL-C after 24 h and down-regulation following 72 h treatment (1, 10 ng/ml). These effects agree well with the finding that TNFα increased GSH content in rat hepatocytes by regulating the expression of γGCL-C [33] and that TNFα 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 TNFα exerted toxicity per se. However, when the cells were challenged with oxidative stress (H_2O_2), increased toxicity was observed in cells treated for 72 h with TNFα whereas 24 h treatment protected against oxidative stress. The protection after 24 h was related to the increased protein levels of γGCL as an inhibitor of this enzyme, BSO, blocked the protective effect. The protective effect of TNFα may partly involve the NF-κB and AP-1 transcription factors. The promoter of γGCL-C contains binding sites for NF-κB subunits and AP-1 [33, 59]. The promoter for rat γGCL-M contains an ARE sequence but no AP-1 or NF-kB binding sites [59]. Activation of γGCL-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 TNFα was confirmed by Nrf2 down-regulating by siRNA technology, which blocked the TNFα-mediated protection against H_2O_2 after 24 h treatment. In an earlier study on the effects of TNFα (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 TNFα 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 TNFα 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 TNFα as time-related sensitization and protection against oxygen-glucose deprivation in organotypic slices by TNFα was elicited via the TNFR1 subtype and not the TNFR2 subtype [30]. Interestingly, TNFα 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 TNFα can induce synthesis and release of other cytokines such as IL-6 [46]. However, it should be noted that the dual effects by TNFα treatment for 24 or 72 h in organotypic slices was lost by genetic deletion of TNFR1, indicating that TNFα indeed is the major player when added exogenously [30]. Studies are in progress to determine if TNFα 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 *t*BHQ to induce the Nrf2 system, elevate GSH and γGCL activity and protect astrocyte-rich cultures against H_2O_2 was preserved after 24 h also in the presence of TNFα. Moreover, the effects of TNFα and *t*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 TNFα or *t*BHQalone. Alternatively, complementary protective functions are elevated by *t*BHQ and TNFα, possibly via AP-1 and/or NF-κB as discussed above.

The effects after 24 h treatment with TNFα 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-κB binding sites) that was coupled to a sequence coding for luciferase. From the experiments using the ARE-LUC plasmid it was obvious that TNFα 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 TNFα-induced increase in ARE-mediated transcription after 24 h. In contrast inhibiting GSK3β 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 phosphorylationsites 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 TNFα on the Nrf2 system was not elucidated here, it is clear that TNFα activates ARE-mediated transcription via Nrf2, although this does not rule out indirect participation of other transcription factors such as AP-1 and NF-κB [59].

Inhibitors of p38 MAPK and GSK3β together increased ARE-stimulated transcription additively after 24 h treatment with TNFα indicating that different sites and/or proteins were phosphorylated. The mechanisms are likely due to that both activated GSK3β 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 TNFα 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 TNFα (1 and 10 ng/ml) for 72 h dramatically reduced Nrf2/γGCL-M/γGCL-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/γGCL-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-κB activity [26] and the degree of Bcl-2 degradation by the proteasome [36].

The decreased levels of Nrf2/γGCL in astrocyte-rich cultures after 72 h treatment with TNFα were counteracted by blockers of p38 MAPK and GSK3β. 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 γGCL-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 GSK3β is that increased activation of Akt, which decreases activation of GSK3β via phosphorylation, decreases HDAC activity whereas activation of GSK3β has the opposite effect [4]. A putative mechanism in our case is thus that activated GSK3β decreases acetylation of Nrf2, which leads to elevated degradation of Nrf2.

Concerning the long-term negative effects of TNFα on Nrf2/γGCL-M/γGCL-C, NFκB may also be involved. Thus, it has been shown that the p65 subunit of NFκB 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 TNFα thus indicate that the “background” kinase activation in combination with effects of TNFα on these kinases can be deterministic for transactivation by Nrf2. The kinase activity may thus be one key to understanding the various effects of TNFα, 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 GSK3β phosphorylation after hypoxia-ischemia induced brain damage in rats [3, 60]. The activation of GSK3β 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 TNFα 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 GSK3β and p38 MAPK signaling pathways. The implications include that TNFα 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 TNFα is lost [41].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

ARE	Antioxidant responsive element
BSO	Buthionine sulfoxide
DMEM	Dulbecco's Modified Eagle's Medium
FBS	Foetal bovine serum
γGCL	Gamma-glutamylcysteine ligase
γGCL-C	Gamma-glutamylcysteine ligase catalytic subunit
γGCL-M	Gamma-glutamylcysteine ligase modulatory subunit
GSH	Glutathione
GSK3β	Glycogen synthase kinase-3 beta
Keap1	Kelch-like ECH-associated protein 1
ERK1/2	Extracellular regulated kinase
FBS	Foetal bovine serum
JNK	c-Jun N-terminal kinase

LPS	Lipopolysaccaride
MAPKs	Mitogen-activated protein kinases
MCM	Microglia-conditioned medium
MEK	Mitogen-activated protein kinase kinase
MEM	Modified Eagle's Medium
MnSOD	Manganese superoxide dismutase
NDA	Naphthalene-2,3-dicarboxaldehyde
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
Nrf2	Nuclear factor-erythroid 2-related factor 2
PBS	Phosphate buffered saline
5-SSA	5-Sulfosalicylic acid
***t*BHQ**	Tert-butylhydroquinone
TNFα	Tumor necrosis factor-alpha

References

1. Allaman I, Belanger M, Magistretti PJ. Astrocyte-neuron metabolic relationships: for better and for worse. Trends Neurosci. 2011; 34:76–87. [PubMed: 21236501]
2. Bruce-Keller AJ, Geddes JW, Knapp PE, McFall RW, Keller JN, Holtsberg FW, Parthasarathy S, Steiner SM, Mattson MP. Anti-death properties of TNF against metabolic poisoning: mitochondrial stabilization by MnSOD. J Neuroimmunol. 1999; 93:53–71. [PubMed: 10378869]
3. Brywe KG, Mallard C, Gustavsson M, Hedtjarn M, Leverin AL, Wang X, Blomgren K, Isgaard J, Hagberg H. IGF-I neuroprotection in the immature brain after hypoxia-ischemia, involvement of Akt and GSK3beta? Eur J Neurosci. 2005; 21:1489–1502. [PubMed: 15845077]
4. Chen S, Owens GC, Makarenkova H, Edelman DB. HDAC6 regulates mitochondrial transport in hippocampal neurons. PLoS ONE. 2010; 5(5):e10848. [PubMed: 20520769]
5. Cheng B, Christakos S, Mattson MP. Tumor necrosis factors protect neurons against metabolic-excitotoxic insults and promote maintenance of calcium homeostasis. Neuron. 1994; 12(1):139–153. [PubMed: 7507336]
6. Correa F, Ljunggren E, Mallard C, Nilsson M, Weber SG, Sandberg M. The Nrf2-inducible antioxidant defense in astrocytes can be both up- and down-regulated by activated microglia: involvement of p38 MAPK. Glia. 2011; 59:785–799. [PubMed: 21351160]
7. Correa F, Mallard C, Nilsson M, Sandberg M. Activated microglia decrease histone acetylation and Nrf2-inducible anti-oxidant defence in astrocytes: restoring effects of inhibitors of HDACs, p38 MAPK and GSK3beta. Neurobiol Dis. 2011; 44:142–151. [PubMed: 21757005]
8. Cui J, Shao L, Young LT, Wang JF. Role of Glutathione in neuroprotective effects of mood stabilizing drugs lithium and valproate. Neuroscience. 2007; 144:1447–1453. [PubMed: 17184924]
9. Cullinan SB, Gordan JD, Jin J, Harper JW, Diehl JA. The Keap1-BTB protein is an adaptor that bridges Nrf2 to a Cul3-based E3 ligase: oxidative stress sensing by a Cul3-Keap1 ligase. Mol Cell Biol. 2004; 24:8477–8486. [PubMed: 15367669]
10. De Lella Ezcurra AL, Chertoff M, Ferrari C, Graciarena M, Pitossi F. Chronic expression of low levels of tumor necrosis factor-alpha in the substantia nigra elicits progressive neurodegeneration, delayed motor symptoms and microglia/macrophage activation. Neurobiol Dis. 2010; 37:630–640. [PubMed: 19969084]
11. Dringen R, Pfeiffer B, Hamprecht B. Synthesis of the antioxidant glutathione in neurons: supply by astrocytes of Cys-Gly as precursor for neuronal glutathione. J Neurosci. 1999; 19:562–569. [PubMed: 9880576]

12. Eftekharpour E, Holmgren A, Juurlink BH. Thioredoxin reductase and glutathione synthesis is upregulated by t-butylhydroquinone in cortical astrocytes but not in cortical neurons. Glia. 2000; 31:241–248. [PubMed: 10941150]

13. Figiel I. Pro-inflammatory cytokine TNF-alpha as a neuroprotective agent in the brain. Acta Neurobiol Exp (Wars). 2008; 68:526–534. [PubMed: 19112477]

14. Fiore M, Angelucci F, Alleva E, Branchi I, Probert L, Aloe L. Learning performances, brain NGF distribution and NPY levels in transgenic mice expressing TNF-alpha. Behav Brain Res. 2000; 112:165–175. [PubMed: 10862948]

15. Gavillet M, Allaman I, Magistretti PJ. Modulation of astrocytic metabolic phenotype by proinflammatory cytokines. Glia. 2008; 56(9):975–989. [PubMed: 18383346]

16. Goven D, Boutten A, Lecon-Malas V, Boczkowski J, Bonay M. Prolonged cigarette smoke exposure decreases heme oxygenase-1 and alters Nrf2 and Bach1 expression in human macrophages: roles of the MAP kinases ERK(1/2) and JNK. FEBS Lett. 2009; 583:3508–3518. [PubMed: 19822148]

17. Hansson E. Cellular composition of a cerebral hemisphere primary culture. Neurochem Res. 1984; 9:153–172. [PubMed: 6204217]

18. Hubbs AF, Benkovic SA, Miller DB, O'Callaghan JP, Battelli L, Schwegler-Berry D, Ma Q. Vacuolar leukoencephalopathy with widespread astrogliosis in mice lacking transcription factor Nrf2. Am J Pathol. 2007; 170:2068–2076. [PubMed: 17525273]

19. Innamorato NG, Rojo AI, Garcia-Yague AJ, Yamamoto M, de Ceballos ML, Cuadrado A. The transcription factor Nrf2 is a therapeutic target against brain inflammation. J Immunol. 2008; 181:680–689. [PubMed: 18566435]

20. Jain A, Lamark T, Sjottem E, Larsen KB, Awuh JA, Overvatn A, McMahon M, Hayes JD, Johansen T. p62/SQSTM1 is a target gene for transcription factor NRF2 and creates a positive feedback loop by inducing antioxidant response element-driven gene transcription. J Biol Chem. 2010; 285:22576–22591. [PubMed: 20452972]

21. Jia JP, Meng R, Sun YX, Sun WJ, Ji XM, Jia LF. Cerebrospinal fluid tau, Abeta1-42 and inflammatory cytokines in patients with Alzheimer's disease and vascular dementia. Neurosci Lett. 2005; 383:12–16. [PubMed: 15936505]

22. Kawai Y, Garduno L, Theodore M, Yang J, Arinze IJ. Acetylation-deacetylation of the transcription factor Nrf2 (nuclear factor erythroid 2-related factor 2) regulates its transcriptional activity and nucleocytoplasmic localization. J Biol Chem. 2011; 286:7629–7640. [PubMed: 21196497]

23. Kensler TW, Wakabayashi N, Biswal S. Cell survival responses to environmental stresses via the Keap1-Nrf2-ARE pathway. Annu Rev Pharmacol Toxicol. 2007; 47:89–116. [PubMed: 16968214]

24. Kim HJ, Vaziri ND. Contribution of impaired Nrf2-Keap1 pathway to oxidative stress and inflammation in chronic renal failure. Am J Physiol Renal Physiol. 2010; 298:F662–F671. [PubMed: 20007347]

25. Kim JE, You DJ, Lee C, Ahn C, Seong JY, Hwang JI. Suppression of NF-kappaB signaling by KEAP1 regulation of IKKbeta activity through autophagic degradation and inhibition of phosphorylation. Cell Signal. 2010; 22:1645–1654. [PubMed: 20600852]

26. Kozlova EN, Takenaga K. A procedure for culturing astrocytes from white matter and the application of the siRNA technique for silencing the expression of their specific marker, S100A4. Brain Res Brain Res Protoc. 2005; 15(2):59–65. [PubMed: 15975528]

27. Lavoie S, Chen Y, Dalton TP, Gysin R, Cuenod M, Steullet P, Do KQ. Curcumin, quercetin, and tBHQ modulate glutathione levels in astrocytes and neurons: importance of the glutamate cysteine ligase modifier subunit. J Neurochem. 2009; 108:1410–1422. [PubMed: 19183254]

28. Liu GH, Qu J, Shen X. NF-kappaB/p65 antagonizes Nrf2-ARE pathway by depriving CBP from Nrf2 and facilitating recruitment of HDAC3 to MafK. Biochim Biophys Acta. 2008; 1783:713–727. [PubMed: 18241676]

29. Lung HL, Leung KN, Stadlin A, Ma CM, Tsang D. Induction of tumor necrosis factor receptor type 2 gene expression by tumor necrosis factor-α in rat primary astrocytes. Life Sci. 2001; 68:2081–2091. [PubMed: 11324713]

30. Markus T, Cronberg T, Cilio C, Pronk C, Wieloch T, Ley D. Tumor necrosis factor receptor-1 is essential for LPS-induced sensitization and tolerance to oxygen–glucose deprivation in murine neonatal organotypic hippocampal slices. J Cereb Blood Flow Metab. 2009; 29:73–86. [PubMed: 18728678]
31. McCoy MK, Tansey MG. TNF signaling inhibition in the CNS: implications for normal brain function and neurodegenerative disease. J Neuroinflamm. 2008; 5:45.
32. Mehindate K, Sahlas DJ, Frankel D, Mawal Y, Liberman A, Corcos J, Dion S, Schipper HM. Proinflammatory cytokines promote glial heme oxygenase-1 expression and mitochondrial iron deposition: implications for multiple sclerosis. J Neurochem. 2001; 77:1386–1395. [PubMed: 11389189]
33. Morales A, García-Ruiz C, Mirandai M, Marí M, Colelli A, Ardite E, Fernández-Checa JC. Tumor necrosis factor increases hepatocellular glutathione by transcriptional regulation of the heavy subunit chain of γ-glutamylcysteine synthetase. J Biol Chem. 1997; 272(48):30371–30379. [PubMed: 9374527]
34. Morgan MJ, Liu ZG. Reactive oxygen species in TNFalpha-induced signaling and cell death. Mol Cells. 2010; 30:1–12. [PubMed: 20652490]
35. Naidu S, Vijayan V, Santoso S, Kietzmann T, Immenschuh S. Inhibition and genetic deficiency of p38 MAPK up-regulates heme oxygenase-1 gene expression via Nrf2. J Immunol. 2009; 182:7048–7057. [PubMed: 19454702]
36. Niture SK, Jaiswal AK. INrf2 (Keap1) targets Bcl-2 degradation and controls cellular apoptosis. Cell Death Differ. 2011; 18:439–451. [PubMed: 20865015]
37. Nodin C, Nilsson M, Blomstrand F. Gap junction blockage limits intercellular spreading of astrocytic apoptosis induced by metabolic depression. J Neurochem. 2005; 94:1111–1123. [PubMed: 16092948]
38. Perry SW, Dewhurst S, Bellizzi MJ, Gelbard HA. Tumor necrosis factor-alpha in normal and diseased brain: conflicting effects via intraneuronal receptor crosstalk? J Neurovirol. 2002; 8:611–624. [PubMed: 12476354]
39. Poizat C, Puri PL, Bai Y, Kedes L. Phosphorylation-dependent degradation of p300 by doxorubicin-activated p38 mitogen-activated protein kinase in cardiac cells. Mol Cell Biol. 2005; 25:2673–2687. [PubMed: 15767673]
40. Popkie AP, Zeidner LC, Albrecht AM, D'Ippolito A, Eckardt S, Newsom DE, Groden J, Doble BW, Aronow B, McLaughlin KJ, White P, Phiel CJ. Phosphatidylinositol 3-kinase (PI3 K) signaling via glycogen synthase kinase-3 (Gsk-3) regulates DNA methylation of imprinted loci. J Biol Chem. 2010; 285:41337–41347. [PubMed: 21047779]
41. Ramsey CP, Glass CA, Montgomery MB, Lindl KA, Ritson GP, Chia LA, Hamilton RL, Chu CT, Jordan-Sciutto KL. Expression of Nrf2 in neurodegenerative diseases. J Neuropathol Exp Neurol. 2007; 66:75–85. [PubMed: 17204939]
42. Rojo AI, Sagarra MR, Cuadrado A. GSK-3beta down-regulates the transcription factor Nrf2 after oxidant damage: relevance to exposure of neuronal cells to oxidative stress. J Neurochem. 2008; 105:192–202. [PubMed: 18005231]
43. Ruedig C, Dringen R. TNFα increases activity of γ-glutamyl transpeptidase in cultured rat astroglial cells. J Neurosci Res. 2004; 75:536–543. [PubMed: 14743437]
44. Saha RN, Liu X, Pahan K. Up-regulation of BDNF in astrocytes by TNF-α: a case for the neuroprotective role of cytokine. J Neuroimmune Pharmacol. 2006; 1(3):212–222. [PubMed: 18040799]
45. Salazar M, Rojo AI, Velasco D, de Sagarra RM, Cuadrado A. Glycogen synthase kinase-3beta inhibits the xenobiotic and antioxidant cell response by direct phosphorylation and nuclear exclusion of the transcription factor Nrf2. J Biol Chem. 2006; 281:14841–14851. [PubMed: 16551619]
46. Sawada M, Suzumura A, Marunouchi T. TNF alpha induces IL-6 production by astrocytes but not by microglia. Brain Res. 1992; 583(1–2):296–299. [PubMed: 1504836]
47. Shen G, Hebbar V, Nair S, Xu C, Li W, Lin W, Keum YS, Han J, Gallo MA, Kong AN. Regulation of Nrf2 transactivation domain activity. The differential effects of mitogen-activated

protein kinase cascades and synergistic stimulatory effect of Raf and CREB-binding protein. J Biol Chem. 2004; 279:23052–23060. [PubMed: 15020583]

48. Shen G, Kong AN. Nrf2 plays an important role in coordinated regulation of Phase II drug metabolism enzymes and Phase III drug transporters. Biopharm Drug Dispos. 2009; 30:345–355. [PubMed: 19725016]
49. Shih AY, Johnson DA, Wong G, Kraft AD, Jiang L, Erb H, Johnson JA, Murphy TH. Coordinate regulation of Glutathione biosynthesis and release by Nrf2-expressing glia potently protects neurons from oxidative stress. J Neurosci. 2003; 23(8):3394–3406. [PubMed: 12716947]
50. Shih AY, Li P, Murphy TH. A small-molecule-inducible Nrf2-mediated antioxidant response provides effective prophylaxis against cerebral ischemia in vivo. J Neurosci. 2005; 25:10321–10335. [PubMed: 16267240]
51. Singh I, Pahan K, Khan M, Singh AK. Cytokine-mediated induction of ceramide production is redox-sensitive. Implications to proinflammatory cytokine-mediated apoptosis in demyelinating diseases. J Biol Chem. 1998; 273(32):20354–20362. [PubMed: 9685387]
52. Song IS, Tatebe S, Dai W, Kuo MT. Delayed mechanism for induction of gamma-glutamylcysteine synthetase heavy subunit mRNA stability by oxidative stress involving p38 mitogenactivated protein kinase signaling. J Biol Chem. 2005; 280:28230–28240. [PubMed: 15946948]
53. Sun X, Erb H, Murphy TH. Coordinate regulation of glutathione metabolism in astrocytes by Nrf2. Biochem Biophys Res Commun. 2005; 326:371–377. [PubMed: 15582588]
54. Tanaka N, Ikeda Y, Ohta Y, Deguchi K, Tian F, Shang J, Matsuura T, Abe K. Expression of Keap1-Nrf2 system and antioxidative proteins in mouse brain after transient middle cerebral artery occlusion. Brain Res. 2011; 1370:246–253. [PubMed: 21075092]
55. Tarkowski E, Liljeroth AM, Minthon L, Tarkowski A, Wallin A, Blennow K. Cerebral pattern of pro- and anti-inflammatory cytokines in dementias. Brain Res Bull. 2003; 61:255–260. [PubMed: 12909295]
56. Vargas MR, Johnson DA, Sirkis DW, Messing A, Johnson JA. Nrf2 activation in astrocytes protects against neurodegeneration in mouse models of familial amyotrophic lateral sclerosis. J Neurosci. 2008; 28:13574–13581. [PubMed: 19074031]
57. Wang X, Svedin P, Nie C, Lapatto R, Zhu C, Gustavsson M, Sandberg M, Karlsson JO, Romero R, Hagberg H, Mallard C. N-acetylcysteine reduces lipopolysaccharide-sensitized hypoxic-ischemic brain injury. Ann Neurol. 2007; 61:263–271. [PubMed: 17253623]
58. Xu C, Yuan X, Pan Z, Shen G, Kim JH, Yu S, Khor TO, Li W, Ma J, Kong AN. Mechanism of action of isothiocyanates: the induction of ARE-regulated genes is associated with activation of ERK and JNK and the phosphorylation and nuclear translocation of Nrf2. Mol Cancer Ther. 2006; 5:1918–1926. [PubMed: 16928811]
59. Yang H, Magilnick N, Ou X, Lu SC. Tumour necrosis factor alpha induces co-ordinated activation of rat GSH synthetic enzymes via nuclear factor kappaB and activator protein-1. Biochem J. 2005; 391:399–408. [PubMed: 16011481]
60. Yin W, Signore AP, Iwai M, Cao G, Gao Y, Johnnides MJ, Hickey RW, Chen J. Preconditioning suppresses inflammation in neonatal hypoxic ischemia via Akt activation. Stroke. 2007; 38:1017–1024. [PubMed: 17272774]
61. Yoon K, Jung EJ, Lee SY. TRAF6-mediated regulation of the PI3 kinase (PI3 K)-Akt-GSK3beta cascade is required for TNF-induced cell survival. Biochem Biophys Res Commun. 2008; 371:118–121. [PubMed: 18407828]
62. Yu M, Li H, Liu Q, Liu F, Tang L, Li C, Yuan Y, Zhan Y, Xu W, Li W, Chen H, Ge C, Wang J, Yang X. Nuclear factor p65 interacts with Keap1 to repress the Nrf2-ARE pathway. Cell Signal. 2011; 23:883–892. [PubMed: 21262351]
63. Yu R, Chen C, Mo YY, Hebbar V, Owuor ED, Tan TH, Kong AN. Activation of mitogen-activated protein kinase pathways induces antioxidant response element-mediated gene expression via a Nrf2-dependent mechanism. J Biol Chem. 2000; 275:39907–39913. [PubMed: 10986282]
64. Zhao Z, Chen Y, Wang J, Sternberg P, Freeman ML, Grossniklaus HE, Cai J. Age-related retinopathy in NRF2-deficient mice. PLoS ONE. 2011; 6:e19456. [PubMed: 21559389]

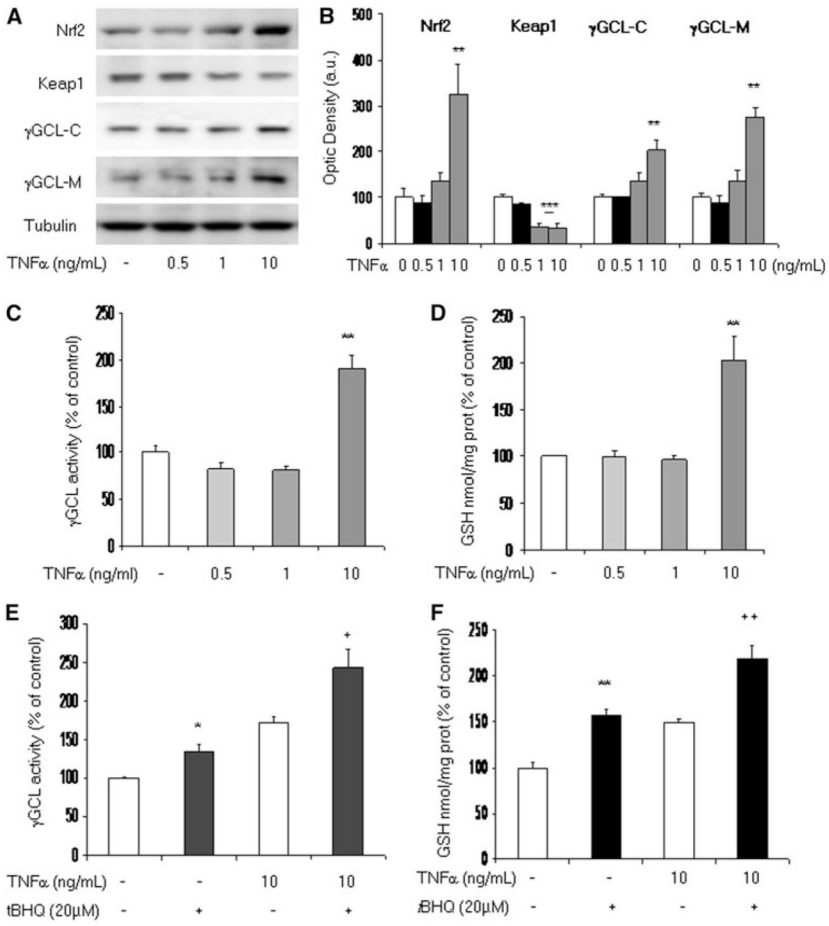

Fig. 1.

TNFα (24 h treatment) increased astroglial antioxidant defense system. Astrocyte-rich cultures were exposed to different concentrations of TNFα (0.5, 1 and 10 ng/mL) for 24 h followed by the protein expression analysis of Nrf2, Keap1, γGCL-C and γGCL-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. TNFα (10 ng/mL) induced an increase in γGCL activity (**c**) and GSH (**d**). Results are shown as mean ± SEM and expressed as percentage of control. Statistics: $^{**}p < 0.01$ versus control. Astrocyte-rich cultures were treated with TNFα in the presence or absence of 20 μM *t*BHQ and the activity of γGCL (**e**) and GSH levels (**f**) were determined. In both cases, results shown are the mean ± SEM and expressed as percentage of control. Statistics: $^{*}p < 0.05$ versus control; $^{**}p < 0.01$ versus control; $^{+}p < 0.05$ versus TNFα 10 ng/mL; $^{++}p < 0.01$ versus TNFα 10 ng/mL. (n = 4–6)

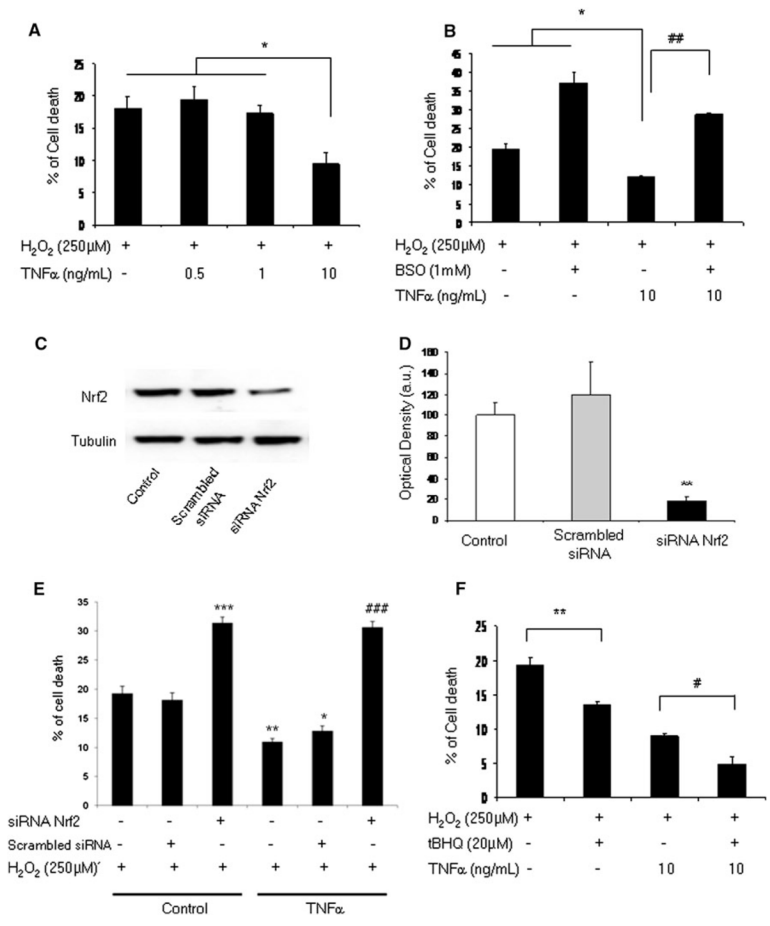

Fig. 2.

TNFα (24 h treatment) protected from cell death induced by 3 h exposure to 250 µM hydrogen peroxide. Astrocyte-rich cultures pre-treated for 24 h with 10 ng/mL TNFα showed higher resistance to oxidative stress (**a**). Inhibition of γGCL activity with 1 mM BSO reversed the protective effects of 10 ng/mL of TNFα (**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 TNFα (24 h) against 250 µM hydrogen peroxide (**e**). Co-treatment with the Nrf2-inducer *t*BHQ 20 µM potentiated the protective effect of 10 ng/mL TNFα (**f**). In all cases, results are shown as the mean ± SEM. Statistics: *$p < 0.05$ versus control; **$p < 0.01$ versus control; ***$p < 0.005$ versus control; #$p < 0.05$ versus TNFα 10 ng/mL; ##$p < 0.01$ versus TNFα 10 ng/mL; ###$p < 0.005$ versus TNFα 10 ng/mL (n = 4–8)

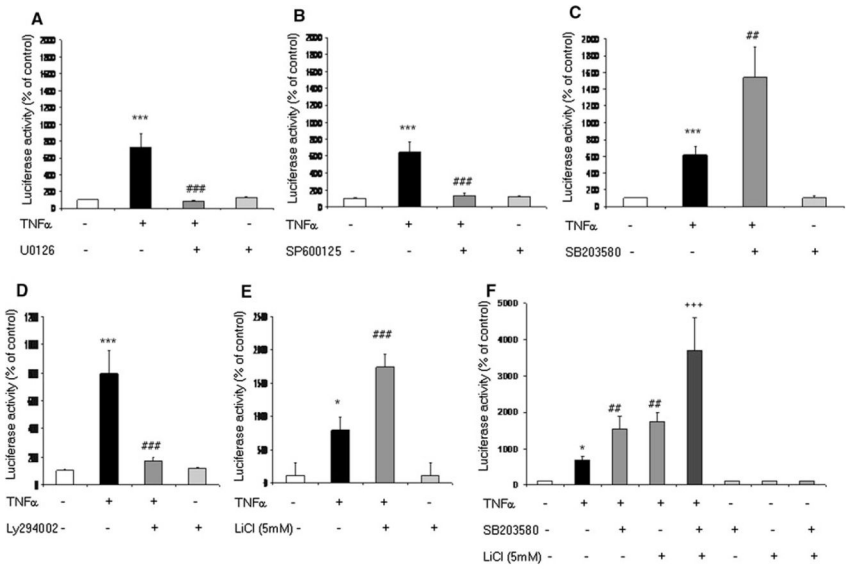

Fig. 3.

Effect of the inhibition of ERK1/2, JNK, p38 MAPK, Akt and GSK3β signalling pathways on the ARE-driven luciferase activity induced by 10 ng/mL TNFα. Astrocyte-rich cultures were transfected with ARE-Luc reporter gene construct and treated for 24 h with 10 ng/mL TNFα in the presence or absence of 10 μM U0126 (**a**), 10 μM SP600125 (**b**), 20 μM SB203580 (**c**), 10 μM Ly294002 (**d**), 5 mM LiCl (**e**) and a combination of 5 mM LiCl and 20 μM SB203580 (**f**). Mean ± 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 TNFα; ###$p < 0.005$ versus TNFα; +++$p < 0.005$ versus TNFα + SB203580 or TNFα + LiCl (n = 9)

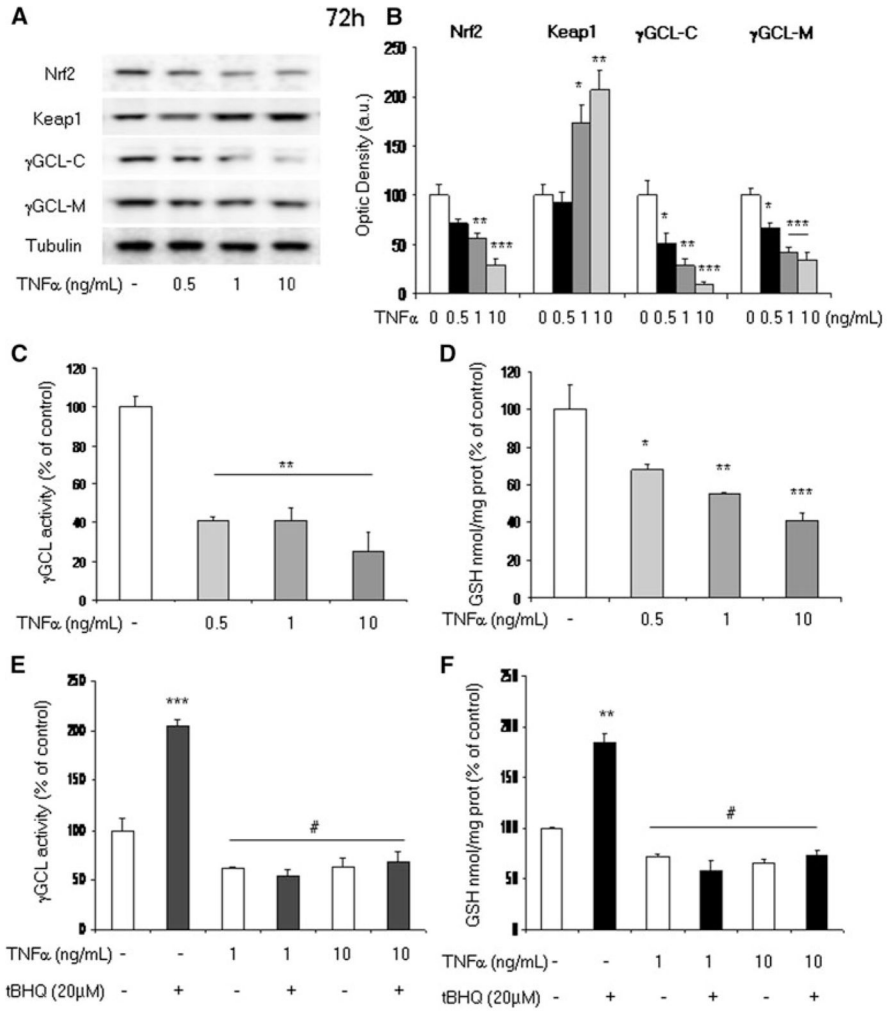

Fig. 4.

TNFα (72 h treatment) decreased the astroglial antioxidant defense system. Astrocyte-rich cultures were exposed to different concentrations of TNFα (0.5, 1 and 10 ng/mL) for 72 h followed by protein expression analysis of Nrf2, Keap1, γGCL-C and γGCL-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. TNFα (10 ng/mL) induced a reduction in γGCL activity (**c**) as well as on the levels of GSH (**d**). Results are shown as mean ± 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 TNFα (10 ng/mL) for 72 h in the presence or absence of 20 μM *t*BHQ and the activity of γGCL (**e**) and GSH levels (**f**) were determined. In this case, *t*BHQ was unable to restore the TNFα-induced down-regulation of γGCL activity and GSH levels. In both cases, results shown are the mean ± 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)

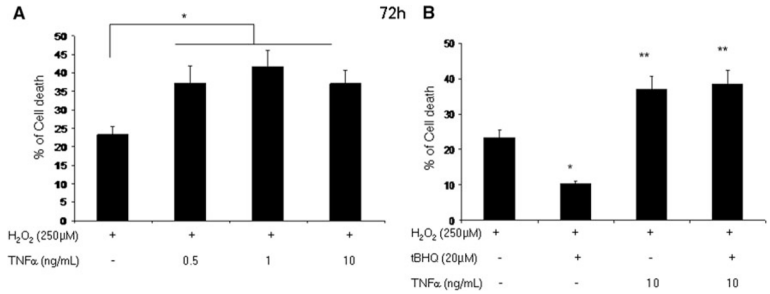

Fig. 5.

TNFα (72 h treatment) increased cell death induced by 3 h exposure to 250 μM hydrogen peroxide. Astrocyte-rich cultures pre-treated for 72 h with 10 ng/mL TNFα showed higher cell death levels when challenged to oxidative stress (**a**). Treatment with *t*BHQ 20 μM was unable to protect from the effects of 72 h treatment with 10 ng/mL of TNFα (**b**). Results are shown as the mean ± SEM. Statistics: *$p < 0.05$ versus control + H_2O_2-treatment; **$p < 0.01$ versus control + H_2O_2-treatment (n = 4–6)

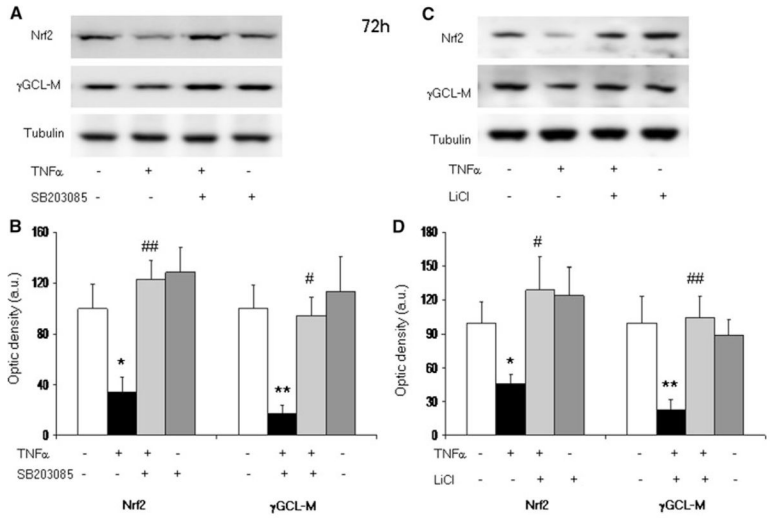

Fig. 6.

Effect of the inhibition of p38MAPK and GSK3β on the down-regulated expression of Nrf2 and γGCL-M induced by 10 ng/mL of TNFα (72 h). Inhibition of p38 MAPK activation with the specific inhibitor SB203580 (20 μM) resulted in a restoration in the down-regulated expression of Nrf2 and γGCL-M induced by 72 h treatment with TNFα (**a**). In (**b**), the densitometric analysis is shown. Statistics: *$p < 0.05$ versus control; **$p < 0.01$ versus control; #$p < 0.05$ versus TNFα; ##$p < 0.01$ versus TNFα. Inhibition of GSK3β activation with LiCl (5 mM) resulted in a reversal of the down-regulated expression of Nrf2 and γGCL-M induced by 72 h treatment with TNFα (**c**). In (**d**), the densitometric analysis is shown. Statistics: *$p < 0.05$ versus control; **$p < 0.01$ versus control; #$p < 0.05$ versus TNFα; ##$p < 0.01$ versus TNFα (n = 6)